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INVESTIGATIONS OF THE MOLECULAR MECHANISMS FOR HOST SPECIFICITY AND INFECTION IN THE *RHIZOBIUM*/LEGUME SYMBIOSIS

By

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ABSTRACT

INVESTIGATIONS OF THE MOLECULAR MECHANISMS FOR HOST SPECIFICITY AND INFECTION IN THE *RHIZOBIUM*/LEGUME SYMBIOSIS

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The *Rhizobium*/legume symbiosis represents an important agricultural and ecological interaction between prokaryotes and eukaryotes. Despite years of study, the molecular mechanisms critical to various aspects of this relationship, especially host specificity, have not yet been fully elucidated. The studies presented here explore the criticality of membrane chemistry in ensuring a viable symbiotic interaction.

Several common links between the structural chemistry of the chitolipooligosaccharides (Nod factors) of *Rhizobium* and the general rhizobial membrane lipid and lipopolysaccharide chemistry of these bacteria are uncovered. Aspects of common chemistry include sulfation, methylation and the position and extent of fatty acyl chain unsaturation. In the case of sulfation, common genetic control is shown by using strains with mutations in the associated *nod* genes. A large degree of spatial overlap between these molecules is also demonstrated since it is revealed that the chitolipooligosaccharides are normal rhizobial membrane

components. These results contradict the current dogma and allow us to begin to develop a comprehension of the highly integrated membrane lipid and glycolipid chemistry of rhizobia.

Novel aspects of the cell surface chemistry of the unusual *Rhizobium* strain *R. leguminosarum* biovar *trifolii* 4S have also been uncovered. Differences in this strain are manifested by a unique LPS core region and O-antigen as well as a distinct extracellular polysaccharide; compared to other *R. trifolii* strains, these cell-surface components are uniquely devoid of galactose. The discovery here of a galactose-containing lipid-linked oligosaccharide with a composition that resembles the typical *R. trifolii* O-antigen may allow us to understand better these abnormalities. These findings suggest that this strain is an effective natural isolate that has surface chemistry similar to some ineffective mutants.

The plant sulfolipid, sulfoquinovosyl diacylglycerol, was identified in the membranes of various strains of the family *Rhizobiaceae*. Until this study, this lipid was thought to be restricted to plants, photosynthetic bacteria or diatoms. The presence of this lipid in rhizobia, therefore, has significant implications to the mechanisms of symbiosis. Investigations into the heretofore unresolved biosynthetic pathway of this lipid were also performed. Two molecules, thiolactaldehyde and thioquinovose, are proposed to be involved.

To Mom and Dad.

Thank you for everything.

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Thanks again everybody,
Rob.

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LIST OF ABBREVIATIONS

ADP	adenosine 5′-diphosphate
APS	adenosine 5′-phosphosulfate
ATP	adenosine 5′-triphosphate
BIII	modified Bergensen's media
CDP	cytidine 5′-diphosphate
CLOS	chitolipooligosaccharide
CM	carboxymethyl cellulose
CPS	capsular polysaccharide
DAG	diacylglycerol
DEPT	distortionless enhancement by polarization transfer
DFQ-COSY	double quantum filtered-correlated spectroscopy
DHAP	dihydroxyacetone phosphate
DPG	diphosphatidylglycerol
EPS	extracellular polysaccharide
FAB-MS	fast-atom bombardment mass spectrometry
FAME	fatty acid methyl ester
FID	free induction decay

GC/MS	gas chromatography / mass spectrometry
GDP	guanosine 5′-diphosphate
GlcNAc	N-acetyl glucosamine
HMQC	heteronuclear multiple-quantum coherence spectroscopy
HOHAHA	homonuclear Hartmann-Hahn spectroscopy
HPLC	high performance liquid chromatography
KDO	2-keto-3-deoxyoctulosonic acid
LPC	lysophosphatidyl choline
LPS	lipopolysaccharide
MDO	membrane derived oligosaccharides
NMR	nuclear magnetic resonance
PAPS	3′-phosphoadenosine-5′-phosphosulfate
PC	phosphatidyl choline
PE	phosphatidylethanolamine
PG	phosphatidyl glycerol
PMME	phosphatidyl monomethyl ethanolamine
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SQDG	sulfoquinovosyl diacylglycerol
TEAE	triethylaminoethyl cellulose
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UDP	uridine 5′-diphosphate

CHAPTER 1

OVERVIEW OF RHIZOBIAL CELL-SURFACE CHEMISTRY AND ITS POSSIBLE ROLES IN SYMBIOTIC NITROGEN FIXATION

INTRODUCTION

Symbiotic nitrogen fixation is a classical illustration of an interaction between eukaryotes and prokaryotes that encompasses many fields of science; biochemistry, chemistry, genetics, microbiology, botany, physiology and agricultural science. The relationship between bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively termed rhizobia) and their host leguminous plants (family *Leguminosae*) has interested scientists for over 100 years. The importance of symbiotic nitrogen fixation to the global nitrogen cycle has been well documented. It has been estimated that the rhizobia/legume symbiosis accounts for over 50% of natural nitrogen fixation and when taking into account the nitrogen fixation of agricultural value, this number jumps to over 70% (1). The interest in this unusual relationship, however, has stemmed not only from the various agricultural ramifications but also because the information gleaned from this system might provide insight into the processes involved in other infections as well.

