RHIZOBIUM LIPID A VERY LONG CHAIN FATTY ACID INFLUENCE ON STUCTURE AND FUNCTION

by

DIANNA VIOLA BOURASSA

(Under the Direction of RUSSELL W. CARLSON)

ABSTRACT

Nitrogen fixation is a biological process essential to life. Atmospheric nitrogen (N_2) must be converted to an available form such as ammonia (NH_3) so that nitrogen is available for use in biological processes. The symbiotic relationship of legume plants and rhizobia bacteria plays a crucial role in this process. The complex interaction between the plant and bacteria from the first recognition and interaction through the fully functional nodule filled with nitrogen-fixing bacteroids involves many factors from both the plants and bacteria. This work takes an in depth look at an unusual very long chain fatty acid (27-hydroxyoctacosanoic acid, VLCFA) bound to the lipid A portion of the lipopolysaccharide (LPS) found in the *Rhizobium leguminosarum* cell membrane. Two genes required for the biosynthesis and transfer of the VLCFA (*acpXL* acyl carrier protein and *lpxXL* acyl transferase) were mutated producing Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$, *lpxXL*$^-$), and EL197 (effective *lpxXL*$^-$). The effects on LPS structure, bacterial phenotype, and symbiotic proficiency were examined. Both *acpXL* and *lpxXL* mutants were unable to add the VLCFA to the lipid A. In the *acpXL* mutant a shorter acyl chain (palmitic or stearic) was transferred in place of the VLCFA. However, in strains without *lpxXL* neither the VLCFA nor the shorter acyl chain was added. It was also noted that for both parent and mutant

strains nodule bacteroids tended to have LPS with longer acyl chains than the laboratory-cultured bacteria. The *acpXL* and *lpxXL* mutants also affected bacterial phenotype. Mutants had diminished membrane integrity, altered sensitivity to cationic peptides, and diminished relative levels of cyclic β-glucans. When the host plant (*Pisum sativum*, Pea) was inoculated with parent and mutant strains changes to the symbiotic phenotype was also observed. Host root infection was less efficient and infected plant nodule cells were disorganized. Bacteroids formed atypical multiply branched structures with multiple bacteroids per symbiosome. The bacteroids were not packed as tightly into the nodule plant cells, and were senescing earlier than normal. The main purpose of symbiosis, nitrogen fixation, was also detrimentally affected. Clearly this unusual VLCFA plays an important role in the symbiotic relationship between *Rhizobium* and pea plants.

INDEX WORDS: Rhizobium leguminosarum, Very long chain fatty acid, Lipopolysaccharide

RHIZOBIUM LIPID A VERY LONG CHAIN FATTY ACID INFLUENCE ON STUCTURE AND FUNCTION

by

DIANNA VIOLA BOURASSA

B.S., The University of Georgia, 2002

M.S., The University of Georgia, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

RHIZOBIUM LIPID A VERY LONG CHAIN FATTY ACID INFLUENCE ON STUCTURE AND FUNCTION

by

DIANNA VIOLA BOURASSA

Major Professor: Russell W. Carlson

Committee: Elmar L. Kannenberg
Debra Mohnen
R. Jeffrey Buhr

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2013

DEDICATION

This work is dedicated to my husband, Jason Bourassa. Thank you for your never ending patience and support throughout this journey. I also dedicate this dissertation to my son, Isaac Bourassa. You have inspired me to do my best to make this world a better place.

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help of my mentors, colleges, friends, and family. I would like to thank my major professor, Russ Carlson, for his thoughtful insights, encouragement, and assistance with completing this work and dissertation. I would also like to thank my committee, Elmar Kannenberg, Deb Mohnen, and Jeff Buhr for their expertise, thoughts, and help along the way. Thank you to my fellow lab mates Dusty Brown, Artur Muszynski, and Scott Forsberg for all of your help with lab techniques and concepts along the way. I would also like to thank my friend Nicole Bartenfeld for your faith in my abilities.

The pursuit of scientific exploration does not begin with an acceptance letter but years before. I would like to thank a wonderful high school science teacher, Holly Villhauer, who inspired me to look further. I would also again like to thank Jeff Buhr for mentoring me starting in my undergraduate career and encouraging me to excel. I wouldn't have come so far without him.

I would like to give a special thanks to my family. My parents Don and Diane Gensler taught me that with hard work and perseverance I can achieve anything. My brothers Marcus, Travis, and David remind me that I am not infallible. My sister Amy reminds me that I should lead by example, since there may be someone looking up to me. I would also like to thank my son, Isaac. He brings joy to my heart and reminds me of the wonder of experiencing things for the first time. Finally, I would like to thank my husband, Jason, for standing by me though this journey. You have been there to support me for the highs as well as the lows. Without you this would not have been possible.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview of the Biology of Rhizobia/Legume Symbiosis

The interactions between rhizobia and legumes have been studied for over 400 years beginning with observations of nodules on roots (Fuchsius 1542). However, it wasn't until 1866 that Woronin discovered that there were bacteria-like bodies within the nodule (Woronin 1866). Since then the inhabitants of symbiotic root nodules have been identified as rhizobia, an inclusive term for the currently 62 species within 12 genera (Weir 2008). These rhizobia enter into a symbiotic relationship with legumes, fixing nitrogen for the plant while receiving carbohydrates, proteins and a unique niche for growth. This relationship has had importance in agriculture for many years, but recently is being utilized as a model for eukaryotic intracellular infections due to similarities in processes such as bacteria/host recognition, evasion of the host defense responses, and differentiation of bacterial and/or host cells. In Figure 1.1 a general scheme for the rhizobia/legume symbiosis is shown.

The symbiotic process is a highly complicated molecular dialogue beginning with flavonoids secreted from the legume root tissues which induce the production of Nod factors in the rhizobia (Schlaman et al. 1998). The root then recognizes Nod factors secreted from the rhizobia by root surface nod factor receptors resulting in the production of a root nodule (Broghammer et al. 2012). This interaction between flavonoids, Nod factors, and the legume recognition mechanism is the first step of a specific recognition between bacteria and host. Flavonoids (luteolin and 7,4' dihydroxyflavone) were first identified in 1986 (Peters et al. 1986;

Redmond et al. 1986). Other plant signals that induce *nod* genes in addition to approximately thirty identified flavonoids include aldonic acids, betaines, jasmonates, simple phenolics, and xanthones (Cooper et al. 2004; Cooper et al. 2007). The release of these plant signals is induced by the presence of compatible rhizobia within the rhizosphere (van Brussel et al. 1990; Schmidt et al. 1994). Nod factor production is in turn activated by these plant signals (Spaink 2000). The first Nod factor structure was determined from *Sinorhizobium meliloti* in 1990 (Lerouge et al. 1990). These Nod factors are a signal chitin lipooligosaccharide and have a general β-1,4 linked *N*-acetyl-D-glucosamine structure with many variations leading to specificity. In the plant host these Nod factors induce root hair curling, membrane depolarization, intracellular calcium oscillations, root hair cytoskeleton alteration, and root cortex cell division (Gage 2004; Cooper 2007). In addition to Nod factors, other compounds including secreted proteins, *N*-acyl homoserine lactones, indole-3-acetic acid, bradyoxetin, hopanoids, and lumichrome affect this molecular dialogue (Cooper 2007).

Once this molecular dialogue has commenced, rhizobia bind to root hair cells first through rhicadhesin and then cellulose fibrils. Following this attachment, the tip of the root hair curls into a "shepherd's crook" configuration where the rhizobia become trapped between two cell walls of the root hair (Callaham and Torrey 1981). This leads to degradation and invagination of the plant cell wall, tip growth of the invagination, and initiation of infection thread formation (Smit et al. 1987; Gage 2004). Exopolysaccharides (EPS) are particularly important in this stage of symbiosis. EPS are variable carbohydrate heteropolymers with repeating unit structures that contain mainly D-glucose, D-galactose, D-mannose, D-glucuronic acid, and D-galacturonic acid with additional modifications including acetyl, pyruvyl, succinyl, and hydroxybutanoyl groups (Spaink 2000). The structure and synthesis of EPS are variable and are affected by osmolarity,

nitrogen starvation, and phosphate limitation (Breedveld et al. 1990; Dusha et al. 1999; Ruberg et al. 1999; Fraysse et al. 2003). EPS is required for nodule invasion in plants that form indeterminate nodules but is not required for invasion of determinate nodules (Gray and Rolfe 1990; Cheng and Walker 1998). *Sinorhizobium meliloti* produce two EPS, succinoglycan (EPSI) and galactoglucan (EPSII), both of which play signaling roles during infection thread extension (Pellock et al. 2000). Succinoglycan affects polarization and root hair cytoskeleton organization while moderating the plant defense response (Cheng and Walker 1998; Pellock et al. 2000; Fraysse et al. 2003). They also are known to be important for survival in the rhizosphere, attachment to surfaces, osmoregulation, and infection of host plant cells (Spaink 2000). After the initiation of the infection thread into the root hair the infection thread extends into the epidermal cell and continues through the distal cell wall into the intercellular space further branching and expanding into the root nodule. During nodule formation, the branched infection network reaches past the nodule meristem into the infection zone (Vasse et al. 1990).

Host plants that harbor rhizobia form two distinct types of nodules, indeterminate and determinate. For indeterminate nodules the infection thread branches and bacteria enter individual dividing meristematic cells in the nodule primordium through infection droplets (Leborgne-Castel et al. 2010). A single bacterium is released and is enveloped by a plant-derived membrane also known as the symbiosomal or peribacteroid membrane. The bacteria and the symbiosomal membrane have synchronous division that restricts symbiosis to one bacterium/bacteroid per symbiosome. The proximity of the rhizobial outer membrane and the plant derived membrane and their interaction may affect this synchronous division (Bhat et al. 1994). Bacteria enveloped in the symbiosomal membrane differentiate into bacteroids and then are able to begin to fix nitrogen. For determinate nodules there is not an infection droplet.

Instead, rhizobia bacteria enter cells by endocytosis that leads to the plant derived symbiosomal membrane around the bacteria. The bacteria then replicate inside each symbiosome leading to symbiosomes with multiple bacteroids inside. Rhizobia mutants that lack the O-chain polysaccharide (OPS) portion of the LPS typically abort symbiosis in the early stage of the infection thread formation where the rhizobia and plant membrane have close contact. In both indeterminate and determinate nodules, thousands of symbiosomes are found within single host cells, which are known to have an acidic, low-oxygen environment (Layzell and Hunt 1990). This low-oxygen environment is required for nitrogenase activity. Leghemoglobin is produced within the nodule by the plant to sequester oxygen allowing for nitrogen fixation to occur. The LPS of rhizobia have been demonstrated to be important throughout the nodulation process including bacterial attachment through to the production of a fully functional nodule.

The LPSs on the surface of the bacterial cells come in direct contact with plant host cells and are thus likely to directly influence interactions between the bacteria and host cells. Lipopolysaccharides have been reported to have some influence on stages of infection including specific binding to lectins on the root hair surface (Dazzo and Brill 1979). However, LPS is more commonly known to be essential in stages of symbiosis including root nodule invasion, infection, release from the infection thread, differentiation into bacteroids, and symbiosome development (Dazzo et al. 1991; Kannenberg et al. 1998; Niehaus et al. 1998; Carlson et al. 1999; Carlson et al. 2010). LPS also has a role in signaling to suppress the plant defense response during invasion (Albus et al. 2001). This role has been hypothesized to include protection against cationic peptides, a protective barrier, and molecular masking of bacterial compounds possibly through shielding by O-antigen polysaccharide or stabilization of the outer membrane by LPS (Breedveld et al. 1993; Raetz 1993; Lepek and D'Antuono 2005). During

differentiation from bacteria to bacteroids the LPS undergoes structural changes increasing surface hydrophobicity that may be related to bacteroid and symbiosome synchronous division (Dazzo et al. 1991; Kannenberg et al. 1998; Kannenberg and Carlson 2001; Fraysse et al. 2003).

Other polysaccharides important for the rhizobia legume symbiosis include the K-antigens and cyclic β-glucans. The K-antigens are capsular polysaccharide containing high proportions of Kdo (3-deoxy-D-*manno*-2-octulosonic acid) and are involved in the protection of the bacteria against the host defense response as well as host recognition (Kannenberg et al. 1998; Carlson et al. 1999; Fraysse et al. 2003). Cyclic β-glucans are molecules of 10-40 glucose residues circularly linked by β-(1,2) glycosidic bonds or β-(1,3) and β-(1,6) glycosidic bonds for *Bradyrhizobium*. They are found within the periplasm and also have a major role in rhizobia-legume symbiosis through the stabilization of the cell membrane protecting the cell against the hypoosmotic conditions found within the plant nodule (Chen et al. 1985; Miller and Wood 1996). Levels of cyclic β-glucans have been shown to be increased within bacteroids likely due to the hypoosmotic conditions found within the root nodule (Gore and Miller 1993).

Structure and Biosynthesis of Lipopolysaccharides

The cellular envelope of gram-negative bacteria consists of an inner membrane phospholipid bilayer, periplasmic space between the inner and outer membranes, and an outer membrane consisting of phospholipids for the inner leaflet and lipopolysaccharides making up the outer leaflet (Figure 1.2). Both the inner and outer membranes as well as the periplasmic space contain proteins important for various functions such as the passage of molecules across membranes and protein folding and assembly.

Lipopolysaccharides consist of three main components including a variable OPS extending from the cell surface, a polysaccharide core (inner and outer) and a lipid A that comprises the

outer leaflet of the outer membrane (Kannenberg et al. 1998). Lipid A biosynthesis is well conserved among gram-negative bacteria and has been well described for *Escherichia coli* (Figure 1.3) (Raetz and Whitfield 2002). Biosynthesis begins with the transfer of a β-hydroxymyristate from an acyl carrier protein to the 3-hydroxyl group on UDP-GlcNAc by LpxA. Next, UDP-3-O-acyl-GlcNAc is deacetylated by LpxC. A second β-hydroxymyristate is then transferred to the $-NH_2$ group by LpxD generating UDP-2,3-diacyl-GlcN. Pyrophosphatase LpxH cleaves off UMP generating lipid X (2,3-diacyl-GlcN-1-P) which is condensed with UDP-2,3-diacyl-GlcN by LpxB to form a tetraacylated GlcN disaccharide-1-Ps. Lipid IV_A is formed by phosphorylation at the 4' position of the tetraacylated GlcN disaccharide-1-P by LpxK. KdtA transfers two Kdo residues to the 6' hydroxyl group of Lipid IV_A generating Kdo_2-Lipid IV_A. Finally, LpxL and LpxM transfer lauroyl and myristoyl chains to the hydroxyl groups of the 2' and 3' β-hydroxylacyl chains forming acyloxyacyl groups. From this moiety (Kdo_2-lipid A) various lipid A structures are synthesized dependent on the gram-negative bacterial species. Some of these lipid A alterations will be discussed in the following section.

LPS core consists of two parts, the inner and outer core, with OPS attached to the outer core. The inner core is generally well conserved within a bacterial genus and usually contains Kdo and other sugars that may be modified with additionsl sugars, phosphate, pyrophosphorylethanolamine, or phosphorylcholine (Raetz and Whitfield 2002). The outer core is more variable within a genus. The *Rhizobium leguminosarum* bv. *viciae* has a core structure that consists of OPS-Kdo-(2-6)-α-D-Gal-(1-6)-α-D-Man-(1-5)-Kdo-Lipid A with α-D-GalA 1,4-linked to Man and two α-D-GalA 1,4 and 1,5-linked to a branching Kdo which is 2,4-linked to the Kdo attached to the lipid A (Carlson et al. 2010). In order to distinguish these three Kdo residues, the proximal Kdo that is attached to the lipid A will be referred to as Kdo-1, the

branching Kdo which is attached to Kdo-1 as Kdo-2, and the distal Kdo that is attached to the core Gal residue as Kdo-3. Core biosynthesis has been described in several reviews (Carlson et al. 2010; Raetz et al. 2007). The core is synthesized on the cytoplasmic side of the inner membrane. Each sugar is assembled onto the Kdo_2-lipid A from nucleotide sugar precursors. LpcC transfers Man from GDP-Man to the proximal Kdo, Kdo-1. LpcA transfers Gal from UDP-Gal to Man and LpcB transfers the distal Kdo-3 from CMP-Kdo to Gal. Once the backbone of the core is complete the lipid A-core is transported across the inner membrane by MsbA flippase. The biosynthesis of the GalA residues added to the core as well as the GalA residue present on lipid A has recently been elucidated (Brown et al. 2012). RgtE was found to transfer GalA from UDP-GalA to dodecaprenyl-phosphate. In the periplasmic space RgtA, RgtB, RgtC, and RgtD transfer GalA from Dod-P-GalA to the core and lipid A in a stepwise manner. GalA must first be 1,4-linked to the lipid A distal GlcN by RgtD. Then RgtA transfers GalA to Kdo-2 at the 5-position, RgtB transfers GalA to Kdo-2 at the 4-position, and RgtC transfers GalA to Man. The LPS core has been shown to have a role in outer membrane stability in Gram-negative bacteria, as have the presence of the GalA residues in *Rhizobium leguminosarum* (Schnaitman and Klena 1993; Brown et al. 2012).

The OPS is the most variable component of the LPS between species and also contains broad heterogeneity in chain length within a species. The OPS is synthesized on the cytoplasmic side of the inner membrane onto undecaprenyl phosphate (und-P) acceptors by glycosyltransferases and sugar nucleotide substrates. Transfer of the OPS is accomplished by one of three possible routes, Wzy-dependent, ABC transporter, or synthase-dependent pathways (Raetz and Whitfield 2002). In the Wzy-dependent pathway repeat-unit structures of the OPS are transported across the inner membrane one at a time and polymerized on the periplasmic side of

the inner membrane. Once the OPS is complete it is ligated to the lipid A-core. For the ABC transporter pathway the entire OPS is synthesized on the cytoplasmic side of the inner membrane before transport across the inner membrane by an ABC-transporter and ligation on the lipid A-core within the periplasm. The synthase-dependent pathway simultaneously extends the length of the OPS while transporting it across the inner membrane. Within the periplasm the OPS is ligated to the lipid A-core by WaaL. Typically OPS heteropolysaccharides are synthesized through the Wzy-dependent pathway and OPS homopolysaccharides are synthesized through the ABC-transporter pathway (Carlson et al. 2010). Transport of LPS across the periplasmic space is less well studied. Two models for LPS translocation include transport in a soluble complex with a chaperone and transport through zones of adhesion between the inner and outer membranes (Bos et al. 2007). Some of the *Rhizobium leguminosarum* bv. *viciae* gene region that is likely responsible for OPS biosynthesis has been defined (Kannenberg et al. 1992), as has the gene region for *Rhizobium etli* CE3 (Ojeda et al. 2013). This region includes genes of unknown function as well as genes for glycosyl transferases and nucleotide sugar synthesis. *Rhizobium etli* has *wzm* and *wzt* and thus appears to use the ABC-transporter method for OPS biosynthesis (Lerouge et al. 2001). While the structure of *Rhizobium leguminosarum* OPS has been determined, the mechanism of *Rhizobium leguminosarum* OPS biosynthesis is still unknown (Forsberg and Carlson 2008). Neither *wzm* and *wzt* genes nor *wzy* and *wzx* homologs are present in *Rhizobium leguminosarum* (Muszynski et al. 2011).

Alternative Structure and Function of Lipid A

The link between LPS and eukaryotic immunity has been established for some time. The lipid A portion of LPS is known to activate the innate immune response through its interaction with TLR4/MD2 via CD14 in eukaryotic cells leading to inflammation by TNF-α and IL-1-β

which, in turn, activate co-stimulatory molecules required for the adaptive immune response (Raetz and Whitfield 2002). Mutations in lipid A biosynthesis genes are known to alter the strength of the immune response against many pathogens. Lipid A from gram-negative bacteria is detected in animal systems by the binding of lipid A to CD14 on animal cell surfaces (Tobias et al. 1986). The lipid A/CD14 complex is then recognized by toll-like receptor 4 (TLR4) on macrophages and endothelial cells that leads to inflammation by TNF-α and IL1-β and the subsequent induction of the adaptive immune response through co-stimulatory molecules (Poltorak et al. 1998; Hoshino et al. 1999; Medzhitov and Janeway 2000). Activation of TLR4 has been shown to depend on lipid A phosphate groups and number and length of fatty acyl chains (Golenbock et al. 1991; Persing et al. 2002). In the presence of larger amounts of lipid A, the inflammation and adaptive immune response can lead to sepsis. Structural analogues of the lipid A portion of bacterial LPS have been used for the development of vaccines for diseases such as Hepatitis B (Kundi 2007). This portion of the LPS has been demonstrated to be a good human vaccine adjuvant creating an immunostimulatory response (Mata-Haro et al. 2007).

Modifications to the lipid A can assist pathogens in cellular protection and in invasion and infection (Figure 1.4). In *Escherichia coli* and *Salmonella* some of these modifications include addition of a phosphoethanolamine, 4-amino-4-deoxy-L-arabinose (L-AraN), and palmitate (Zhou et al. 1999). Phosphoethanolamine and L-AraN protect against acidic conditions and cationic antibacterial peptides and the addition of the palmitate in the outer membrane aids in pathogenesis. Addition of the palmitate (hepta-acylated lipid A) can block signaling of the innate immune response by hexa-acylated lipid A molecules (Tanamoto and Azumi 2000; Janusch et al. 2002; Muroi et al. 2002; Bishop et al. 2005). In addition, *Salmonella* is able to hydroxylate the 3' secondary acyl chain as well as remove the 3 position β-hydroxymyristoyl chain (Raetz and

Whitfield 2002; Reynolds et al. 2006). The function of the hydroxylation is unknown but may be related to regulation of outer membrane permeability (Nikaido 2003; Murata et al. 2007; Gibbons et al. 2008). Deacylation is thought to reduce the ability of lipid A to stimulate the TLR4-MD-2 complex (Kawasaki et al. 2012). Mutation of the *lpxM* gene that adds a secondary acyl chain in *Escherichia coli* and *Salmonella* lipid A has attenuated pathogenicity in comparison to the wild type strains (Khan et al. 1998). Under cold shock conditions *lpxP* adds a palmitoleate in place of the laurate to protect the cell against cold temperatures (Carty et al. 1999). *Pseudomonas aeruginosa* produces shorter hydroxyacyl chains, is able to hydroxylate the 2 and 2' secondary acyl chains, can deacylate the 3 position, and add L-Ara4N (Kulshin et al. 1991; Ernst et al. 1999). The secondary acyl chains on lipid A have been shown to be important for pathogenic infection. *Aquifex prophilus* lacks the phosphate residues, contains GalA residues, and replaces GlcN backbone with 2,3-diamino-2,3-dideoxy-D-glucose residues (Plotz et al. 2000). *Leptospira interrogans*, *Acidithiobacillus ferrooxidans*, and *Campyloabacter jejuni* also can convert UDP-GlcNAc to UDP-GlcNAc3N during lipid A biosynthesis (Moran et al. 1991; Sweet et al. 2004a; Sweet et al. 2004b). Pathogens also modify the Kdo portion of the lipid A. *Hemophilus influenzae, Vibrio cholerae,* and *Bordetella pertussis* incorporate only one Kdo with a phosphate group instead of the second Kdo and *Chlamydia trachomatis* incorporates three Kdo residues (Belunis et al. 1995; White et al. 1997). *Francisella* and *Helicobacter pylori* lipid A lack the 4' phosphate and the 3' acyl chain. *Francisella* adds a GalN bound to the 1-phosphate (Phillips et al 2004; Wang et al. 2006).

The rhizobia/legume symbiotic system is an excellent system for the study of pathogen/host interactions. The symbiotic relationship is similar to the pathogenic relationship in that bacteria evade the host immune response and the host is altered due to the presence of the

bacteria. *Brucella abortus* parallels the rhizobia/plant interaction in that *Brucella* persists in a membrane-bound compartment within the host cells (Corbel 1997; Porte et al. 1999). *Brucella* and rhizobia have similar mechanisms to enable them to persist their respective hosts (LeVier et al. 2000). Also, the genes required for synthesis and transfer of a very long chain fatty acid (VLCFA) can also be found in some intracellular pathogens (Vedam et al. 2003). The importance of the VLCFA fatty acid during symbiosis may parallel its importance during pathogen intracellular infection. The rhizobia/legume symbiosis model is beneficial to study because rhizobia are not lethal to the host enabling the study of the entire symbiotic process. Study of specific lipid A/host interactions using the rhizobia/legume symbiotic interaction will help elucidate the importance of these molecules and their modifications in disease. The symbiotic relationship between rhizobia and their host plants is influenced by the unusual structures found on the lipid A component of their lipopolysaccharides. Some of these unusual structures are also present on pathogens that live intracellularly within their plant or animal hosts.

When compared to *Escherichia coli*, lipid A of rhizobia has some unique features (Figure 1.5). These unique features are thought to be important for symbiosis. In order to make this unusual lipid A structure, rhizobia express unique genes in addition to those needed for the constitutive biosynthesis pathway. The unique genes encode products required for modifying the lipid A structure. In the constitutive biosynthesis pathway, the *Escherichia coli* lipid A structure in Figure 1.5 is synthesized on the cytoplasmic side of the inner membrane and is transported across the inner membrane following the addition of the core sugars (Bos et al. 2007). Rhizobia use the same biosynthetic pathway as *Escherichia coli* prior to flipping of the lipid A precursor across the inner membrane, except that the VLCFA is added instead of the secondary myristic

acid on the 2' acyl chain and the secondary lauric acid on the 3' acyl chain is not added at all. It is thought that the addition of the VLCFA occurs at the cytoplasmic face of the inner membrane. The distant ortholog of *Escherichia coli lpxL* that adds a secondary lauryl chain to lipid A, *lpxXL*, is also presumed to face the cytoplasm because its co-substrates are cytoplasmic. The locus that is required for biosynthesis and addition of the VLCFA to the lipid A is shown in Figure 1.6. The *acpXL* gene encodes the acyl carrier protein on which the VLCFA is built. Open reading frames 1 through 4 encode genes required for the biosynthesis of the VLCFA on the acyl carrier protein (Tobias et al. 1986; Bhat et al. 1994; Poltorak et al. 1998; Que et al. 2000). The *lpxXL* gene encodes for an acyl transferase that transfers the VLCFA from the acyl carrier protein to the 2'-3OH-myristic acid on the lipid A (Basu et al. 2002). The VLCFA (27OCH28:0) sometimes has a hydroxybutyrate moiety at C27OH and is attached to the 2'-3OH myristic acid.

The remaining unique features of the rhizobial lipid A are thought be synthesized within the periplasm or at the outer membrane. The biosynthesis of the unique rhizobial lipid A has been described in several reviews (Raetz et al. 2007; Carlson et al. 2010). While most gram-negative bacteria have 1- and 4'- phosphate groups, rhizobia such as *Rhizobium leguminosarum* and *Rhizobium etli* have late functioning phosphatases (LpxE and LpxF) that remove the phosphate groups following lipid A transport across the inner membrane (Bhat et al. 1994; Price et al. 1995). Following removal of the 1-phosphate, in the outer membrane of some rhizobia, LpxQ forms an aminogluconate moiety by oxidizing the anomeric carbon of the proximal GlcN (Que-Gewirth et al. 2003; Ingram et al. 2010). Also, GalA is attached to the 4'- position of the lipid A by RgtD (Brown et al. 2012). Other members of the *Rhizobiaceae* can have different modifications (see review by Carlson et al. 2010). For example, the lipid A of *Bradyrhizobium*

strains has multiple VLCFA residues and its glycosyl disaccharide consists of GlcN3N residues rather than GlcN. *Sinorhizobium* species have lipid A which consist of the "normal" *bis*-phosphorylated GlcN disaccharide but has the pentaacylation pattern found in *Rhizobium leguminosarum* lipid A. *Rhizobium galegae* lipid A contains the *Rhizobium leguminosarum* lipid A structure but lacks the GalA residue, while in *Mesorhizobium* an additional GalA is added to the lipid A proximal GlcN by RgtF (Brown et al. 2013a).

VLCFAs in Bacterial Lipopolysaccharides

There are two different types of fatty acid synthesis (FAS) processes. FASI is a single gene system found in animals while FASII is found in bacteria, plants, and parasites and utilizes multiple genes. Having multiple genes to produce fatty acids leads to the ability to produce a multitude of different fatty acids within the cell or organism. FASII biosynthesis has been described previously in *Escherichia coli* and is separated into initiation and elongation phases (White et al. 2005). The FASII initiation begins with a 4'-phosphopantheteine group from acetyl coenzyme A (CoA) being transferred to the acyl carrier protein (ACP) by an ACP synthase (Figure 1.7). Next a malonyl group is transferred from malonyl-CoA to the sulfur on the end of the ACP 4'-phosphopantheteline group by a transacylase. Then an ACP synthase condenses the malonyl group using acetyl-CoA as a primer to form an acetoacetyl-ACP. The elongation cycle has four enzymes. The acetoacetyl-ACP is acted upon by a reductase that produces β-hydroxyacyl-ACP which is then dehydrated by a β-hydroxyacyl-ACP dehydratase to form an enoyl-ACP that is reduced by another reductase to complete the cycle.

Most gram-negative bacteria use this FASII pathway characterized in *Escherchia coli*, however some have additional differing ACP structures. While the α-helices have similar structure there are additional ACP C-terminal extensions found in some bacteria that produce

VLCFAs such as C70-C90 α-alkyl, β-hydroxy fatty acids in *Mycobacterium tuberculosis* and β-hydroxy C28 fatty acids in *Rhizobium leguminosarum* (Barry et al. 1998). It is hypothesized that this C-terminal extension may affect the "hydrocarbon ruler" that determines the length of fatty acids. Figure 1.8 shows the difference between *Escherchia coli* AcpP structure (Roujeinikova et al. 2002) and calculated *Rhizobium leguminosarum* bv. *viciae* AcpXL structure (Valafar and Carlson, personnel communication). Recently, the solution NMR structure of the AcpXL from *Rhodobacter palustris* was determined (Ramelot et al. 2012) and confirmed the calculated structure of *Rhizobium leguminosarum* bv. *viciae* AcpXL.

Several bacteria other than *Rhizobium* form a VLCFA on their lipid A similar to that on *Rhizobium* in Figure 1.5. *Rhodopseudomonas, Nitrobacter, Pseudomonas, Brucella, Bartonella,* and *Thiobacillus* all form 27OHC28:0 (Bhat et al. 1991). The VLCFAs of the pathogens *Bartonella*, *Legionella*, and *Chlamydia* may be the reason why there is a low endotoxic response in the potential animal host (Zahringer et al. 2004). The lipid A structure of *Agrobacterium tumefaciens* was characterized and determined to have a VLCFA similar to symbiotic *Rhizobium* species (Silipo et al. 2004). This similar lipid A structure between a pathogenic and symbiotic bacterium is hypothesized to aid the pathogen in evading the plant immune response (Silipo et al. 2004).

Most members of the *Rhizobiaceae* family have the VLCFA (27-OHC-28:0) as a component of the lipid A with one exception to this rule being *Azorhizobium caulinodans* (Dreyfus et al. 1988). *Bradyrhizobium* has a variety of VLCFAs including straight-chain and mono- and dimethyl branched fatty acids with 26-34 carbons (Choma and Komaniecka 2011). Examination of the role of the lipid A VLCFA in symbiosis has been investigated in several

different *Rhizobiaceae* and the answers are still unclear. Most work has been done with *Sinorhizobium meliloti* and *Rhizobium leguminosarum.*

In *Sinorhizobium meliloti* mutants of the acyl carrier protein gene (*acpXL*), acyl transferase gene (*lpxXL*), and both genes simultaneously (*acpXL/lpxXL*) have previously been made and characterized (Sharypova et al. 2003; Ferguson et al. 2005). In free-living cells the VLCFA was not present on these mutants. However, the *acpXL* mutant was able to add C18:0 in place of the VLCFA on some of the lipid A. It was also suggested that the *lpxXL* and *acpXL/lpxXL* mutants had compromised outer membranes due to susceptibility to sodium dodecyl sulfate (SDS) and deoxycholate (DOC) and inability to grow without supplementation with $MgSO_4$ and $CaCl_2$ to the media. *Sinorhizobium meliloti* mutants of *acpXL, lpxXL,* and *acpXL/lpxXL* were all able to form successful symbiosis with their host plants although less successfully than wild type. *Sinorhizobium meliloti* mutants without the VLCFA were still able to suppress the oxidative burst from plant cells but not in non-host plant cells (Scheidle et al. 2005). When mutant bacteroids were analyzed, restoration of the VLCFA within the plant was indicated for a *Sinorhizobium meliloti acpXL* mutant (Haag et al. 2009). However, the GC-MS chromatogram peak indicating that VLCFA may have been restored within bacteroids appears extremely small and may be due to background noise. Further investigation should be done to strengthen this conclusion. *Sinorhizobium meliloti lpxXL* and *acpXL* mutants have also been shown to have a reduced ability to compete with the parent strain in alfalfa (Sharypova et al. 2003; Ferguson et al. 2005). These carrier protein and transferase mutants also have delayed infection droplet release and premature nodule senescence. Bacteroid development also differs in that the bacteroids are misshapen and multiple bacteroids are found per symbiosome (Kannenberg and Carlson 2001; Haag et al. 2009).

A mutation in the gene which encodes the acyl carrier protein AcpXL, on which 27OHC28:0 is made, has been created and has been shown to significantly influence nodule and bacteroid development in *Rhizobium leguminosarum* bv. *viciae* (Vedam et al. 2003). It was found that without the acyl carrier protein AcpXL, the VLCFA was not added to the lipid A of the free-living bacteria. These free-living bacteria were sensitive to increased osmolarity, presence of detergent, and increased acidity indicating that the lack of the VLCFA may decrease membrane stability in comparison to the wild type. This increased osmolarity and acidity is found in the nodule environment and indicates that bacteria lacking the VLCFA may be less capable of surviving within the nodule. This indication was confirmed by evidence showing a delay in nodule development and tissue invasion. Upon further study, Vedam et al. (2004) observed that the bacteroids within nodules infected by the *acpXL* mutant were irregularly shaped and sometimes more than one were present within a symbiosome. This irregular shape and number indicated that the interaction between the bacteria/bacteroid membrane and the symbiosomal membrane may have been affected. A delay in the onset of nitrogenase production and reduced nitrogenase activity were also observed. Following analysis of lipid A in bacteroids harvested from nodules, it was determined that the VLCFA was partially restored to the lipid A (Vedam et al. 2006). It was suggested that the restoration of the VLCFA on the lipid A was due to an alternative mechanism containing a different acyl carrier protein. Following passage through the host plant these *acpXL* mutants were restored to parent strain levels of salt and pH tolerance even though the VLCFA was not present (Vedam et al. 2006).

Other VLCFA mutants of *Rhizobium leguminosarum* bv. *viciae* were made in the oxo-acyl synthase genes *fabF1* and *fabF2* (later defined at *fabF1XL* and *fabF2XL*) (Vanderlinde et al. 2009). Mutant characterization indicated that the *fabF2/F1* mutants did not have VLCFA on

their lipid A. Previously, the *acpXL* mutant LPS was found to have a similar banding pattern to that of the wild type 3841 except for a small shift to lower molecular weight due to the lack of the VLCFA when separated by DOC-PAGE (Vedam et al. 2003). However, in work by Vanderlinde et al. (2009) the *fabF2/F1*, *fabF1*, and *fabF2* mutants were shown to lack the ladder pattern indicating an alteration in the LPS O-antigen. This resulted in diminished membrane stability of these mutants was indicated by a need for increased concentrations of Ca^{2+} in growth media and sensitivity to the detergents deoxycholate and sarcosyl (Vanderlinde et al. 2009). Additionally, *fabF2/F1*, *fabF1*, and *fabF2* mutants were found to be more sensitive to desiccation and hyper- and hypo-osmotic stress. An increase in excretion of cyclic β-glucans was also observed in the mutants and shown to likely be due to an increase in membrane permeability and not to an increase in production. Vanderlinde et al. (2009) hypothesized that the loss of the cyclic β-glucans within the periplasm may be a factor in the increased sensitivity to osmolarity. Alterations in biofilm formation were also observed. Mutants formed large, tightly organized microcolonies with cells attached to the surface by their sides while wild type cells were loosely organized and attached to the surface by their poles. These differences were hypothesized to be due to asymmetrical distribution of cell-surface polysaccharides. Loss of motility was also observed for the *fabF1* mutant (Vanderlinde et al. 2009).

Sinorhizobium meliloti mutants of the genes required for VLCFA biosynthesis have also been examined. The *acpXL* and *lpxXL* genes are required for biosynthesis of VLCFA-modified lipid A in free-living bacteria and bacteroids. These mutants were also less competitive and more sensitive to membrane stressors (Sharypova et al. 2003; Ferguson et al. 2005; Haag et al. 2009). Mutants of the 3-oxoacyl synthases (*fabF1XL, fabF2XL*) and alcohol dehydrogenase (*adhA2XL*) also lacked the VLCFA while the hydroxyacyl dehydrogenase mutant (*fabZXL*) had a mixture of

lipid A species including the VLCFA (Haag et al. 2011). These mutants also lacked high molecular weight LPS, were slightly more sensitive to high osmolarity, had a minimum salt requirement for growth, and had a reduced ability to induce cytokine production from human monocytes (Haag et al. 2011).

An *acpXL* mutant of *Rhizobium leguminosarum* bv. *phaseoli* has also been developed and characterized (Brown et al. 2011). The biovar *phaseoli* differs from the biovar *viciae* in that *phaseoli* infects bean plants, which form determinate nodules, while *viciae* infects pea plants, which form indeterminate nodules. LPS separation on DOC-PAGE gels showed similar results to that of *Rhizobium leguminosarum* bv. *viciae* with similar banding patterns and a shift in migration due to the lack of a VLCFA (Vedam et al. 2003). The *acpXL* mutants did not have lipid A VLCFA, were more sensitive than the wild type to deoxycholate, sodium dodecyl sulfate, polymyxin B, and sodium chloride. The *Rhizobium phaseoli acpXL* mutant was able to form nitrogen-fixing nodules. Mutant-infected plant cells were smaller and contained fewer bacteria at 21 dpi but were similar to wild type at 28 dpi. However, the mutant symbiosomes had larger numbers of bacteroids than wild type, excessive matrix material, and loosely associated peribacteroid membranes unlike the closely associated wild type peribacteroid membranes (Brown et al. 2011).

Rhizobium etli has been shown to contain tetraacylated lipid A similar to that of *Salmonella, Pseudomonas, and Bordatella* (Basu et al. 1999; Trent et al. 2006). PagL, the 3-O-deacylase that removes an acyl group from the lipid A to form tetraacylated lipid A, was recently identified in *Rhizobium etli* and determined to be responsible for lipid A deacylase activity (Brown et al. 2013b). It is thought that the *R. etli* PagL may be more active in bacteroids and play an important role in symbiosis since the lipid A from *R. etli* bacteroids consists exclusively

of tetraacylated lipid A (D'Haeze et al. 2007). The mutation of *pagL* also affected symbiotic infection including yellowing of host plant leaves, smaller nodule size, and impaired nitrogen fixation early on in symbiosis.

While a large body of work has been completed on *acpXL* mutants, less has been done with *lpxXL* (transferase) mutation. As described above, mutations in different genes required for the addition of VLCFA can have different effects on bacterial LPS. Analysis of a mutant of *lpxXL* is needed in *Rhizobium leguminosarum* as well.

Osmotic Tolerance and Cyclic B-Glucans

Cyclic β-glucans are molecules that are found in the periplasm of *Proteobacteria* (Breedveld and Miller 1994; Bohin et al. 2000). In *Rhizobium* and *Agrobacterium* species cyclic β-glucans have a (1,2)-linked D-glucosyl circular backbone. Four classes of these cyclic β-glucans have been identified based on ring size, representative species, and main substituents and include rings ranging from 17 to 40 glucans (Hisamatsu et al. 1983; Benincasa et al. 1987; Hisamatsu et al. 1987; Breedveld and Miller 1994). *Bradyrhizobium* produce cyclic β-glucans made up of 10 to 13 glucans with both β-(1,3) and β-(1,6) glycosidic linkages (Dudman and Jones 1980; Miller et al. 1990).