One of the most studied aspects of the symbiotic relationship, and also one of the most intriguing, is the specificity in host range exhibited by most species of rhizobia. With a few notable exceptions, each species or biovar (i.e., subspecies) of bacteria is only able to infect a very limited number of plants. For instance, *Rhizobium meliloti* strains are capable of

infecting alfalfa plants but lack the ability to form a symbiosis with clover (Table 1.1). Conversely, strains belonging to *Rhizobium leguminosarum* biovar *trifolii* (hereafter referred to as *R. trifolii*) infect clover but not alfalfa. Fundamental differences must therefore exist between the

Table 1.1: Some rhizobial species and their associated host plants.

Rhizobial Species	Host Plant
Rhizobium meliloti	Alfalfa (*Medicago*)
R. leguminosarum biovar *trifolii*	Clover (*Trifolium*)
biovar *phaseoli*	Bean (*Phaseolus*)
biovar *viciae*	Vetch (*Vicia*), Pea (*Pisum*)
Rhizobium species strain NGR234	Tropical legumes, *Parasponia* (non-legume)
Bradyrhizobium japonicum	Soybean (*Glycine*)

various species of rhizobia with regard to host recognition. Many theories have been advanced over the years to explain the mechanisms responsible for initial as well as other necessary interactions throughout the rhizobia/legume symbiosis. The studies performed in this dissertation were aimed at testing some of these theories and hopefully unraveling the molecular mechanisms involved in host specificity as well as other important aspects of the infection process.

The literature review that is presented in Chapter 1 is a general overview on the history of rhizobial research. An emphasis is placed on

the role of membrane components as well as diffusable metabolites in the process of nitrogen fixation. Evidence of common structural and biosynthetic characteristics between these two cell surface related elements is presented in Chapter 2. It is shown that rhizobial chitolipooligosaccharides and their general membrane lipid components have several common structural features and that the same genes are involved. Chapter 3 presents the discovery of a new class of lipid from a strain of *R. trifolii* with unusual membrane chemistry. This lipid may answer questions as to why this strain is able to infect with a membrane that is so unique to its species. The isolation and characterization of sulfoquinovosyl diacylglycerol from *R. meliloti* is shown in Chapter 4. This is a lipid that heretofore was thought to be virtually nonexistent outside of photosynthetic organisms. The occurrence of this lipid amongst various rhizobial species as well as implications for a role in symbiosis are addressed. Experiments aimed at deciphering the biosynthesis of this lipid are given in Chapter 5. Based on the data presented in this chapter, two compounds are suggested that may be involved in one of the several possible synthetic pathways that have been delineated over the years. The final chapter encompasses all of these studies by offering a broad perspective on the role of the membrane in assuring a viable infection in the *Rhizobium*/legume symbiosis.

LITERATURE REVIEW

The Process of Nodulation

The process of symbiotic nitrogen fixation is characterized by the ability of the Gram-negative soil inhabiting rhizobia to invade and participate in a mutualistic relationship with a legume host. A wide variety of microorganisms, both free-living and symbiotic, possess the ability to fix nitrogen. The rhizobia/legume system is by far the most studied, however, due to the agricultural importance of legumes. The infection by rhizobia culminates in the formation of a novel structure on the root, termed nodule, within which the bacterium transforms into a nitrogen fixing bacteroid state fueled by the plant. This reduction of molecular nitrogen into ammonia that is performed by the bacteroid is of utmost agronomical and ecological importance. Nitrogen is one of the most limiting nutrients to plants since it is not readily available in most soils. Atmospheric nitrogen is not assimilable by plants and the only other usable sources besides biological fixation are the small amounts in rainwater, the waste and decay products of other organisms or artificial fertilizers (1). The latter of these, although accounting for increased agricultural productivity, has had an adverse ecological impact. Run-off from fertilized lands has contaminated drinking water supplies with nitrates and contributed to the eutrophication of lakes and rivers (1). The

application of liquid ammonium fertilizers and the associated release of excess ammonia also increases the load of greenhouse gases in the atmosphere. An additional problem related to fertilizers is that the reduction of nitrogen by abiological means is an extremely energy-expensive procedure. With the growing world population and subsequent demand for food, these concerns will become increasingly more and more significant. Both the economic as well as environmental expense of nitrogen fertilizers will eventually make them prohibitive and biological nitrogen fixation that much more important.

A basic diagrammatical representation of the events leading to nitrogen fixation is shown in Figure 1.1. The first obvious indication of an impending infection occurs with the appearance of distinct morphological changes in the root. These changes begin with deformations and curling of the root hairs (Shepard crooks) (2, 3) along with simultaneous mitogenic activity in the cortical cells of the root (4). The bacteria, which were bound to the root after being attracted by plant chemotaxants (5, 6), become trapped by the contorted root hair. These trapped bacteria then begin to degrade (or induce the degradation of) the plant cell wall and subsequently influence the development of a new structure called the infection thread (7). The infection thread, which can be compared to a

Figure 1.1: Representation of the events leading to nitrogen fixation in the rhizobia/legume interaction. After chemotaxis to the host root hair, the bacterium binds and the plant cell wall degrades. At this point begins the formation of the infection thread through which the bacteria are able to traverse to the rapidly dividing cortical cells. The bacterium is released from the infection thread into the plant cell simultaneously becoming surrounded by a plant derived peribacteroid membrane. When the plant cell is nearly full of bacteria, the outer membrane and peptidoglycan of the bacteria are lost and the enlarged bacteroid form is able to reduce nitrogen. Adapted from references 1 and 10.

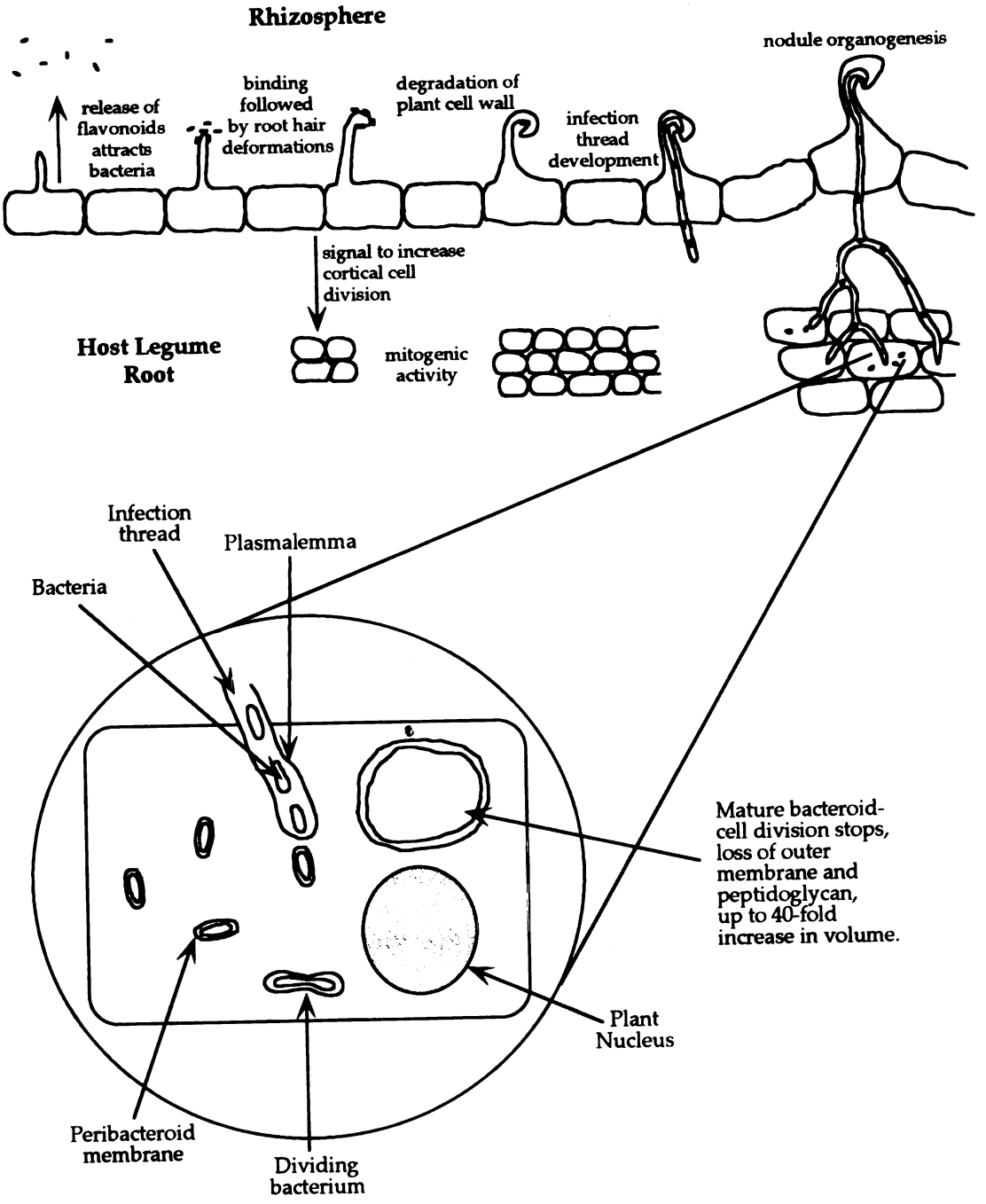

Rhizosphere
nodule organogenesis
release of flavonoids attracts bacteria
binding followed by root hair deformations
degradation of plant cell wall
infection thread development
signal to increase cortical cell division
Host Legume Root
mitogenic activity
Infection thread
Plasmalemma
Bacteria
Mature bacteroid-cell division stops, loss of outer membrane and peptidoglycan, up to 40-fold increase in volume.