Cyclic β-glucans are localized within the periplasm and are secreted into the extracellular medium dependent on factors such as growth stage and culture conditions (Miller et al. 1986; Geiger et al 1991; Miller and Gore 1992). Substituents including *sn*-1 phosphoglycerol, succinic acid, and methylmalonic acid for *Agrobacterium* and *Rhizobium* and phosphocholine for *Bradyrhizobium* can be added to the glucan ring and the degree of substitution varies widely (Batley et al. 1987; Zevenhuizen et al. 1990; Rolin et al. 1992).

Some bacteria other than *Rhizobiaceae* that also produce cyclic β-glucans are *Brucella*, *Alcaligenes*, *Xanthomonas,* and *Pseudomonas*. *Brucella* (a close relative of *Rhizobium*) and *Alcaligenes* form the same cyclic β-(1,2)-glucans as *Rhizobium* while *Xanthomonas* forms a slightly different structure (Hisamatsu et al. 1982; Amemura and Cavrera-Crespo 1986; Bundle et al. 1988). A cyclic β-(1,3) glucan was recently identified in *Pseudomonas aeruginosa* (Coulon et al. 2010; Sadovskaya et al. 2010). Linear β-glucans with phosphoglycerol and phosphoethanolamine residue substituents have also been found in a few *Rhizobium* as well as *Escherichia coli*, *Acetobacter*, and *Xanthomonas* (Amemura et al. 1985a; Amemura et al. 1985b; Amemura and Cabrera-Crespo 1986).

Cyclic β-glucans are synthesized using UDP-glucose and transported by NdvA and NdvB proteins (Geremia et al. 1987; Stanfield et al. 1988). NdvB is a cytoplasmic membrane protein required for synthesis of the cyclic β-glucan while NdvA is responsible for transport across the membrane to the periplasm (Stanfield et al. 1988; Cangelosi et al. 1989). In addition to the cyclic β-glucan backbone, nonglycosidic modifications such as phosphoglycerol and succinic acid can be added (Miller et al. 1987; Roset et al. 2006; Kawaharada et al. 2008; Crespo-Rivas et al. 2009).

An important function of these cyclic β-glucans is for hypoosmotic adaptation. Mutation studies of *ndvA* and *ndvB* have demonstrated that under hypoosmotic conditions these mutants have impaired growth rates (Cangelosi et al. 1990; Dylan et al. 1990a; Dickstein et al. 1988). Additionally, the biosynthesis of cyclic β-glucans is also osmoregulated (Dylan et al. 1990a; Tully et al. 1990; Zorreguieta et al. 1990; Breedveld et al. 1990; Miller and Gore 1992).

The ability to infect a host plant is also affected by cyclic β –glucans. *Rhizobium meliloti ndv* mutants induce the formation of pseudonodules in their host plant and have only a small

number of infection thread initiations with no bacteroid formation (Dylan et al. 1986; Dylan et al. 1990b). Cyclic β-glucans are also involved in signaling during plant infection (Mithofer 2002). Nodule number and nodule formation kinetics are increased when exogenous cyclic β-glucan is introduced (Abe et al. 1982; Bhagwat et al. 1992; Dylan et al. 1990b). This increase in nodulation may be due to the formation of inclusion complexes with hydrophobic guest molecules such as flavonoids. The cyclic β –glucans may also have a role in attachment of the bacteria to root hair cells (Dylan et al. 1990b). Although cyclic β-glucans are needed for root hair infection they are also present during later stages of infection.

Acyl carrier proteins have previously been shown to have a function in the synthesis of periplasmic glucans. In *Escherichia coli* an acyl carrier protein was determined to be required for transglycosylation of the linear β-(1,2) glucans (Therisod et al. 1986). The phosphopantetheine arm of acyl carrier proteins that is required for lipid biosynthesis is not necessary for β-glucan biosynthesis (Therisod and Kennedy 1987). It has been suggested that this apo-ACP (lacking phosphopantetheine arm) is created by a phosphodiesterase that creates a group of ACPs needed for functions other than lipid biosynthesis (Vagelos and Larrabes 1967; Therisod and Kennedy 1987). However, it has been stated that in the *Rhizobiaceae* an acyl carrier protein is not utilized for cyclic β-glucan synthesis (Platt et al. 1990; Tang et al. 1997). However, the acyl carrier proteins studied were the constitutive ACP and NodF, which are necessary for lipochitooligosaccharide (i.e. nod factor) production. *Rhizobium leguminosarum* contains three additional acyl carrier proteins, Acp4, Acp5, and AcpXL, one of which may have an effect on cyclic β-glucan biosynthesis.

The goals of this dissertation were to examine the VLCFA acyl transferase role in structure and function in *Rhizobium leguminosarum* bv. *viciae* 3841. While *lpxXL* mutants have

been examined in other *Rhizobiaceae*, a *Rhizobium leguminosarum lpxXL* mutant has not been examined. In addition, this work focuses more strongly on the phenotypic and symbiotic effects seen when the lipid A lack the unique VLCFA attachment.

Figure Legends

Figure 1.1. Diagram depicting the symbiotic process beginning with crosstalk between host plant root flavonoids and rhizobial bacteria nod factors, leading to root hair curling and bacterial invasion, continuing to nodule formation and bacteroid differention, competed with the full rhizobial-legume symbiosis.

Figure 1.2. Diagram of the cellular envelope of gram-negative bacteria (adapted from Raetz and Whitfield 2002).

Figure 1.3. Diagram of the proteins and intermediates required for the biosynthesis of lipid A by gram-negative bacteria starting with UDP-GlcNAc and ending with the Kdo_2LipidIVA lipopolysaccharide precursor.

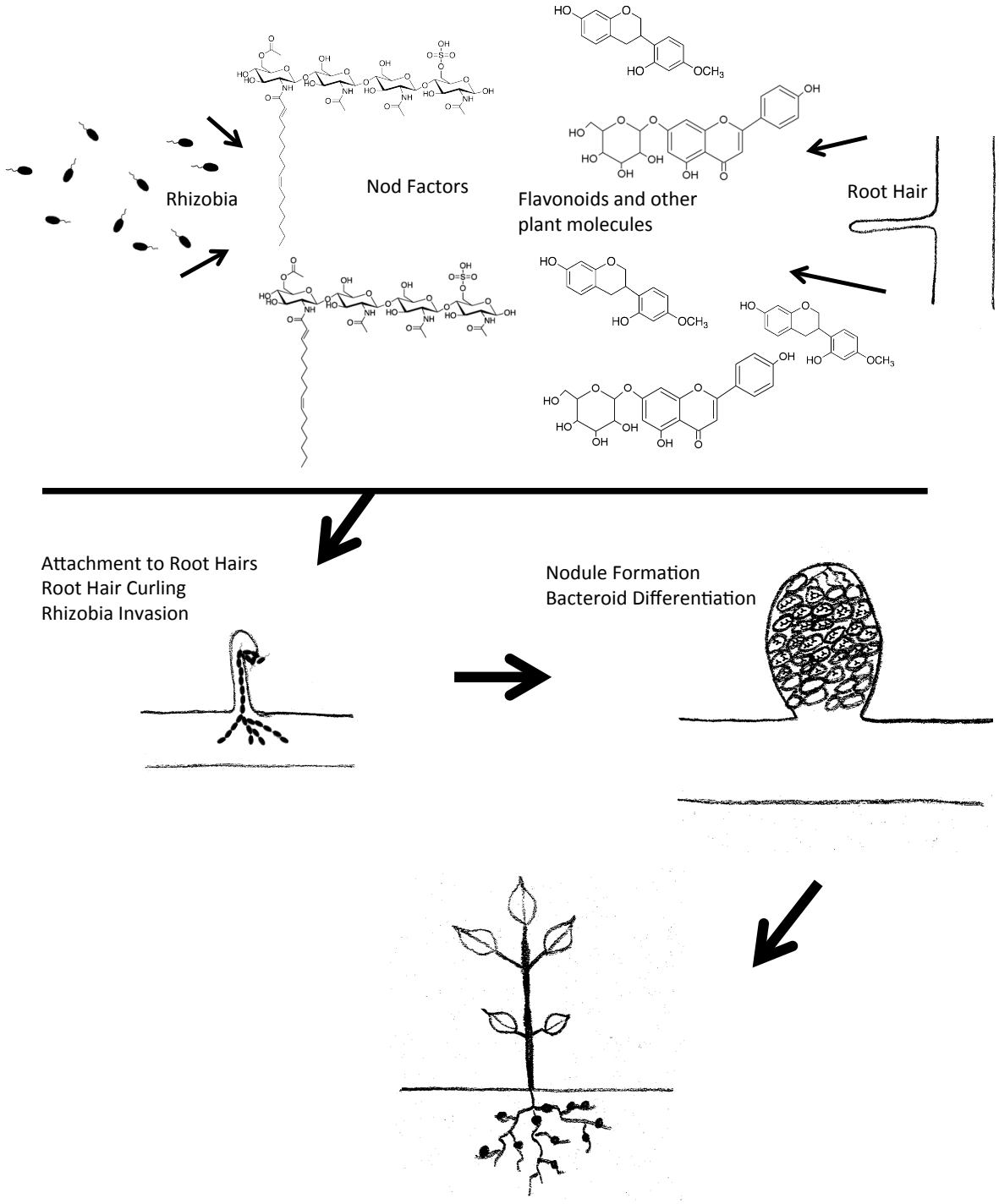
Figure 1.4 Examples of bacterial lipid A modifications. These lipid A modifications can be observed on different gram negative bacteria.

Figure 1.5. Typical lipopolysaccharide lipid A structures of *Escherichia coli* and *Rhizobium leguminosarum* illustrating the major differences observed between the two structures.

Figure 1.6. Diagram of the chromosomal gene region required for VLCFA biosynthesis.

Figure 1.7. Diagram of fatty acid synthesis II of *Escherichia coli*.

Figure 1.8. Computational structure comparison of *Escherichia coli* AcpP and *Rhizobium leguminosarum* bv *viciae* AcpXL. The serine residue site of pantothenic acid attachment on both proteins and N-terminal protein extension of the AcpXL are highlighted.



Rhizobia – Legume Symbiosis

Figure 1.1

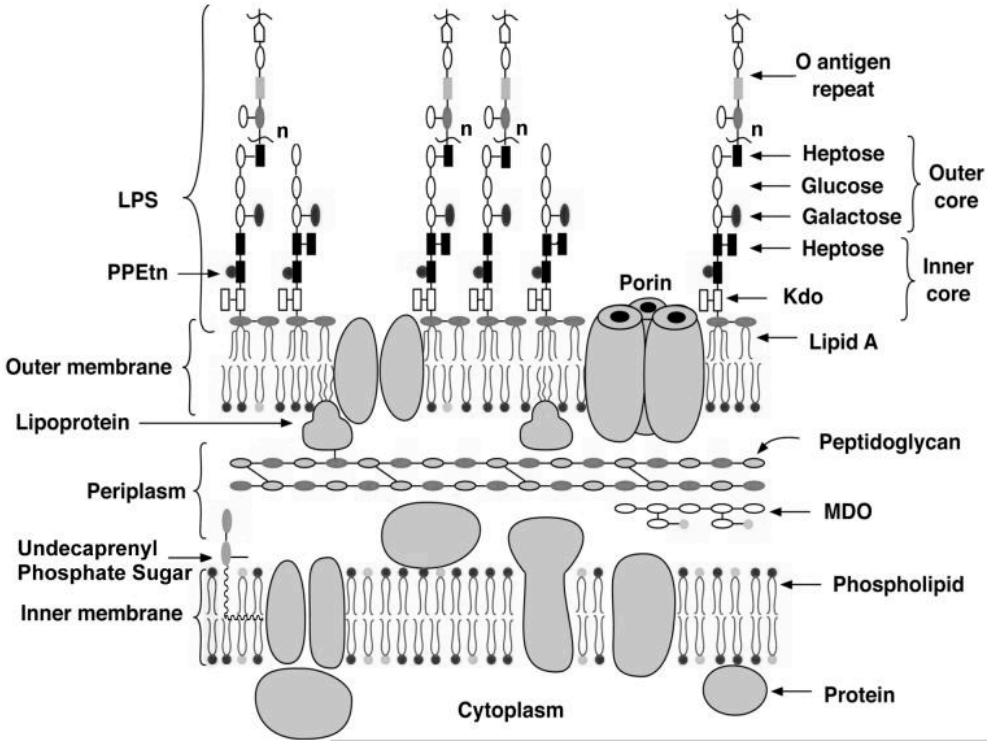

O antigen
repeat
n
Heptose
Glucose
Galactose
Outer
core
Heptose
Inner
core
Kdo
LPS
PPEtn
Porin
Lipid A
Outer membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Undecaprenyl
Phosphate Sugar
Phospholipid
Inner membrane
Protein
Cytoplasm

Figure 1.2

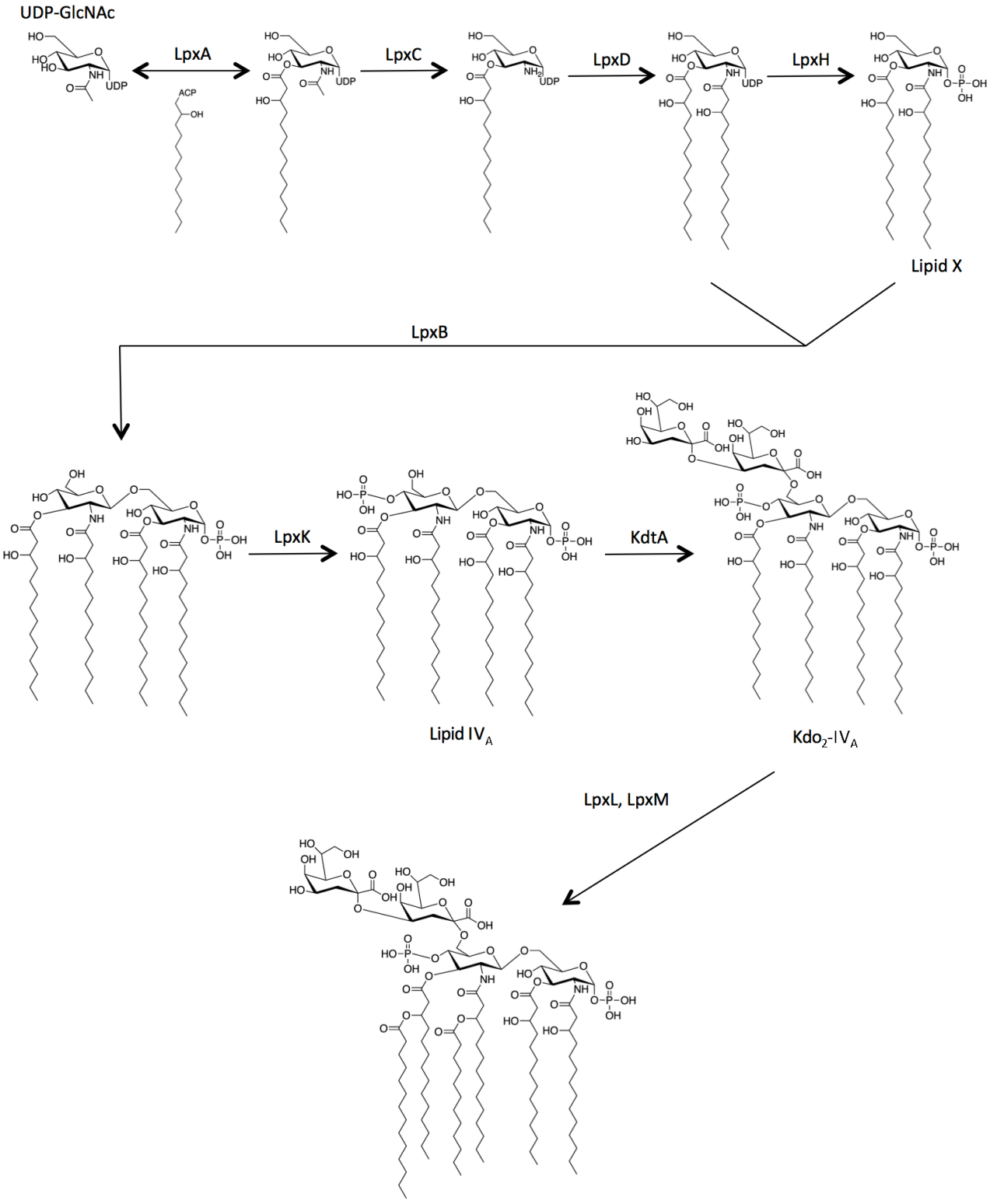
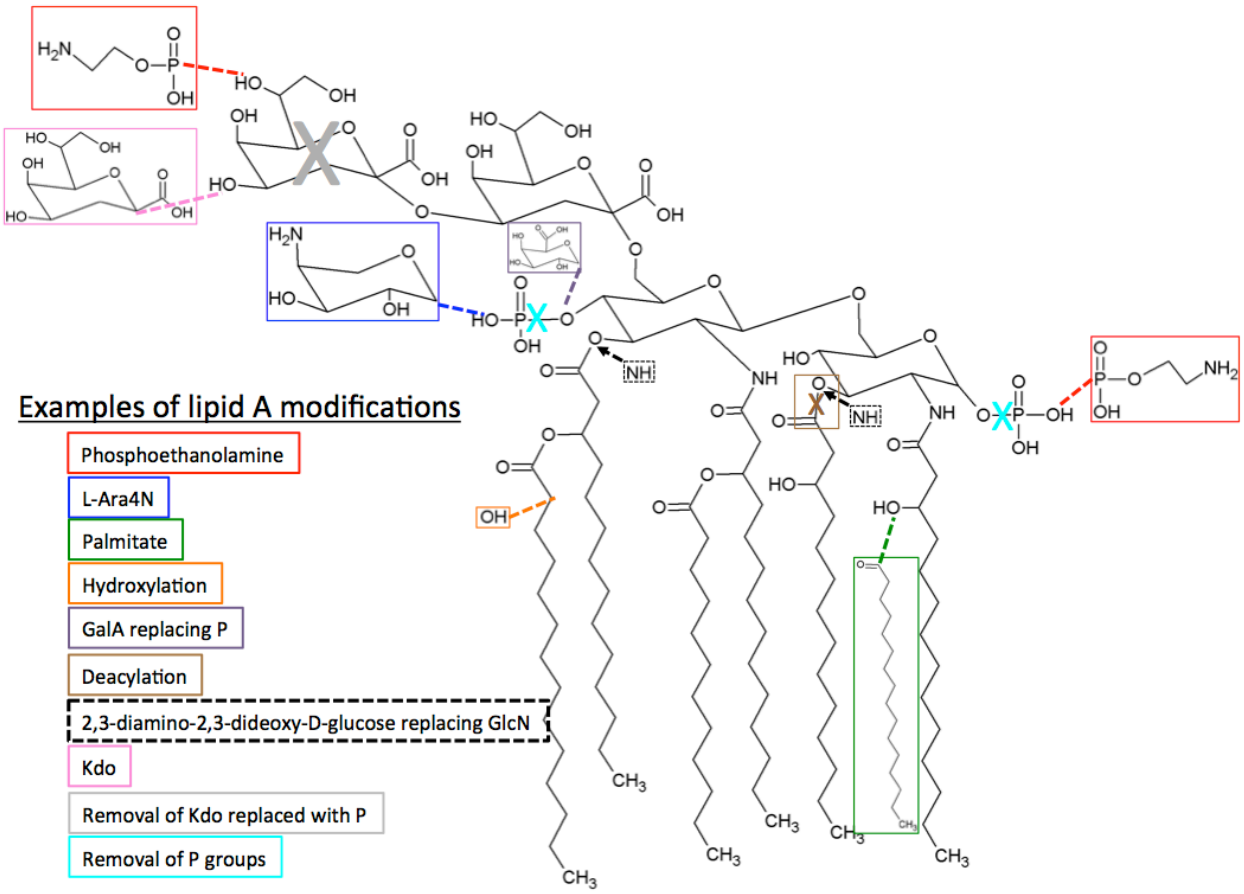
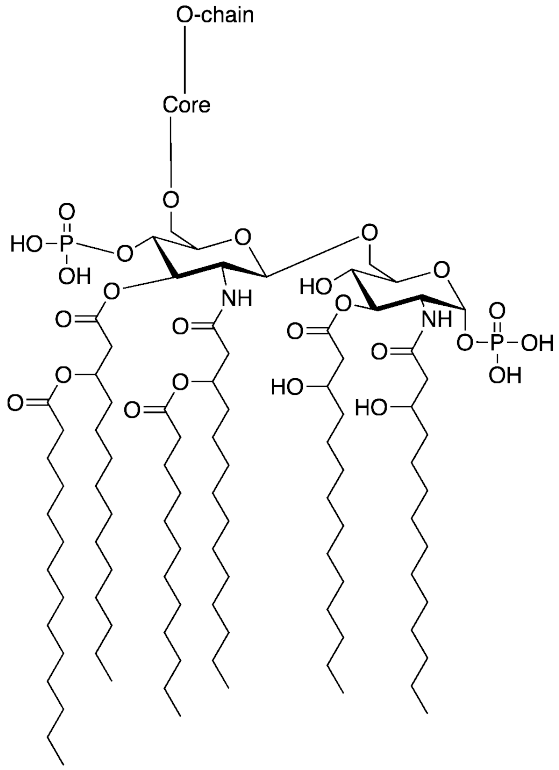

UDP-GlcNAc
LpxA
ACP
LpxC
LpxD
LpxH
Lipid X
LpxB
LpxK
KdtA
Lipid IV$_A$
Kdo$_2$-IV$_A$
LpxL, LpxM

Figure 1.3

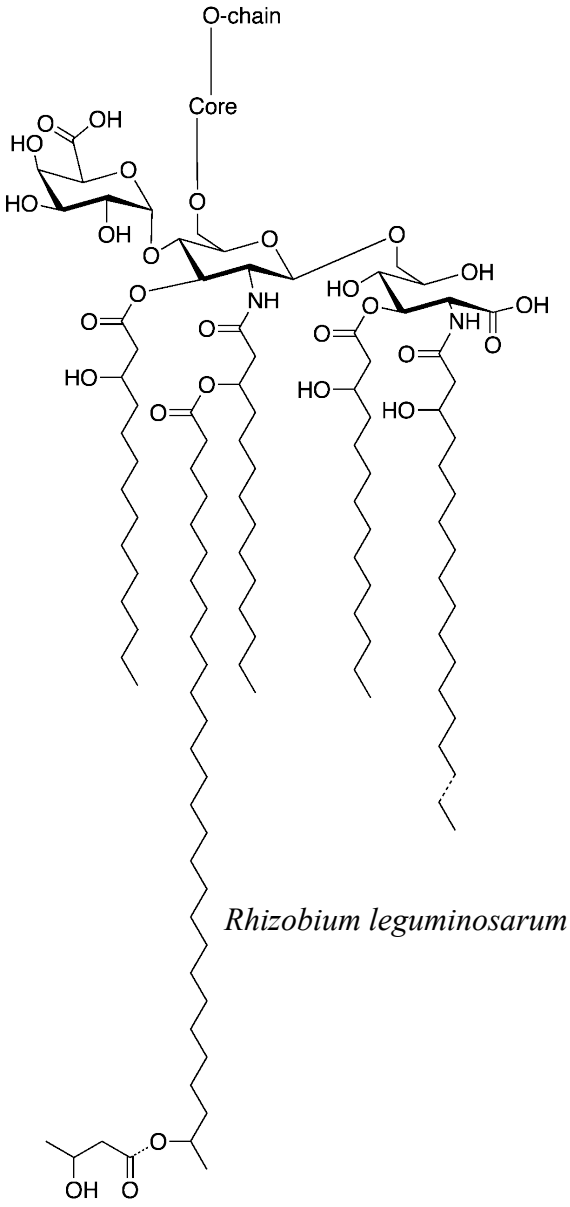


Typical *Escherichia coli* Kdo_2 lipid A

Figure 1.4



Escherichia coli



Rhizobium leguminosarum

Figure 1.5

- *acpXL* - Lipid A 27OHC28:0 acyl carrier protein
- *fabZXL* - Hydroxyacyl-(*acpXL*) dehydratase
- *fabF2XL* - 3-oxoacyl-(*acpXL*) synthase
- *fabF1XL* - 3-oxoacyl-(*acpXL*) synthase
- *adhA2XL* - Alcohol dehydrogenase
- *lpxXL* - Lipid A 27OHC28:0 acyl transferase

Figure 1.6

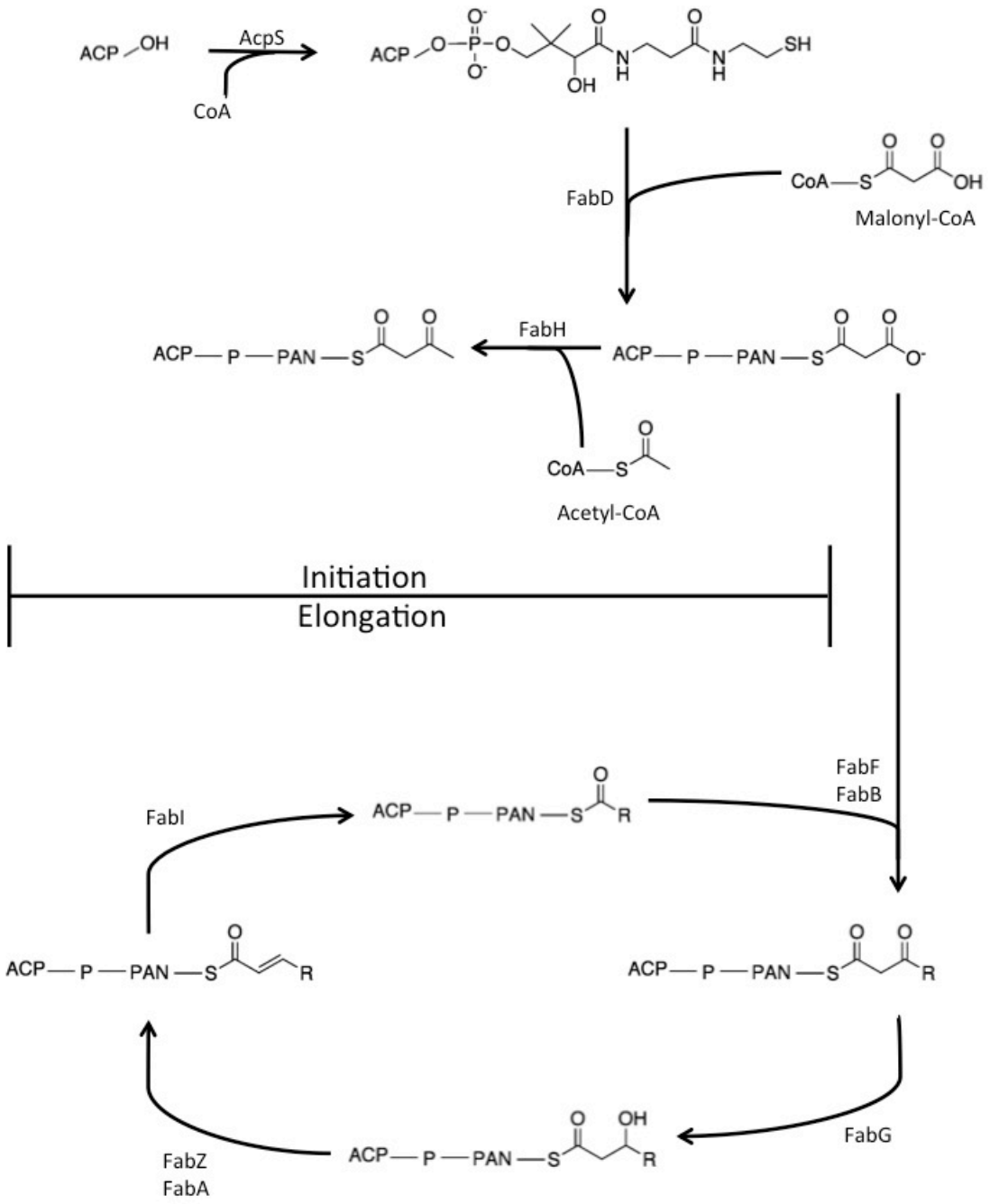

ACP OH
AcpS
CoA
ACP O P O
O
N H
O
N H
SH
OH
FabD
CoA S O O OH
Malonyl-CoA
FabH
ACP P PAN S O O
ACP P PAN S O O O
CoA S O
Acetyl-CoA
Initiation
Elongation
FabF
FabB
FabI
ACP P PAN S O R
ACP P PAN S O O R
ACP P PAN S O R
ACP P PAN S O OH R
FabG
FabZ
FabA

Figure 1.7

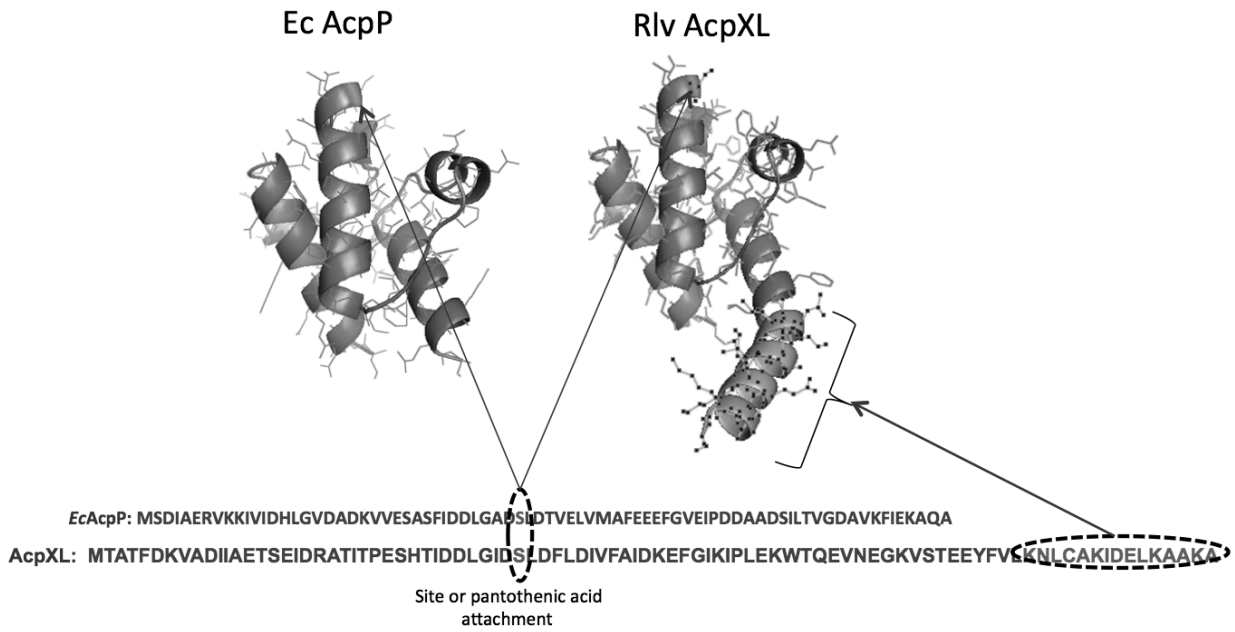

Ec AcpP
Rlv AcpXL
EcAcpP: MSDIAERVKKIVIDHLGVDADKVVESASFIDDLGADSLDTVELVMAFEEEFGVEIPDDAADSILTVGDAVKFIEKAQA
AcpXL: MTATFDKVADIIAETSEIDRATITPESHTIDDLGIDSLDFLDIVFAIDKEFGIKIPLEKWTQEVNEGKVSTEEYFVSKNLCAKIDELKAAKA
Site or pantothenic acid attachment

Figure 1.8

References

Abe, M., Amemura, A., and Higashi, S. (1982) Studies on cyclic β-(1,2)-glucan obtained from periplasmic space of *Rhizobium trifolii* cells. *Plant Soil*., 64: 315-324.

Albus, U., Raier, R., Holst, O., Huhler, A., and Niehaus, K. (2001) Suppression of an elicitor-induced oxidative burst reaction in *Medicago sativa* cell cultures by *Sinorhizobium meliloti* lipopolysaccharides. *New Phytol.,* 151: 597-606.

Amemura, A., Hashimoto, T., Koizumi, K., and Utamura, T. (1985a) Occurrence of extracellular (1,2)-β-D-glucans and (1,2)-β-D-gluco-oligosaccharides in *Acetobacter*. *J. Gen. Microbiol*., 131: 301-307.

Amemura, A., Praphaisri, F., Kyoko, K., Toshiko, U., and Hisaharu, T. (1985b) Isolation of (1,2)-β-D-glucans from tropical strains of *Rhizobium* and determination of their degrees of polymerization. *J. Ferment. Technol.,* 63: 115-120.

Amemura, A. and Cabrera-Crespo, J. (1986) Extracellular oligosaccharides and low-Mr polysaccharides containing (1----2)-beta-D-glucosidic linkages from strains of *Xanthomonas*, *Escherichia coli* and *Klebsiella pneumonia*e. *J. Gen. Microbiol.,* 132: 2443-2452.

Barry, C. E., 3rd, Lee, R. E., Mdluli, K., Sampson, A. E., Schroeder, B. G., Slayden, R. A., and Yuan, Y. (1998) Mycolic acids: structure, biosynthesis and physiological functions. *Prog. Lipid Res.,* 37: 143-179.

Basu, S. S., White, K. A., Que, N. L. S., and Raetz, C. R. H. (1999) A deacylase in *Rhizobium leguminosarum* membranes that cleaves the 3-O-linked beta-hydroxymyristoyl moiety of lipid A precursors. *J. Biol. Chem.,* 274: 11150-11158.

Basu, S. S., Karbarz, M. J., and Raetz, C. R. (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in Rhizobium leguminosarum. *J. Biol. Chem.,* 277: 28959-28971.

Batley, M., Redmond, J. W., Djordjevic, S. P., and Rolfe, B. G. (1987) Characterization of glycerophosphorylated cyclic β-(1,2)-glucans from a fast-growing *Rhizobium* species. *Biochim. Biophys. Acta,* 901: 119-126.

Belunis, C. J., Clementz, T., Carty, S. M., and Raetz, C. R. (1995) Inhibition of lipopolysaccharide biosynthesis and cell growth following inactivation of the kdtA gene in *Escherichia coli*. *J. Biol. Chem.,* 270: 27646-27652.

Benincasa, M., Cartoni, G. P., Coccioli, F., Rizzo, R., and Zevenhuizen, L. P. T. M. (1987) High-performance liquid chromatography of cyclic β-(1,2)-D-glucans (cyclosophoraoses) produced by *Rhizobium meliloti* and *Rhizobium trifolii*. *J. Chromatogr.,* 393: 263-271.

Bhagwat, A. A., Tully, R. E., and Keister, D. L. (1992) Isolation and characterization of an *ndvB* locus from *Rhizobium fredii*. *Mol. Microbiol.,* 6: 2159-2165.

Bhat, U. R., Carlson, R. W., Busch, M., and Mayer, H. (1991) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria. *Int. J. Syst. Bacteriol.,* 41: 213-217.

Bhat, U. R., Forsberg, L. S., and Carlson, R. W. (1994) Structure of lipid A component of *Rhizobium leguminosarum* bv. *phaseoli* lipopolysaccharide. Unique nonphosphorylated lipid A containing 2-amino-2-deoxygluconate, galacturonate, and glucosamine. *J. Biol. Chem.,* 269: 14402-14410.

Bishop, R. E., Kim, S. H., and El Zoeiby, A. (2005) Role of lipid A palmitoylation in bacterial pathogenesis. *J. Endotoxin Res.,* 11: 174-180.

Bohin, J. P. (2000) Osmoregulated periplasmic glucans in Proteobacteria. *FEMS Microbiol. Lett.,* 186: 11-19.

Bos, M. P., Robert, V., and Tommassen, J. (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annu. Rev. Microbiol.,* 61: 191-214.

Breedveld, M. W., Zevehuizen, L. P. T. M., and Zehnder, A. J. B. (1990) Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47. *J. Gen. Microbiol.,* 136: 2511-2519.

Breedveld, M. W., Cremers, H. C., Batley, M., Posthumus, M. A., Zevenhuizen, L. P., Wijffelman, C. A., and Zehnder, A. J. (1993) Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum. J. Bacteriol.,* 175: 750-757.

Breedveld, M. W. and Miller, K. J. (1994) Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiol. Rev.,* 58: 145-161.

Broghammer, A., Krusell, L., Blaise, M., Sauer, J., Sullivan, J. T., Maolanon, N., Vinther, M., Lorentzen, A., Madsen, E. B., Jensen, K. J., Roepstorff, P., Thirup, S., Ronson, C. W., Thygesen, M. B., and Stougaard, J. (2012) Legume receptors perceive the rhizobial lipochitin oligosaccharide signal molecules by direct binding. *P. Natl. Acad. Sci. USA,* 109: 13859-13864.

Brown, D. B., Huang, Y. C., Kannenberg, E. L., Sherrier, D. J., and Carlson, R. W. (2011) An *acpXL* mutant of *Rhizobium leguminosarum* bv. *phaseoli* lacks 27-hydroxyoctacosanoic acid in its lipid A and is developmentally delayed during symbiotic infection of the determinate nodulating host plant *Phaseolus vulgaris*. *J. Bacteriol.,* 193: 4766-4778.

Brown, D. B., Forsberg, L. S., Kannenberg, E. L., and Carlson, R. W. (2012) Characterization of galacturonosyl transferase genes *rgtA*, *rgtB*, *rgtC*, *rgtD*, and *rgtE* responsible for

lipopolysaccharide synthesis in nitrogen-fixing endosymbiont *Rhizobium leguminosarum*: lipopolysaccharide core and lipid galacturonosyl residues confer membrane stability. *J. Biol. Chem.,* 287: 935-949.

Brown, D. B., Muszynski, A., and Carlson, R. W. (2013a) Elucidation of a novel lipid A alpha-(1,1)-GalA transferase gene (*rgtF*) from *Mesorhizobium loti*: Heterologous expression of *rgtF* causes *Rhizobium etli* to synthesize lipid A with alpha-(1,1)-GalA. *Glycobiology,* 23: 546-558.

Brown, D. B., Muszynski, A., Salas, O., Speed, K., and Carlson, R. W. (2013b) Elucidation of the 3-O-deacylase gene, *pagL*, required for the removal of primary beta-hydroxy fatty acid from the lipid A in the nitrogen-fixing endosymbiont *Rhizobium etli* CE3. *J. Biol. Chem.,* 288: 12004-12013.

Bundle, D. R., Cherwonogrodzky, J. W., and Perry, M. B. (1988) Characterization of *Brucella* polysaccharide B. *Infect. Immun.,* 56: 1101-1106.

Callaham, D. A. and Torrey, J. G. (1981) The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. *Can. J. Bot.,* 59: 1647-1664.

Cangelosi, G. A., Martinetti, G., Leigh, J. A., Lee, C. C., Thienes, C., and Nester, E. W. (1989) Role for *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. *J. Bacteriol.,* 171: 1609-1615.

Cangelosi, G. A., Martinetti, G., and Nester, E. W. (1990) Osmosensitivity phenotypes of *Agrobacterium tumefaciens* mutants that lack periplasmic beta-1,2-glucan. *J. Bacteriol.,* 172: 2172-2174.

Carlson, R. W., Reuhs, B. L., Forsberg, L. S., and Kannenberg, E. L. (1999) Rhizobial cell surface carbohydrates: Their structures, biosynthesis, and functions. In J. B. Goldberg (ed.), *Genetics of Bacterial Polysaccharides* (Ann Arbor: Ann Arbor Press), 53-99.

Carlson, R. W., Forsberg, L. S., and Kannenberg, E. L. (2010) Lipopolysaccharides in *Rhizobium*-legume symbioses. In P. J. Quinn and X. Wang (eds.), *Subcellular Biochemistry: Endotoxins: Structure, function, and recognition* (Springer), 339-386.

Carty, S. M., Sreekumar, K. R., and Raetz, C. R. (1999) Effect of cold shock on lipid A biosynthesis in *Escherichia coli*. Induction At 12 degrees C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J. Biol. Chem.,* 274: 9677-9685.