Plant Nucleus
Peribacteroid membrane
Dividing bacterium

Figure 1.1

root hair growing inward, is composed of plant derived material and acts as a tunnel which allows the bacterium to navigate its way to the inner cortex of the root. These densely populated cortical cells are then penetrated by the ramified infection thread and infected by the bacterium (8). As they leave the infection thread, the rhizobia are enveloped by a plant-like plasma membrane that is at this point designated as the peribacteroid membrane (9). Eventually the plant cells in this area have divided and become infected to such a great extent that they create a bulb-like structure on the root. This structure is called the nodule and this entire process termed nodulation. At this stage the invading organisms are in their bacteroid state and capable of relieving the host plant of the need for external sources of nitrogen.

Membrane Associated Carbohydrates of Rhizobia

The general cell envelope organization of rhizobia is fairly typical of Gram-negative bacteria. It consists of an inner cytoplasmic membrane, a periplasmic space in which resides the peptidoglycan and an outer membrane of which the outer leaflet is mainly composed of lipopolysaccharide (LPS). The LPS is generally composed of three structural domains: the lipid A, which acts as the membrane anchor, the core region, an oligosaccharide that is fairly conserved between species and the O-antigen, a repeating polysaccharide of varying length that is

Figure 1.2: Basic structure of typical enteric bacterial lipopolysaccharide. Three structural domains exist in normal LPS: the lipid A membrane anchor, the inner and outer core regions that are fairly conserved amongst species and typically contain KDO and heptose as marker carbohydrates, and an O-antigen repeat that is highly strain specific. Adapted from reference 137.