Chen, H., Batley, M., Redmond, J., and Rolfe, B. G. (1985) Alteration of the effective nodulation properties of a fast-growing broad host range *Rhizobium* due to changes in exopolysaccharide synthesis. *J. Plant Physiol.,* 120: 331-349.

Cheng, H. P. and Walker, G. C. (1998) Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. *J. Bacteriol.,* 180: 5183-5191.

Choma, A. and Komaniecka, I. (2011) Straight and branched (omega-1)-hydroxylated very long chain fatty acids are components of *Bradyrhizobium* lipid A. *Acta Biochim. Pol.,* 58: 51-58.

Cooper, J. E. (2004) Multiple responses of rhizobia to flavonoids during legume root infection. *Adv. Bot. Res.,* 41: 1-62.

Cooper, J. E. (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. *J. Appl. Microbiol.,* 103: 1355-1365.

Corbel, M. J. (1997) Brucellosis: an overview. *Emerg. Infect. Dis.,* 3: 213-221.

Coulon, C., Vinogradov, E., Filloux, A., and Sadovskaya, I. (2010) Chemical analysis of cellular and extracellular carbohydrates of a biofilm-forming strain *Pseudomonas aeruginosa* PA14. *PLoS One,* 5: e14220.

Crespo-Rivas, J. C., Margaret, I., Hidalgo, A., Buendia-Claveria, A. M., Ollero, F. J., Lopez-Baena, F. J., del Socorro Murdoch, P., Rodriguez-Carvajal, M. A., Soria-Diaz, R. E., Reguera, M., Lloret, J., Sumpton, D. P., Mosely, J. A., Thomas-Oates, J. E., van Brussel, A. A., Gil-Serrano, A., Vinardell, J. M., Ruiz-Sainz, J. E. (2009) *Sinorhizobium fredii* HH103 cgs mutants are unable to nodulate determinate- and indeterminate nodule-forming legumes and overproduce an altered EPS. *Mol. Plant Microbe Interact.,* 22: 575-588.

D'Haeze, W., Leoff, C., Freshour, G., Noel, K. D., and Carlson, R. W. (2007) *Rhizobium etli* CE3 bacteroid lipopolysaccharides are structurally similar but not identical to those produced by cultured CE3 bacteria. *J. Biol. Chem.,* 282: 17101-17113.

Dazzo, F. B. and Brill, W. J. (1979) Bacterial polysaccharide which binds *Rhizobium trifolii* to clover root hairs. *J. Bacteriol.,* 137: 1362-1373.

Dazzo, F. B., Truchet, G. L., Hollingsworth, R. I., Hrabak, E. M., Pankratz, H. S., Philip-Hollingsworth, S., Salzwedel, J. L., Chapman, K., Appenzeller, L., and Squartini, A. (1991) *Rhizobium* lipopolysaccharide modulates infection thread development in white clover root hairs. *J. Bacteriol.,* 173: 5371-5384.

Dickstein, R., Bisseling, T., Reinhold, V. N., and Ausubel, F. M., (1988) Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. *Genes Dev.,* 2: 677-687.

Dreyfus, B., Garcia, J. L., and Gillis, M. (1988) Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int. J. Sys. Bacteriol.,* 38: 89-98.

Dudman, W. F. and Jones, A. J. (1980) The extracellular glucans of *Bradyrhizobium japonicum* strain 3I1b71a. *Carbohydr. Res.,* 84: 358-364.

Dusha, I., Olah, B., Szegletes, Z., Erdei, L., and Kondorosi, A. (1999) *syrM* is involved in the determination of the amount and ratio of the two forms of the acidic exopolysaccharide EPSI in *Rhizobium meliloti*. *Mol. Plant Microbe Interact.,* 12: 755-765.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E., Helinski, D. R., and Ditta, G. (1986) *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *PNAS,* 83: 4403-4407.

Dylan, T., Helinski, D. R., and Ditta, G. S. (1990a) Hypoosmotic adaptation in *Rhizobium meliloti* requires beta-(1----2)-glucan. *J. Bacteriol.,* 172: 1400-1408.

Dylan, T., Nagpal, P., Helinski, D. R., and Ditta, G. S. (1990b) Symbiotic pseudorevertants of *Rhizobium meliloti ndv* mutants. *J. Bacteriol.,* 172: 1409-1417.

Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M., and Miller, S. I. (1999) Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science,* 286: 1561-1565.

Ferguson, G. P., Datta, A., Carlson, R. W., and Walker, G. C. (2005) Importance of unusually modified lipid A in *Sinorhizobium* stress resistance and legume symbiosis. *Mol. Microbiol.,* 56: 68-80.

Forsberg, L. S. and Carlson, R. W. (2008) Structural characterization of the primary O-antigenic polysaccharide of the *Rhizobium leguminosarum* 3841 lipopolysaccharide and identification of a new 3-acetimidoylamino-3-deoxyhexuronic acid glycosyl component: a unique O-methylated glycan of uniform size, containing 6-deoxy-3-O-methyl-D-talose, n-acetylquinovosamine, and rhizoaminuronic acid (3-acetimidoylamino-3-deoxy-D-gluco-hexuronic acid). *J. Biol. Chem.,* 283: 16037-16050.

Fraysse, N., Couderc, F., and Poinsot, V. (2003) Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. *Eur. J. Biochem.,* 270: 1365-1380.

Fuchsius, L. (1542) De historia stirpium commentarii isignes. *Michael Insingrin*.

Gage, D. J. (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.,* 68: 280-300.

Geiger, O., Weissborn, A. C., and Kennedy, E. P. (1991) Biosynthesis and excretion of cyclic glucans by *Rhizobium meliloti* 1021. *J. Bacteriol.,* 173: 3021-3024.

Geremia, R. A., Cavaignac, S., Zorreguieta, A., Toro, N., Olivares, J., and Ugalde, R. A. (1987) A *Rhizobium meliloti* mutant that forms ineffective pseudonodules in alfalfa produces exopolysaccharide but fails to form β-(1,2)-glucan. *J. Bacteriol.,* 169: 880-884.

Gibbons, H. S., Reynolds, C. M., Guan, Z., and Raetz, C. R. (2008) An inner membrane dioxygenase that generates the 2-hydroxymyristate moiety of *Salmonella* lipid A. *Biochemistry,* 47: 2814-2825.

Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K., and Raetz, C. R. (1991) Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J. Biol. Chem.,* 266: 19490-19498.

Gore, R. S. and Miller, K. J. (1993) Cyclic [beta]-1,6 -1,3 glucans are synthesized by *Bradyrhizobium japonicum* bacteroids within soybean (*Glycine max*) root nodules. *Plant Physiol.,* 102: 191-194.

Gray, J. X. and Rolfe, B. G. (1990) Exopolysaccharide production in *Rhizobium* and its role in invasion. *Mol. Microbiol.,* 4: 1425-1431.

Haag, A. F., Wehmeier, S., Beck, S., Marlow, V. L., Fletcher, V., James, E. K., and Ferguson, G. P. (2009) The *Sinorhizobium meliloti* LpxXL and AcpXL proteins play important roles in bacteroid development within alfalfa. *J. Bacteriol.,* 191: 4681-4686.

Haag, A. F., Wehmeier, S., Muszynski, A., Kerscher, B., Fletcher, V., Berry, S. H., Hold, G. L., Carlson, R. W., and Ferguson, G. P. (2011) Biochemical characterization of *Sinorhizobium meliloti* mutants reveals gene products involved in the biosynthesis of the unusual lipid A very long-chain fatty acid. *J. Biol. Chem.,* 286: 17455-17466.

Hisamatsu, M., Amemura, A., Matsuo, T., Matsuda, H., and Harada, T. (1982) Cyclic β-(1,2)-D-glucan and the octasaccharide repeating-unit of succinoglycan produced by *Agrobacterium*. *J. Gen. Microbiol.,* 128: 1873-1879.

Hisamatsu, M., Amemura, A., Koizumi, K., Utamura, T., and Okada, Y. (1983) Structural studies on cyclic (1,2)-β-D-glucans (cyclosophoraoses) produced by *Agrobacterium* and *Rhizobium*. *Carbohydr. Res.,* 121: 31-40.

Hisamatsu, M., Yamada, T., Higashiuri, T., and Ikeda, M. (1987) The production of acidic, O-acetylated cyclosophorans (cyclic β-(1,2)-D-glucans) by *Agrobacterium* and *Rhizobium* species. *Carbohydr. Res.,* 163: 115-122.

Hoshino, K., Takeuchi, O., Kawai, T., Sonjo, H., Ogawa, T., Takeda, Y., and Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to

lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.,* 162: 3749-3752.

Ingram, B. O., Sohlenkamp, C., Geiger, O., and Raetz, C. R. (2010) Altered lipid A structures and polymyxin hypersensitivity of *Rhizobium etli* mutants lacking the LpxE and LpxF phosphatases. *Biochim. Biophys. Acta,* 1801: 593-604.

Janusch, H., Brecker, L., Lindner, B., Alexander, C., Sronow, S., Heine, H., Ulmer, A. J., Rietschel, E. T., and Zahringer, U. (2002) Structural and biological characterization of highly purified hepta-acyl lipid A present in the lipopolysaccharide of the *Salmonella enterica* sv. Minnesota Re deep rough mutant strain R595. *J. Endotoxin Res.,* 8: 343-356.

Kannenberg, E. L., Rathbun, E. A., and Brewin, N. J. (1992) Molecular dissection of structure and function in the lipopolysaccharide of *Rhizobium leguminosarum* strain 3841 using monoclonal antibodies and genetic analysis. *Mol. Microbiol.,* 6: 2477-2487.

Kannenberg, E. L., Reuhs, B. L., Forsberg, S., and Carlson, R. W. (1998) Lipopolysaccharides and K-antigens: Their structures, biosynthesis, and function. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (eds.), *The Rhizobiaceae; Molecular Biology of Model Plant-Associated Bacteria* (Dordrecht/Boston/London: Kluwer Academic Publishers), 119-154.

Kannenberg, E. L. and Carlson, R. W. (2001) Lipid A and O-chain modifications cause *Rhizobium* lipopolysaccharides to become hydrophobic during bacteroid development. *Mol. Microbiol.,* 39: 379-391.

Kawaharada, Y., Kiyota, H., Eda, S., Minamisawa, K., and Mitsui, H. (2008) Structural characterization of neutral and anionic glucans from *Mesorhizobium loti*. *Carbohydr. Res.,* 343: 2422-2427.

Kawasaki, K., Teramoto, M., Tatsui, R., and Amamoto, S. (2012) Lipid A 3'-O-deacylation by *Salmonella* outer membrane enzyme LpxR modulates the ability of lipid A to stimulate Toll-like receptor 4. *Biochem. Bioph. Res. Co.,* 428: 343-347.

Khan, S. A., Everest, P., Servos, S., Foxwell, N., Zahringer, U., Brade, H., Rietschel, E. T., Dougan, G., Charles, I. G., and Maskell, D. J. (1998) A lethal role for lipid A in *Salmonella* infections. *Mol. Microbiol.,* 29: 571-579.

Kulshin, V. A., Zahringer, U., Linder, B., Jager, K. E., Dmitriev, B. A., and Rietschel, E. T. (1991) Structural characterization of the lipid A component of *Pseudomonas aeruginosa* wild-type and rough mutant lipopolysaccharides. *Eur. J. Biochem.,* 198: 697-704.

Kundi, M. (2007) New hepatitis B vaccine formulated with an improved adjuvant system. *Expert Rev. Vaccines,* 6: 133-140.

Layzell, D. B. and Hunt, S. (1990) Oxygen and the regulation of nitrogen fixation in legume nodules. *Plant Physiol*. 80: 322-327.

Leborgne-Castel, N., Adam, T., and Bouhidel, K. (2010) Endocytosis in plant-microbe interactions. *Protoplasma*, 247: 177-193.

Lepek, V.C. and D'Antuono, A.L. (2005) Bacterial surface polysaccharides and their role in the rhizobia-legume association. *Lotus Newsletter,* 35: 93-105.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature,* 344: 781-784.

Lerouge, I., Laeremans, T., Verreth, C., Vanderleyden, J., Van Soom, C., Tobin, A., and Carlson, R. W. (2001) Identification of an ATP-binding cassette transporter for export of the O-antigen across the inner membrane in *Rhizobium etli* based on the genetic, functional, and

structural analysis of an lps mutant deficient in O-antigen. *J. Biol. Chem.,* 276: 17190-17198.

LeVier, K., Phillips, R. W., Grippe, V. K., Roop, R. M., Jr., and Walker, G. C. (2000) Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. *Science,* 287: 2492-2493.

Mata-Haro, V., Cekic, C., Martin, M., Chilton, P. M., Casella, C. R., and Mitchell, T. C. (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science,* 316: 1628-1632.

Medzhitov, R. and Janeway, C., Jr. (2000) Innate immunity. *N. Engl. J. Med.,* 343: 338-344.

Miller, K. J., Kennedy, E. P., and Reinhold, V. N. (1986) Osmotic adaptation by Gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science,* 231: 48-51.

Miller, K. J., Reinhold, V. N., Weissborn, A. C., and Kennedy, E. P. (1987) Cyclic glucans produced by *Agrobacterium tumefaciens* are substituted with sn-1-phosphoglycerol residues. *Biochim. Biophys. Acta,* 901: 112-118.

Miller, K. J., Gore, R. S., Johnson, R., Benesi, A. J., and Reinhold, V. N. (1990) Cell-associated oligosaccharides of *Bradyrhizobium* spp. *J. Bacteriol.,* 172: 136-142.

Miller, K. J. and Gore, R. S. (1992) Cyclic beta-1,6-1,3 glucans of *Bradyrhizobium*: functional analogs of the cyclic beta-1,2-glucans of *Rhizobium*?. *Curr. Microbiol.,* 24: 101-104.

Miller, K. J. and Wood, J. M. (1996) Osmoadaptation by rhizosphere bacteria. *Annu. Rev. Microbiol.,* 50: 101-136.

Mithofer, A. (2002) Suppression of plant defence in rhizobia-legume symbiosis. *Trends Plant Sci.,* 7: 440-444.

Moran, A. P., Zahringer, U., Seydel, U., Scholz, D., Stutz, P., and Rietschel, E. T. (1991) Structural analysis of the lipid A component of *Campylobacter jejuni* CCUG 10936 (serotype O:2) lipopolysaccharide. Description of a lipid A containing a hybrid backbone of 2-amino-2-deoxy-D-glucose and 2,3-diamino-2,3-dideoxy-D-glucose. *Eur. J. Biochem.,* 198: 459-469.

Murata, T., Tseng, W., Guina, T., Miller, S. I., and Nikaido, H. (2007) PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.,* 189: 7213-7222.

Muroi, M., Ohnishi, T., and Tanamoto, K. (2002) MD-2, a novel accessory molecule, is involved in species-specific actions of *Salmonella* lipid A. *Infect. Immun.,* 70: 3546-3550.

Muszynski, A., Laus, M., Kijne, J. W., and Carlson, R. W. (2011) Structures of the lipopolysaccharides from *Rhizobium leguminosarum* RBL5523 and its UDP-glucose dehydrogenase mutant (exo5). *Glycobiology,* 21: 55-68.

Niehaus, K., Lagares, A., and Puhler, A. (1998) A *Sinorhizobium meliloti* lipopolysaccharide mutant induces effective nodules on the host plant *Medicago sativa* (alfalfa) but fails to establish a symbiosis with *Medicago trancatula. Mol. Plant Microbe Interact.,* 11: 906-914.

Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. R.,* 67: 593-656.

Ojeda, K. J., Simonds, L., and Noel, K. D. (2013) Roles of predicted glycosyltransferases in the biosynthesis of the *Rhizobium etli* CE3 O antigen. *J. Bacteriol.,* 195: 1949-1958.

Pellock, B. J., Cheng, H. P., and Walker, G. C. (2000) Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. *J. Bacteriol.,* 182: 4310-4318.

Persing, D. H., Coler, R. N., Lacy, M. J., Johnson, D. A., Baldridge, J. R., Hershberg, R. M., and Reed, S. G. (2002) Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol.,* 10(10 Suppl): S32-37.

Peters, N. K., Frost, J. W., and Long, S. R. (1986) A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. *Science,* 233: 977-980.

Phillips, N. J., Schilling, B., McLendon, M. K., Apicella, M. A., and Gibson, B. W. (2004) Novel modification of lipid A of *Francisella tularensis*. *Infect. Immun.,* 72: 5340-5348.

Platt, M. W., Miller, K. J., Lane, W. S., and Kennedy, E. P. (1990) Isolation and characterization of the constitutive acyl carrier protein from *Rhizobium meliloti*. *J. Bacteriol.,* 172: 5440-5444.

Plotz, B. M., Linder, B., Stetter, K. O., and Holst, O. (2000) Characterization of a novel lipid A containing D-galacturonic acid that replaces phosphate residues. The structure of the lipid a of the lipopolysaccharide from the hyperthermophilic bacterium *Aquifex pyrophilus*. *J. Biol. Chem.,* 275: 11222-11228.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science,* 282: 2085-2088.

Porte, F., Liautard, J. P., and Kohler, S. (1999) Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. *Infect. Immun.,* 67: 4041-4047.

Price, N. P., Jeyaretnam, B., Carlson, R. W., Kadrmas, J. L., Raetz, C. R., and Brozek K. A. (1995) Lipid A biosynthesis in *Rhizobium leguminosarum*: role of a 2-keto-3-deoxyoctulosonate-activated 4' phosphatase. *P. Natl. Acad. Sci. USA,* 92: 7352-7356.

Que, N. L., Ribeiro, A. A., and Raetz, C. R. (2000) Two-dimensional NMR spectroscopy and structures of six lipid A species from *Rhizobium etli* CE3. Detection of an acyloxyacyl residue in each component and origin of the aminogluconate moiety. *J. Biol. Chem.,* 275: 28017-28027.

Que-Gewirth, N. L., Lin, S., Cotter, R. J., and Raetz, C. R. (2003) An outer membrane enzyme that generates the 2-amino-2-deoxy-gluconate moiety of *Rhizobium leguminosarum* lipid A. *J. Biol. Chem.,* 278: 12109-12119.

Raetz, C. R. (1993) Bacterial endotoxins: extraordinary lipids that activate eucaryotic signal transduction. *J. Bacteriol.,* 175: 5745-5753.

Raetz, C. R. and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.,* 71: 635-700.

Raetz, C. R., Reynolds, C. M., Trent, M. S., and Bishop, R. E. (2007) Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.,* 76: 295-329.

Ramelot, T. A., Rossi, P., Forouhar, F., Lee, H. W., Yang, Y., Ni, S., Unser, S., Lew, S., Seetharaman, J., Xiao, R., Acton, T. B., Everett, J. K., Prestegard, J. H., Hunt, J. F., Montelione, G. T., and Kennedy, M. A. (2012) Structure of a specialized acyl carrier protein essential for lipid A biosynthesis with very long-chain fatty acids in open and closed conformations. *Biochemistry,* 51: 7239-7249.

Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L., and Rolfe, B. G. (1986) Flavones induce expression of nodulation genes in *Rhizobium. Nature,* 323: 632-635.

Reynolds, C. M., Riveiro, A. A., McGrath, S. C., Cotter, R. J., Raetz, C. R., and Trent, M. S. (2006) An outer membrane enzyme encoded by *Salmonella typhimurium lpxR* that removes the 3'-acyloxyacyl moiety of lipid A. *J. Biol. Chem.,* 281: 21974-21987.

Rolin, D. B., Pfeffer, P. E., Osman, S. F., Szwergold, B. S., Kappler, F., and Benesi, A. J. (1992) Structural studies of a phosphocholine substituted beta-(1,3);(1,6) macrocyclic glucan from *Bradyrhizobium japonicum* USDA 110. *Biochim. Biophys. Acta,* 1116: 215-225.

Roset, M. S., Ciocchini, A. E., Ugalde, R. A., and Inon de lannino, N. (2006) The *Brucella abortus* cyclic beta-1,2-glucan virulence factor is substituted with O-ester-linked succinyl residues. *J. Bacteriol.,* 188: 5003-5013.

Roujeinikova, A., Baldock, C., Simon, W. J., Gilroy, J., Baker, P. J., Stuitje, A. R., Rice, D. W., Slabas, A. R., and Rafferty, J. B. (2002) X-ray crystallographic studies on butyryl-ACP reveal flexibility of the structure around a putative acyl chain binding site. *Structure,* 10: 825-835.

Ruberg, S., Puhler, A., and Becker, A. (1999) Biosynthesis of the exopolysaccharide galactoglucan in *Sinorhizobium meliloti* is subject to a complex control by the phosphate-dependent regulator PhoB and the proteins ExpG and MucR. *Microbiol.,* 145(Pt 3): 603-611.

Sadovskaya, I., Vinogradov, E., Li, J., Hachani, A., Kowalska, K., and Filloux, A. (2010) High-level antibiotic resistance in *Pseudomonas aeruginosa* biofilm: the *ndvB* gene is involved

in the production of highly glycerol-phosphorylated beta-(1->3)-glucans, which bind aminoglycosides. *Glycobiol.,* 20: 895-904.

Scheidle, H., Gross, A., and Niehaus, K. (2005) The Lipid A substructure of the *Sinorhizobium meliloti* lipopolysaccharides is sufficient to suppress the oxidative burst in host plants. *New Phytol.,* 165: 559-565.

Schlaman, H. R. M., Phillips, D. A., and Kondorosi, E. (1998) Genetic organizaiton and transcriptional regulation of rhizobial nodulation genes. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (eds.), *The Rhizobiaceae* (Dordrecht/Boston/London: Kluwer Acad.), 361-386.

Schmidt, P. E., Broughton, W. J., and Werner, D. (1994) Nod factors of *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR234 induce flavonoid accumulation in soybean root exudate. *Mol. Plant Microbe Interact.,* 7: 384-390.

Schnaitman, C. A. and Klena, J. D. (1993) Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol. Rev.,* 57: 655-682.

Sharypova, L. A., Niehaus, K., Scheidle, H., Holst, O., and Becker, A. (2003) *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *J. Biol. Chem.,* 278: 12946-12954.

Silipo, A., De Castro, C., Lanzetta, R., Molinaro, A., and Parrilli, M. (2004) Full structural characterization of the lipid A components from the *Agrobacterium tumefaciens* strain C58 lipopolysaccharide fraction. *Glycobiology,* 14: 805-815.

Smit, G., Kijne, J. W., and Lugtenberg, B. J. (1987) Involvement of both cellulose fibrils and a Ca2+-dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *J. Bacterol.,* 169: 4294-4301.

Spaink, H. P. (2000) Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.,* 54: 257-288.

Stanfield, S. W., Ielpi, L., O'Brochta, D., Helinski, D. R., and Ditta, G. S. (1988) The *ndvA* gene product of *Rhizobium meliloti* is required for beta-(1----2)glucan production and has homology to the ATP-binding export protein HlyB. *J. Bacteriol.,* 170: 3523-3530.

Sweet, C. R., Ribeiro, A. A., and Raetz, C. R. (2004a) Oxidation and transamination of the 3"-position of UDP-N-acetylglucosamine by enzymes from *Acidithiobacillus ferrooxidans*. Role in the formation of lipid a molecules with four amide-linked acyl chains. *J. Biol. Chem.,* 279: 25400-25410.

Sweet, C. R., Williams, A. H., Karbarz, M. J., Werts, C., Kalb, S. R., Cotter, R. J., and Raetz, C. R. (2004b) Enzymatic synthesis of lipid A molecules with four amide-linked acyl chains. LpxA acyltransferases selective for an analog of UDP-N-acetylglucosamine in which an amine replaces the 3"-hydroxyl group. *J. Biol. Chem.,* 279: 25411-25419.

Tanamoto, K. and Azumi, S. (2000) *Salmonella*-type heptaacylated lipid A is inactive and acts as an antagonist of lipopolysaccharide action on human line cells. *J. Immunol.,* 164: 3149-3156.

Tang, L., Weissborn, A. C., and Kennedy, E. P. (1997) Domains of *Escherichia coli* acyl carrier protein important for membrane-derived-oligosaccharide biosynthesis. *J. Bacteriol.,* 179: 3697-3705.

Therisod, H., Weissborn, A. C., and Kennedy, E. P. (1986) An essential function for acyl carrier protein in the biosynthesis of membrane-derived oligosaccharides of *Escherichia coli*. *PNAS,* 83: 7236-7240.

Therisod, H. and Kennedy, E. P. (1987) The function of acyl carrier protein in the synthesis of membrane-derived oligosaccharides does not require its phosphopantetheine prosthetic group. *PNAS,* 84: 8235-8238.

Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1986) Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med.,* 164: 777-793.

Trent, M. S., Stead, C. M., Tran, A. X., and Hanking, J. V. (2006) Diversity of endotoxin and its impact on pathogenesis. *J. Endotoxin Res.* 12: 205-223.

Tully, R. E., Keister, D. L., and Gross, K. C. (1990) Fractionation of the beta-linked glucans of *Bradyrhizobium japonicum* and their response to osmotic potential. *Appl. Environ. Microbiol.,* 56: 1518-1522.

Vagelos, P. R. and Larrabes, A. R. (1967) Acyl carrier protein. IX. Acyl carrier protein hydrolase. *J. Biol. Chem.,* 242: 1776-1781.

van Brussel, A. A., Recourt, K., Pees, E., Spaink, H. P., Tak, T., Wijffelman, C. A., Kijne, J. W., and Lugtenberg, B. J. (1990) A biovar-specific signal of *Rhizobium leguminosarum* bv. *viciae* induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. nigra. *J. Bacteriol.,* 172: 5394-5401.

Vanderlinde, E. M., Harrison, J. J., Muszynski, A., Carlson, R. W., Turner, R. J., and Yost, C. K. (2009) Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. *FEMS Microbiol Ecol*. 71: 327-340.

Vasse, J., de Billy, F., Camut, S., and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.,* 172: 4295-4306.

Vedam, V., Kannenberg, E. L., Haynes, J. G., Sherrier, D. J., Datta, A., and Carlson, R. W. (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J. Bacteriol.,* 185: 1841-1850.

Vedam, V., Haynes, J. G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. (2004) A *Rhizobium leguminosarum* lipopolysaccharide lipid-A mutant induces nitrogen-fixing nodules with delayed and defective bacteroid formation. *Mol. Plant Microbe Interact.,* 17: 283-291.

Vedam, V., Kannenberg, E. L., Datta, A., Brown, D., Haynes-Gann, J. G., Sherrier, D. J., and Carlson, R. W. (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide *acpXL* mutant to add 27-hydroxyoctacosanoic acid to its lipid A. *J. Bacteriol.,* 188: 2126-2133.

Wang, X., Ribeiro, A. A., Guan, Z., McGrath, S. C., Cotter, R. J., and Raetz, C. R. (2006) Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. *Biochem.,* 45: 14427-14440.

Weir, B. S. The current taxonomy of rhizobia. *New Zealand rhizobia website* (updated September 13th, 2008) <http://www.rhizobia.co.nz/taxonomy/rhizobia.html>.

White, K. A., Kaltashov, I. A., Cotter, R. J., and Raetz, C. R. (1997) A mono-functional 3-deoxy-D-manno-octulosonic acid (Kdo) transferase and a Kdo kinase in extracts of *Haemophilus influenzae*. *J. Biol. Chem.,* 272: 16555-16563.

White, S. W., Zheng, J., Zhang, Y. M., and Rock, C. O. (2005) The structural biology of type II fatty acid biosynthesis. *Annu. Rev. Biochem.,* 74: 791-831.

Woronin, M. S. (1866) Uber die bei der Schwarzerle (*Alnus glutinosa*) und bei der gewohnlichen Gartenlupine (*Lupinus mutabilis*) auftretenden Wurzelanchwellungen. *Memoires de l'Academie Imperiale des Sciences de St. Petersbourg,* VII Series (Vol. X).

Zahringer, U., Linder, B., Knirel, Y. A., van den Akker, W. M., Hiestand, R., Heine, H., and Dehio, C. (2004) Structure and biological activity of the short-chain lipopolysaccharide from *Bartonella henselae* ATCC 49882T. *J. Biol. Chem.,* 279: 21046-21054.

Zevenhuizen, L. P., van Veldhuizen, A., and Fokkens, R. H. (1990) Re-examination of cellular cyclic beta-1,2-glucans of Rhizobiaceae: distribution of ring sizes and degrees of glycerol-1-phosphate substitution. *Anton. Van. Lee.,* 57: 173-178.

Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. (1999) Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH4VO3 in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. *J. Biol. Chem.,* 274: 18503-18514.

Zorreguieta, A., Cavaignac, S., Geremia, R. A., and Ugalde, R. A. (1990) Osmotic regulation of beta(1-2) glucan synthesis in members of the family Rhizobiaceae. *J. Bacteriol.,* 172: 4701-4704.

CHAPTER 2

MUTATION OF THE ACYL TRANSFERASE LpxXL PREVENTS THE ADDITION OF 27-HYDROXYOCTACOSANOIC ACID AND PALMITIC ACID TO *RHIZOBIUM LEGUMINOSARUM* LIPID A[1]

[1] Bourassa, D.V., Carlson, R.W., Buhr, R.J., and Kannenberg, E.L. To be submitted to MPMI.

Abstract

Lipopolysaccharides (LPS) of many rhizobial species differ from other gram-negative bacteria in that an acyloxyacyl 27-hydroxyoctacosanoic acid (VLCFA) is bound to lipid A. This long chain fatty acid is also present in lipopolysaccharides of intracellular pathogens such as *Brucella abortus*, *Bartonella henselae*, and *Legionella pneumophila*. A previously characterized *Rhizobium leguminosarum* 3841 mutant of AcpXL (VLCFA acyl carrier protein) was used to generate an AcpXL/LpxXL (acyl transferase) double mutant (EL196) by inserting a gentamicin cassette into *lpxXL*. The *Rhizboium leguminosarum* 3841 *acpXL*$^{-}$*lpxXL*$^{-}$ double mutant was partially complemented with cloned wild type *acpXL* creating a clone (EL197) expected to have the same phenotypic traits as an LpxXL single mutant. EL196 (*acpXL*$^{-}$*lpxXL*$^{-}$) and EL197 (acpXL^{-}*lpxXL*$^{-}$*acpXL*$^{+}$) were examined as free-living bacteria, bacteroids, and as isolates obtained from host root nodules (i.e. ex-nodule isolates). The LPS from both mutants had tetraacylated lipid A without 27OCH28:0. Previous work showed that an AcpXL mutant could replace VLCFA with palmitic/stearic acid (Vedam et al. 2003; Haag et al. 2011). Here, we show that without a functional LpxXL transferase, palmitic/stearic acid cannot be added to the lipid A; a result that had also been shown for *Sinorhizobium meliloti* (Haag et al. 2011). For both parent and mutant strains lipid A tends to have longer acyl chains in bacteroid form than in the free-living bacteria. Also, bacteroid LPS from *acpXL* mutants has higher molecular weight LPS, i.e. more LPS containing the O-chain polysaccharide, during symbiosis. Although presence of the 27OCH28:0 acyl chain is not absolutely required for symbiosis in the laboratory, it may still have more subtle effects on the symbiotic phenotype and possibly be important for survival and proliferation in the rhizosphere.

Introduction

The *Rhizobiaceae* are a family of gram-negative bacteria that form an important symbiotic relationship with legume plants. These soil bacteria infect legume roots that form nodules in which the bacteria differentiate into bacteroids that can fix atmospheric nitrogen into ammonia for the plant while the plant provides nutrients for the bacteria. This complicated symbiotic relationship involves a number of secreted and cell surface polysaccharides. Early in the infection process flavonoids released by the plant attract rhizobia and induce nodulation (*nod*) genes that encode proteins required for the synthesis of lipochito-oligosaccharides known as Nod factors (Lerouge et al. 1990). These Nod factors induce root nodule formation and are important factors in determining host plant specificity (Demont-Caulet et al. 1999). The rhizobia then bind to root hairs and exopolysaccharides are thought to protect the rhizobia from the plant defense response (Niehaus et al. 1993; Parniske et al. 1994). Rhizobial lipopolysaccharides (LPSs) are important in later symbiotic stages involving the infection of root nodule cells to form symbiosomes, differentiation into nitrogen-fixing bacteroids, and possibly modulating the plant defense response to protect the bacterium (Perotto et al. 1994; Kannenberg et al. 1998; Carlson et al 2010). Cyclic glucans are also important for the symbiotic relationship due to their use in protecting against hypoosmotic conditions thought to be present within the root nodule cells and possible suppression of the host defense response (Kennedy 1982; Bhagwat et al. 1999). The following work focuses on the function of the LPS biosynthetic proteins AcpXL and LpxXL in the symbiotic relationship between *Rhizobium leguminosarum* biovar *viciae* (Rlv) and the pea.

Lipopolysaccharides consist of three main components; O-chain polysaccharide (OPS), core (outer and inner), and the lipid A. Rhizobial LPSs have several unique features in comparison with other gram-negative bacteria. The lipid A portion of the LPS has four distinct

structural characteristics including 1) the absence of two phosphate groups typically found on the 1- and 4'-positions of the glucosamine (GlcN) disaccharide lipid A backbone, 2) addition of a galacturonic acid (GalA) to the 4'-position once the phosphate has been removed, 3) oxidation of the proximal glucosaminosyl residue to 2-aminogluconic acid (GlcNonate), and 4) a very long chain fatty acid (VLCFA), 27-hydroxyoctacosanoate, linked as an acyloxyacyl ester to the β-hydroxyl group of the β-hydroxyacyl chain at the 2'-position and a β-hydroxybutyrate modification of the 27-hydroxyl group of the VLCFA (Bhat et al. 1994) Figure 2.1A.

The role of the VLCFA in either the symbiotic or pathogenic infection process is not yet understood. This VLCFA is found in most *Rhizobiaceae* as well as some intracellular pathogens such as *Brucella, Bartonella,* and *Legionella* (Bhat et al. 1991; Zahringer et al. 1995; Corbel et al. 1997). The formation and addition of the VLCFA to the LPS lipid A requires a gene region encoding an acyl carrier protein (*acpXL*), fatty acid synthesis proteins (*fabF1XL* and *fabF2XL*), beta-hydroxyacyl-acyl carrier protein (ACP) dehydratase (fabZXL), an alcohol dehydrogenase, and an acyl transferase (*lpxXL*) (Figure 2.1B). Homologs of this gene region are present in members of the *Rhizobiaciae* as well as in the above mentioned pathogens. *Rhizobium leguminosarum* mutants disrupted in *acpXL* and *fabF1XL* and *fabF2XL* have previously been created and characterized (Vedam et al. 2003; Vedam et al. 2004; Vedam et al. 2006; Vanderlinde et al. 2009). Mutations in the acyl carrier protein and acyl synthase genes led to a lack of VLCFA in the lipid A. However a palmitoyl residue replaced VLCFA in a portion of the lipid A (Vedam et al. 2003; Vanderlinde et al. 2009). This previous work also demonstrated partial restoration of VLCFA in the lipid A from bacteroids of the *acpXL* mutant (Vedam et al. 2006). Similar results have also been reported for *Sinorhizobium meliloti* mutants of *acpXL* and *lpxXL* (Sharypova et al. 2003; Haag et al. 2009). These *Sinorhizobium meliloti* mutants were

determined to lack VLCFA in their lipid A and were able to infect nodules and fix nitrogen but have been shown to be less competitive than the parent strain (Sharypova et al. 2003; Haag et al. 2009). In contrast, an *acpXL* mutant was constructed in *Rhizobium* sp. strain NGR234 (a *Rhizobium* with a broad host range) and it also lacked the VLCFA (Ardissone et al. 2011). However, unlike the *acpXL* mutants of *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, it was severely impaired in its symbiotic ability (Ardissone et al. 2011).

The goal of this work was to begin to assess the function of VLCFA in *Rhizobium leguminosarum*-pea symbiosis by mutation of the acyl transferase, LpxXL, and characterization of its lipid A structure.

Materials and Methods

Bacterial strains and growth conditions

Strains of bacteria and plasmids are listed in Table 2.1. Strains of *Escherichia coli* were grown on Luria-Bertani (LB) medium at 37°C with appropriate antibiotics. *Rhizobium leguminosarum* strains were grown at 30°C on tryptone-yeast extract (TY) media with added calcium and appropriate antibiotics. Antibiotics were used at concentrations of 10 μg/mL tetracycline, 30 μg/mL kanamycin, 25 μg/mL gentamicin, 200 μg/mL streptomycin, and 50 μg/mL ampicillin. For β-galactosidase blue/white colony screening 100 μL of 40 mM isopropylthiogalactopyranoside (IPTG) and 100 μL of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were spread onto each plate.

Plant growth and inoculation conditions

For germination Early Alaska pea seeds were sterilized with 70% ethanol, washed 3x with sterile dH_2O, sterilized with 6% sodium hypochlorite, washed 10x with sterile dH_2O and

were germinated for 10-13 days at 22°C for 14 hours of light and 18°C for 10 hours in darkness each day at ambient relative humidity in 500 mL foam stoppered flasks with 300 mL of nitrogen-free Fahraeus medium (Nutman 1970). Following germination, stems were freed from the flask and secured with the foam stopper. Each plant was inoculated with 1 mL of an approximately 0.15 OD_{600} log phase *Rhizobium* culture (approximately 10^8 cfu/mL) by dripping onto the seed and emerged roots. Each flask was then covered with a brown paper bag and taped closed to minimize light at the roots while leaving the foam stopper and stem/leaves of the plant exposed. The pea plants were returned to the growth chamber for 21 dpi.

Bacteroid isolation

Following plant inoculation and nodule growth bacteroids were collected at 21 dpi from plant nodules for chemical analysis. Plant nodules from approximately 50 plants were removed from the roots with forceps and collected in a 0.5M sucrose buffer (50mM Tris, 10mM DTT, 0.5M sucrose, pH 7.4). Following collection the nodules were removed from the buffer and ground with a mortar and pestle with a small amount of fresh 0.5M sucrose buffer with added 5% polyvinylpolypyrrolidone and 5mM p-aminobenzamidine. Plant material from the crushed nodules was filtered through a syringe packed with 1 cm of glass wool. Bacteroids from the filtrate were further isolated through sucrose gradient ultracentrifugation. The sucrose gradients consisted of 2mL of each of 70%, 52%, 35%, 25%, and 17.5% sucrose in buffer (50mM Tris, 10mM DTT, pH 7.4). The bacteroid filtrate was layered on top of the sucrose gradient and ultracentrifuged at 100,000g for 4 hours. The bacteroid layer, a dark pink section between the 70% and 52% sucrose layers, was removed from the gradient, diluted in 30mL buffer (50mM Tris, 10mM DTT, pH 7.4), and centrifuged at 4000g for 30 minutes. The pellet was resuspended

in extraction buffer (0.05M $Na_2PO_4*7H_2O$, 0.005M EDTA, 0.05% $NaNO_3$, pH 7) and stored at -20°C.

Ex-nodule bacterial isolation

For each mutant, an isolate was obtained from the nodule. This isolate is referred to as the "ex-nodule" or ExN isolate. Several nodules were picked from a root system and each nodule was individually washed with 6% sodium hypochlorite for 30 s, then washed 4x with sterile dH_2O. Each nodule was crushed and streaked onto a TY plate with the appropriate antibiotics. Resulting colonies were restreaked for purity and stored in glycerol stocks. Each ex-nodule isolate was confirmed to retain the antibiotic resistance cassettes within the correct gene through PCR.

Mutant construction

The plasmid pSSB-1 containing a *Rhizobium leguminosarum* chromosomal gene region with the cluster of genes required for very long chain fatty acid biosynthesis and attachment to the lipid A was used for isolation of the *lpxXL* gene, RL2812 (Basu et al. 2002). pSSB-1 was digested with the restriction enzyme PstI creating a 2.6 kb fragment including *lpxXL* with flanking regions 1271-bp upstream (including the alcohol dehydrogenase RL2813 and a small fragment of *fabF1XL*) and 423-bp downstream (encoding a small fragment of RL2811, a hypothetical protein). This fragment containing *lpxXL* was subcloned into pBluescript pBSII SK(+) creating pDVB1 (Figure 2.2). The pDVB1 plasmid was used to transform XL1-B cells and grown on LB plates with ampicillin, X-Gal and IPTG. White colonies were selected and plasmid preparations were restriction digested and viewed on an agarose gel to confirm presence of the construct.

To interrupt the *lpxXL* gene a gentamicin resistance cassette (Becker et al. 1995) was introduced into the *lpxXL* gene. Fragments from restriction digestions of pDVB1 with AccIII and pMS255 with XmaI were ligated to form pDVB2. DH5α was transformed with pDVB2 and selected for on LB with gentamicin. Transformants were confirmed through restriction digestion and gel electrophoresis. Both forward and reverse orientations of the gentamicin cassette (*aacC1*) were obtained and orientation opposite the *lpxXL* gene was used.

The broad host range suicide vector pEX18Tc (Hoang et al. 1998) and pDVB2 were digested with EcoRI and SacI. The pDVB2 fragment containing *lpxXL::Gm* was gel extracted and ligated with the digested pEx18Tc to form pDVB3 which was transformed into DH5α and selected for antibiotic resistance on LB with gentamicin and tetracycline. Transformants were confirmed by restriction digestion and gel electrophoresis.

pDVB3 was introduced into *Rhizobium leguminosarum* bv. *viciae* 3841 and 22 through triparental mating with DH5α/pDVB3 as the donor, Rlv3841 or Rlv22 as the recipient, and pRK2013 (Ditta et al. 1980) as the helper. Single recombinants were selected on TY with gentamicin and streptomycin and then replica plated onto TY with gentamicin, streptomycin, and 10% sucrose for selection of double recombinants. Three double *acpXL::Km/ lpxXL::Gm* mutants were isolated (EL196, DM13, DM15). However, no *lpxXL::Gm* single mutants could be constructed. Mutations were confirmed by PCR with primers for *lpxXL* (AATGAGGGTGGACGTTCTTG and GTTCGACTACGATCCCGAAA) and *acpXL* (ATTGCAGAAACCAGCGAGAT and AGAAACCTTGCCTTCGTTGA).

Due to the inability to isolate a *lpxXL::Gm* mutant the *acpXL::Km/ lpxXL::Gm* double mutant, EL196, was complemented with the parental *acpXL*. The pVV5 plasmid (Vedam et al. 2003) containing the *acpXL* gene was introduced into EL196 through *Escherichia coli* triparental

mating with pVV5 the donor, EL196 the recipient, and pRK600 as the helper. Transformants were selected on TY with gentamicin, kanamycin, and tetracycline. The plasmid wild type *acpXL* gene should compensate for the chromosomal mutated *acpXL* therefore creating a mutant EL197 that is phenotypically like a *lpxXL*$^-$ mutant; i.e. *acpXL*$^-$*lpxXL*$^-$*acpXL*$^+$. The EL196 mutant was fully complemented by the *Escherichia coli* triparental mating of pSSB1 as donor, EL196 as acceptor and pRK2013 as helper creating EL198. Transformants were selected on TY agar with tetracycline and streptomycin and confirmed with PCR.

LPS extraction and purification

Each mutant and its isolate obtained from within host root nodules (ex-nodule, ExN, isolate) were grown under laboratory conditions, collected by centrifugation from 4 L cultures (~1.0 OD_{600}), washed with 0.9% NaCl, washed 3x with dH_2O, and the pellet suspended in 20 mL dH_2O. LPS was isolated by hot phenol-water extraction (Westphal and Jann 1965). Briefly, the cell pellet in 20 mL dH_2O and 20 mL hot phenol was heated at 65°C for 45 minutes, cooled for 5 minutes in ice water, centrifuged (7-10K for 25 minutes) to separate the water and phenol layers, and the water layer collected. Then 18 mL dH_2O was added to the phenol layer and the extraction procedure was repeated twice more after which pooled water layers and the phenol layers were dialyzed (MWCO:2000). Following dialysis against dH_2O LPS was lyophilized and further purified with RNase, DNase, and proteinase treatments.

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

Lipopolysaccharide characterization

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

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

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

Results

Cloning of acyl transferase mutants

Rhizobium leguminosarum bv. *viciae* 3841 acyl transferase mutants were cloned by double recombination mutagenesis as described (Figure 2.2). Several isolates were obtained from the mutagenesis of Rlv22 leading to the *acpXL*$^{-}$*lpxXL*$^{-}$ double mutant (EL196). However, multiple attempts using both recombination as described for EL196 and phage transduction methods (Buchanan-Wollaston 1979) to create a single *lpxXL*$^{-}$ mutant in Rlv 3841 were

unsuccessful. PCR products from Rlv3841, Rlv22 (*acpXL::Km),* EL196 (*lpxXL::Gm/acpXL::Km*), EL197 (*lpxXL::Gm/acpXL::Km,* pVV5), and EL198 (full complement) are shown in Figure 2.3. Figure 2.3A shows PCR products from reactions with *acpXL* specific primers, Figure 2.3B shows PCR products from reactions with *lpxXL* specific primers, Figure 2.3C shows PCR products from reactions with *acpXL::Km* specific primers and *lpxXL::Gm* specific primers. In Figure 2.3A and B, lane 1, the 177 bp PCR product from within *acpXL* is shown as expected for Rlv3841. In Figure 2.3A, lane 2 (Rlv22), a 1454 bp PCR product indicates that the kanamycin cassette is present within the *acpXL* gene. In Figure 2.3B, lane 2 (Rlv22), a 725 bp PCR product indicates that the *lpxXL* gene is present and wild type. As a control the pDVB3 PCR reactions were loaded into lanes 3 if Figures 2.3A and B where no *acpXL* PCR product (Figure 2.3A) was expected or seen and where the *lpxXL* gene with gentamycin cassette inserted (Figure 2.3B) was expected and seen. In lane 4 of Figure 2.3A the 177 bp PCR product for *acpXL* was seen but in Figure 2.3B the *lpxXL* product was 725 bp indicating that the putitive *lpxXL* mutant tested did not contain the gentamicin cassette insert. In lane 5 (EL196) of Figure 2.3A, a 1454 bp product confirms that the kanamycin cassette is present in the *acpXL* gene. Lane 5 (EL196) of Figure 2.3B had the 1425 bp product confirming insertion of the gentamicin cassette into the *lpxXL* gene. Multiple attempts for obtaining a single *lpxXL* mutant were made through homologous recombination and phage transduction, however, all attempts failed. Due to the inability to clone a *lpxXL*$^-$ strain after multiple attempts, it was decided to create a mutant, EL197, with a *lpxXL*$^-$ phenotype by transfecting a wild type *acpXL* gene on a plasmid into EL196 to complement the *acpXL*$^-$ chromosomal gene mutation. In Figure 2.3C EL197 has a 177 bp fragment in lane 1 indicating a wild type *acpXL* and a 1425 bp product in lane 2 indicating *lpxXL::Gm*. The lack of a PCR product for the chromosomal *acpXL::Km* is

thought to be due to the increased copy number of the plasmid borne wild type *acpXL*. Although a PCR product was not visualized, resistance to kanamycin remained. Additional PCR using primers that would bind within the antibiotic resistance cassettes and target genes was done to confirm the presence of strains with the cassette insertions.

LPS and lipid A isolation and characterization

LPS isolated by phenol/water extraction from free-living bacteria, bacteroids, and ex-nodule isolates of wild type and mutant strains was analyzed by DOC-PAGE gel electrophoresis (Figure 2.4). By weight the majority (>90%) of the LPS was extracted into the water layers for laboratory culture and bacteroid preparations. The LPS II band is due to LPS that lacks OPS, while the LPS I bands contain various lengths of OPS; i.e. the faster to slower mobility bands have short to long OPS, respectively. The LPS from laboratory cultured parent strain Rlv3841 and from Rlv3841 bacteroids both show LPS I and LPS II; however, the Rlv3841 bacteroid LPS has a shift toward molecular species with longer OPS (Figure 2.4, compare panel 1, lane 1 with panel 4, lane1). The LPS from the laboratory cultured *acpXL* mutant, Rlv22, exhibited the same banding pattern as observed for that from Rlv3841 but with all bands slightly shifted to a lower molecular weight likely due to the loss of the VLCFA from the lipid A (described further below) (Figure 2.4, panel 1, lane 2). In contrast, the LPS from Rlv22 bacteroids contained only two of the higher molecular weight bands of LPS I (Figure 2.4, panel 1, lane 3); i.e. there was either none or barely detectable levels of LPS II. The LPS from laboratory cultured ExN Rlv22 gave the same banding pattern as that from laboratory cultured Rlv22 (Figure 2.4, panel 1, lane 4). The LPS from laboratory cultured EL196 (*acpXL/lpxXL* mutant) and its ExN isolate also had a similar banding pattern to that from laboratory cultured Rlv3841 with a slight shift to lower molecular weight LPS forms due to the loss of VLCFA. The LPS from EL196 bacteroids was

similar to Rlv22 bacteroid LPS with only two higher molecular weight bands of LPS I. The EL196 ExN isolate lacked a low molecular weight band of LPS I that was visible in the EL196 laboratory culture isolate. The LPS from laboratory cultured EL197 (*acpXL/lpxXL* mutant complemented with *acpXL*) also had a similar banding pattern to that from Rlv3841 with the shift to lower molecular weight as observed for Rlv22 and EL196 LPS except that a low molecular weight band of LPS I was not visible. However, unlike Rlv22 and EL196, LPS from EL197 bacteroids had a similar banding pattern to parent Rlv3841 bacteroid LPS in that it contained the various LPS I bands as well as LPS II with the same shift toward higher molecular weight LPS I bands as observed for Rlv3841. These results show that bacteroid LPS from those mutants without the acyl carrier protein AcpXL, Rlv22 and EL196, appear to require, almost exclusively, the two higher molecular forms of LPS I, while bacteroids from the parent Rlv3841 and mutant EL197 have LPS with a LPS I having a variety of OPS chain lengths as well as LPS II without OPS.

Relative fatty acid compositions of the lipid A from the parent strain, each mutant, and its ExN isolate; both from laboratory cultured preparations and from bacteroids are shown in Table 2.2. When comparing the lipid A from laboratory cultured parent Rlv3841 with its laboratory cultured ExN isolate and bacteroids, the percentages of C16:0 and C18:0 increased from a total of 9% in Rlv3841 to 17% and 18% for its laboratory cultured ExN isolate and bacteroids. Five different 3OH fatty acids were detected (3OHC14:0, 3OHC15:0, 3OHC16:0, 3OHC18:0, and 3OHC18:1). Of these 3OHC14:0 was present in the largest amount in the LPS from laboratory cultured Rlv3841 (46%) and its ExN isolate (37%) and lowest in the LPS from Rlv3841 bacteroids (20%). The remaining four 3OH fatty acids were found at low percentages from 2%-13%. The VLCFA (27OHC28:0) showed the opposite trend with bacteroid VLCFA being higher

(41%) than the laboratory grown Rlv3841 (16%) and its ExN isolate (25%). For the Rlv22 mutant C16:0 and C18:0 total percentage was higher in bacteroid lipid A (25%) than for Rlv22 and its ExN laboratory cultures, (2%) and (8%), respectively. The relative amounts of 3OHC14:0, 3OHC16:0, and 3OHC18:0 fatty acids decreased in the lipid A from Rlv22 bacteroids compared to that from laboratory cultures of Rlv22 and its ExN isolate while both 3OHC15:0 and 3OHC18:1 increased in bacteroid lipid A. No VLCFA was detected in any of the Rlv22 LPS preparations. As with Rlv22, bacteroids from the double mutant EL196 also had lipid A with a higher percentage of C16:0 and C18:0 (47%) than laboratory grown cultures (2%). However, laboratory cultures of the EL196 ExN isolate contained low levels of C16:0 (2%) while C18:0 had an intermediate amount (14%). A similar trend as was observed for EL196 lipid A 3OH fatty acids as was seen for Rlv22; i.e. the overall levels of 3OHC14:0, 3OHC16:0, and 3OHC18:0 fatty acids decreased in bacteroid lipid A compared to the lipid A from laboratory cultures and its ExN isolate. However the 3OHC18:1 fatty acid percentage was higher in bacteroid lipid A than laboratory cultures of EL196 and its ExN isolate. The 3OHC15:0 from the EL196 ExN lipid A remained similar to the percentage in bacteroids. As with Rlv22, no VLCFA was detected in any of the EL196 lipid A preparations. The *acpXL* complemented double mutant, EL197, showed different trends in comparison to the other mutants. The C16:0 and C18:0 fatty acid percentages were low for the lipid A from both laboratory grown EL197 (2%) and bacteroids (4%), while laboratory cultures of the EL197 ExN isolate still had a low percentage of C16:0 (3%) but had an increased amount of C18:0 (17%). The 3OHC14:0 had a similar trend to the lipid A from the other mutants with a decreased percentage in bacteroid lipid A. With regard to the 3OH fatty acids, however, the level of 3OHC18:0 was much higher in the bacteroid lipid A (41%) than for the lipid A from laboratory grown cultures of EL197 (24%) and its ExN isolate

(15%). The remaining 3OH fatty acids, 3OHC15:0, 3OHC16:0, and 3OHC18:1, remained at similar percentages for all of the EL197 lipid A preparations. Interestingly, 3% of the total fatty acids of EL197 bacteroid lipid A was VLCFA. Although VLCFA was found in the EL197 lipid A preparation, there was no lipid A containing VLCFA detected using MALDI-TOF MS analysis (described further below), i.e. all ions observed were consistent with lipid A species that lacked the VLCFA (Figure 2.5A). It is possible that VLCFA-AcpXL may have been isolated during the phenol/water extraction and, therefore, the VLCFA is not part of the lipid A. Larger amounts of VLCFA-AcpXL may be present since this strain contains multiple copies of plasmid pVV5, which carries the *acpXL* gene.

Lipid A preparations from laboratory cultured bacteria and bacteroids were further analyzed by MALDI-TOF/MS (Figure 2.5-2.8). The structures I.A, I.B, II, III.A, III.B, IV, V.A, and V.B predicted from the MALDI-TOF MS spectra are shown in Figure 2.9 and described in Table 2.3. Each structure is heterogeneous in fatty acid chain length and/or proximal GlcN structure and consists of a cluster of ions. The lipid A from the laboratory cultured parent strain Rlv3841 has four major clusters of ions consistent with structures I.A, I.B, II, III.A, III.B, and IV in Figure 2.9 indicating an intact lipid A with the VLCFA and either GlcN or GlcNonate proximal sugars (structures I.A, I.B) and a lipid A with the VLCFA but missing 3OHC14:0 on the 3 position glucosamine (structure II). The loss of this fatty acid is an artifact of the mild acid hydrolysis procedure used to isolate lipid A (Price et al. 1995). Structures III.A, III.B, and IV were observed to a lesser extent. These structures are similar to structures I and II but lack the VLCFA. The Rlv3841 bacteroid lipid A had structures I.A, I.B, II, and IV. However, these structures appeared to have a shift towards longer chain fatty acids in each ion cluster. For example, ion m/z 1914.4 is a longer fatty acid chain variant of m/z 1886.5, and ions m/z 2012.7

and 1984.6 are longer fatty acid chain variants of ion m/z 1956.5, etc. The structures I.A and I.B with the shortest chain lengths (ions m/z 1870.5 and 1886.5, respectively) were only observed in the laboratory cultured parent and not the bacteroids. Both the laboratory cultures and bacteroid Rlv3841 had the same ions in structure II. Structure III was only observed in laboratory cultured Rlv3841 and not in the bacteroids. The ions observed for structure III were of the shorter fatty acyl chains (only 1448.1, 1476.1, 1464.0, and 1492.1) in comparison to ions observed from the mutants. For structure IV, Rlv3841 laboratory cultured lipid A lacked two of the ions suspected to have hydroxylated structures (1259.9 and 1301.9). The bacteroids contained all of the observed ions for structure IV except for the three with the shortest acyl chain lengths (1173.8, 1201.8, and 1229.9). Laboratory cultured Rlv22 (*acpXL*$^{-}$) had structures III.A, III.B, IV, and V.B., all of which lack the VLCFA. Structure V is similar to structure III except that a C16:0 or C18:0 is added instead of the VLCFA. Based on peak intensity the structure V was less abundant than the structures III and IV in the laboratory cultured bacteria and structure V.A may be present at insufficient levels for detection. The Rlv22 bacteroid lipid A showed a similar trend as Rlv3841 in that ion clusters tended towards having longer chain fatty acids. Structure V with the additional C16:0 or C18:0 was also more abundant. The proximal-GlcN form of structure III was not observed in Rlv22 bacteroid lipid A as was observed for Rlv3841 bacteroids. Only the shorter chain ions (m/z 1448.1 and 1476.1) were observed for laboratory cultured Rlv22 structure III.A. Rlv22 structure III.B. had two observed ions thought to be of sodiated structures that were not present in any other strains, one (1513.9) present only in the laboratory culture and one (1577.9) observed in both laboratory culture and bacteroid Rlv22. For structure IV, the laboratory cultured Rlv22 lacked the ions thought to be hydroxylated or sodiated while these ions m/z 1273.9 and above were observed in the bacteroids. The proximal-GlcN form of structure V

was not observed in Rlv22 laboratory culture and only the ions corresponding to the proximal-GlcNonate form with shorter chain length structures were observed (m/z 1786.2 and below). EL196 (*acpXL*$^{-}$/*lpxXL*$^{-}$) and EL197 (*acpXL*$^{-}$/*lpxXL*$^{-}$/*acpXL*$^{+}$) both had structures III.A, III.B, and IV in both free-living and bacteroid lipid A (Figure 2.7, 2.8). EL196 bacteroids had structure III.A lipid A with longer chain length fatty acyl residues (ion 1560.2) than that from the laboratory cultured strain and did not have the ions thought to be sodiated as observed for Rlv22. The EL196 bacteroid lipid A did not have the ions for the predicted shortest chain length structures that were observed for the laboratory culture (1173.8 and 1201.8). Neither the laboratory strain nor the bacteroids had the ions for structures suspected to be hydroxylated in structure IV. Both laboratory cultured and bacteroid EL197 lipid A had all of the observed ions for structure III.A. but for structure III.B the bacteroid lipid A had an additional ion predicted to have longer acyl chains. EL197 bacteroids had ions predicted to be hydroxylated or sodiated in addition to the ions observed for the laboratory culture. Both the EL196 and EL197 bacteroid lipid A were shifted towards longer chain fatty acid structures.

Discussion

This study assessed a mutation in an acyl transferase required for the transfer of a secondary fatty acid to the lipid A of *Rhizobium leguminosarum* bv. *viciae* 3841. An acyl carrier protein (AcpXL)/acyl transferase (LpxXL) double mutant (EL196) was successfully constructed. However, an LpxXL mutant could not be isolated so a partially complemented double mutant was constructed (EL197 (*acpXL*$^{-}$/*lpxXL*$^{-}$/*acpXL*$^{+}$). Except for a slight shift in molecular weight due to loss of the VLCFA, LPS from *acpXL* and *lpxXL* mutants from free-living and ex-nodule bacteria did not appear to be significantly altered. However, in EL196 ExN isolate and EL197

laboratory culture isolate the lowest molecular weight band of LPS I was not visible. Rlv22 and EL196 bacteroid LPS only contained higher molecular weight bands of LPS I, which contain OPS. A shift to longer chain fatty acids was observed in the lipid A from all bacteroid preparations compared to that from laboratory cultured bacteria.

It is presently unknown why, after multiple attempts using double recombination and phage transduction mutagenesis methods, we were unable to obtain a single mutant in LpxXL. The inability to create a single *lpxXL* mutant may indicate that this mutation is lethal or that the media used were inadequate for growth; e.g. a *fabF2XL/fabF1XL* double mutant was unable to grow on TY media, however, with added calcium this mutant was able to grow (Vanderlinde et al. 2009). Therefore, we tested whether or not an Rlv3841 *lpxXL* mutant could be isolated on TY medium with added calcium or on minimal medium plates but this also was unsuccessful. We do not know why it is possible to create an Rlv3841 *acpXL/lpxXL* double mutant but not an *lpxXL* single mutant. The importance of *lpxXL* expression for growth is not the same among different *Rhizobium* species since, unlike Rlv3841, a single *lpxXL* mutant was viable in *Sinorhizobium meliloti,* as were the *acpXL*, and *acpXL/lpxXL* mutants (Ferguson et al. 2005)**.** Even though an Rlv3841 *lpxXL* single mutant could not be isolated, the *acpXL/lpxXL* double mutant could be complemented with a wild type *acpXL* and viable clones were isolated. Thus, for Rlv3841, the only *lpxXL* mutants that can grow are EL196, which also lacks AcpXL, or EL197 which contains multiple copies of *acpXL*. This may indicate that *acpXL* expression acts in some type of regulatory manner in conjunction with *lpxXL* in addition to providing the substrate for the LpxXL transferase, and perhaps for other genes. For example, it has been reported that both mutation of *acpXL* and *fabF2XL/fabF1XL* down regulate expression of an outer membrane

protein *ropB* (Vanderlinde and Yost 2012). It is not known what pleotropic effects might be caused by the loss of the *lpxXL* expression.

Examination of the mutant LPS preparations by DOC-PAGE showed that there was a general shift of all visible bands to a lower molecular weight can be explained by the loss of the VLCFA from lipid A. It was also observed that all bacteroid LPS preparations showed a shift toward higher molecular weight LPS (i.e. more LPS containing longer OPS). This shift was particularly dramatic in the bacteroid LPS from mutants containing a defective *acpXL*, i.e. mutants Rlv22 and EL196, which showed only two higher molecular weight LPS I bands and were almost totally devoid of LPS II. An increase in the amount of bacteroid LPS I has previously been observed for Rlv3841 and was also seen in this work (Kannenberg and Carlson 2001). The even greater shift to the higher molecular weight LPS and therefore longer OPS in the Rlv22 and EL196 mutants (*acpXL*$^-$ and *acpXL*$^-$/*lpxXL*$^-$) may be due to a requirement for increased hydrophobicity (provided by the OPS) during symbiosis that was lost due to the lack of the VLCFA. In the EL197 mutant (*acpXL*$^-$,*lpxXL*$^-$,*acpXL*$^+$) it appears that presence of AcpXL alleviates the requirement that bacteroid LPS be restricted to only those LPS molecules with the longer OPS. It may be that the membrane is stabilized by the presence of VLCFA on the untransferred AcpXL protein. Another possibility is that AcpXL has a function in the membrane other than carrying the VLCFA.

Our results for Rlv3841 show that mutants which are devoid of *acpXL* but still contain *lpxXL* produce lipid A species in which C16:0 or C18:0 can be added in place of the VLCFA while mutants devoid of *lpxXL* are unable to replace VLCFA with C16:0 or C18:0. This result has been previously shown for the *acpXL* mutant (Vedam et al. 2003). The mutants of the acp synthases (FabF1XL, FabF2XL) for the VLCFA of *Rhizobium leguminosarum* were previously

reported to have LPS that lack the VLCFA but had a palmitic or stearic acid in place of the VLCFA (Vedam et al. 2003; Vanderlinde et al. 2009). Similar results were also observed in *Sinorhizobium meliloti* for acyl carrier protein (AcpXL), acp synthases (FabF1XL, FabF2XL), and alcohol dehydrogenase (AdhA2XL) (Ferguson et al. 2005; Haag et al. 2011). The transferase, LpxXL, is likely required not only for transfer of the VLCFA but also the C16:0 or C18:0 to the lipid A as seen in the other Acp-VLCFA mutants. In *Sinorhizobium meliloti* the LpxXL transferase is also required for pentaacylated lipid A (Ferguson et al. 2005).

It has been previously reported that the *acpXL* mutant of *Rhizobium leguminosarum* is able to partially restore the addition of the VLCFA while in the plant nodules (Vedam et al. 2006). In addition to *Rhizobium leguminosarum,* VLCFA partial restoration was also observed for bacteroids of the *Sinorhizobium meliloti* AcpXL mutant (Haag et al. 2009). The restoration of the VLCFA was not observed in this work. Multiple attempts were conducted including varying factors such as method of growth (in flasks vs. cassions), bacteroid isolation (ultracentrifugation in sucrose gradients vs. washing crushed nodules with sucrose), and LPS extraction (hot water/phenol vs. TEA/EDTA/phenol). It is unknown why the restoration of VLCFA was not observed in this work under any of these conditions. VLCFA was detected in lipid A preparations from EL197 bacteroids. However, following MALDI analysis a lipid A structure containing the VLCFA was not detected indicating that this VLCFA was not a component of the lipid A. It is possible that the VLCFA was biosynthesized on the acyl carrier protein but without a functional transferase the VLCFA was unable to be transferred and remained on the ACP. This ACP-VLCFA may have been isolated along with LPS during the hot phenol-water extraction and then again with the lipid A during the mild acid hydrolysis.

When comparing laboratory cultured versus bacteroid lipid A structures a general shift towards more hydrophobic structures, i.e. toward more lipid A with longer chain fatty acyl residues, was observed. This increase in hydrophobicity is important in the nodule environment and symbiosis and may have an important influence on the interaction between plant and bacterial cell surface interactions during bacteroid differentiation (Kannenberg and Carlson 2001; Carlson et al. 2010). Lack of the VLCFA or palmitic/stearic acid bound to the lipid A of *Rhizobium leguminosarum* still allows for the symbiotic relationship to proceed, but attenuation of that relationship indicates that the VLCFA does influence symbiosis. This lipid A component may have importance for the rhizobia surviving with the rhizophere which has not yet been examined. The growth and symbiotic phenotypes of these mutants and the host pea plant nodules are elucidated in detail in Chapters 3 and 4.

Acknowledgements

The authors would like to thank Chris Raetz for supplying the pSSB-1 plasmid. This work was supported, in part, by National Institutes of Health Grant GM39583 (to R.W.C.) and by the Department of Energy Grant DE-FG-02-93ER20097 to the CCRC.

Table 2.1. Bacterial strains and plasmids used in this study.

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*^{q}*Z* ΔM15, ::Tn10(Tet^R)]	Stratagene Corp.
DH5α	Φ80d*lacZ* ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (rk-, mk+), *supE*44, *relA*1, *deoR*, Δ(*lacZYAargF*) U169	Stratagene Corp.
Rhizobium leguminosarum* bv. *viciae		
Rlv3841	Strain 300 Str^R, Fix$^+$	(VandenBosch et al. 1989)
Rlv22	Strain 3841 *acpXL::kan* Str^R, Kan^R, Fix$^+$	(Vedam et al. 2003)
EL196	Strain Rlv22 *lpxXL::gen* Str^R, Kan^R, Gen^R, Fix$^+$	This study
EL197	pVV5 (*acpXL*) in Strain EL196, Str^R, Kan^R, Gen^R, Tet^R, Fix$^+$	This study, (Vedam et al. 2003)
EL198	pSSB-1 (*acpXL,lpxXL*) in Strain EL196, Str^R, Kan^R, Gen^R, Tet^R, Fix$^+$	This study, (Basu et al. 2002)
Plasmids		
pSSB-1	pLAFR-1 with 22 kb *Rhizobium leguminosarum* genomic DNA (RL2810 – RL2832)	(Basu et al. 2002)
pBS II SK+	2.96 kb phagemid derived from pUC19, lacZ, Amp^R	Stratagene Corp.
pVV5	pVV1 derivative cloned into pBBR1MCS-3 containing *acpXL*	(Vedam et al. 2003)
pDVB1	pBS SK derivative with 2.6 kb insert from pSSB-1 containing *lpxXL*	This study
pDVB2	pDVB1 containing aacC1 cassette from pMS255	This study
pDVB3	pDVB2 derivative cloned into pEx18Tc	This study
pMS255	*Gen* resistance cassette in pSVB30	(Becker et al. 1995)
pEX18Tc	Broad host range suicide vector, Tc^R *sacB*$^+$	(Hoang et al. 1998)
pRK600	Mobilizing plasmid	(Finan et al. 1986)
pRK2013	Mobilizing plasmid	(Ditta et al. 1980)

Table 2.2 Lipid A relative fatty acid compositions of major fatty acid constituents from Rlv3841, Rlv22, EL196, and EL197 free-living bacteria, bacteroids, and ex-nodule bacteria.

	Fatty Acid Percentages[1]							
			3OH					27OH
	C16:0	C18:0	C14:0	C15:0	C16:0	C18:0	C18:1	C28:0
Strain								
Rlv3841	4	5	46	2	9	13	4	16
Rlv22	2	nd	57	7	13	18	1	nd
EL196	1	1	57	5	12	22	3	nd
EL197	1	1	53	5	12	24	4	nd
Rlv3841 Bac	8	9	20	4	4	8	7	41
Rlv22 Bac	14	11	36	12	6	9	11	nd
EL196 Bac	21	26	26	11	5	5	6	nd
EL197 Bac	1	3	30	4	12	41	6	3*
Rlv3841 ExN	6	12	37	2	7	9	2	25
Rlv22 ExN	4	4	43	4	13	29	4	nd
EL196 ExN	2	14	32	10	11	29	2	nd
EL197 ExN	3	17	46	9	9	15	2	nd

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

[1] Fatty acid percentages are calculated based on the ratios of GC area values.

Table 2.3 MALDI-TOF major ions of the lipid A from laboratory cultures of *Rhizobium leguminosarum* Rlv3841, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$,*lpxXL*$^-$), and EL197 (*acpXL*$^-$,*lpxXL*$^-$,*acpXL*$^+$) and their ExN isolates, and from their bacteroid preparations isolated from pea nodules.

Structure	Obs.	Calc.	Proposed Composition[a]	Strain							
Figure 2.9	$[M-H]^-$	$[M-H]^-$		Rlv3841	Bac	Rlv22	Bac	EL196	Bac	EL197	Bac
I.A[b]	1870.5	1870.4	GalAGlcN(3OHC14:0)$_3$(3OHC16:0)$_1$ (27OHC28:0)$_1$	+	-	-	-	-	-	-	-
	1898.5	1898.4	GalAGlcN(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$	+	+	-	-	-	-	-	-
	1956.5	1956.4	GalAGlcN(3OHC14:0)$_3$(3OHC16:0)$_1$ (27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	1984.6	1984.5	GalAGlcN(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	1998.7	1998.5	GalAGlcN(3OHC14:0)$_2$(3OHC15:0)$_1$ (3OHC18:0)$_1$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	2012.7	2012.5	GalAGlcN(3OHC14:0)$_2$(3OHC16:0)$_1$ (3OHC18:0)$_1$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-

	2054.7	2054.5	GalAGlcN(3OHC14:0)$_1$(3OHC15:0)$_1$ (3OHC18:0)$_2$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	2068.7	2068.6	GalAGlcN(3OHC14:0)$_1$(3OHC16:0)$_1$ (3OHC18:0)$_2$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
I.B	1886.5	1886.4	GalAGlcNonate(3OHC14:0)$_3$(3OHC16:0)$_1$ (27OHC28:0)$_1$	+	-	-	-	-	-	-	-
	1914.5	1914.4	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$	+	+	-	-	-	-	-	-
	1936.5	1936.4	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$(Na)	+	+	-	-	-	-	-	-
	2000.5	2000.4	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	2022.5	2022.4	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (27OHC28:0)$_1$(3OHC4:0)$_1$(Na)	+	+	-	-	-	-	-	-
II.	1624.3	1624.2	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_2$ (3OHC16:0)$_1$(27OHC28:0)$_1$	+	+	-	-	-	-	-	-
	1652.3	1652.2	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_2$	+	+	-	-	-	-	-	-

		$(3OHC18:0)_1(27OHC28:0)_1$								
1710.3	1710.2	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_2$	+	+	-	-	-	-	-	-
		$(3OHC16:0)_1(27OHC28:0)_1(3OHC4:0)_1$								
1724.5	1724.2	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	-	-	-	-	-	-
		$(3OHC15:0)_1(3OHC16:0)_1(27OHC28:0)_1$								
		$(3OHC4:0)_1$								
1738.4	1738.2	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_2$	+	+	-	-	-	-	-	-
		$(3OHC18:0)_1(27OHC28:0)_1(3OHC4:0)_1$								
1752.5	1752.3	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	-	-	-	-	-	-
		$(3OHC15:0)_1(3OHC18:0)_1(27OHC28:0)_1$								
		$(3OHC4:0)_1$								
1766.5	1766.3	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	-	-	-	-	-	-
		$(3OHC16:0)_1(3OHC18:0)_1(27OHC28:0)_1$								
		$(3OHC4:0)_1$								
1780.4	1780.3	GalAGlcN2eneGlcNonolactone$(3OHC15:0)_1$	+	+	-	-	-	-	-	-
		$(3OHC16:0)_1(3OHC18:0)_1(27OHC28:0)_1$								
		$(3OHC4:0)_1$								

	1794.4	1794.3	GalAGlcN2eneGlcNonolactone(3OHC16:0)$_2$ (3OHC18:0)$_1$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	1808.5	1808.3	GalAGlcN2eneGlcNonolactone(3OHC15:0)$_1$ (3OHC18:0)$_2$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
	1822.4	1822.3	GalAGlcN2eneGlcNonolactone(3OHC16:0)$_1$ (3OHC18:0)$_2$(27OHC28:0)$_1$(3OHC4:0)$_1$	+	+	-	-	-	-	-	-
III.A	1448.1	1448.0	GalAGlcN(3OHC14:0)$_3$(3OHC16:0)$_1$	+	-	+	-	-	-	+	+
	1476.1	1476.0	GalAGlcN(3OHC14:0)$_3$(3OHC18:0)$_1$	+	-	+	-	+	+	+	+
	1504.1	1504.0	GalAGlcN(3OHC14:0)$_2$(3OHC16:0)$_1$ (3OHC18:0)$_1$	-	-	-	-	+	+	+	+
	1532.2	1532.1	GalAGlcN(3OHC14:0)$_2$(3OHC18:0)$_2$	-	-	-	-	+	+	+	+
	1560.2	1560.1	GalAGlcN(3OHC14:0)$_1$(3OHC16:0)$_1$ (3OHC18:0)$_2$	-	-	-	-	-	+	+	+
III.B	1464.0	1464.0	GalAGlcNonate(3OHC14:0)$_3$(3OHC16:0)$_1$	+	-	+	+	+	+	+	+
	1492.1	1492.0	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$	+	-	+	+	+	+	+	+
	1513.9	1513.9	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (Na)	-	-	+	-	-	-	-	-

	1520.0	1520.0	GalAGlcNonate(3OHC14:0)$_2$(3OHC16:0)$_1$ (3OHC18:0)$_1$	-	-	+	+	+	+	+	+
	1548.0	1548.1	GalAGlcNonate(3OHC14:0)$_2$(3OHC18:0)$_2$	-	-	+	+	+	+	+	+
	1576.3	1576.1	GalAGlcNonate(3OHC14:0)$_1$(3OHC16:0)$_1$ (3OHC18:0)$_2$	-	-	-	-	-	-	-	+
	1577.9	1578.0	GalAGlcNonate(3OHC14:0)$_1$(3OHC15:0)$_1$ (3OHC16:0)$_1$(3OHC18:0)$_1$(Na)$_2$	-	-	+	+	-	-	-	-
IV.	1173.8	1173.7	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_3$	+	-	+	+	+	-	+	+
	1201.8	1201.8	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_2$ (3OHC16:0)$_1$	+	-	+	-	+	-	+	+
	1229.9	1229.8	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_2$ (3OHC18:0)$_1$	+	-	+	+	+	+	+	+
	1245.8	1245.8	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_2$ (3OHC18:0)$_1$(OH)	+	+	-	-	-	-	-	-
	1257.9	1257.8	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_1$ (3OHC16:0)$_1$(3OHC18:0)$_1$	+	+	+	+	+	+	+	+
	1259.9	1259.8	GalAGlcN2eneGlcNonolactone(3OHC14:0)$_1$	-	+	-	-	-	-	-	-

			$(3OHC15:0)_1(3OHC18:0)_1(OH)$										
	1273.9	1273.8	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	-	+	-	-	-	+
			$(3OHC16:0)_1(3OHC18:0)_1(OH)$										
	1286.0	1285.8	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	+	+	+	+	+	+
			$(3OHC18:0)_2$										
	1301.9	1301.8	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	-	+	-	+	-	-	-	+
			$(3OHC18:0)_2(OH)$										
	1323.9	1323.8	GalAGlcN2eneGlcNonolactone$(3OHC14:0)_1$	+	+	-	+	-	-	-	+
			$(3OHC18:0)_2(OH)(Na)$										
V.A	1742.0	1742.3	GalAGlcN$(3OHC14:0)_2(3OHC16:0)_1$	-	-	-	+	-	-	-	-
			$(3OHC18:0)_1(C16:0)_1$										
	1770.1	1770.3	GalAGlcN$(3OHC14:0)_2(3OHC16:0)_1$	-	-	-	+	-	-	-	-
			$(3OHC18:0)_1(C18:0)_1$										
	1798.1	1798.3	GalAGlcN$(3OHC14:0)_2(3OHC18:0)_2(C16:0)_1$	-	-	-	+	-	-	-	-
	1826.2	1826.4	GalAGlcN$(3OHC14:0)_2(3OHC18:0)_2(C18:0)_1$	-	-	-	+	-	-	-	-
V.B	1730.1	1730.2	GalAGlcNonate$(3OHC14:0)_3(3OHC18:0)_1$	-	-	+	+	-	-	-	-
			$(C16:0)_1$										

1758.2	1758.3	GalAGlcNonate(3OHC14:0)$_3$(3OHC18:0)$_1$ (C18:0)$_1$	-	-	+	+	-	-	-	-
1786.2	1786.3	GalAGlcNonate(3OHC14:0)$_2$(3OHC16:0)$_1$ (3OHC18:0)(C16:0)$_1$	-	-	+	+	-	-	-	-
1814.1	1814.3	GalAGlcNonate(3OHC14:0)$_2$(3OHC16:0)$_1$ (3OHC18:0)(C18:0)$_1$	-	-	-	+	-	-	-	-
1814.1	1814.3	GalAGlcNonate(3OHC14:0)$_2$(3OHC18:0)$_2$ (C16:0)$_1$	-	-	-	+	-	-	-	-
1842.2	1842.4	GalAGlcNonate(3OHC14:0)$_2$(3OHC18:0)$_2$ (C18:0)$_1$	-	-	-	+	-	-	-	-

[a] The proposed structures were derived from the calculated mass-to-charge ratios from monoisotopic $[M-H]^-$ ions from negative reflecton mode. The proposed structure for a single ion may be due to different fatty acyl substitutions that would have the same calculation and the structures proposed may not include all possible combinations.

[b] Proposed structures are shown in Figure 2.9.

Figure Legends

Figure 2.1 A. Structures of the Lipid A of *Escherichia coli* and *Rhizobium leguminosarum*. B. Schematic of the gene region present in *Rhizobium leguminosarum* required for the biosynthesis of VLCFA onto the acyl carrier protein and transfer to the lipid A during LPS biosynthesis.

Figure 2.2 Schematic for the cloning of plasmids used for *lpxXL* mutant development. The first plasmid pDVB1 was created by insertion of a DNA fragment containing the *lpxXL* gene into pBluescript. A gentamicin cassette was inserted into the *lpxXL* gene creating pDVB2 and a DNA region containing the interrupted *lpxXL* gene was inserted into the broad host range vector pEx18Tc creating pDVB3 to be used for mutant development.

Figure 2.3 PCR to confirm the presence of mutations. A) Primers for *acpXL* gene. Lanes 1 and 4 show 177 bp fragment indicating wild type *acpXL.* Lanes 2 and 5 show 1454 bp fragment indicating the *acpXL* gene with kanamycin cassette insert. Lane 3 is a negative control where the *acpXL* gene is not present. B) Primers for *lpxXL* gene. Lanes 1, 2, and 4 show 725 bp fragment indicating wild type *lpxXL.* Lanes 3 and 5 show 1425 bp fragment indicating the *lpxXL* gene with gentamicin cassette insert. C) Primers for *acpXL* gene and *lpxXL* genes for EL197 mutant. Lane 1 shows a 177 bp fragment indicating wild type *acpXL.* Lane 2 shows a 1425 bp fragment indicating the *lpxXL* gene with gentamicin cassette insert.