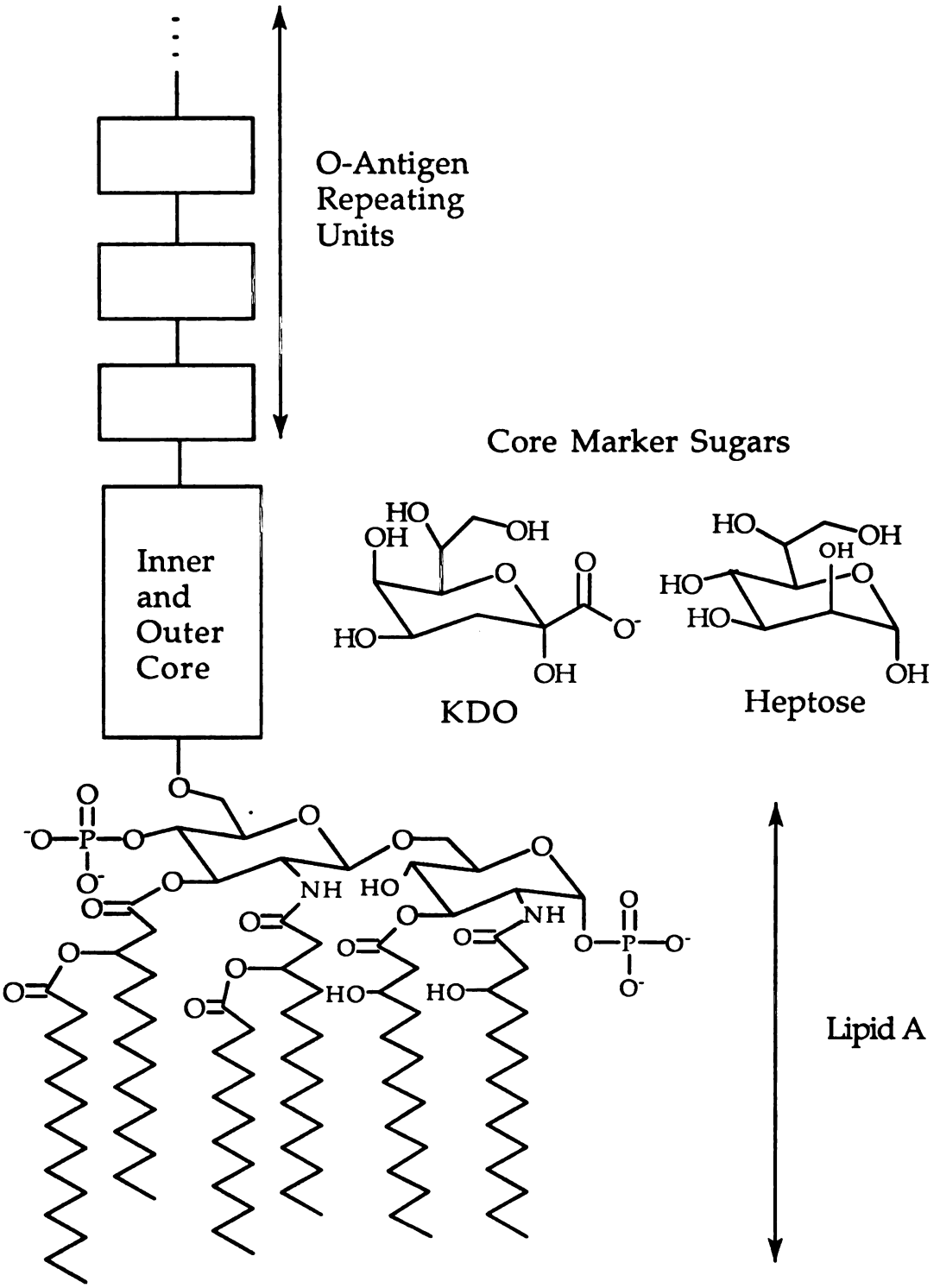

O-Antigen
Repeating
Units
Inner
and
Outer
Core
Core Marker Sugars
KDO
Heptose
Lipid A

Figure 1.2

highly variable in composition between species (137). In the "typical" enteric Gram-negative bacteria, the LPS has been very well characterized (Figure 1.2). The lipid A in these bacteria consists of a disaccharide of 1-6 ß-linked glucosamine substituted with 3-OH fatty acids in either ester or amide linkages at the 2, 3, 2′ and 3′ positions (137). There are also fatty acids in ester linkages at the 3-OH functionalities. Additionally, there is a net negative charge on lipid A resulting from phosphate esters at the 1 and 4′ positions. The core region is generally characterized by the presence of two marker carbohydrates, 2-keto-3-deoxyoctulosonic acid (KDO) and heptose (137).

The structural elucidations performed thus far on rhizobial LPS have shown it to contain certain features distinct from the "typical" LPS. For instance, the lipid A from *R. trifolii* ANU 843 was found to contain an unusual long chain fatty acid accounting for about 50% of the total fatty acid content (11). This fatty acid, 27-hydroxyoctacosanoic acid, was subsequently found to be a constitutive component in members of the *Rhizobiaceae* (12). Another unusual fatty acid, 29-hydroxytriacontanoic acid, was also isolated from a rhizobial species (13). Besides the fatty acid composition, the headgroup of lipid A in rhizobial strains has been shown to differ from that found in the enteric bacteria. It was found that the lipid A from *R. trifolii* ANU 843 lacks the phosphate groups and instead a carboxyl group accounts for the requisite negative charge (14, 15). This