Figure 2.4 Alcian blue stained DOC-PAGE gels. Separation of water layer lyophilized LPS from phenol/water extractions. Section 1 shows separation of Rlv3841 free-living LPS compared to Rlv22 free-living, bacteroid, and ex-nodule LPS. Section 2 shows separation of Rlv3841 free-

living LPS compared to EL196 free-living, bacteroid, and ex-nodule LPS. Section 3 shows separation of Rlv3841 free-living LPS compared to EL197 free-living, bacteroid, and ex-nodule LPS. Section 4 shows separation of Rlv3841 bacteroid and EL198 free-living LPS for banding pattern comparison. In each sample well 1 μg dry weight of lyophilized LPS material from the water layer of phenol/water extractions in 1 μL loading buffer was added.

Figure 2.5 MALDI-TOF MS spectrum of the lipid A from Rlv3841 laboratory culture (top) and bacteroids (bottom). The ions in the blue boxes, generally, have longer fatty acyl chains than in the red boxes. Each cluster of ions corresponds to a lipid A structure depicted in Figure 2.9. Ions between 1173.3 and 1323.9 m/z represent Structure IV. Ions between 1448.1 and 1492.1 m/z represent Structure III. Ions between 1624.3 and 1822.4 m/z represent Structure II. Ions between 1870.5 and 1068.7 m/z represent Structure IV.

Figure 2.6 MALDI-TOF MS spectrum of the lipid A from Rlv22 (*acpXL*$^{-}$) laboratory culture (top) and bacteroids (bottom). The ions in the blue boxes, generally, have longer fatty acyl chains than in the red boxes. Each cluster of ions corresponds to a lipid A structure depicted in Figure 2.9. Ions between 1173.7 and 1323.9 m/z represent Structure IV. Ions between 1447.9 and 1577.9 m/z represent Structure III. Ions between 1730.1 and 1842.2 m/z represent Structure V.

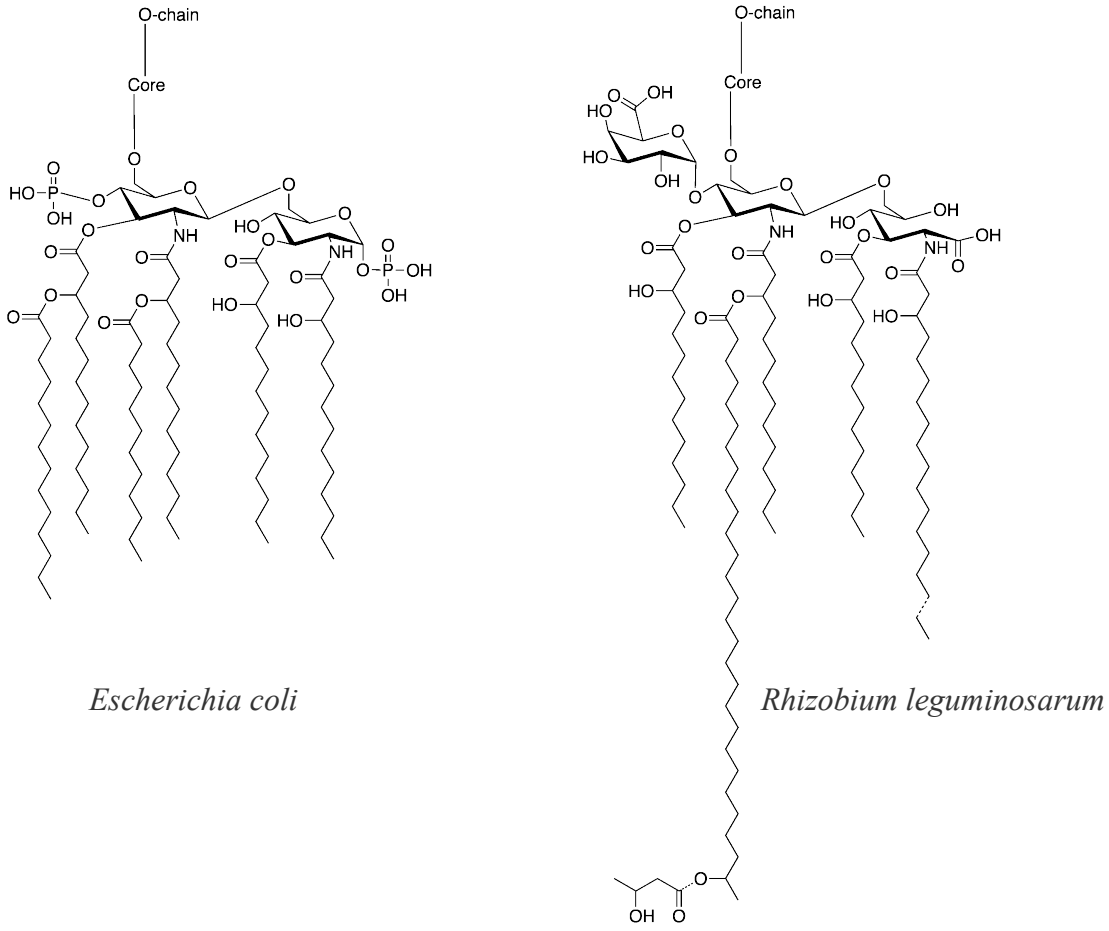
Figure 2.7 MALDI-TOF MS spectrum of the lipid A from EL196 (*acpXL*$^{-}$/*lpxXL*$^{-}$) laboratory culture (top) and bacteroids (bottom). The ions in the blue boxes, generally, have longer fatty acyl chains than in the red boxes. Each cluster of ions corresponds to a lipid A structure depicted

in Figure 2.9. Ions between 1173.6 and 1285.9 m/z represent Structure IV. Ions between 1464.3 and 1560.6 m/z represent Structure III.

Figure 2.8 MALDI-TOF MS spectrum of the lipid A from EL197 (*acpXL*$^{-}$/*lpxXL*$^{-}$/*acpXL*$^{+}$) laboratory culture (top) and bacteroids (bottom). The ions in the blue boxes, generally, have longer fatty acyl chains than in the red boxes. Each cluster of ions corresponds to a lipid A structure depicted in Figure 2.9. Ions between 1173.7 and 1323.9 m/z represent Structure IV. Ions between 1448.0 and 1576.3 m/z represent Structure III.

Figure 2.9 Proposed lipid A structures for ions from MALDI-TOF analysis (Figures 2.5-2.8) of Rlv3841, Rlv22 (*acpXL*$^{-}$), EL196 (*acpXL*$^{-}$/*lpxXL*$^{-}$), and EL197 (*acpXL*$^{-}$/*lpxXL*$^{-}$/*acpXL*$^{+}$) free-living and bacteroid lipid A. Structures I.A, I.B, II, III.A, III.B, and IV were observed in Rlv3841 free-living lipid A. Structures I.A, I.B, II, and IV were observed in Rlv3841 bacteroid lipid A. Structures III.A, III.B, IV, V.A, and V.B were observed in Rlv22 free-living and bacteroid lipid A. Structures III.A, III.B, and IV were observed in EL196 and EL197 free-living and bacteroid lipid A. Lipid A structure proposed compositions are given in Table 2.3.

A.

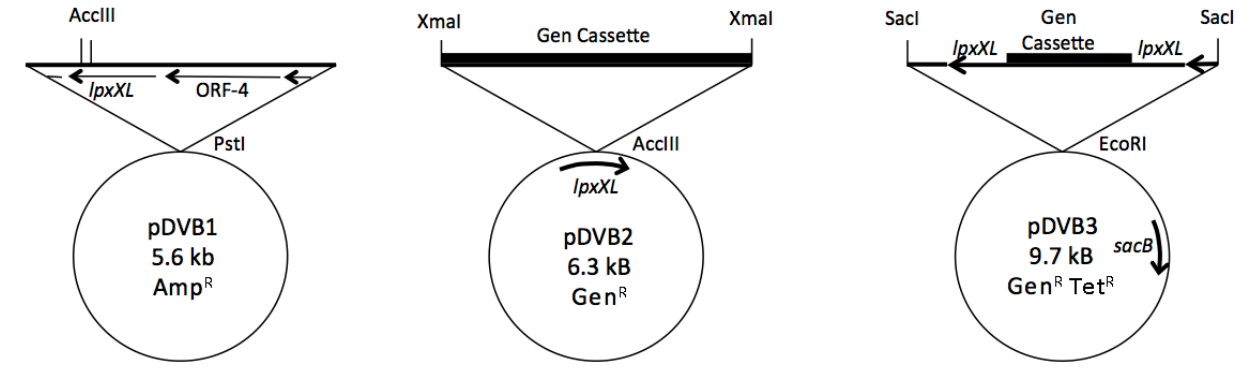


Escherichia coli *Rhizobium leguminosarum*

B.

- *acpXL* - Lipid A 27OHC28:0 acyl carrier protein
- *fabZXL* - Hydroxyacyl-(*acpXL*) dehydratase
- *fabF2XL* - 3-oxoacyl-(*acpXL*) synthase
- *fabF1XL* - 3-oxoacyl-(*acpXL*) synthase
- *adhA2XL* - Alcohol dehydrogenase
- *lpxXL* - Lipid A 27OHC28:0 acyl transferase

Figure 2.1



lpxXL in pBS II SK+ *lpxXL:Gm* in pBS II SK+ *lpxXL:Gm* in pEx18Tc

Figure 2.2

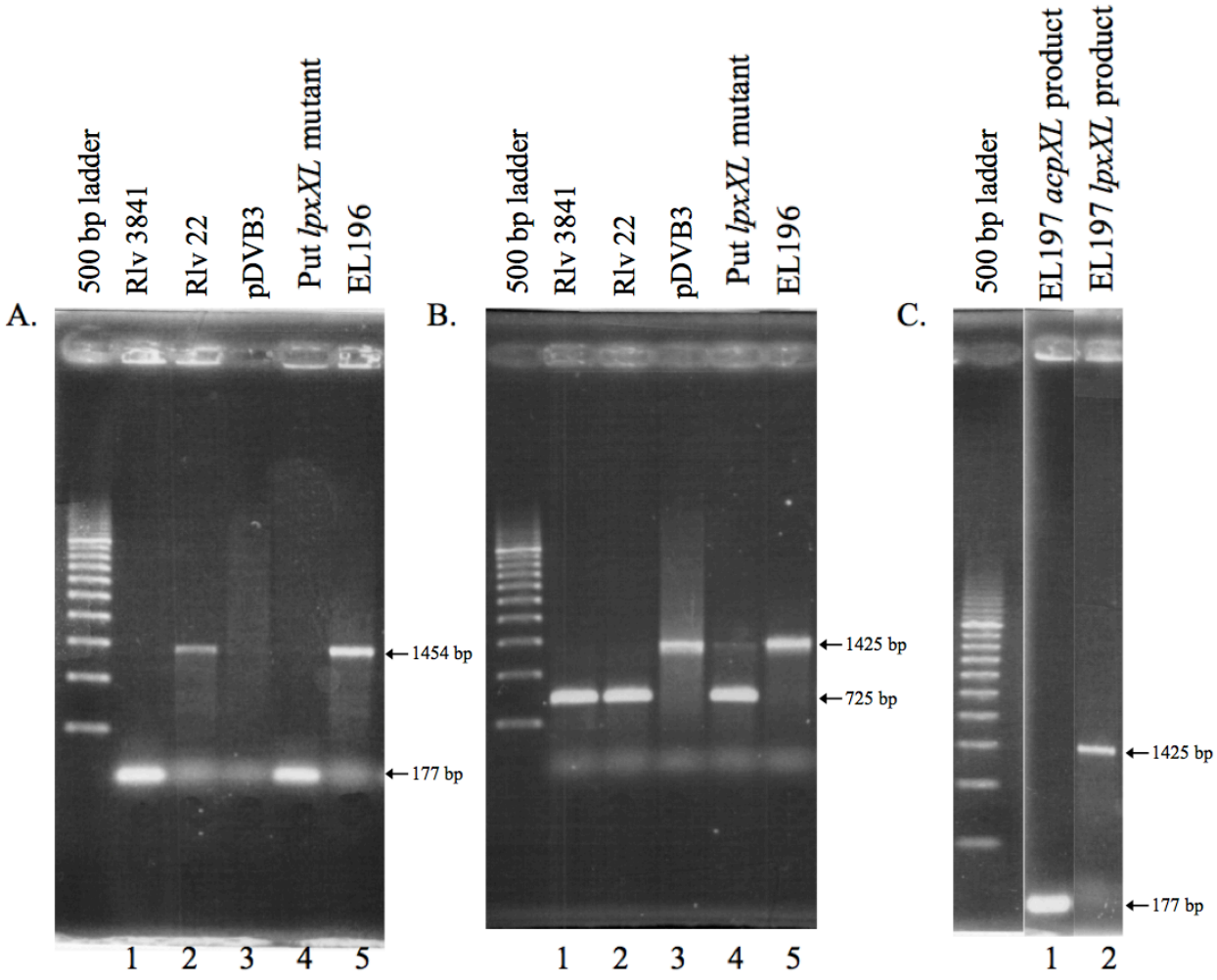

A.
500 bp ladder
Rlv 3841
Rlv 22
pDVB3
Put lpxXL mutant
EL196
1454 bp
177 bp
1 2 3 4 5
B.
500 bp ladder
Rlv 3841
Rlv 22
pDVB3
Put lpxXL mutant
EL196
1425 bp
725 bp
1 2 3 4 5
C.
500 bp ladder
EL197 acpXL product
EL197 lpxXL product
1425 bp
177 bp
1 2

Figure 2.3

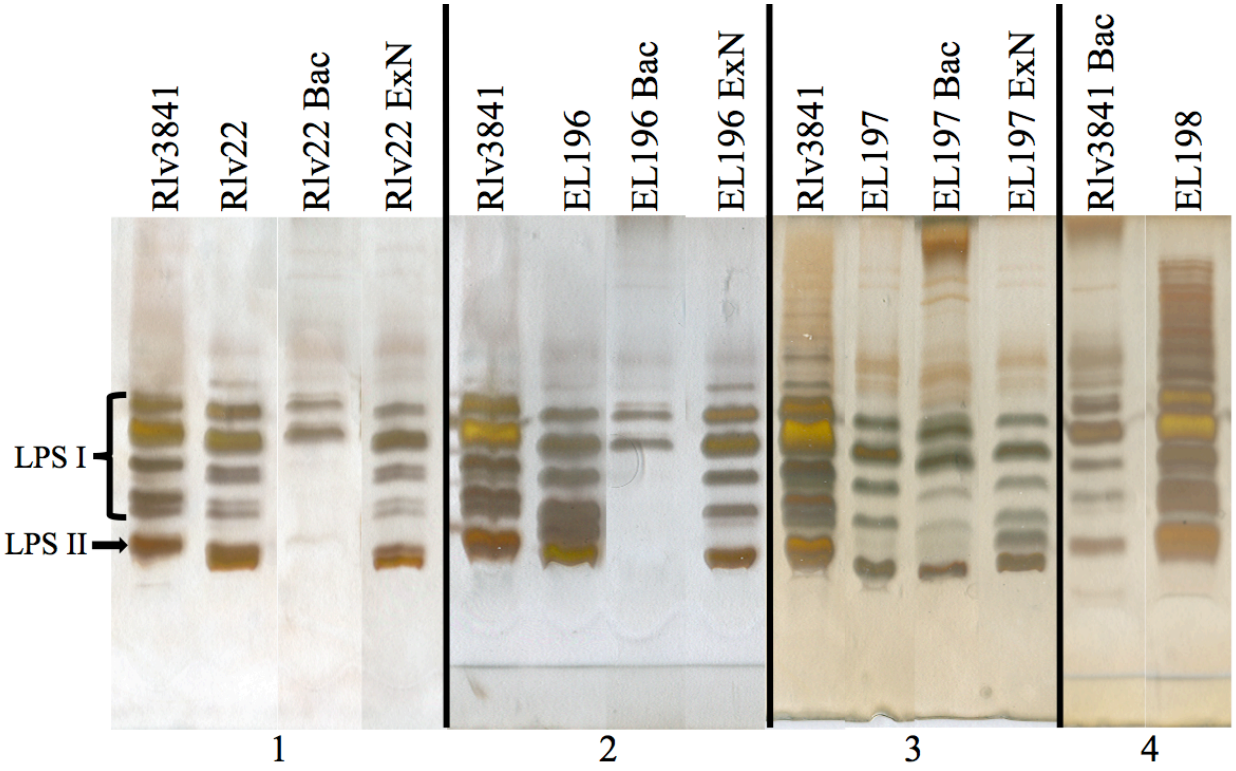

Rlv3841
Rlv22
Rlv22 Bac
Rlv22 ExN
Rlv3841
EL196
EL196 Bac
EL196 ExN
Rlv3841
EL197
EL197 Bac
EL197 ExN
Rlv3841 Bac
EL198
LPS I
LPS II
1
2
3
4

Figure 2.4

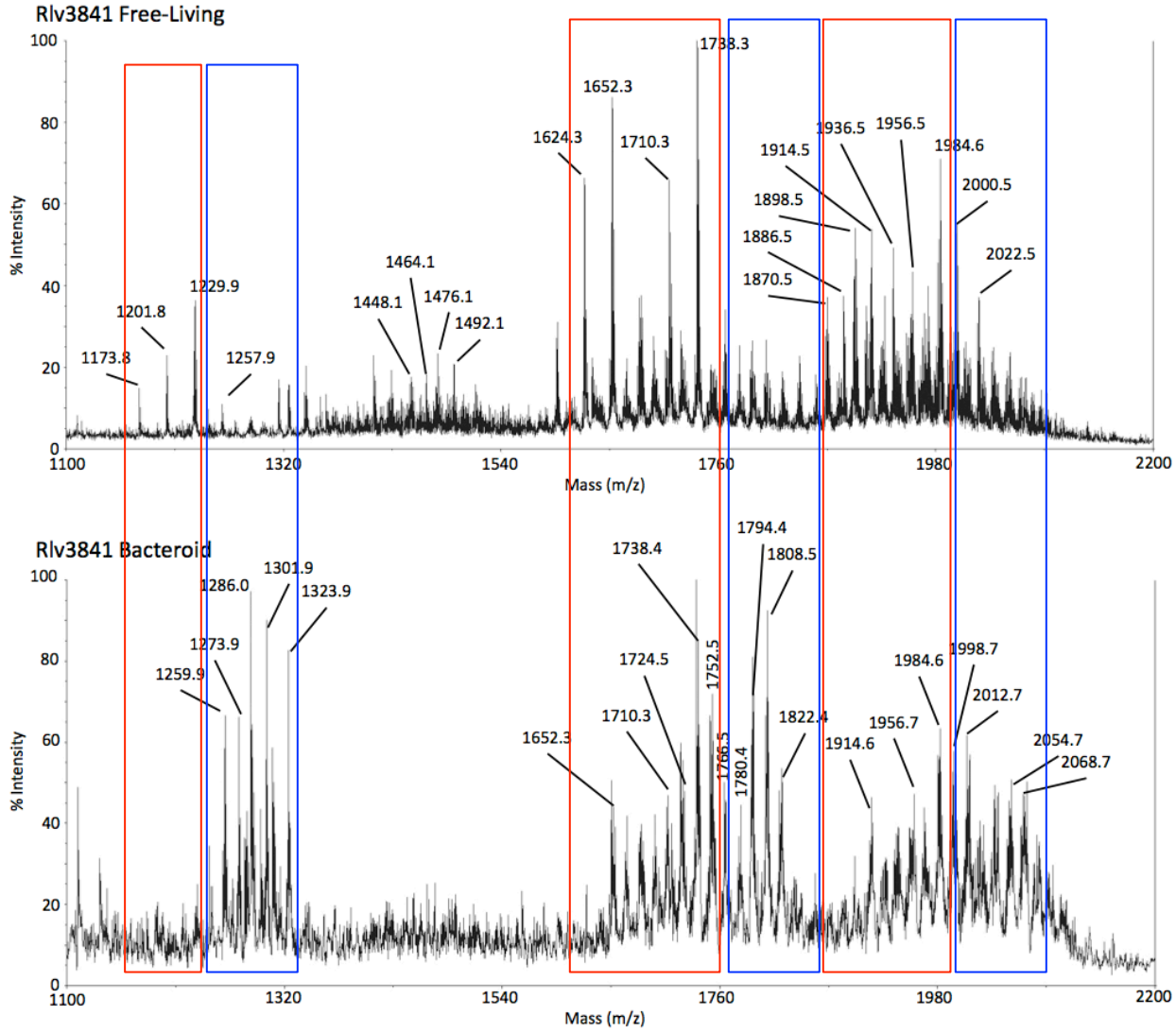

Rlv3841 Free-Living
% Intensity
Mass (m/z)
1100
1320
1540
1760
1980
2200
1173.8
1201.8
1229.9
1257.9
1448.1
1464.1
1476.1
1492.1
1624.3
1652.3
1710.3
1738.3
1870.5
1886.5
1898.5
1914.5
1936.5
1956.5
1984.6
2000.5
2022.5
Rlv3841 Bacteroid
1259.9
1273.9
1286.0
1301.9
1323.9
1652.3
1710.3
1724.5
1738.4
1752.5
1766.5
1780.4
1794.4
1808.5
1822.4
1914.6
1956.7
1984.6
1998.7
2012.7
2054.7
2068.7

Figure 2.5

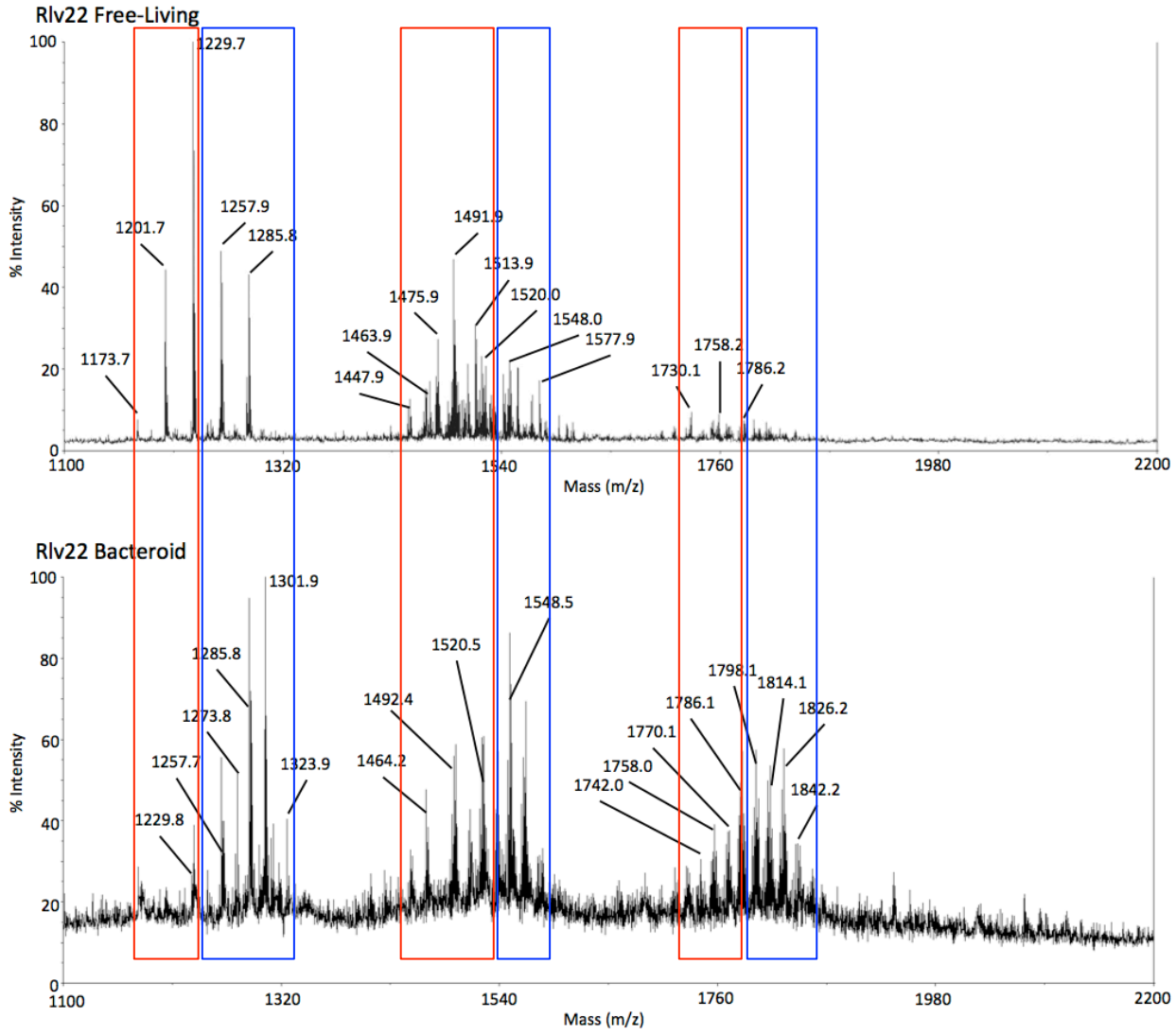

Rlv22 Free-Living
% Intensity
Mass (m/z)
1229.7
1201.7
1257.9
1285.8
1491.9
1513.9
1475.9
1520.0
1548.0
1463.9
1577.9
1758.2
1730.1
1786.2
1173.7
1447.9
Rlv22 Bacteroid
% Intensity
Mass (m/z)
1301.9
1548.5
1285.8
1520.5
1798.1
1814.1
1786.1
1826.2
1273.8
1492.4
1770.1
1257.7
1323.9
1464.2
1758.0
1742.0
1842.2
1229.8

Figure 2.6

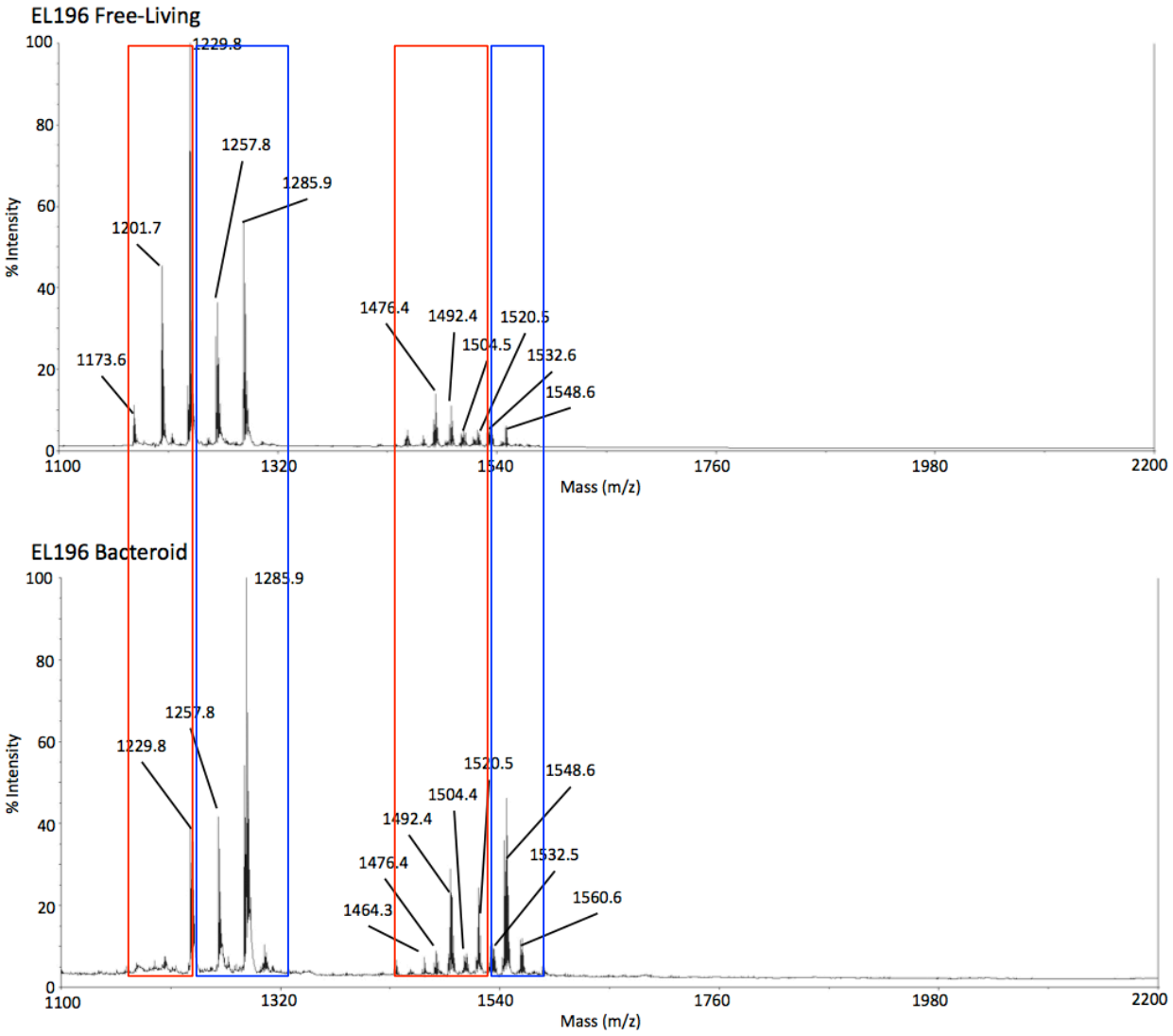

EL196 Free-Living
% Intensity
Mass (m/z)
1229.8
1257.8
1285.9
1201.7
1173.6
1476.4
1492.4
1520.5
1504.5
1532.6
1548.6
EL196 Bacteroid
1285.9
1257.8
1229.8
1520.5
1548.6
1504.4
1492.4
1532.5
1476.4
1560.6
1464.3

Figure 2.7

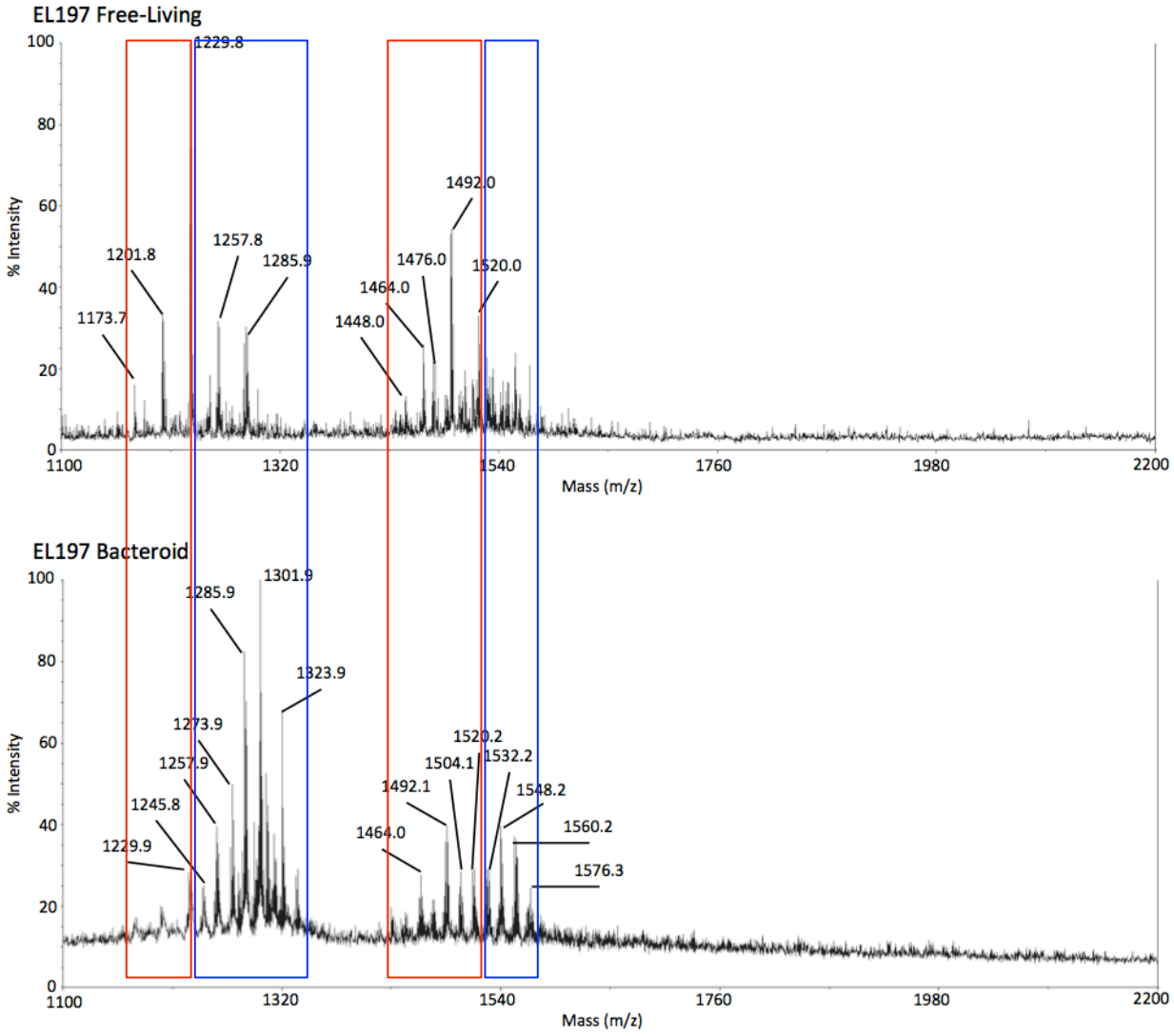

EL197 Free-Living
% Intensity
Mass (m/z)
1229.8
1201.8
1257.8
1285.9
1173.7
1492.0
1476.0
1520.0
1464.0
1448.0
EL197 Bacteroid
1301.9
1285.9
1323.9
1273.9
1257.9
1245.8
1229.9
1520.2
1504.1
1532.2
1492.1
1548.2
1560.2
1464.0
1576.3

Figure 2.8

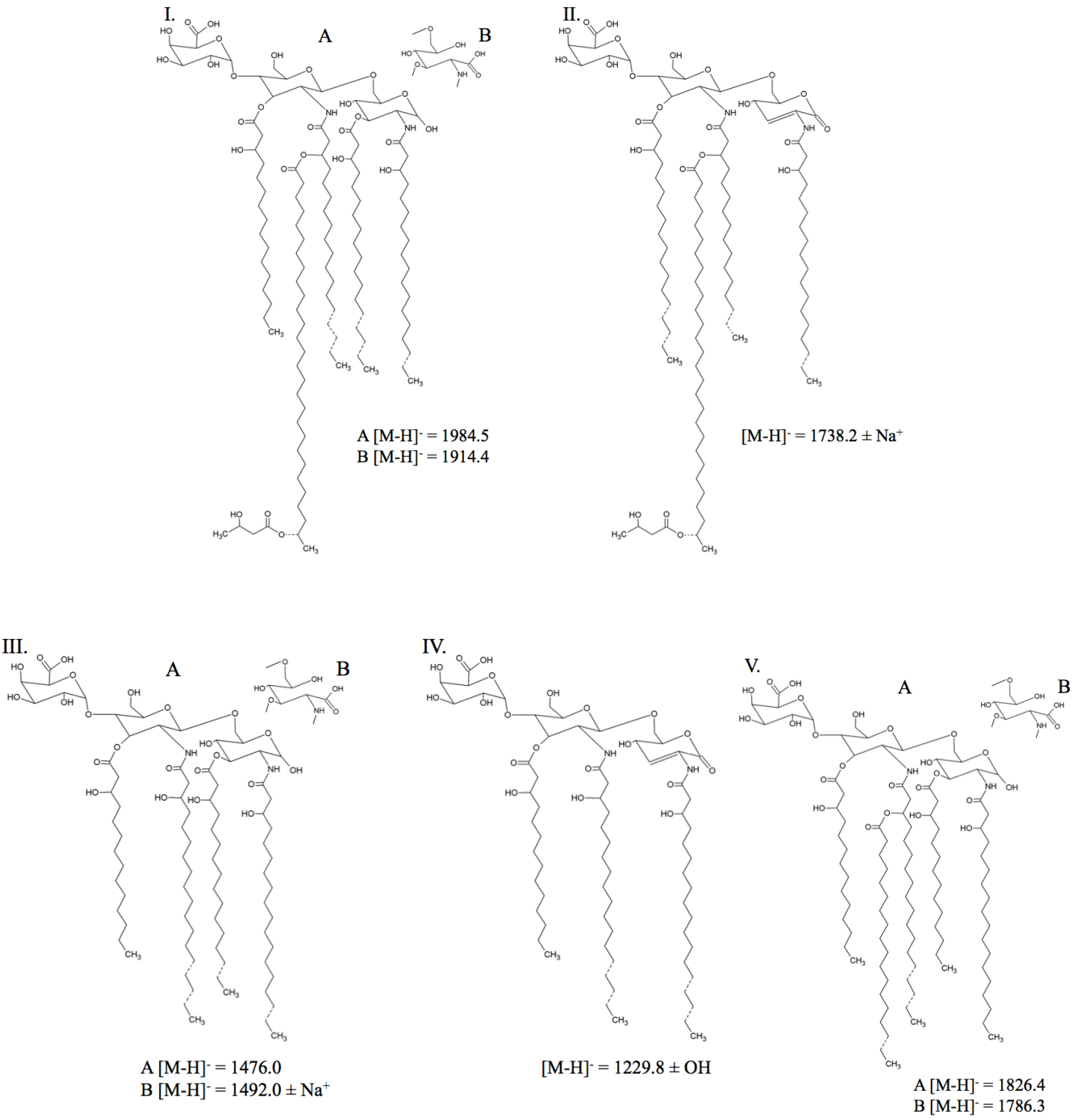

I.
A
B
A [M-H]- = 1984.5
B [M-H]- = 1914.4
II.
[M-H]- = 1738.2 ± Na+
III.
A
B
A [M-H]- = 1476.0
B [M-H]- = 1492.0 ± Na+
IV.
[M-H]- = 1229.8 ± OH
V.
A
B
A [M-H]- = 1826.4
B [M-H]- = 1786.3

Figure 2.9

References

Ardissone, S., Kobayashi, H., Kambara, K., Rummel, C., Noel, K. D., Walker, G. C., Broughton, W. J., and Deakin, W. J. (2011) Role of BacA in lipopolysaccharide synthesis, peptide transport, and nodulation by *Rhizobium* sp. strain NGR234. *J. Bacteriol.,* 193: 2218-2228.

Bhagwat, A. A., Mithofer, A., Pfeffer, P. E., Kraus, C., Spickers, N., Hotchkiss, A., Ebel, J., and Keister, D. L. (1999) Further studies of the role of cyclic β-glucans in symbiosis. An *ndvC* mutant of *Bradyrhizobium japonicum* synthesizes cyclodecakis-(1-3)-β-glucosyl. *Plant Physiol.,* 119: 1057-1064.

Basu, S. S., Karbarz, M. J., and Raetz, C. R. (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum.* *J. Biol. Chem.,* 277: 28959-28971.

Becker, A., Schmidt, M., Jager, W., and Puhler, A. (1995) New gentamicin-resistance and *lacZ* promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. *Gene,* 162: 37-39.

Bhat, U. R., Carlson, R. W., Busch, M., and Mayer, H. (1991) Distribution and phylogenetic significance of 27-hydroxy-octacosanoic acid in lipopolysaccharides from bacteria belonging to the alpha-2 subgroup of Proteobacteria. *Int. J. Syst. Bacteriol.,* 41: 213-217.

Bhat, U. R., Forsberg, L. S., and Carlson, R. W. (1994) Structure of lipid A component of *Rhizobium leguminosarum* bv. *phaseoli* lipopolysaccharide. Unique nonphosphorylated lipid A containing 2-amino-2-deoxygluconate, galacturonate, and glucosamine. *J. Biol. Chem.,* 269: 14402-14410.

Buchanan-Wollaston, V. (1979) Generalized transduction in *Rhizobium leguminosarum*. *Microbiol.,* 112: 135-142.

Carlson, R. W., Forsberg, L. S., and Kannenberg, E. L. (2010) Lipopolysaccharides in *Rhizobium*-legume symbioses. In P. J. Quinn and X. Wang (eds.), *Subcellular Biochemistry: Endotoxins: Structure, function, and recognition* (Springer), 339-386.

Caroff, M., Tacken, A., and Szabo, L. (1988) Detergent-accelerated hydrolysis of bacterial endotoxins and determination of the anomeric configuration of the glycosyl phosphate present in the "isolated lipid A" fragment of the *Bordetella pertussis* endotoxin. *Carbohydr. Res.,* 175: 273-282.

Corbel, M. J. (1997) Brucellosis: an overview. *Emerg. Infect. Dis.,* 3: 213-221.

Demont-Caulet, N., Maillet, F., Tailler, D., Jacquinet, J., Prome, J., Nicolaou, K. C., Truchet, G., Beau, J., and Denarie, J. (1999) Nodule-inducing activity of synthetic *Sinorhizobium meliloti* nodulation factors and related lipo-chitooligosaccharides on alfalfa. Importance of the acyl chain structure. *Plant Physiol.*, 120: 83-92.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *P. Natl. Acad. Sci. USA,* 77: 7347-7351.

Ferguson, G. P., Datta, A., Carlson, R. W., and Walker, G. C. (2005) Importance of unusually modified lipid A in *Sinorhizobium* stress resistance and legume symbiosis. *Mol. Microbiol.,* 56: 68-80.

Haag, A. F., Wehmeier, S., Beck, S., Marlow, V. L., Fletcher, V., James, E. K., and Ferguson, G. P. (2009) The *Sinorhizobium meliloti* LpxXL and AcpXL proteins play important roles in bacteroid development within alfalfa. *J. Bacteriol.,* 191: 4681-4686.

Haag, A. F., Wehmeier, S., Muszynski, A., Kerscher, B., Fletcher, V., Berry, S. H., Hold, G. L., Carlson, R. W., and Ferguson, G. P. (2011) Biochemical characterization of *Sinorhizobium meliloti* mutants reveals gene products involved in the biosynthesis of the unusual lipid A very long-chain fatty acid. *J. Biol. Chem.,* 286: 17455-17466.

Hoang, T. T., Kakhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer H. P. (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomanas aeruginosa* mutants. *Gene,* 212: 77-96.

Kannenberg, E. L., Reuhs, B. L., Forsberg, S., and Carlson, R. W. (1998) Lipopolysaccharides and K-antigens: Their structures, biosynthesis, and function. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (eds.), *The Rhizobiaceae; Molecular Biology of Model Plant-Associated Bacteria* (Dordrecht/Boston/London: Kluwer Academic Publishers), 119-154.

Kannenberg, E. L. and Carlson, R. W. (2001) Lipid A and O-chain modifications cause *Rhizobium* lipopolysaccharides to become hydrophobic during bacteroid development. *Mol. Microbiol.,* 39: 379-391.

Kennedy, E. P. (1982) Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *P. Natl. Acad. Sci. USA,* 79: 1092-1095.

Lerouge, P., Roche, P., Raucher, C., Maillet, F., Truchet, G., Prome, J. C., and Denarie, J. (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature*, 344: 781-784.

Niehaus, K., Kapp, D., and Puhler, A. (1993) Plant defense and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS-I)-deficient *Rhizobium meliloti* mutant. *Planta*, 190: 415-425.

Nutman, P. S. (1970) Appendix III: The modified Fahraeus slide technique. In J. M. Vincent (ed.), *A manual for the practical study of root-nodule bacteria* (Oxford and Edinburgh: Blackwell Scientific Publications), 144-145.

Parniske, M., Schmidt, P. E., Kosch, K., and Muller, P. (1994) Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. *Mol. Plant Microbe Interact.,* 7: 631-638.

Perotto, S., Brewin, N. J., and Kannenberg, E. L. (1994) Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. *Mol. Plant Microbe Interact.,* 7: 99-112.

Price, N. P., Jeyaretnam, B., Carlson, R. W., Kadrmas, J. L., Raetz, C. R., and Brozek, K. A. (1995) Lipid A biosynthesis in Rhizobium leguminosarum: role of a 2-keto-3-deoxyoctulosonate-activated 4' phosphatase. *P. Natl. Acad. Sci. USA,* 92: 7352-7356.

Reuhs, B. L., Carlson, R. W., and Kim, J. S. (1993) *Rhizobium fredii* and *Rhizobium meliloti* produce 3-deoxy-D-manno-2-octulosonic acid-containing polysaccharides that are structurally analogous to group II K antigens (capsular polysaccharides) found in *Escherichia coli*. *J. Bacteriol.,* 175: 3570-3580.

Sharypova, L. A., Niehaus, K., Scheidle, H., Holst, O., and Becker, A. (2003) *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does

not express a slow migrating form of lipopolysaccharide. *J. Biol. Chem.,* 278: 12946-12954.

Vanderlinde, E. M., Harrison, J. J., Muszynski, A., Carlson, R. W., Turner, R. J., and Yost, C. K. (2009) Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. *FEMS Microbiol. Ecol*. 71: 327-340.

Vanderlinde, E. M. and Yost, C. K. (2012) Genetic analysis reveals links between lipid A structure and expression of the outer membrane protein gene, *ropB*, in *Rhizobium leguminosarum. FEMS Microbiol. Lett.,* 335: 130-139.

Vedam, V., Kannenberg, E. L., Haynes, J. G., Sherrier, D. J., Datta, A., and Carlson, R. W. (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J. Bacteriol.,* 185: 1841-1850.

Vedam, V., Haynes, J. G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. (2004) A *Rhizobium leguminosarum* lipopolysaccharide lipid-A mutant induces nitrogen-fixing nodules with delayed and defective bacteroid formation. *Mol. Plant Microbe Interact.,* 17: 283-291.

Vedam, V., Kannenberg, E. L., Datta, A., Brown, D., Haynes-Gann, J. G., Sherrier, D. J., and Carlson, R. W. (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide *acpXL* mutant to add 27-hydroxyoctacosanoic acid to its lipid A. *J. Bacteriol.,* 188: 2126-2133.

Westphal, O. and Jann, K. (1965) Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.,* 5: 83-91.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., and Albersheim, P. (1985) Isolation and characterization of plant cell walls and cell wall components. *Methods Enzymol.,* 118: 3-40.

Zahringer, U., Knirel, Y. A., Linder, B., Helbig, J. H., Sonesson, A., Marre, R., and Rietschel, E. T. (1995) The lipopolysaccharide of *Legionella pneumophila* serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. *Prog. Clin. Biol. Res.,* 392: 113-139.

CHAPTER 3

PHENOTYPIC CHARACTERISTICS OF THE ACYL TRANSFERASE LpxXL MUTANT OF *RHIZOBIUM LEGUMINOSARUM* LIPID A[2]

[1] Bourassa, D.V., Carlson, R.W., Buhr, R.J., and Kannenberg, E.L. To be submitted to MPMI.

Abstract

Lipopolysaccharide (LPS) gene mutations of many rhizobial species lead to alterations in the bacterial and symbiotic phenotypes. A *Rhizobium leguminosarum* 3841 *acpXL*$^{-}$ mutant (AcpXL, 27OHC28:0 acyl carrier protein), Rlv22, a constructed *acpXL*$^{-}$*lpxXL*$^{-}$ (LpxXL, acyl transferase), double mutant (EL196), and the *acpXL*$^{-}$*lpxXL*$^{-}$ double mutant partially complemented with cloned wild type *acpXL* (*acpXL*$^{-}$*lpxXL*$^{-}$*acpXL*$^{+}$, EL197), i.e. an effective *lpxXL*$^{-}$ single mutant, were analyzed for several bacterial growth phenotypes that have implications for symbiosis. The membrane integrity of Rlv22, EL196 and EL197 was examined by growth at high salt, growth on media with added detergents, growth in the presence of polymyxin B, and by sensitivity to desiccation. Rlv22, EL196, and EL197 mutants were more sensitive to salt, detergents, and desiccation than Rlv3841 or the fully complemented double mutant (EL198). Following passage through the plant, the ex-nodule (ExN) isolates of all strains were obtained and characterized. ExN isolates of Rlv22 and EL196 regained parental levels of resistance to salt and detergents but not EL197. When grown on polymyxin B gradients, Rlv22 and EL196 laboratory-cultured bacteria were more sensitive than the parent strain while EL197 was slightly more resistant. ExN isolates of Rlv22, EL196 and EL197 were all more sensitive than ExN Rlv3841. Relative amounts of 2-linked glucose (cyclic β-glucans) were also examined in all strains and their ExN isolates. The level of 2-Glc was much lower for the VLCFA mutants. However, 2-Glc remained low in all ExN isolates. These results are discussed with regard to their implications for symbiosis with the host legume.

Introduction

The 27-hydroxyoctacosanoic acid (very long chain fatty acid, VLCFA) on the *Rhizobium leguminosarum* lipid A is an unusual feature found in only a few families of gram-negative bacteria including these plant cell symbionts and some intracellular pathogens. Figure 3.1A shows the lipid A structure of *Escherichia coli* in comparison to *Rhizobium leguminosarum*. This VLCFA is biosynthesized through a gene region including an acyl carrier protein *acpXL*, a hydroxyacyl dehydratase *fabZXL*, two 3-oxoacyl synthases *fabF1XL* and *fabF2XL*, an alcohol dehydrogenase, and an acyl transferase *lpxXL* (Figure 3.1B). *Rhizobium leguminosarum* mutants disrupted in *acpXL* and *fabF1XL* and *fabF2XL* have previously been created and characterized (Vedam et al. 2003; Vedam et al. 2004; Vedam et al. 2006; Vanderlinde et al. 2009). *Rhizobium* mutants in these genes were able to form nodules and fix nitrogen but are unable to add the VLCFA to the LPS lipid A. Instead a palmitic or stearic acid is added to the lipid A. In addition to a change in LPS structure, phenotypic factors are affected by the lack of the VLCFA. *Rhizobium leguminosarum* mutants of *acpXL*, *fabF1XL*, and *fabF2XL* are more susceptible to osmolarity, detergent, and acidity than the parent strain (Vedam et al. 2003; Vanderlinde et al. 2009). Mutations in the oxo-acyl synthase genes *fabF1XL* and *fabF2XL* also show diminished membrane stability through the need for increased concentrations of Ca^{2+} in the medium and sensitivity to detergents as well as sensitivity to desiccation and osmotic stress (Vanderlinde et al. 2009). Membrane integrity has important implications for symbiosis since when the bacterium undergoes infection of the host via endocytosis it must adjust to the low osmotic environment of the host root nodule cell as well as survive interactions with its host innate immune response including interaction with antimicrobial nodule cationic peptides (NCPs) (Kannenberg and Brewin 1989; Brewin 1991; Van de Velde et al. 2010).

Various mutants in the VLCFA gene cluster from other *Rhizobiaceae* have also been constructed. Mutations in *acpXL* in *Rhizobium leguminosarum* bv. *phaseoli* and *Sinorhizobium meliloti* also lacked the VLCFA but were able to add a palmitic or stearic acid (Sharypova et al. 2003; Ferguson et al. 2005; Brown et al. 2011). These mutants are able to infect nodules and fix nitrogen but have been shown to be less competitive than the parent strain (Ferguson et al. 2005). In *Sinorhizobium meliloti* the *acpXL*$^-$*, lpxXL*$^-$*,* and *acpXL*$^-$*lpxXL*$^-$ mutants were more susceptible to detergents and were also unable to grow without increased amounts of $MgSO_4$ and $CaCl_2$ supplementation in the TY medium (Sharypova et al. 2003; Ferguson et al. 2005). However, unlike the *acpXL* mutants of *Rhizobium leguminosarum* and *Sinorhizobium meliloti*, an *acpXL* of *Rhizobium* sp. strain NGR234 (a *Rhizobium* with a broad host range) was severely impaired in its symbiotic ability (Ardissone et al. 2011). This *Rhizobium* sp. strain NGR234 *acpXL* mutant was able to form white pseudonodules but was unable to form functional pink nodules on most host plants. This is also true for an *lpxXL* mutant of the soybean symbiont, *Bradyrhizobium japonicum* (Artur Muszynski, personal communication).

For some of these VLCFA mutants following symbiosis with the host plant, bacteria that are isolated from root nodules (i.e. the ExN isolates) and cultured in the laboratory have altered phenotypic characteristics compared to laboratory cultures prior to passage through the plant. *Rhizobium leguminosarum* bv. *viciae* and bv. *phaseoli acpXL*$^-$ mutant ExN isolates are restored in their tolerance to detergents, osmotic, and acid stressors (Vedam et al. 2006; Brown et al. 2011). Previous work demonstrated a partial restoration of VLCFA in the lipid A from bacteroids of the *acpXL* mutants of *Rhizobium leguminosarum* bv. *viciae* and *Sinorhizobium meliloti* (Haag et al. 2009; Vedam et al. 2006), but not *Rhizobium leguminosarum* bv. *phaseoli* (Brown et al. 2011). However, the restored VLCFA was not present in laboratory-cultured ExN

isolates. Vanderlinde and Yost demonstrated that an overexpression of the *ropB* gene was able to restore detergent, hyperosmotic, and acid tolerance to *Rhizobium leguminosarum acpXL* and *fabF1XL, fabF2XL* mutants (Vanderlinde and Yost 2012). It was hypothesized that *ropB* expression could be the reason for the restoration of phenotypic tolerances, however, they determined that there was no increased *ropB* expression after passage through the plant and that the loss of a functioning RopB did not affect phenotype restoration. Furthermore, Vanderlinde and Yost went on to determine that ExN isolates are stable after multiple generations indicating that the restored tolerance is due to an inherited mechanism and not an adaptive mechanism while in symbiosis with the host (Vanderlinde and Yost 2012). The genetic change required for this restoration of tolerance remains unknown.

The goal of this work was to assess the function of VLCFA in *Rhizobium leguminosarum* through mutation of the acyl transferase, LpxXL, by analyzing phenotypic characteristics of mutants lacking the acyl transferase and VLCFA, and the ability to infect the host plant.

Materials and Methods

Bacterial strains and growth conditions

Strains of bacteria are listed in Table 3.1. *Rhizobium leguminosarum* strains were grown at 30°C on TY medium with added calcium and appropriate antibiotics. Antibiotics were used at concentrations of 10 μg/mL tetracycline, 30 μg/mL kanamycin, 25 μg/mL gentamicin, 200 μg/mL streptomycin, and 50 μg/mL ampicillin.

Plant growth and inoculation conditions

For germination Early Alaska pea seeds were sterilized with 70% ethanol, washed 3x with sterile dH_2O, sterilized with 6% sodium hypochlorite, washed 10x with sterile dH_2O. The

sterilized seeds were germinated for 10-13 days at 22°C for 14 hours of light and 18°C for 10 hours in darkness each day at ambient relative humidity in 500 mL foam stoppered flasks with 300 mL of nitrogen-free Fahraeus medium (Nutman 1970). Following germination, stems were manipulated out of the top of the flask the foam stopper was gently reinserted allowing the stem/leaves of the plant to grow outside the flask. Each plant was inoculated with 1 mL of an approximately 0.15 OD_{600} log phase culture (approximately 10^8 cfu/mL) by dripping onto the seed and emerged roots. Each flask was then covered with a brown paper bag and taped closed to minimize light at the roots while leaving the foam stopper and stem/leaves of the plant exposed to light. The pea plants were grown at 22°C for 14 hours of light and 18°C for 10 hours of darkness each day for 21 dpi.

Ex-nodule bacterial isolation

Following plant inoculation and nodule growth the infecting bacteria were isolated from plant nodules for further evaluation. Several nodules were picked from a root system and each nodule was individually surface washed with 6% sodium hypochlorite for 30 s, then washed 4x with sterile dH_2O. Each nodule was then smashed with sterile forceps and streaked onto a TY plate with the appropriate antibiotics. Resulting colonies were restreaked for purity and stored in glycerol stocks. Each ex-nodule isolate was confirmed to retain the antibiotic resistance cassettes within the correct gene through PCR.

Mutant sensitivity phenotype analysis

Growth rates of Rlv3841, Rlv22, EL196, and EL197 were compared using a BioscreenC Microbiology Reader. Liquid cultures of each strain were grown to 1.0 OD_{600} and 10 µL of each were used to inoculate replicate wells containing 100 µL TY. Plates were incubated with shaking for 60 hours and OD_{600} values were recorded. To assess osmotic tolerance, bacterial strains were

streaked onto TY plates containing 0.25%, 0.50% and 0.75% concentrations of NaCl. Sensitivity to detergents was determined by streaking strains on TY agar containing sodium dodecylsulfate (SDS) at 0.17, 0.35, 0.52, and 0.69 mM or sodium deoxycholate (DOC) at 0.51, 0.64, 0.76, 1, 2, and 3 mM. Following 3 days of incubation at 30°C the relative amounts of growth were visually assessed and assigned a + or - value. Resistance to polymyxin B (PmxB) was analyzed by streaking strains onto TY gradient plates containing 0 to 50 μg/mL PmxB.

Desiccation sensitivity

Sensitivity to desiccation was determined using a method similar to that described by Gilbert et al. (2007) with some modifications. Liquid cultures in TY media were diluted to approximately 10^8 cfu/mL and 500 μL added to 50 mL sterile dH_2O. This 10^6 cfu/mL culture was filtered through a S-Pak 0.45 μm TypeHA membrane (Millipore) in a glass funnel. The filter was cut in half with one half placed in an empty petri dish and the other half on a water agar plate (12.5% agar). All plates were incubated for 6 hours at 30°C in a glass desiccator with freshly charged desiccant. Those filters on agar plates remained moist while in the desiccator. Filters were then vortexed in 2 mL sterile dH_2O for 30 seconds. Serial dilutions were plated onto TY plates and incubated 3 days. CFUs were determined, converted to $\log_{10}$ CFU and percentage survival between paired wet and dry filters was calculated.

Cyclic β-glucan analysis

Each mutant and the parent strain and their ExN isolates were grown under laboratory conditions, collected by centrifugation from 1 L cultures (~1.0 OD_{600}), washed with 0.9% NaCl, washed 3x with dH_2O, and the pellet suspended in 20 mL dH_2O. After centrifugation, the cell pellets were extracted with hot phenol/water (Westphal and Jann 1965). Briefly, the cell pellet in 20 mL dH_2O and 20 mL hot phenol was heated at 65°C for 45 minutes, cooled for 5 minutes in

ice water, centrifuged (7-10K for 25 minutes) to separate the water and phenol layers, and the water layer collected. Then 18 mL dH_2O was added to the phenol layer and the extraction procedure was repeated twice more after which pooled water layers and the phenol layers were dialyzed (MWCO:1000).

The amount of total carbohydrate in the water layer was analyzed by a phenol sulfuric acid test. For each strain 100 μg of lyophilized material extracted into the water layer was diluted in 100 μL of dH_2O. Then 100 μL of 5% phenol in water and 500 μL concentrated H_2SO_4 was added to each sample. Samples were heated at 99°C for 5 minutes then cooled to room temperature. Absorbance was measured at 460 nm and total carbohydrate for each sample was calculated based on a glucose standard curve from 5-60 μg glucose.

Linkage analysis of neutral sugars (particularly 2-linked glucose, 2-Glc) was determined by permethylation (Ciucanu method, (Ciucanu and Kerek 1984)) and conversion to partially methylated alditol acetates as described previously (Darvill et al. 1985). Briefly, permethylation was accomplished by dissolving 200 μg of carbohydrate based on total carbohydrate as measured by the phenol sulfuric acid test in 400 μL DMSO at 35°C for 24 hours. Base was freshly prepared by mixing 200 μL of aqueous 50/50 NaOH and 400 μL dry MeOH. The base was washed with DMSO five times and 1 mL DMSO was added following washing. The prepared base (approximately 15 drops) was added to each sample and stirred at room temperature for 2 hours. Iodomethane was added in two steps, 500 μL for 30 minutes and 200 μL for 1 hour. Next, 1 mL chloroform was added and stirred 20 minutes. Each sample was washed with 1 mL dH_2O three times and dried down. For hydrolysis, 150 μL dH_2O and 150 μL 4N trifluoroacetic acid were added to each sample and heated at 121°C for 2 hours. Samples were dried down and 100 μL dH_2O and 10 drops isopropanol were added. Samples were dried down again and 10 drops

isopropanol were added and dried down two more times. At the reduction step 2 µg inositol were added to each sample. To each sample 200 µL sodium borodeuteride ($NaBD_4$ 10 mg/mL) in 1 M ammonium hydroxide was added and incubated overnight. After neutralization with glacial acetic acid, 20 drops of 9:1 MeOH:HOAc were added and evaporated. This 9:1 MeOH:HOAc addition and evaporation was repeated two times and then 10 drops of MeOH were added and the sample dried down. This evaporation from MeOH was repeated until a crusty white residue was apparent. Samples were O-acetylated by adding 7 drops of pyridine and acetic anhydride and heating at 80°C for 1 hour. Samples were dried down and 1 mL dichloromethane and 1 mL dH_2O were added, vortexed, and centrifuged. The water layer was removed and the organic layer was washed twice more. The organic layer was filtered through glass wool with sodium sulfate. Samples were dried down and 100 µL of dichloromethane was added. Analysis was performed using combined gas chromatography/mass spectrometry (GC/MS). A known inositol standard was used for quantification of 2-Glc.

Results

Sensitivites to stressors

Growth rates in TY media were determined in order to examine the general fitness of the mutant strains. The growth rates of Rlv22, EL196, and EL197 were somewhat diminished in comparison with the parent strain Rlv3841, and stationary phase growth was reached at a lower OD value for each of the mutants indicating that growth stopped at lower bacterial levels (0.55-0.65) than for the parent strain (0.85) (Figure 3.2). This may have been due to a decrease in membrane stability. Membrane stability was tested by growing the strains at high osmolarity (increased levels of NaCl), with detergents (SDS or DOC, Table 3.2), or with the antibiotic

PmxB (Figure 3.3), known to affect growth and presumably gram-negative cell membrane architecture. The mutant strains Rlv22, EL196, and EL197 were all sensitive to NaCl at 0.50% and did not grow on the medium while the parent strain Rlv3841 and the complemented double mutant EL198 were able to grow. This was also the case for growth on detergents SDS and DOC. At 0.35 mM SDS Rlv22, EL196, and EL197 were unable to grow normally. On DOC plates EL197 was slightly more susceptible to DOC at 0.64 mM than Rlv22 and EL196 which were able to grow at 0.64 mM but unable to grow at 0.76 mM. The parent Rlv3841 strain was sensitive to DOC at 3 mM. Following passage through the plant it has been shown previously that the Rlv22 ExN isolate regained parental levels of tolerance to NaCl (Vedam et al. 2003). This result was repeated for Rlv22 ExN and was also found to be true for EL196 ExN. However, the EL197 ExN isolate did not regain tolerance to NaCl. Similarly to the NaCl salt sensitivity, the Rlv22 ExN and EL196 ExN isolates both showed increased tolerance to SDS and DOC but not the EL197 ExN isolate. However, none of the mutant ExN isolates regained resistance to DOC to the same level as the parent strain or complemented double mutant. When grown on PmxB gradient plates Rlv22 and EL196 had diminished growth in comparison with the parent Rlv3841, EL197, and EL198 (Figure 3.3). The ExN isolates did not exhibit increased tolerance to PmxB as was observed for SDS, DOC, and NaCl. The EL197 ExN was found to be more susceptible to PmxB than the parent Rlv3841.

Sensitivity to desiccation for parent and mutant strains is shown in Figure 3.4. Following exposure to desiccant for 6 hours at 30°C, 54% of the 10^6 cfu/mL starter culture from parent strain Rlv3841 survived. Each of the mutants Rlv22, EL196, and EL197 were more sensitive to desiccation than the parent strain with survival percentages of 6%, 12%, and 6%. The complemented double mutant EL198 showed an intermediate survival of 32%.

Analysis of cyclic β-glucan

Rhizobium is known to use cyclic β-glucans for hypoosmotic adaptation and their synthesis is osmoregulated (Breedveld et al. 1990). Thus, the ability of Rlv22 and EL196 to regain resistance to NaCl and detergents after passage through the plant may have been due to a change in the amount of cyclic β-glucans found in the periplasm. Since the cyclic β-glucan is 2-linked, 2-Glc levels were determined for the parent, mutants, and their ExN isolates as described in the Methods. The results are shown in Figure 3.5. Rlv3841 and EL198 laboratory culture bacteria had greater amounts of 2-Glc (2-Glc/Ino ratios of 30.9 and 18.9, respectively) compared to mutants Rlv22 and EL197 (3.4 and 2.1 ratios, respectively). EL196 had a barely detectable amount of 2-Glc (a 0.5 ratio). For both Rlv3841 and EL198, their ExN isolates both showed a dramatic decrease in the 2-Glc to Ino ratio, i.e. 13 and 4-fold decreases, respectively. Only the ExN isolate of Rlv22 showed a slight increase in the amount of 2-Glc. The EL196 and EL197 ExN isolates were unchanged in their levels of 2-Glc. Thus, the parent and fully complemented double mutant both had high levels of 2-Glc compared to all of the mutants, while all of the mutants showed, comparably, dramatic decreases in 2-Glc. It was interesting that the ExN isolates from the parent and fully complemented double mutant showed dramatic decreases in 2-Glc. These results clearly show that the restoration of salt and detergent tolerance in the ExN isolates is not due to increased production of cyclic β-glucan.

Nodulation assay

To obtain ExN isolates for analysis, each strain was used to inoculate host pea plants. During this process a general visual examination of nodulation was conducted (Figure 3.6). For each bacterial strain examined pink nodules were formed on the host pea plant roots. However, a general observation in variability in onset of nodulation, nodule number, and nodule size was

observed. In Figure 3.6 the parent strain Rlv3841 appears to have fewer and larger nodules than those inoculated with the mutant strains. Based on these general observations further investigation was warranted and was performed to fully characterize the symbiotic phenotypes of these mutants. This work is described in Chapter 4 of this dissertation.

Discussion

During the process of plant infection and symbiosis rhizobia encounters stressors within the plant environment. Some of these changes include changes in osmolarity, oxygen concentration, exposure to nodule-specific cysteine-rich plant peptides (NCRs), and possible suppression of the plant innate immune response (Kannenberg and Brewin 1989; Brewin 1991; Van de Velde et al. 2010; Erbs and Newman 2012). Mutations of the acyl carrier protein *acpXL* and transferase *lpxXL* affect the phenotypic characteristics of *Rhizobium leguminosarum* in laboratory culture and upon culture following nodulation (i.e. ex-nodule). All of the mutants, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$*lpxXL*$^-$), and EL197 (*acpXL*$^-$*lpxXL*$^-$*acpXL*$^+$), had a slower growth rate, were more sensitive to salt, detergents, and desiccation, and had lower levels of 2-Glc than the parent strain Rlv3841. Ex-nodule isolates of Rlv22 and EL196 were restored in salt and detergent tolerance while EL197 was not.

Slow growth and susceptibility to salt, detergents, and desiccation are predicted to be due to a loss of membrane stability caused by the lack of a VLCFA on the lipid A and/or a diminished amount of β-cyclic glucans. In previous work sensitivity to high osmolarity, detergents, and desiccation was observed in other VLCFA mutant laboratory-cultured bacteria (Vedam et al. 2003; Vanderlinde et al. 2009; Brown et al. 2011). Sensitivity to osmolarity and detergent results agree with those seen previously for Rlv22 and other *acpXL* and *lpxXL* mutants

of *Rhizobium* and *Sinorhizobium* (Sharypova et al. 2003; Vedam et al. 2003; Ferguson et al. 2005; Brown et al. 2011). It has been shown in *Bradyrhizobium* and *Rhizobium leguminosarum* that changes to LPS affect desiccation tolerance (Cytryn et al. 2007; Vanderlinde et al. 2009). Vanderlinde et al. (2009) suggest that susceptibility of the Fab1XL/Fab2XL mutant to desiccation may have been due to alteration in the O-chain component of the lipopolysaccharide. However, based on DOC-PAGE analysis of LPS (Chapter 2) the O-chain from the Rlv22, EL196, and EL197 mutants appears to be similar to that of the parent Rlv3841, and for these LPS, the observed band shift to lower molecular weight is due to the lack of the VLCFA. For these mutants the alteration in lipid A leads to an increased sensitivity to desiccation. The cause of this sensitivity could be the effect on membrane stability or the lack of the osmoprotectant cyclic β-glucan. Cyclic β-glucans are constructed of 2-linked glucose and are known to play in important role in hypoosmotic adaptation and are osmoregulated (Dickstein et al. 1988; Cangelosi et al. 1990; Dylan et al. 1990). The VLCFA mutants had a 9-fold or more decrease in the amount of 2-linked glucose in comparison to the parent strain, possibly leading to the inability of the mutants to protect themselves against these membrane stressors. Another possible explanation for the sensitivity to osmolarity, detergents, and desiccation is the expression of an outer membrane protein, RopB. Expression of *ropB* in a *Rhizobium leguminosarum* VLCFA mutants (*acpXL* and *fabF2XL, fabF1XL*) has also been shown to contribute to osmotic and detergent tolerance (Vanderlinde and Yost 2012).

In this and previous work it has been demonstrated that ExN isolates of *acpXL* mutants of *Rhizobium leguminosarum* and *acpXL* and *lpxXL* mutants of *Sinorhizobium meliloti* have restored tolerance to detergents (Sharypova et al. 2003; Vedam et al. 2006; Brown et al. 2011). However, the mechanism for this restoration has not yet been identified. The previous work

concluded that RopB expression did not influence detergent tolerance restoration (Vanderlinde and Yost 2012). They hypothesized that restored tolerance was due to an inherited mechanism and not an adaptive mechanism while in symbiosis with the host. To further examine a possible mechanism for restoration of detergent and osmotic tolerance the amounts of 2-linked glucose were measured for *Rhizobium leguminosarum* parent strain and mutant laboratory-cultured and ExN isolates. It is expected that the 2-linked glucose analyzed was present within the periplasm and not secreted 2-linked glucose due to washing of the cells removing secreted 2-linked glucose prior to LPS isolation. The amount of 2-linked glucose did not appear to be correlated to the restoration of osmotic tolerance seen in the mutant ExN isolates. While the amount of 2-linked glucose was much lower for VLCFA mutants from laboratory-cultured bacteria, 2-Glc levels remained low for all strains following passage through the plant. Since there is no increase in either the glucan or RopB expression (Vanderlinde and Yost 2012), the mechanism for restoration of osmotic and detergent tolerance in mutant ExN isolates remains unknown. It has been suggested that the plant may select for salt-tolerant bacterial variants during the symbiotic process (Brown et al. 2011). It is possible that the unknown mechanism for the restoration of osmotic and detergent tolerance in ExN isolates may be related to the expression of *acpXL*. When there is no AcpXL (in the case of Rlv22 and EL196) this unknown mechanism appears to be activated. However, when *acpXL* is expressed or possibly overexpressed there was no restoration of tolerance for EL197, indicating that the unknown mechanism was not activated.

The Rlv22 (*acpXL*$^-$) and EL196 (*acpXL*$^-$,*lpxXL*$^-$) double mutant were sensitive to polymyxin B but the EL197 (*acpXL*$^-$,*lpxXL*$^-$,*acpXL*$^+$) mutant was slightly more resistant than the parent Rlv3841. After passage through the plant EL197 also became PmxB sensitive. The complemented double mutant (EL198, *acpXL*$^-$,*lpxXL*$^-$,pSSB1) was more resistant to PmxB than

Rlv3841 in both laboratory-cultured and ExN isolates. Antimicrobial peptides (AMPs) have been shown to increase membrane permeability and inhibit cell division (Brogden 2005). Nodule-specific cysteine-rich (NCR) peptides (also AMPs) from the plant are required for terminal differentiation of bacteria into bacteroids (Kereszt et al. 2011). PmxB is an antimicrobial cationic peptide (AMP) that functions by binding to negative charges and the hydrophobic acyl portion of LPS and disrupting membrane integrity within the lipid portion of the molecule. It has been previously demonstrated that lipid A modifications such as the addition of cationic groups to the lipid A or changes in fatty acylation can affect sensitivity to AMPs (Brown et al. 2012). Deacylation of *Rhizobium leguminosarum* bv. *phaseoli* and *Salmonella* mutants also lead to PmxB sensitivity (Brown et al. 2011; Kong et al. 2012). One main difference between the PmxB-sensitive mutants (Rlv22, EL196) and the resistant mutants (EL197, EL198) is the presence or overexpression of AcpXL. The AcpXL-VLCFA may decrease the ability of PmxB to bind to LPS or perhaps the AcpXL has an entirely different function within the membrane that affects PmxB binding or function.

Lack of a VLCFA in *Rhizobium leguminosarum* while not essential for symbiosis does affect the phenotype of the organism and its symbiosis. The phenotypic deficiencies observed in this work lead us to ask additional questions regarding effects on the symbiotic process. An in depth analysis of this symbiotic relationship is described in Chapter 4.

Acknowledgements

This work was supported, in part, by National Institutes of Health Grant GM39583 (to R.W.C.) and by the Department of Energy Grant DE-FG-02-93ER20097 to the CCRC.

Table 3.1. Bacterial strains and plasmids used in this study

Strain/Plasmid	**Characteristics**	**Source**
Rhizobium leguminosarum* bv. *viciae		
Rlv3841	Strain 300 Str^R, Fix^+	(VandenBosch et al. 1989)
Rlv22	Strain 3841 *acpXL::kan* Str^R, Kan^R, Fix^+	(Vedam et al. 2003)
EL196	Strain 3841 *acpXL::kan lpxXL::gen* Str^R, Kan^R, Gen^R, Fix^+	This study
EL197	pVV5 (*acpXL*) in Strain EL196, Str^R, Kan^R, Gen^R, Tet^R, Fix^+	This study, (Vedam et al. 2003)
EL198	pSSB-1 (*acpXL,lpxXL*) in Strain EL196, Str^R, Kan^R, Gen^R, Tet^R, Fix^+	This study, (Basu et al. 2002)

Table 3.2. Osmotic tolerance and detergent sensitivity of laboratory-cultured and ex-nodule strains[1]

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

[1]Strains were grown on TY plus calcium media with the indicated concentrations of NaCl, SDS, or DOC. Plus signs and minus signs indicate whether that strain of *Rhizobium* was able to grow on the media.

Figure Legends

Figure 3.1 A. Structures of the Lipid A of *Escherichia coli* and *Rhizobium leguminosarum*. B. Schematic of the gene region present in *Rhizobium leguminosarum* required for the biosynthesis of 27OHC28:0 onto the acyl carrier protein and transfer to the lipid A during LPS biosynthesis.

Figure 3.2 Growth rates of laboratory-cultured Rlv3841, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$*,lpxXL*$^-$), and EL197 (*acpXL*$^-$*,lpxXL*$^-$*,acpXL*$^+$) strains measured with a BioscreenC Microbiology Reader in 96-well plates. Optical density was measured at 600nm over 56 hours at 30°C.

Figure 3.3 Growth of laboratory-cultured and ex-nodule isolates of Rlv3841, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$*,lpxXL*$^-$), EL197 (*acpXL*$^-$*,lpxXL*$^-$*,acpXL*$^+$), and EL198 (double mutant fully complemented with pSSB1) isolates on tryptone-yeast extract gradient plates ranging from 0 µg/mL to 50 µg/mL of polymyxin B. Solid black lines indicate the approximate distance of growth of each isolate.

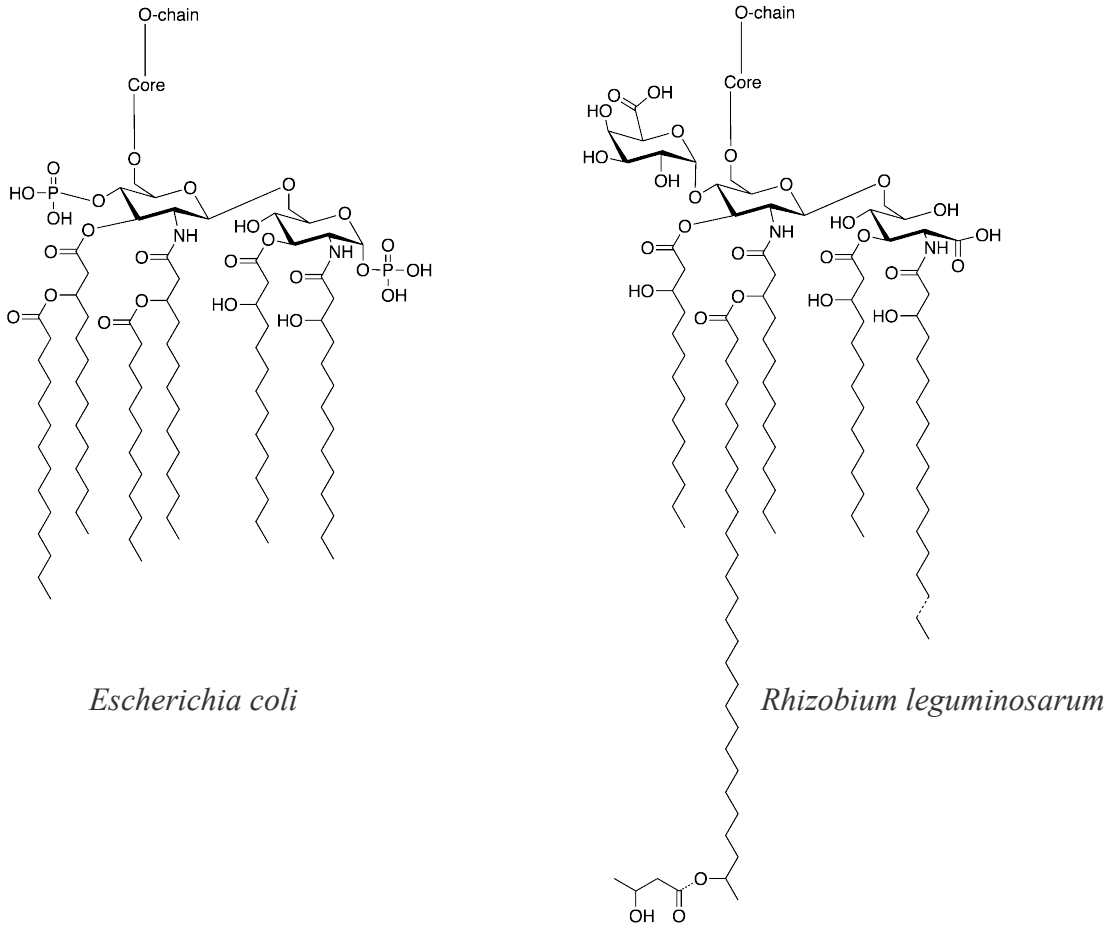
Figure 3.4 Percentage survival of laboratory-cultured Rlv3841, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$*,lpxXL*$^-$), EL197 (*acpXL*$^-$*,lpxXL*$^-$*,acpXL*$^+$), and EL198 (complemented double mutant) after exposure to 6 hours of desiccation at 30°C. Percentages were calculated by comparison of bacterial survival on filter paper in either dry petri dishes or placed on water agar.

Figure 3.5 Comparison of the levels of 2-linked glucose measured by partially methylated alditol acetate analysis. Rlv3841, Rlv22 (*acpXL*$^-$), EL196 (*acpXL*$^-$*,lpxXL*$^-$), EL197 (*acpXL*$^-$*,lpxXL*$^-$

,*acpXL*$^{+}$), and EL198 (complemented double mutant) from laboratory-cultured (black bars) and ex-nodule (grey bars) isolates were measured.

Figure 3.6 Photographs of nodules from Rlv3841, Rlv22 (*acpXL*$^{-}$), EL196 (*acpXL*$^{-}$,*lpxXL*$^{-}$), EL197 (*acpXL*$^{-}$,*lpxXL*$^{-}$,*acpXL*$^{+}$), and EL198 (complemented double mutant) inoculated pea plants following 21 days post inoculation. All mutants were able to interact with the plant for formation of nodules.

A.



Escherichia coli *Rhizobium leguminosarum*

B.