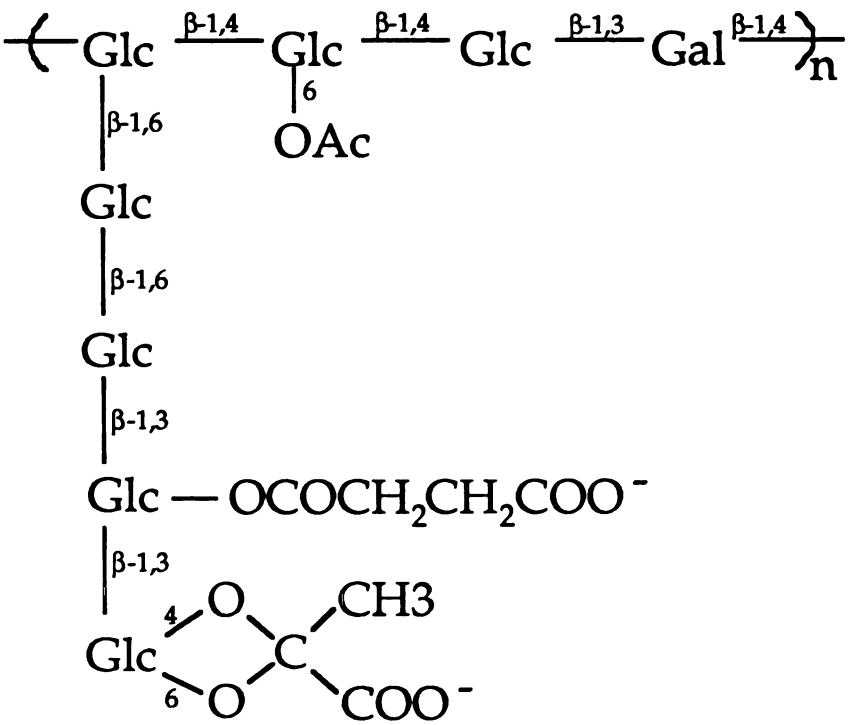
may not be a critical structural feature, however, since another study found the typical phosphorylated diglucosamine backbone in this species (16). In subsequent studies, glucosamine and galacturonic acid were identified as the lipid A sugars in this strain (12, 15). Yet another unusual rhizobial lipid A backbone was identified as a trisaccharide containing two uronic acids and a glucosamine (17). A later study indicated that this structure was incorrect though, and the purported glucosamine aldonic acid component was not present (23).

The LPS core region is usually conserved between strains of the classical Gram-negative bacteria and this is thought to be generally true of rhizobia as well. Two distinct core oligosaccharides are known to exist simultaneously in the biovars of *R. leguminosarum* (*trifolii, phaseoli* and *viciae*) (18-22). One is a trisaccharide consisting of two galacturonic acid residues and a KDO while the other is a tetrasaccharide containing galactose, galacturonic acid, mannose and KDO. Neither contains heptose, a "typical" core marker (along with KDO) found in the *Enterobacteriaceae*.

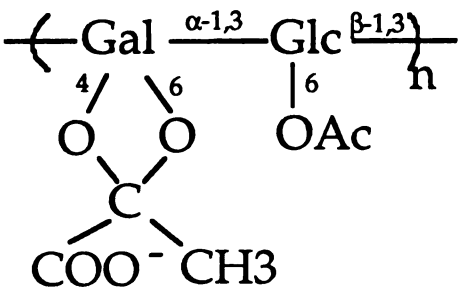
The basic O-antigen structure of rhizobial LPS is very different from the classical Gram-negative bacteria as evidenced by its behavior in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enteric LPS appears as a "ladder"; discrete bands which reflect various degrees of polymerization of linear oligosaccharide subunits (24, 25). With rhizobial

LPS there are typically only two major bands. These have been attributed to the so-called LPS I, with an intact O-antigen (smooth), and LPS II, with an attenuated O-antigen (rough) (26-31). The reason for this major difference is not known but it may be that the rhizobial O-antigen is a branched polysaccharide as opposed to a linear repeat. The only rhizobial O-antigen characterized to date has been from the unusual *R. trifolii* 4S strain (32). The question of branching was not addressed in this study.

Most rhizobial strains also synthesize a capsule or capsular polysaccharide (CPS) as well as an excreted acidic extracellular polysaccharide (EPS). The structure of these two glycoconjugates are generally similar but the difference between the two lies in the fact that the CPS is associated with the cell surface while the EPS is sloughed off into the extracellular milieu. Much of the earlier work done on rhizobial EPS was performed on *R. meliloti*. The major form of *R. meliloti* EPS, also known as succinoglycan or EPS I (Figure 1.3), is an acidic repeating octasaccharide carrying certain non-carbohydrate substituents (a succinate, pyruvate and O-acyl group) that account for its acidity. A family of genes (*exo*) important for the synthesis and export of the succinoglycan has been well characterized (for review see reference 33). Certain mutant strains of *R. meliloti* have been found to produce an alternate EPS, termed EPS II, that has a structure considerably distinct from the succinoglycan (Figure 1.3) (34, 35). The EPS of the biovars of *R.*



Succinoglycan



EPS II

Figure 1.3: Glycosyl sequences of the two major EPS structures found in *R. meliloti*, succinoglycan, or EPS I, and EPS II. OAc = acyl group, Glc = glucose and Gal = galactose. Adapted from reference 33.

leguminosarum have been found to be fairly similar to that of *R. meliloti*. These bacteria typically produce an octasaccharide repeating EPS consisting of glucose, glucuronic acid, and galactose (5:2:1) with a pyruvate acetal attached to the terminal galactose (36). Another non-carbohydrate substituent, an ether linked 3-hydroxybutanoic acid group, has also been found in certain strains of *R. trifolii* (37, 38).