- *acpXL* - Lipid A 27OHC28:0 acyl carrier protein
- *fabZXL* - Hydroxyacyl-(*acpXL*) dehydratase
- *fabF2XL* - 3-oxoacyl-(*acpXL*) synthase
- *fabF1XL* - 3-oxoacyl-(*acpXL*) synthase
- *adhA2XL* - Alcohol dehydrogenase
- *lpxXL* - Lipid A 27OHC28:0 acyl transferase

Figure 3.1

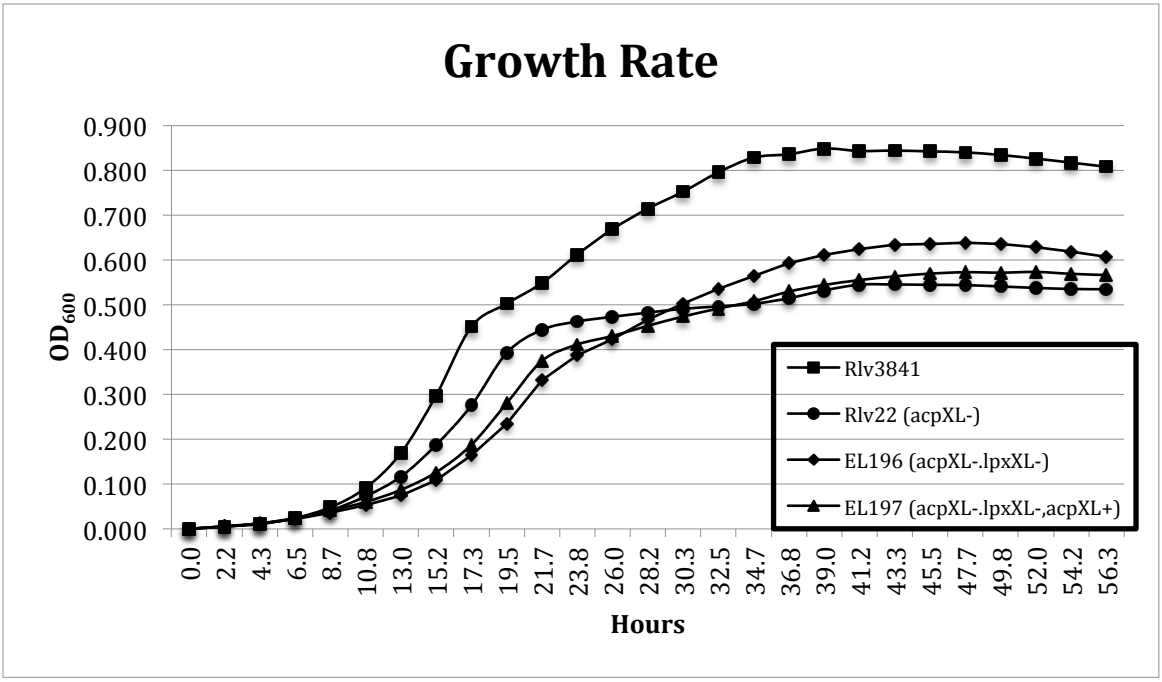


Figure 3.2

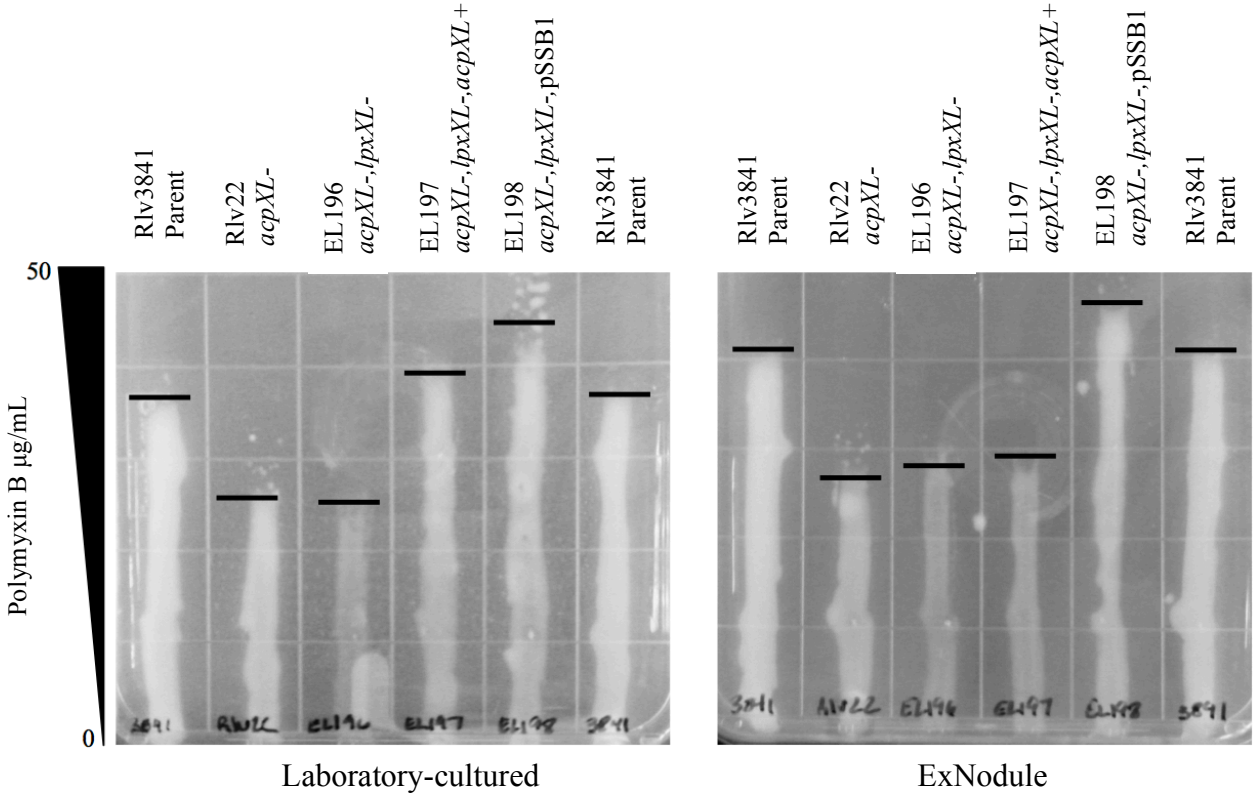


Figure 3.3

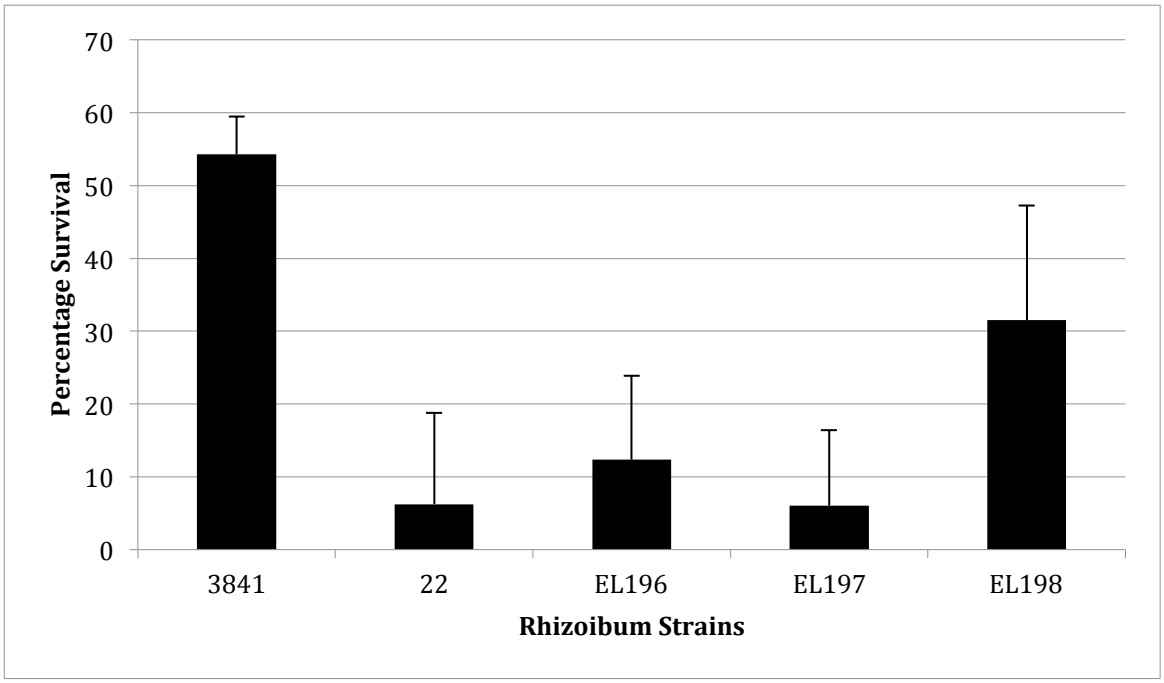


Figure 3.4

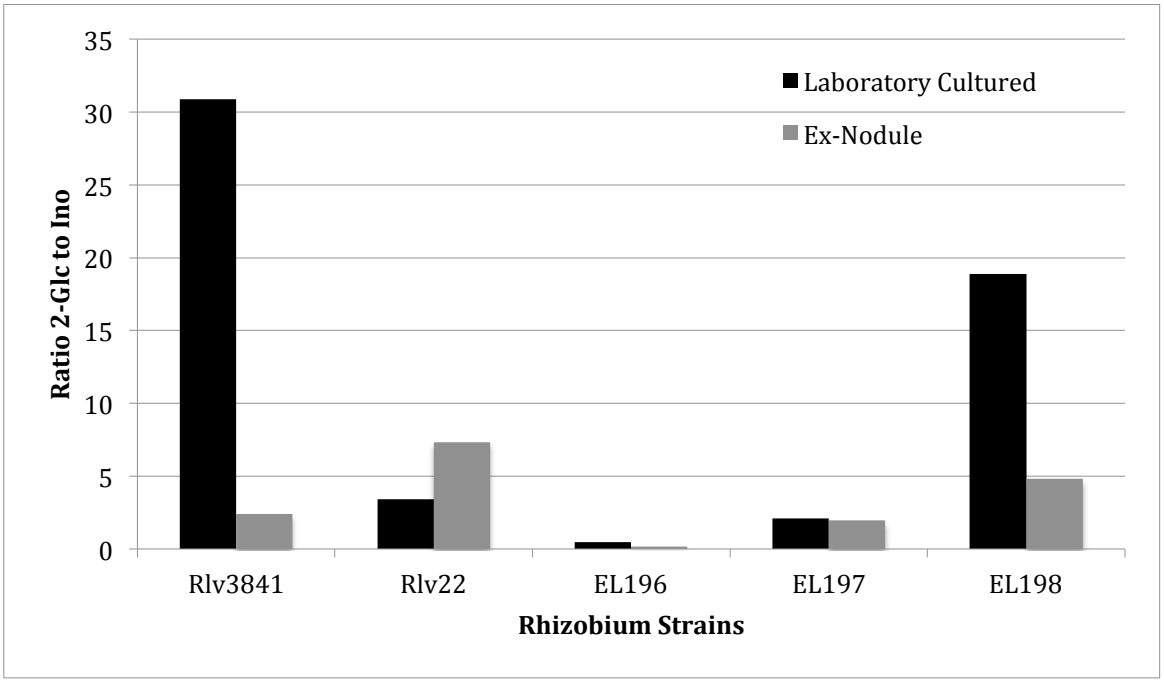


Figure 3.5

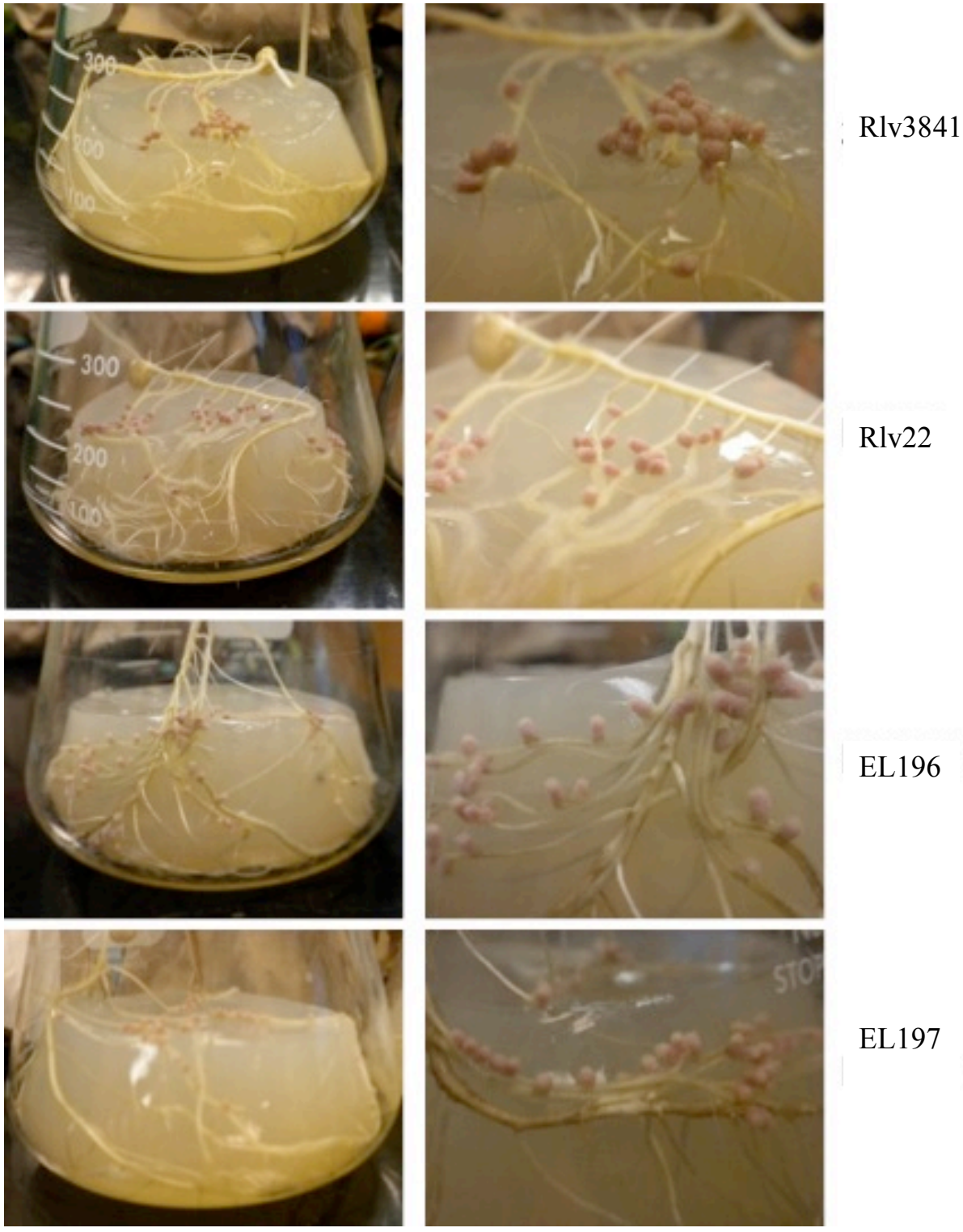


Figure 3.6

References

Ardissone, S., Kobayashi, H., Kambara, K., Rummel, C., Noel, K. D., Walker, G. C., Broughton, W. J., and Deakin, W. J. (2011) Role of BacA in lipopolysaccharide synthesis, peptide transport, and nodulation by *Rhizobium* sp. strain NGR234. *J. Bacteriol.,* 193: 2218-2228.

Basu, S. S., Karbarz, M. J., and Raetz, C. R. (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum.* *J. Biol. Chem.,* 277: 28959-28971.

Breedveld, M. W., Zevehuizen, L. P. T. M., and Zehnder, A. J. B. (1990) Osmotically induced oligo- and polysaccharide synthesis by *Rhizobium meliloti* SU-47. *J. Gen. Microbiol.,* 136: 2511-2519.

Brewin, N. J. (1991) Development of the legume root nodule. *Ann. Rev. Cell Biol.,* 7: 191-226.

Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nature Rev. Microbiol.,* 3: 238-250.

Brown, D. B., Huang, Y. C., Kannenberg, E. L., Sherrier, D. J., and Carlson, R. W. (2011) An *acpXL* mutant of *Rhizobium leguminosarum* bv. *phaseoli* lacks 27-hydroxyoctacosanoic acid in its lipid A and is developmentally delayed during symbiotic infection of the determinate nodulating host plant *Phaseolus vulgaris*. *J. Bacteriol.,* 193: 4766-4778.

Brown, D. B., Forsberg, L. S., Kannenberg, E. L., and Carlson, R. W. (2012) Characterization of galacturonosyl transferase genes *rgtA*, *rgtB*, *rgtC*, *rgtD*, and *rgtE* responsible for lipopolysaccharide synthesis in nitrogen-fixing endosymbiont *Rhizobium leguminosarum*: lipopolysaccharide core and lipid galacturonosyl residues confer membrane stability. *J. Biol. Chem.,* 287: 935-949.

Cangelosi, G. A., Martinetti, G., and Nester, E. W. (1990) Osmosensitivity phenotypes of *Agrobacterium tumefaciens* mutants that lack periplasmic beta-1,2-glucan. *J. Bacteriol.,* 172: 2172-2174.

Ciucanu, L. and Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res,* 131: 209-217.

Cytryn, E. J., Sangurdekar, D. P., Streeter, J. G., Franck, W. L., Chang, W. S., Stacey, G., Emerich, D. W., Joshi, T., Xu, D., and Sadowsky, M. J. (2007) Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.,* 189: 6751-6762.

Darvill, A. G., Albersheim, P., McNeil, M., Lau, J. M., York, W. S., Stevenson, T. T., Thomas, J., Doares, S., Gollin, D. J., Chelf, P., and Davis, K. (1985) Structure and function of plant cell wall polysaccharides. *J. Cell Sci., Suppl.,* 2: 203-17.

Dickstein, R., Bisseling, T., Reinhold, V. N., and Ausubel, F. M. (1988) Expression of nodule-specific genes in alfalfa root nodules blocked at an early stage of development. *Genes Dev.,* 2: 677-687.

Dylan, T., Helinski, D. R., and Ditta, G. S. (1990) Hypoosmotic adaptation in *Rhizobium meliloti* requires beta-(1----2)-glucan. *J. Bacteriol.,* 172: 1400-1408.

Erbs, G. and Newman, M. A. (2012) The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. *Mol. Plant Pathol.,* 13: 95-104.

Ferguson, G. P., Datta, A., Carlson, R. W., and Walker, G. C. (2005) Importance of unusually modified lipid A in *Sinorhizobium* stress resistance and legume symbiosis. *Mol. Microbiol.,* 56: 68-80.

Gilbert, K. B., Vanderlinde, E. M., and Yost, C. K. (2007) Mutagenesis of the carboxy terminal protease CtpA decreases desiccation tolerance in *Rhizobium leguminosarum*. *FEMS Microbiol. Lett.,* 272: 65-74.

Haag, A. F., Wehmeier, S., Beck, S., Marlow, V. L., Fletcher, V., James, E. K., and Ferguson, G. P. (2009) The *Sinorhizobium meliloti* LpxXL and AcpXL proteins play important roles in bacteroid development within alfalfa. *J. Bacteriol.,* 191: 4681-4686.

Kannenberg, E. L. and Brewin, N. J. (1989) Expression of a cell surface antigen from *Rhizobium leguminosarum* 3841 is regulated by oxygen and pH. *J. Bacteriol.,* 171: 4543-4548.

Kereszt, A., Mergaert, P., and Kondorosi, E. (2011) Bacteroid development in legume nodules: Evolution of mutual benefit or of sacrificial victims?. *Mol. Plant Microbe Interact.*, 24: 1300-1309.

Kong, Q., Six, D. A., Liu, Q., Gu, L., Wang, S., Alamuri, P., Raetz, C. R., and Curtiss, R. III. (2012) Phosphate groups of lipid A are essential for *Salmonella enterica* serovar Typhimurium virulence and affect innate and adaptive immunity. *Infect. Immun.,* 80: 3215-3224.

Nutman, P. S. (1970) Appendix III: The modified Fahraeus slide technique. In J. M. Vincent (ed.), *A manual for the practical study of root-nodule bacteria* (Oxford and Edinburgh: Blackwell Scientific Publications), 144-145.

Sharypova, L. A., Niehaus, K., Scheidle, H., Holst, O., and Becker, A. (2003) *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *J. Biol. Chem.,* 278: 12946-12954.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H., Satiat-Jeunemaitre, B., Alunni, B., Bourge, M., Kucho, K., Abe, M., Kereszt, A., Maroti, G., Uchiumi, T., Kondorosi, E., and Mergaert, P. (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science,* 327: 1122-1126.

VandenBosch, K. A., Brewin, N. J., and Kannenberg, E. L. (1989) Developmental regulation of a *Rhizobium* cell surface antigen during growth of pea root nodules. *J. Bacteriol.,* 171: 4537-4542.

Vanderlinde, E. M., Harrison, J. J., Muszynsi, A., Carlson, R. W., Turner, R. J., and Yost, C. K. (2009) Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. *FEMS Microbiol. Ecol*. 71: 327-340.

Vanderlinde, E. M. and Yost, C. K. (2012) Genetic analysis reveals links between lipid A structure and expression of the outer membrane protein gene, *ropB*, in *Rhizobium leguminosarum*. *FEMS Microbiol. Lett.,* 335: 130-139.

Vedam, V., Kannenberg, E. L., Haynes, J. G., Sherrier, D. J., Datta, A., and Carlson, R. W. (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J. Bacteriol.,* 185: 1841-1850.

Vedam, V., Haynes, J. G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. (2004) A *Rhizobium leguminosarum* lipopolysaccharide lipid-A mutant induces nitrogen-fixing nodules with delayed and defective bacteroid formation. *Mol. Plant Microbe Interact.,* 17: 283-291.

Vedam, V., Kannenberg, E. L., Datta, A., Brown, D., Haynes-Gann, J. G., Sherrier, D. J., and Carlson, R. W. (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide *acpXL* mutant to add 27-hydroxyoctacosanoic acid to its lipid A. *J. Bacteriol.,* 188: 2126-2133.

Westphal, O. and Jann, K. (1965) Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.,* 5: 83-91.

CHAPTER 4

CHARACTERIZATION OF SYMBIOTIC PHENOTYPE OF THE ACYL TRANSFERASE LpxXL MUTANT OF *RHIZOBIUM LEGUMINOSARUM*[3]

[1] Bourassa, D.V., Carlson, R.W., Buhr, R.J., Sherrier, J., and Kannenberg, E.L. To be submitted to MPMI.

Abstract

Rhizobial lipopolysaccharides (LPS) are an important factor in the bacteria-plant interaction required to carry out a successful symbiotic relationship. Acyl carrier protein (*acpXL*$^-$), effective acyl transferase (*lpxXL*$^-$), and double mutant (*acpXL*$^-$,*lpxXL*$^-$) strains of *Rhizobium leguminosarum* were used to infect Early Alaska pea plant roots to examine the symbiotic effects when the LPS lacks the very long chain fatty acid (VLCFA), acyloxyacyl 27-hydroxyoctacosanoic acid (27OHC28:0), bound to lipid A. Nodule symbiosis was assessed at 14, 21, and 28 days post inoculation (dpi). Plants infected with VLCFA mutants were less able to fix nitrogen and tended to have fewer large and more small nodules than those inoculated with the parent strain. Plant nodule cells infected with bacteroids without the VLCFA often did not present the large vacuoles seen in cells infected with the parent strain. Bacteroid distribution within the plant cell was also altered. Host cells infected with the mutants were affected in visible bacteroid density (μm^2 of bacteroids/400 μm^2) and number of bacteroids per 400 μm^2. Plants infected with the *acpXL*$^-$ mutant or *acpXL*$^-$,*lpxXL*$^-$ double mutant had lower bacteroid densities than the parent strain Rlv3841 at 14 dpi and plants infected with the *acpXL*$^-$,*lpxXL*$^-$ double mutant and the acyl transferase mutant *acpXL*$^-$,*lpxXL*$^-$,*acpXL*$^+$ had lower bacteroid densities than the parent strain at 28 dpi. At 14 dpi plants infected with the *acpXL*$^-$,*lpxXL*$^-$,*acpXL*$^+$ mutant had a higher number of bacteroids than the parent strain. VLCFA mutant bacteroids also displayed multiply-branched shapes as well as possible signs of senescence or degradation. Although *Rhizobium leguminosarum* acyl carrier protein and acyl transferase mutants were able to infect host plant root cells the efficiency of the symbiosis was significantly affected by the lack of the VLCFA bound to lipid A.

Introduction

Symbiotic nitrogen fixation occurs in a relationship between rhizobia and host legume plants where the host forms a nodule that is infected by rhizobia bacteria. This relationship begins with the production and secretion plant flavonoids which induce the production of Nod factors (lipochitooligosaccharides) from rhizobia bacteria that are recognized by the host legume (Denarie et al. 1996). Recognition of Nod factors leads the host to initiation of a nodule primordium while the rhizobia enter the plant through infection threads in the root hairs (Denarie et al. 1996). Infecting bacteria are endocytosed into the plant cells surrounded by the peribacteroid membrane where they mature into nitrogen-fixing bacteroids. *Rhizobium leguminosarum* forms a symbiotic relationship with a variety of legumes including pea plants and lipopolysaccharides (LPS) from the bacterial symbiont play in important role in this process (Fraysse et al. 2003; Carlson et al. 2010).

There are two main types of nodules, determinate and indeterminate. Determinate nodules are spherical, meristematic activity is terminated early, bacteroids are elongated but remain viable, and multiple bacteroids are present with the peribacteroid membrane. Indeterminate nodules are cylindrical with an active meristem, bacteroids have a branched Y-shaped structure that is terminally differentiated, and only one bacteroid is present within a peribacteroid membrane. Infected plant cells in both determinate and indeterminate nodules are enlarged and have undergone endoreduplication (Kondorosi et al. 2000; Vinardell et al. 2003, Mergaert et al. 2006). This work deals with pea plants, which form indeterminate nodules.

Nodule development is a process that has been categorized into eight developmental stages: root hair curling, infection thread growth initiation, infection thread growth in root hair cells, infection thread growth inside root cortical tissue, infection thread differentiation inside

nodule tissue, infection droplet differentiation, bacteroid differentiation, and nodule persistence (Borisov et al. 1999). The mature indeterminate nitrogen-fixing nodule can be divided into zones (Figure 4.1). Zone I is the persistent apical meristem. Zone II is the infection zone where plant cells are infected, cell cycle is arrested, and bacterial differentiation including endoreduplication begins and plant and bacterial cells simultaneously mature. Zone III is the nitrogen fixation zone where differentiation is completed to form bacteroids within symbiosomes. Zone IV is the senescent zone (Luyten and Vanderleyden 2000). In zone II bacteria are endocytosed into an enlarged plant cell from the tip of the infection thread. The bacteria is enclosed within the plant derived peribacteroid membrane which undergo simultaneous division until differentiation into a nitrogen fixing bacteroid (Kereszt et al. 2011a). Four stages of bacteroid maturation in indeterminate nodules have been described (Vasse et al. 1990). In stage 1 bacteria are similar to free-living bacteria. Stage 2 bacteroids are elongating, no longer divide, and are in the proximal region of the infection zone II. Stage 3 bacteroids are at mature size, fill the plant cell cytoplasm, and are at the border of zones II and III where the environment is becoming microaerobic and nitrogen-fixation genes are starting to be induced (Soupene et al. 1995). Stage 4 bacteroids are fixing nitrogen and have a heterogeneous cytoplasm. Maturation from bacteria to mature bacteroid involves cell surface structure changes and major differences in gene expression (Kereszt et al. 2011a).

Mutations in LPS affect the symbiotic process and bacteroid maturation. Delayed nodulation, abnormally branched bacteroid formation, decreased competitiveness, and susceptibility to salt, detergent, and antimicrobial peptides can be caused by changes to the LPS (Campbell et al. 2002; Vedam et al. 2004; Haag et al. 2009; Brown et al. 2011; Haag et al. 2011). Mutants lacking the VLCFA on the lipid A have previously been shown to effect the symbiotic

process. The gene region required to make VLCFA and add it to the lipid A includes acyl carrier protein (AcpXL), hydroxyacyl dehydratase (FabZXL), two 3-oxoacyl synthases (FabF1XL, FabF2XL), alcohol dehydrogenase (AdhA2XL), and acyl transferase (LpxXL). *Rhizobium leguminosarum* AcpXL and *Sinorhizobium meliloti* AcpXL and LpxXL mutants both lead to the formation of nitrogen-fixing nodules although less successfully than their parent strain bacteria (Vedam et al. 2004; Ferguson et al. 2005). Lack of a VLCFA has been shown to cause a delay in infection and nitrogen fixing activitiy and a decrease in competitiveness (Sharypova et al. 2003; Vedam et al. 2004; Ferguson et al. 2005; Brown et al. 2011). Bacteroids were also found to have an aberrant structure with multiple bacteroids per symbiosome in VLCFA mutants (Vedam et al. 2004; Haag et al. 2009).

In this work host pea plants were infected with parent strain Rlv3841, *acpXL*$^-$ mutant (Rlv22), *acpXL*$^-$, *lpxXL*$^-$ mutant (EL196), the effective *lpxXL*$^-$ mutant (EL197), and fully complemented mutant (EL198) strains to assess the importance of the acyl transferase LpxXL during symbiosis. Symbiotic factors including nitrogen fixation, nodule number and size, plant cell and bacteroid appearance, and bacteroid density and number were assessed.

Materials and Methods

Bacterial strains and growth conditions

Strains of bacteria are listed in Table 4.1. *Rhizobium leguminosarum* strains were grown at 30°C on TY medium with added calcium and appropriate antibiotics. Antibiotics were used at concentrations of 10 μg/mL tetracycline, 30 μg/mL kanamycin, 25 μg/mL gentamicin, 200 μg/mL streptomycin, and 50 μg/mL ampicillin.

Plant growth and inoculation conditions

For germination, Early Alaska pea seeds were sterilized with 70% ethanol, washed 3x with sterile dH_2O, sterilized with 6% sodium hypochlorite and washed 10x with sterile dH_2O. The sterilized seeds were germinated for 10-13 days at 22°C for 14 hours of light and 18°C for 10 hours in darkness each day at ambient relative humidity in 500 mL foam stoppered flasks with 300 mL of nitrogen-free Fahraeus medium (Nutman et al. 1970). Following germination, stems were manipulated out of the top of the flask the foam stopper was gently reinserted allowing the stem/leaves of the plant to grow outside the flask. Each plant was inoculated with 1 mL of an approximately 0.15 OD_{600} log phase culture (approximately 10^8 cfu/mL) by dripping onto the seed and emerged roots. Each flask was then covered with a brown paper bag and taped closed to minimize light on the roots while leaving the foam stopper and stem/leaves of the plant exposed to light. The pea plants were grown at 22°C for 14 hours of light and 18°C for 10 hours of darkness each day for 21 dpi.

Acetylene reduction assay

To assess nitrogen fixation the amount of acetylene reduced to ethylene was determined. For each plant, the root system was removed at the seed coat and placed inside a 100 mL glass bottle with a septum stopper. Acetylene gas (1 mL) was injected into each bottle. After one hour 0.3 mL of gas was removed from the bottle through the diaphragm and injected into a gas chromatograph (isothermal at 70°C, with a 25 m by 0.53 mm, 10 μm Al_2O_2 KCl column). The ratio of ethylene to acetylene (area under the peak) was calculated for determination of acetylene reduction.

Nodule preparation for microscopy

Plant roots were removed from Fahraeus medium and smaller sections of roots with nodules were harvested. Nodules to be used for confocal microscopy were placed into a 4%

formaldehyde, 100 mM PIPES fixative solution and nodules to be used for transmission electron microscopy were placed into a 4% formaldehyde, 1% glutaraldehyde, 100 mM PIPES fixative solution. Under a stereo microscope nodules were dissected longitudinally perpendicular to the outgrowth at the root with a double-edged razor blade while submerged in the fixative solution and a section containing the middle of the nodule attached to the root was collected (Figure 4.2). Sections were transferred to 8 mL of fixative in a scintillation vial and fixed overnight at 4°C.

Confocal microscopy

Nodule sections in fixative were dyed with Syto-13 (5 μM in DMSO). Nodules were placed on a glass slide cartridge and covered with a glass coverslip. Confocal images were acquired on a Zeiss Axiovert 200 M equipped with a LSM 510 NLO laser-scanning Microscope using a Zeiss 5x, 40x, or 100x objective lenses. Multi-channel images of Syto-13 were acquired as individual optical sections or as z-stacks of optical sections.

Transmission electron microscopy

Nodule sections fixed in 4% formaldehyde, 1% glutaraldehyde, and 100 mM PIPES were rinsed 3 x 15 minutes with distilled water and post-fixed in 1% osmium tetroxide for 2 hours with rotation. Post fixed samples were then rinsed 3 x 15 minutes with distilled water with rotation. Samples were then dehydrated stepwise solutions of acetone (10, 30, 50, 70, 90, 100, and 100%) for 30 minutes each with rotation at room temperature. Samples were then infiltrated stepwise with solutions of epon araldite (25, 50, 75%, in acetone and 100, and 100%) for 1 hour each with rotation at room temperate. The final 100% epon araldite infiltration resin was kept overnight with rotation at room temperature. The next day the resin was replaced with fresh 100% epon araldite for infiltration with rotation for 8 hours. Resin was again replaced with 100% epon araldite and transferred with nodule sections into an aluminum weigh boat. Samples were

polymerized at 65°C for 72 hours. Once dry nodule sections were cut out and mounted onto acrylic mounting stubs for trimming on a microtome. Once trimmed 70 nm parallel sections were cut with a diamond knife and collected onto gold hex mesh grids. Grids were submerged into 2% uranyl acetate for 15 minutes and rinsed 8x with distilled water. Sections were then stained with lead citrate for 10 minutes and washed 8x with distilled water. Once dry grids with sections were carbon coated. Sections were imaged with a Zeiss Libra 120 TEM.

Nodule and bacteroid measurements

Nodule size was determined from 5-15 pea plants per bacterial strain. All nodules on each plant root system were categorized as small (1 mm^2 or smaller), medium (between 1 mm^2 and 4 mm^2), or large (greater than 4 mm^2). TEM images were analyzed with ImageJ. For bacteroid density a 400 μm^2 area within zone III not including the plant cell membrane or vacuole was selected within an infected plant cell. The combined area of all bacteroids within the 400 μm^2 area was divided by the total area to calculate bacteroid density. For the number of bacteroids per area the same TEM images were used and the number of bacteroids was divided by the 400 μm^2.

Statistical significance was determined using the GLM procedure of SAS. Mean separation was conducted by ANOVA and Tukey's test. Significance was determined at $P<0.05$.

Results

Each of the mutants was able to form a symbiotic relationship with the host pea plant. However the efficiency of the interaction appeared to be variable. Mutations in *acpXL* and/or *lpxXL* affected nitrogen fixation, nodule size and number, infected nodule plant cell appearance and internal organization, and bacteroid elements such as shape, degradation, density, and size.

Nitrogen fixation was measured by exposing the plant root system to acetylene and measuring the percentage of ethylene evolved (Table 4.2). At 14 dpi the parent strain Rlv3841 had significantly higher percentage of ethylene than EL196 (*acpXL*$^{-}$, *lpxXL*$^{-}$) and EL197 (*acpXL*$^{-}$, *lpxXL*$^{-}$, *acpXL*$^{+}$). Rlv22 (*acpXL*$^{-}$) and EL198 (complemented double mutant) had intermediate percentages of ethylene that were not significantly different than Rlv3841 or EL196. At 21 dpi a similar trend was seen. Rlv3841 had the highest percentage of ethylene that was significantly higher than EL196 and EL197. Rlv22 and EL198 were again intermediate but not different than Rlv3841. At 28 dpi both Rlv3841 and EL198 had depleted the nutrient agar of moisture therefore a reading was unable to be taken. Rlv22 had a significantly higher acetylene reduction than either EL196 or EL197. Thus, at all all time points plants infected with the acyl transferase mutants were adversely affected in nitrogen fixation compared to plants infected with the parent strain.

Nodule number and size was also affected by VLCFA acyl carrier protein and acyl transferase mutation (Table 4.3). At 14 dpi plants infected with EL197 had significantly fewer medium sized nodules than those infected with Rlv3841 or Rlv22. However, the overall total number of nodules did not significantly differ. At 21 dpi plants infected with EL196 or EL197 had significantly more small nodules than those infected with Rlv3841, however, there was no significant variation in the number of medium and large nodules. Overall, plants infected with EL196 had significantly more nodules those infected with Rlv3841. At 28 dpi plants infected with either Rlv3841 or EL198 could not be assessed for nodule number due to depletion of water and nutrients within the flask-media system. However, plants infected with EL196 had significantly more small nodules and a higher overall nodule number than those infected with either Rlv22 or EL197.

Confocal microscopy shows variations in infected plant cell structure (Figure 4.3). While whole nodules appear similar, higher magnification shows visible differences. Plant cells infected with Rlv3841, Rlv22, and EL198 have clearly defined large vacuoles and plant nuclei. Plant nodule cells infected with EL196 or EL197 appear more sporadically infected. This phenomenon is further demonstrated in Figure 4.4. Light colored spaces appear between bacteroids that are not seen in the nodule cells infected with the parent or *acpXL*$^{-}$ mutant, and fully-complemented strains. These light colored areas may be related to the lack of a distinct plant cell vacuole. In addition, plants infected with all three mutant strains show the production of starch granules indicating an inability to efficiently process nutrients. These abnormalities appear at each time point observed. In addition it was generally observed that very few of the E197 bacteroids were branched at 14 dpi.

Bacteroid shape is also affected by mutation of the VLCFA acyl carrier protein and transferase. Figure 4.5 shows examples of bacteroids from 14, 21, and 28 dpi for Rlv3841, Rlv22, EL196, EL197, and EL198. Both Rlv3841 and EL198 formed mostly normally shaped elongated Y-structure bacteroids while Rlv22, EL196, and EL197 formed bacteroids with increased branching and nodule cells containing symbiosomes with multiple bacteroids. Some bacteroids of Rlv22, EL196, and EL197 also showed regions of dark spots, which has been suggested to be a sign of bacteroid senescence (Figure 4.6, (Melino et al. 2012). Bacteroid density was measured by selecting a 400 μm^2 area within the nitrogen fixation zone of an infected plant cell not including the cell vacuole or nucleus and measuring the area of the visible bacteroids within that section. At 14 dpi nodule cells infected with Rlv22 or EL196 have significantly less bacteroid density than those infected with Rlv3841, EL197, or EL198 (Figure 4.7). At 21 dpi there were no significant differences between strains. At 28 dpi nodule cells

infected with EL196 or EL197 have a lower bacteroid density than those infected with Rlv3841, Rlv22, or EL198. Overall, bacteroids are less tightly packed at 14 dpi for nodule cells infected with the strains containing the *acpXL*$^-$ mutation (Rlv22 and EL196) and less tightly packed at 28 dpi for the *lpxXL*$^-$ mutation (EL196 and EL197). The number of visible bacteroids per 400 μm^2 area was also determined (Figure 4.8). The only significant difference was that nodule cells infected with EL197 had more visible bacteroids per area at 14 dpi than those infected with the parent strain Rlv3841. Nodule cells infected with EL197 appeared to have a majority of small unbranched bacteroids at the 14 dpi timepoint (Figure 4.4).

Discussion

Although *Rhizobium leguminosarum acpXL* and *lpxXL* mutants are able form a symbiotic relationship with pea plants leading to nitrogen fixation, that relationship is altered. The ability to form nitrogen-fixing nodules suggests that the early stages of infection were not significantly affected. Typically in indeterminate nodules extracellular polysaccharides (EPS) are essential for infection thread formation while LPS is needed for persistent intracellular colonization (Kannenberg et al. 1998). Although significant effects on the initial infection events were not observed, plant innate immunity likely plays a role in some of the symbiotic changes observed for each of these mutants. LPS is known to suppress the plant defense response and has been suggested to play an important role in the later stages of symbiosis (Fraysse et al. 2003). Host plants that were infected with VLCFA mutants were slower to form mature nodules, tended to have more small nodules, and had irregular infected nodule cell organization. These phenotypes may be due to a decreased ability to attenuate the plant defense response. The ability of the host to autoregulate nodule number may have led to an increased number of nodule initiations when

individual nodules are not fully functional (Vasse et al. 1993). This host autoregulation of nodule number could account for the increased number of small nodules and large number of total nodules found in the *lpxXL*$^{-}$ mutants. In addition to the plant innate immune response being important for controlling infection it is also required for bacteroid differentiation. Interactions of infecting bacteria with nodule-specific cysteine-rich (NCR) peptides are important for induction of terminal bacteroid differentiation (Van de Velde et al. 2010; Kondorosi et al. 2013). These NCRs are similar to defensin-like antimicrobial peptides known to be effector molecules of the host innate immune system. The signs of early senescence seen within the bacteroids may also be due to the plant defense response. Bacteroids are normally degraded by the plant in Zone IV of older nodules. However, degradation was seen in Zone III and in relatively young nodules.