Other classes of carbohydrate-containing molecules have been found to be membrane associated in the rhizobia. One class, the cyclic ß-(1,2)-glucans, is related to the membrane derived oligosaccharides (MDO) of *E. coli*. These cyclic oligosaccharides are composed entirely of glucose and are localized in the periplasmic space (39, 40). It has been shown that these components are related to osmotic balance (41). Another *E. coli* related rhizobial polysaccharide recently characterized is one containing a high degree of KDO that is not excreted (i.e. not an EPS) and is structurally distinct from the LPS (42, 43). Isolated from *R. meliloti* and *R. fredii*, this polysaccharide consists of repeating units of galactose and KDO and is structurally analogous to the group II K antigens (CPS) of *E. coli*. Another class of glycolipid, the digycosyl diacylglycerols, has been recently found in strains of *R. trifolii* (44, 45). Although their appearance in Gram-negative bacteria is rare, they are widespread components of plant cell membranes.

Involvement of Cell Surface Components in Host Specificity

The earliest models presented to implicate cell surface chemistry in the host recognition process involved lectin/carbohydrate binding. The first paper on this subject, by Hamblin and Kent, presented evidence that the plant lectins might bind the bacteria to the roots in a general fashion (46). Using a single *Rhizobium* strain these researchers found that bacteria treated with lectins were capable of erythrocyte agglutination. However, the question of host specificity was not addressed. In a subsequent study, Bohlool and Schmidt used a fluorescently labelled soybean lectin to bind specifically to all but 3 of 25 soybean nodulating *Rhizobium* strains tested (47). In addition, the lectin did not bind to 23 strains from 5 various species in which soybean is not a host. Therefore they postulated that legume lectins presented a site on the root surface which recognized a specific polysaccharide on the appropriate bacteria. The studies that followed attempted to identify the bacterial polysaccharide receptor that was binding to the lectin. The first such series of experiments, by Dazzo *et al.*, implicated the capsular polysaccharide (CPS) as the determinant of symbiotic specificity. It was shown that the CPS of the clover symbiont *R. trifolii* carried determinants which were antigenically cross-reactive with a surface component on clover seedling roots (48, 49). These researchers proposed that the host lectin was a diffusable component excreted from the plant that joined *R.*

trifolii to clover root hairs by recognizing common antigenic determinants on each. In later studies, they were able to identify the clover lectin trifoliin A, show it to be present at discrete sites near the tip of the root hair cells, and show that the specific encapsulated rhizobial symbiont bound at these sites (50-52). Various workers were subsequently able to identify other specific bacterial capsular polysaccharides and their associated lectins in the *R. meliloti*/alfalfa (50), *R. leguminosarum*/pea (53) and *B. japonicum*/soybean (54, 55) systems.

The role of the acidic extracellular polysaccharide (EPS) in host specificity has proven to be somewhat controversial. One series of experiments has shown that the O-acylation pattern of the EPS of *R. trifolii* was influenced by host specificity genes and therefore the interactions of the EPS with lectins may contribute to the specificity (36, 58). The authors make the claim that "these alterations would be pronounced in the host root environment and contribute to host specificity" (58). This data was refuted by others, however, who found no differences in the structures of EPS repeating subunits from the various biovars of *R. leguminosarum* (59-61). These authors state unequivocally that "the discernible structural features of the acidic polysaccharides secreted by *Rhizobium* biovars cannot be the determinant of host specificity" (59). This debate has not been settled.

Besides the CPS and EPS, the lipopolysaccharide (LPS) has also been shown to be involved in host specific lectin binding. Experiments by Wolpert and Albersheim implicated the O-antigen containing LPS by showing binding to specific lectin columns (56). A later study explored the growth phase dependence of trifoliin A binding and LPS structure (57). It was found that the relative amount of various glycosyl components in the LPS changed from the exponential to stationary phases of growth and these changes correlated to the ability of the bacteria to bind trifoliin A and to infect.

Most of the recent studies in the area of membrane polysaccharides and host specificity have been aimed at determining precise structural features required by the carbohydrate receptors as well as determining the particular genes involved. Researchers have characterized the complete structure of the CPS that binds trifoliin A (62) and demonstrated a correlation between relative levels of non-carbohydrate side groups in the CPS and their effects on lectin binding (63, 64). Studies have also been performed relating the bacterial culture age to surface polysaccharide structure and bacterial attachment to roots (65-67). In an important experiment, Diaz *et al.* were able to isolate a key pea lectin gene. After introducing the gene into white clover roots, the transgenic plants were able to be nodulated by a strain usually specific for peas (68). Similar results were obtained by altering the bacterial genes. Introduction of host

specificity genes from *R. trifolii* into a another *Rhizobium* species resulted in a hybrid strain with efficient nodulation of the *R. trifolii* host clover, production of the *R. trifolii* EPS and an increase in binding to the trifoliin A lectin (58).