It is interesting to note the increased severity of abnormalities from the strains without a functional acyl transferase. When lipid A mutants were unable to replace the VLCFA with a palmitic or stearic acid an additional consequence of disorganization of the infected plant cell was observed. Altered organization of host nodule cells may also be due to the plant defense response and early senescence. During infection both the plant and bacterial cells undergo endoreduplication (Mergaert et al. 2006). Change to the LPS acylation pattern, i.e. from penta- to tetraacylated lipid A, may significantly affect the plant immune response such that normal endoreduplication does not occur and the host cell begins to break down (e.g. no clear vacuole or nucleus). Another possible explanation for the altered plant nodule cell organization could be that the plant cells are slower to fill with bacteroids due to less efficient bacteroid-peribacteroid membrane division. The altered plant cell organization may also affect the microaerophilic environment. Oxygen concentration affects *Rhizobium* gene expression within the nodule

(Soupene et al. 1995). Possible alterations to the host plant nodule cells could have many important implications on the bacteroids they contain.

The relationship of the bacteroid membrane and peribacteroid membrane is also a key component of the symbiotic relationship. During bacteroid differentiation bacteria undergo elongation from 1-2 μm to 5-10 μm as well as differentiation into branched Y-shaped bacteroids (Kereszt et al. 2011b). The bacteria undergo endoreduplication during differentiation and have significant changes to cellular metabolism and gene expression (Soupene et al. 1995; Mergaert et al. 2006; Prell and Poole 2006; Kereszt et al. 2011a). The peribacteriod membrane is also crucial for nutrient exchange between the plant and bacteroid (Udvardi and Day 1997; White et al. 2007). VLCFA mutants were found to have unusual shape with multiple branches, multiple bacteroids per symbiosome, less density in the plant cell, and to show signs of early degradation. Synchronous division of the bacteroid within the peribacteroid membrane, which occurs during bacteroid maturation is altered with the absence of the VLCFA as evidenced by the of multiple bacteroids per symbiosome and multiply branched, and aberrantly shaped bacteroid structures. Bacteroids of indeterminate nodules typically have weaker membrane integrity than bacteria but bacteroids of the VLCFA mutants are likely to be even more affected as evidenced by susceptibility to salt and detergents (Chapter 3, (Sutton and Paterson 1980)). A disruption in the transport of nutrients across membranes and between the plant and bacteroid may lead to early senescence. Failure to complete the bacteroid maturation process generally leads to bacterial cell death and lysis (Werner et al. 1984). The decrease in bacteroid density may be due to either the inability of the bacteria to be endocytosed into the plant cells as efficiently or a lack of replication of the bacteroids within the peribacteroid membrane prior to terminal differentiation.

Multiple factors including fewer large nodules per plant root system, disorganization of the infected nodule cells, and decreased bacteroid density may account for decreased nitrogen fixation. *Rhizobium leguminosarum* and *Sinorhizobium meliloti* mutants that do not have a VLCFA have been shown to retain the ability to infect plant nodules and fix nitrogen, albeit at an attenuated ability (Vedam et al. 2006; Haag et al. 2009; Vanderlinde et al. 2009). Other factors such as nutrient exchange between the plant and bacteroids and general bacteroid fitness may also influence the overall levels of nitrogen fixation.

Although *Rhizobium leguminosarum* bv. *viciae* VLCFA mutants are able to form a symbiotic relationship with pea plants leading to nitrogen fixation the efficiency of this interaction has clearly been diminished. The results observed in this work confirm the importance of a pentacylated lipid A for symbiosis.

Acknowledgements

This work was supported, in part, by National Institutes of Health Grant GM39583 (to R.W.C.) and by the Department of Energy Grant DE-FG-02-93ER20097 to the CCRC.

Table 4.1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristics	Source
Rhizobium leguminosarum* bv. *viciae		
Rlv3841	Strain 300 Str^R, Fix^+	(VandenBosch et al. 1989)
Rlv22	Strain 3841 *acpXL::kan* Str^R, Kan^R, Fix^+	(Vedam et al. 2003)
EL196	Strain 3841 *acpXL::kan lpxXL::gen* Str^R, Kan^R, Gen^R, Fix^+	This study
EL197	pVV5 (*acpXL*) in Strain 3841 *acpXL::kan lpxXL::gen*, Str^R, Kan^R, Gen^R, Tet^R, Fix^+	This study, (Vedam et al. 2003)
EL198	pSSB-1 (*acpXL,lpxXL*) in Strain 3841 *acpXL::kan lpxXL::gen*, Str^R, Kan^R, Gen^R, Tet^R, Fix^+	This study, (Basu et al. 2002)

Table 4.2. Percentage ethylene evolved from exposure of plant root systems to acetylene

	Percentage Ethylene		
Strain	**14 dpi (n[1])**	**21 dpi (n)**	**28 dpi (n)**
Rlv3841	9.93^{A} (7)	5.81^{A} (10)	NA[2]
Rlv22	7.24^{ABC} (6)	4.66^{AB} (14)	12.32^{A} (5)
EL196	4.63^{BCD} (6)	2.73^{C} (23)	9.67^{B} (5)
EL197	2.85^{D} (6)	3.28^{BC} (20)	7.96^{B} (5)
EL198	7.64^{AB} (5)	4.55^{AB} (22)	NA

^{ABCD} Differing superscripts within a column indicate significance ($P<0.05$).

[1] n is the number of plant root systems analyzed for acetylene reduction

[2] NA indicates that acetylation reduction was unable to be determined due to drying out of the plant growth medium

Percentages are calculated from the ratio of the area under the peak for acetylene to the area under the peak for ethylene.

Table 4.3 Number of small, medium, and large nodules for Rlv3841, Rlv22, EL196, EL197, and EL198 at 14, 21, and 28 days post inoculation.

Strain	n	Small[1]	Medium	Large	Total
14 dpi					
Rlv3841	7	75	47^{A}	25	147
Rlv22	6	61	32^{A}	22	114
EL196	6	74	25^{AB}	12	111
EL197	6	86	4^{B}	0	90
EL198	5	68	24^{AB}	14	105
21 dpi					
Rlv3841	14	14^{C}	49^{AB}	43^{AB}	106^{B}
Rlv22	15	27^{BC}	43^{AB}	44^{AB}	114^{B}
EL196	13	82^{A}	69^{A}	26^{B}	178^{A}
EL197	12	50^{AB}	41^{AB}	30^{B}	121^{AB}
EL198	14	10^{C}	22^{B}	54^{A}	85^{B}
28 dpi[2]					
Rlv22	5	12^{B}	38	35	86^{B}
EL196	5	100^{A}	72	61	233^{A}
EL197	5	36^{B}	29	63	127^{B}

^{ABC} Differing superscripts within a column indicate significance ($P<0.05$).

[1] Small nodules = 1 mm^2, Medium nodules = 1-4 mm^2, Large nodules = >4 mm^2

[2] Rlv3841 and EL198 plant nodules were unable to be counted/measured due to fully desiccated plant media.

Figure Legends

Figure 4.1 Mature nitrogen-fixing plant nodule zones. Zone I – Apical meristem elongation. Zone II – Infection of plant cells. Zone III – Nitrogen fixing zone. Zone IV – Senescence zone.

Figure 4.2 Illustration of tissue dissection for obtaining nodule sections for processing and use in confocal and transmission electron microscopy.

Figure 4.3 Confocal imaging of 21 days post inoculation Syto13 stained nodule sections infected with Rlv3841, Rlv22, EL196, EL197, and EL198. Plant cell vacuole is labeled “v”. Uninfected plant cell is labeled “e”. Plant cell nucleus is labeled “n”. The area of the plant cell filled with bacteroids is labeled “b”.

Figure 4.4 TEM images of plant host cells and bacteroids. Bacteroids are evenly distributed within the plant cell in Rlv3841, Rlv22, and EL198. Larger light-colored areas are visible between bacteroids for EL196 and EL197 at all timepoints. Scale bars equal 5 μm.

Figure 4.5 TEM images of individual Rlv3841, Rlv22, EL196, EL197, and EL198 bacteroids. Scale bar equals 2 μm.

Figure 4.6 TEM images of individual mature bacteroids exhibiting degradation. Arrows are pointing to areas of degradation.

Figure 4.7 Bacteroid density from 14, 21, and 28 dpi root nodules infected with Rlv3841, Rlv22, EL196, EL197, and EL198. Density was measured by calculating the visible bacteroid area within a 400 μm^2 area within and infected plant cell not including the plant vacuole or nucleus.

Figure 4.8 The number of bacteroids per bacteroid μm^2 were counted within a 400 μm^2 area to determine the relative size of visible bacteroids.

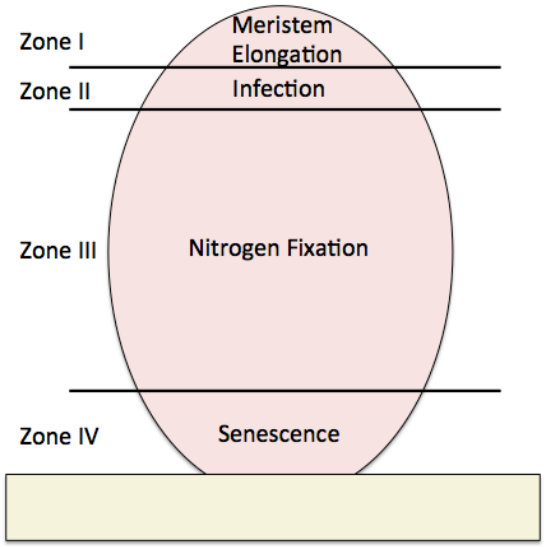


Figure 4.1

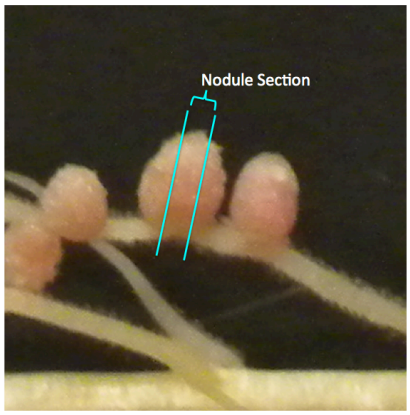


Figure 4.2

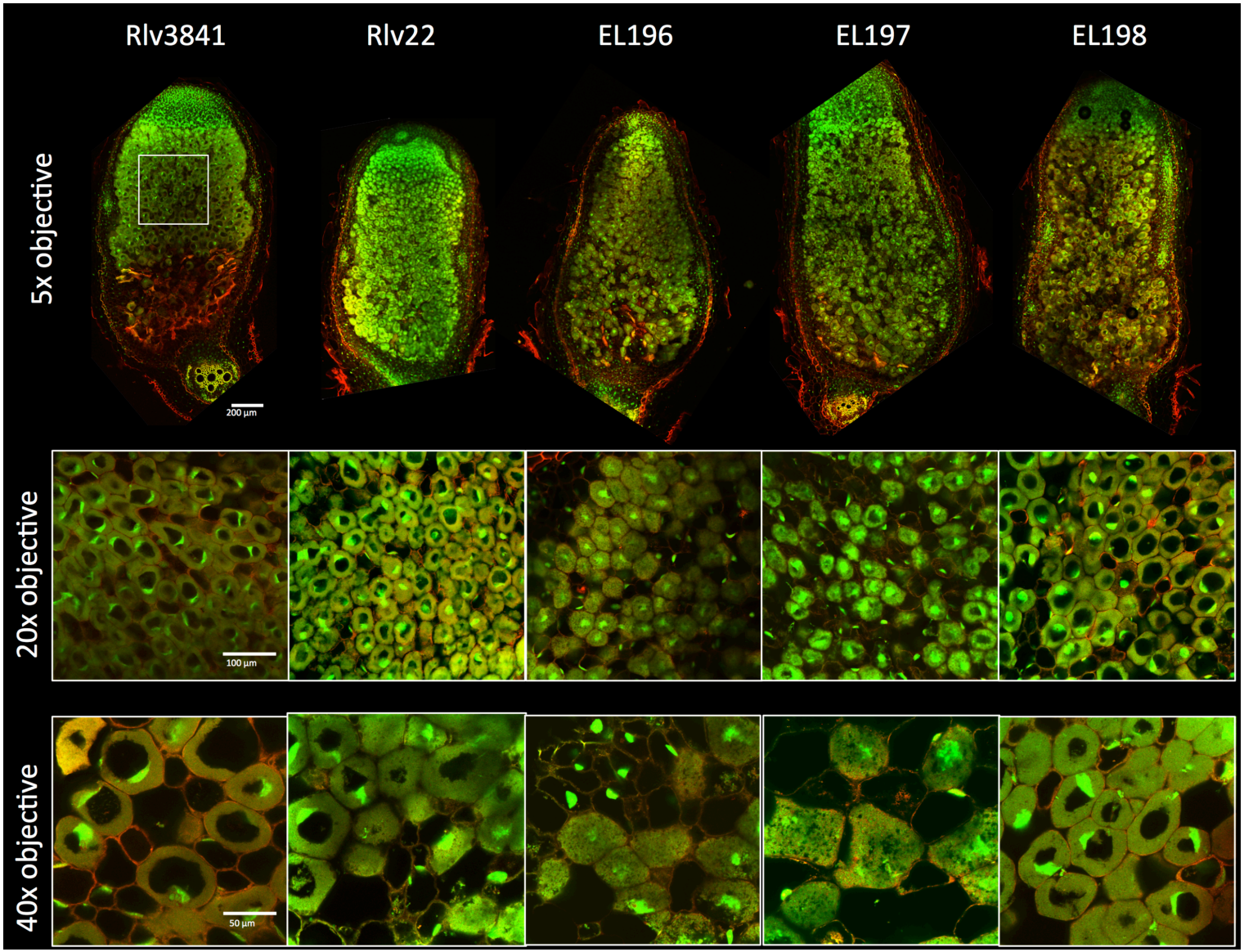

Rlv3841
Rlv22
EL196
EL197
EL198
5x objective
200 µm
20x objective
100 µm
40x objective
50 µm

Figure 4.3

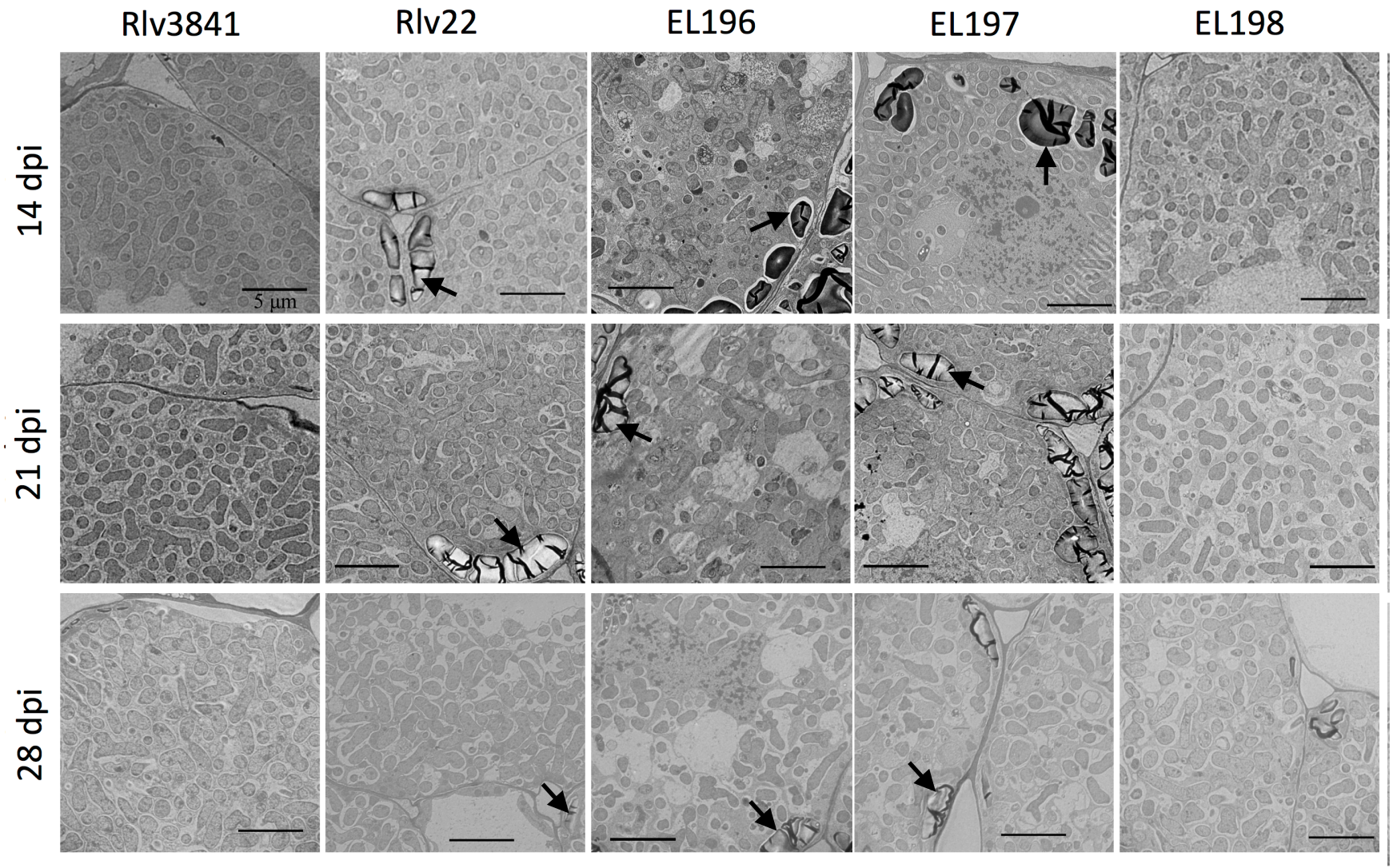


Figure 4.4

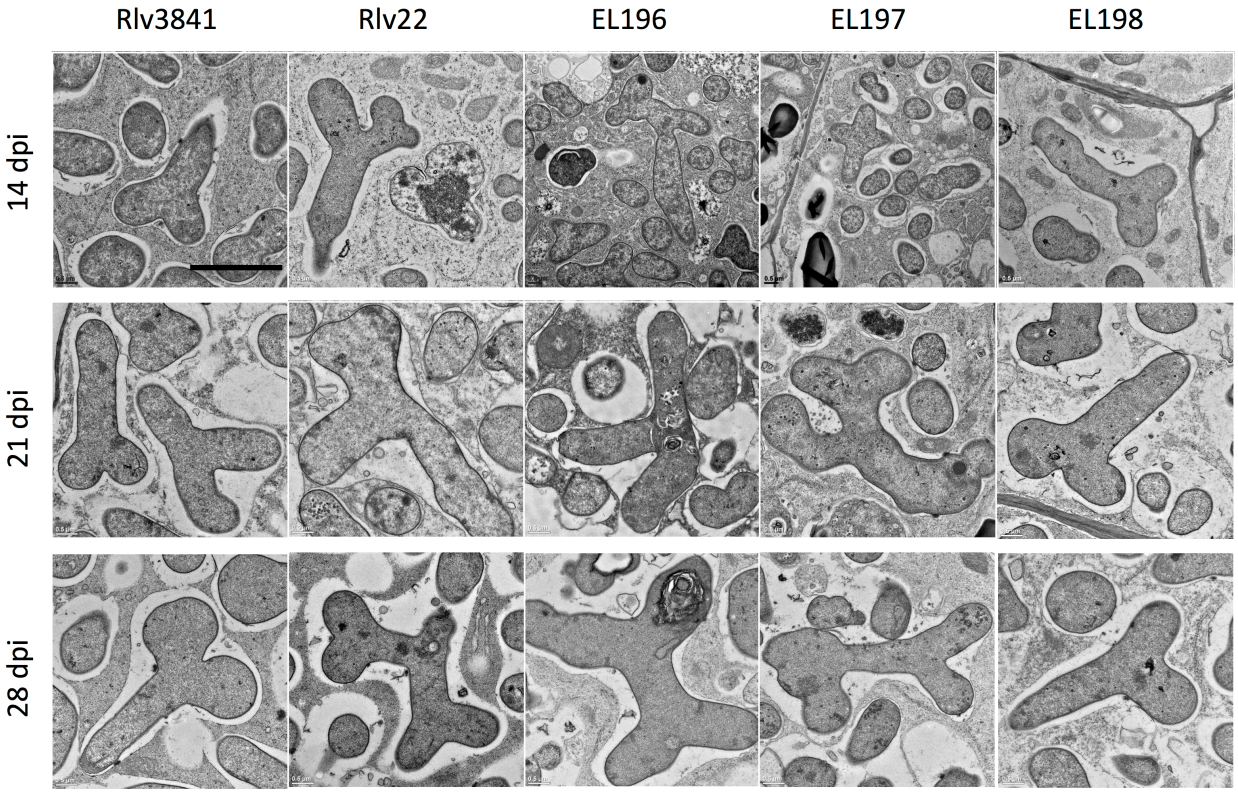


Figure 4.5

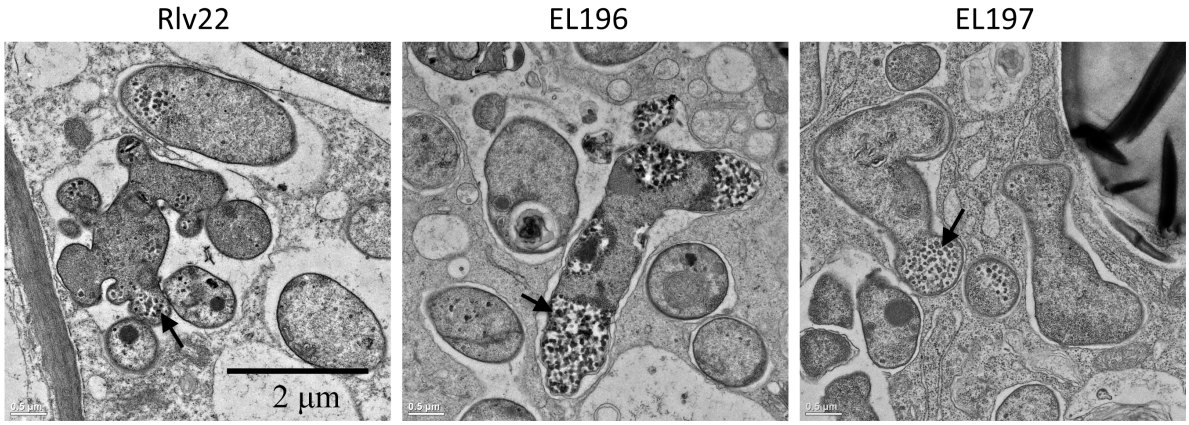


Figure 4.6

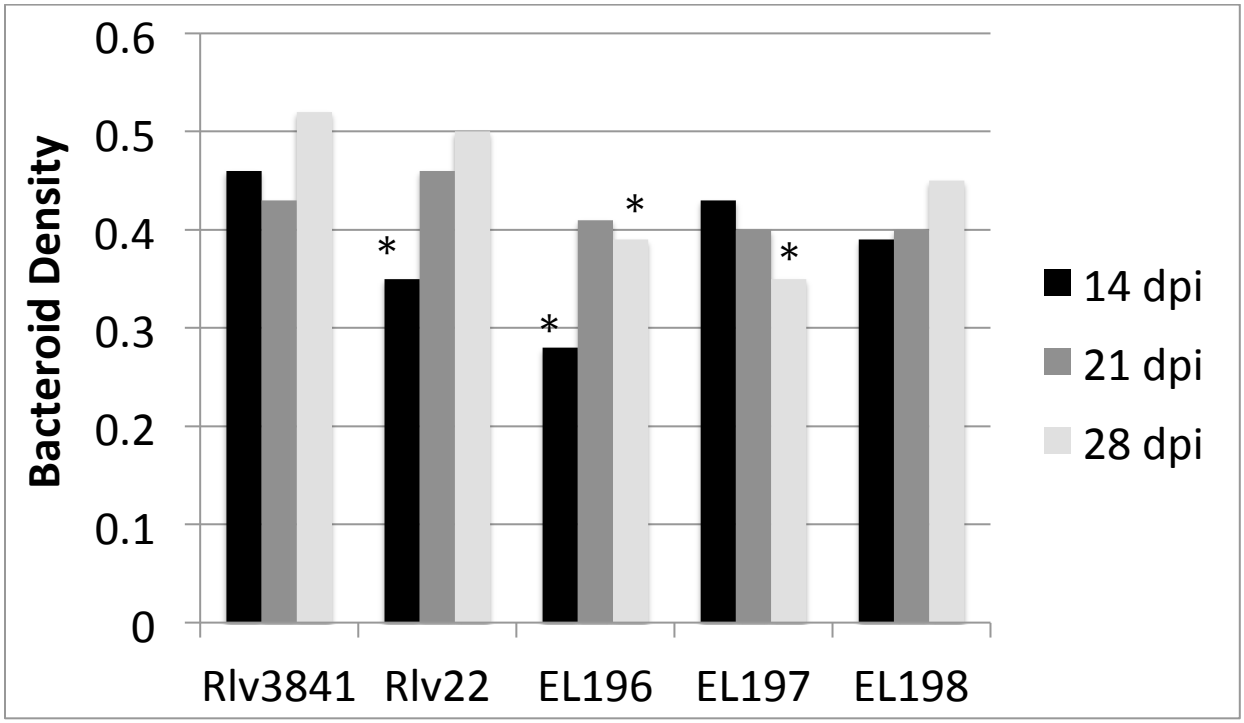


Figure 4.7

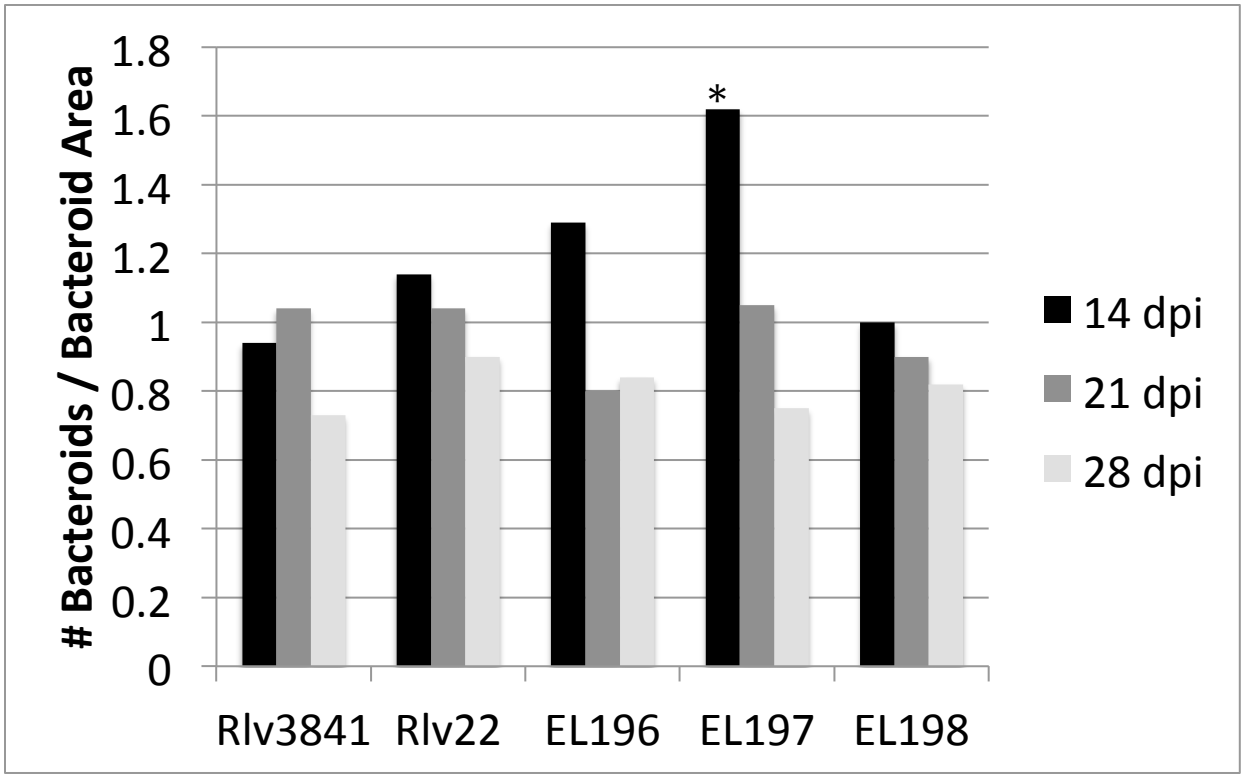


Figure 4.8

References

Basu, S. S., Karbarz, M. J., and Raetz, C. R. (2002) Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum*. *J. Biol. Chem.,* 277: 28959-28971.

Borisov, A. Y., Jacobi, L. M., Lebsky, V. K., Morzhina, E. V., Tsyganov, V. E., Voroshilova, V. A., and Tikhonovich, I. A. (1999) Genetic system controlling development of nitrogen-fixing nodules and arbuscular mycorrhiza. *Pisum Genet.,* 31: 40-44.

Brown, D. B., Huang, Y. C., Kannenberg, E. L., Sherrier, D. J., and Carlson, R. W. (2011) An *acpXL* mutant of *Rhizobium leguminosarum* bv. *phaseoli* lacks 27-hydroxyoctacosanoic acid in its lipid A and is developmentally delayed during symbiotic infection of the determinate nodulating host plant *Phaseolus vulgaris.* *J. Bacteriol.,* 193: 4766-4778.

Campbell, G. R., Reuhs, B. L., and Walker, G. C. (2002) Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. *PNAS,* 99: 3938-3943.

Carlson, R. W., Forsberg, L. S., and Kannenberg, E. L. (2010) Lipopolysaccharides in *Rhizobium*-legume symbioses. *Sub-Cell Biochem.,* 53: 339-386.

Denarie, J., Debelle, F., and Prome, J. C. (1996) *Rhizobium* lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. *Ann. Rev. Biochem.,* 65: 503-535.

Ferguson, G. P., Datta, A., Carlson, R. W., and Walker, G. C. (2005) Importance of unusually modified lipid A in *Sinorhizobium* stress resistance and legume symbiosis. *Mol. Microbiol.,* 56: 68-80.

Fraysse, N., Couderc, F., and Poinsot, V. (2003) Surface polysaccharide involvement in establishing the *rhizobium*-legume symbiosis. *Eur. J. Biochem.,* 270: 1365-1380.

Haag, A. F., Wehmeier, S., Beck, S., Marlow, V. L., Fletcher, V., James, E. K., and Ferguson, G. P. (2009) The *Sinorhizobium meliloti* LpxXL and AcpXL proteins play important roles in bacteroid development within alfalfa. *J. Bacteriol.,* 191: 4681-4686.

Haag, A. F., Wehmeier, S., Muszynski, A., Kerscher, B., Fletcher, V., Berry, S. H., Hold, G. L., Carlson, R. W., and Ferguson, G. P. (2011) Biochemical characterization of *Sinorhizobium meliloti* mutants reveals gene products involved in the biosynthesis of the unusual lipid A very long-chain fatty acid. *J. Biol. Chem.,* 286: 17455-17466.

Kannenberg, E. L., Reuhs, B. L., Forsberg, S., and Carlson, R. W. (1998) Lipopolysaccharides and K-antigens: Their structures, biosynthesis, and function. In H. P. Spaink, A. Kondorosi, and P. J. J. Hooykaas (eds.), *The Rhizobiaceae; Molecular Biology of Model Plant-Associated Bacteria* (Dordrecht/Boston/London: Kluwer Academic Publishers), 119-154.

Kereszt, A., Mergaert, P., and Kondorosi, E. (2011a) Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims?. *MPMI,* 24: 1300-1309.

Kereszt, A., Mergaert, P., Maroti, G., and Kondorosi, E. (2011b) Innate immunity effectors and virulence factors in symbiosis. *Curr. Opin. Microbiol.,* 14: 76-81.

Kondorosi, E., Roudier, F., and Gendreau, E. (2000) Plant cell-size control: growing by ploidy?. *Curr. Opin. Plant Biol.,* 3: 488-492.

Kondorosi, E., Mergaert, P., and Kereszt, A. (2013) A paradigm for endosymbiotic life: cell differentiation of *Rhizobium* bacteria provoked by host plant factors. *Ann. Rev. Microbiol.,* 67: 611-628.

Luyten, E. and Vanderleyden, J. (2000) Survey of genes identified in *Sinorhizobium meliloti* spp., necessary for the development of an efficient symbiosis. *Eur. J. Soil Biol.,* 36: 1-26.

Melino, V. J., Drew, E. A., Ballard, R. A., Reeve, W. G., Thomson, G., White, R. G., and O'Hara, G. W. (2012) Identifying abnormalities in symbiotic development between *Trifolium* spp. and *Rhizobium leguminosarum* bv. *trifolii* leading to sub-optimal and ineffective nodule phenotypes. *Ann. Bot.,* 110: 1559-1572.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A. E., Barloy-Hubler, F., Galibert, F., Kindorosi, A., and Kondorosi, E. (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. *PNAS,* 103: 5230-5235.

Nutman, P.S. (1970) Appendix III: The modified Fahraeus slide technique. In J.M. Vincent (ed.), *A manual for the practical study of root-nodule bacteria* (Oxford and Edinburgh: Blackwell Scientific Publications), 144-145.

Prell, J. and Poole, P. (2006) Metabolic changes of rhizobia in legume nodules. *Trends Microbiol.,* 14: 161-168.

Sharypova, L. A., Niehaus, K., Scheidle, H., Holst, O., and Becker, A. (2003) *Sinorhizobium meliloti acpXL* mutant lacks the C28 hydroxylated fatty acid moiety of lipid A and does not express a slow migrating form of lipopolysaccharide. *J. Biol. Chem.,* 278: 12946-12954.

Soupene, E., Foussard, M., Boistard, P., Truchet, G., and Batut, J. (1995) Oxygen as a key developmental regulator of *Rhizobium meliloti* N2-fixation gene expression within the alfalfa root nodule. *PNAS,* 92: 3759-3763.

Sutton, W. D. and Paterson, A. D. (1980) Effects of the plant host on the detergent sensitivity and viability of *Rhizobium* bacteroids. *Planta,* 148: 287-292.

Udvardi, M. K. and Day, D. A. (1997) Metabolite transport across symbiotic membranes of legume nodules. *Annu. Rev. Plant Phys.,* 48: 493-523.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H., Satiat-Jeunemaitre, B., Alunni, B., Bourge, M., Kucho, K., Abe, M., Kereszt, A., Maroit, G., Uchiumi, T., Kondorosi, E., and Mergaert, P. (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science,* 327: 1122-1126.

VandenBosch, K. A., Brewin, N. J., and Kannenberg, E. L. (1989) Developmental regulation of a *Rhizobium* cell surface antigen during growth of pea root nodules. *J. Bacteriol.,* 171: 4537-4542.

Vanderlinde, E. M., Muszynski, A., Harrison, J. J., Koval, S. F., Foreman, D. L., Ceri, H., Kannenberg, E. L., Carlson, R. W., and Yost, C. K. (2009) *Rhizobium leguminosarum* biovar *viciae* 3841, deficient in 27-hydroxyoctacosanoate-modified lipopolysaccharide, is impaired in desiccation tolerance, biofilm formation and motility. *Microbiol.,* 155(Pt 9): 3055-3069.

Vasse, J., de Billy, F., and Truchet, G. (1993) Abortion of infection during the *Rhizobium meliloti*-alfalfa symbiotic interaction is accompanied by a hypersensitive reaction. *Plant J.,* 4: 555-566.

Vasse, J., de Billy, F., Camut, S., and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J. Bacteriol.,* 172: 4295-4306.

Vedam, V., Kannenberg, E. L., Haynes, J. G., Sherrier, D. J., Datta, A., and Carlson, R. W. (2003) A *Rhizobium leguminosarum* AcpXL mutant produces lipopolysaccharide lacking 27-hydroxyoctacosanoic acid. *J. Bacteriol.,* 185: 1841-1850.

Vedam, V., Haynes, J. G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. (2004) A *Rhizobium leguminosarum* lipopolysaccharide lipid-A mutant induces nitrogen-fixing nodules with delayed and defective bacteroid formation. *Mol. Plant Microbe Interact.,* 17: 283-291.

Vedam, V., Kannenberg, E. L., Datta, A., Brown, D., Haynes-Gann, J. G., Sherrier, D. J., and Carlson, R. W. (2006) The pea nodule environment restores the ability of a *Rhizobium leguminosarum* lipopolysaccharide *acpXL* mutant to add 27-hydroxyoctacosanoic acid to its lipid A. *J. Bacteriol.,* 188: 2126-2133.

Vinardell, J. M., Fedorova, E., Cebolla, A., Kevei, Z., Horvath, G., Kelemen, Z., Tarayre, S., Roudier, F., Mergaert, P., Kondorosi, A., and Kondorosi, E. (2003) Endoreduplication mediated by the anaphase-promoting complex activator CCS52A is required for symbiotic cell differentiation in *Medicago truncatula* nodules. *Plant Cell,* 15: 2093-2105.

Werner, D., Morschel, E., Mellor, R. B., and Bassarab, S. (1984) The host cell nucleus oriented lysis of bacteroid is an ineffective (Fix-) type of *Glycine max* nodule. *Planta,* 162: 8-16.

White, J., Prell, J., James, E. K., and Poole, P. (2007) Nutrient sharing between symbionts. *Plant Physiol.,* 144: 604-614.

CHAPTER 5

SUMMARY

The unusual very long chain fatty acid (VLCFA) found on the lipid A of *Rhizobium leguminosarum* bv. *viciae* is an important structural component in the bacteria-legume symbiotic relationship. In this work it was demonstrated that without the acyl carrier protein, AcpXL, and acyl transferase, LpxXL, the VLCFA was not added to the lipid A. Also, without the LpxXL the VLCFA could not be replaced with a shorter palmitic or stearic fatty acid. We observed that during symbiosis bacteroids tended to have long chain fatty acids, which lead to a more hydrophobic membrane. Without the VLCFA the overall hydrophobicity as well as interactions between membrane components may be affected. These changes led to the bacterial cell membrane being compromised. Not only were the laboratory-cultured bacteria more susceptible to salt, detergents, and desiccation due to loss of membrane integrity but this cell membrane alteration also led to defects in the symbiotic process including poor plant nodule cell organization, deformation and senescence of bacteroids, and fewer bacteroids per plant cell. Alteration of the relationship between the bacterial and bacteroid membranes and the plant cell membranes may explain the changes to bacteroid structure and lack of synchronous division with the peribacteroid membrane observed for these mutants. The poor plant nodule cell organization, decreased uptake of infecting bacteria, and early bacteroid senescence observed for these mutants could be due to an increased host defense response that may be the result of an inability to attenuate the plant innate defense mechanism. Combined, these phenotypes led to diminished nitrogen fixation, which is the major function of the rhizobia-legume symbiotic

relationship. The fixing of atmospheric nitrogen to an available form such as ammonia is a crucial process to life on Earth. In addition to its role in nitrogen fixation, VLCFAs are also found in intracellular pathogens. As we increase our understanding of how bacteria alter the cell surface to evade both plant and animal immune responses we can continue to use that knowledge for our benefit in agriculture and in human and animal disease prevention and treatment.

Studying the role of LPS has been an important avenue of research in understanding prokaryotic and eukaryotic interactions. Clearly LPS play a crucial role in the complex rhizobia-legume symbiotic relationship. Future work examining the relationship of lipid A fatty acid chain length with the environment in which the bacteroid is exposed would be beneficial to add to our understanding of how the bacteria and plant interact. Work is currently being conducted not only with the lipid A portion but also concerning core and OPS portions of LPS. Additional work identifying the specific changes that occur when the bacteria and bacteroid interact with the plant would significantly add to our knowledge and lead to future discoveries in both symbiotic and pathogenic interactions.