Although there is overwhelming evidence for the involvement of carbohydrate/lectin interaction in host specificity, other results seem to suggest that this interaction is necessary but not sufficient for an effective symbiosis. For instance, it was found that a genetically engineered strain of *Azotobacter vinelandii*, carrying the trifoliin A receptor from *R. trifolii*, acquired the ability to specifically adhere to clover roots but not infect them (69, 70). In addition, *R. trifolii* mutants were found that were able to bind the host lectin and attach specifically to clover roots but were also non-infective (71, 72). However, even though the carbohydrate/lectin interaction may not exclusively account for host specificity, the plethora of positive results from over twenty years of research implicating various cell surface components in the recognition process cannot be ignored.

Involvement of Cell Surface Components Throughout Infection

Besides the recognition and binding stages of infection, various defects in normal membrane chemistry have been correlated with a dysfunction at other stages of the nodulation process as well. It was first determined that the presence of LPS was critical for a viable symbiosis in a

study by Maier and Brill in which they noticed the absence of a surface antigen associated with the LPS O-antigen in non-nodulating mutants of *Bradyrhizobium japonicum* (73). Russa, *et al.*, found that LPS isolated from closely related nodulating and non-nodulating strains of *R. trifolii* were vastly different in carbohydrate composition (74). A later study showed that in a non-nodulating mutant strain of *B. japonicum* the LPS seemed to lack an O-antigen (75). These results were echoed in another study of a similar mutant of *R. phaseoli* (30). In this case, it was demonstrated that the symbiotic defect was a flaw in infection thread development. Subsequently, the ability of the bacteria to synthesize the correct LPS structure was implicated in seemingly every stage of the symbiotic process. These stages included competitiveness for nodulation (76), infection thread development (77), bacterial release from the infection thread (27), avoidance of the plant defense mechanisms (44) and the development of complete nitrogen fixing nodules (22, 26, 29, 78). Despite the overwhelming evidence, however, one study has proclaimed that LPS does not play a major role in symbiosis (28). Using various strains of *R. meliloti* with mutations in all of the major LPS genes these researchers found that none of the mutant strains were defective in symbiosis.

A definitive involvement of EPS in a viable symbiosis has been shown as well. Most of the work in this area has centered upon resolving

phenotypic and structural changes from various mutations in the *exo* genes (i.e., the genes responsible for EPS synthesis). The first such study showed that a mutant strain of *R. meliloti* which failed to succinylate its EPS I and did not produce any EPS II was defective in nodule invasion (79). The ability of these strains to nodulate was partially rescued by the addition of exogenous EPS II (45, 80). In a similar experiment performed by Djordjevic *et al.*, it was shown using both *Rhizobium* species strain NGR234 and *R. trifolii* that the EPS or even the EPS oligosaccharide from the parent strain of the Exo$^-$ mutant partially restored nodulation ability (81). Many other studies have been performed demonstrating the significance of the EPS to infection. These have included the importance of the regulation of EPS production (82), the variation of the infection ability of Exo$^-$ mutants depending on the host plant genotype (83) and the effect of pH on restoring nodulation ability to Exo$^-$ mutants (84). The general consensus on the role of the EPS in infection is that it ensures bacterial release from the infection thread and nodule invasion.

Rhizobial Genetics

It is generally thought that several major classes of genes exist in rhizobia that are related to the infection process. Some of these, (i.e. *exo*, *nif* and *fix*) are important in the formation of functional nodules but supposedly not in the determination of host range. Another family of

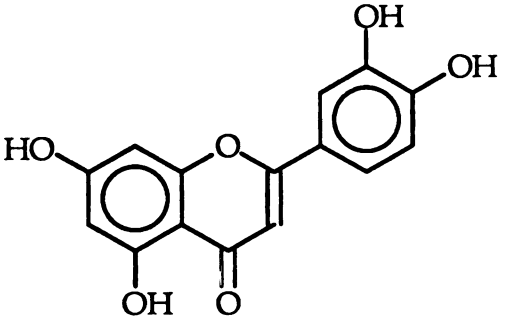
genes, known as the *nod* genes, have been proposed to be solely responsible for specificity. These genes have been classified as either common or host specific. Common *nod* genes, such as *nodA*, *nodB* and *nodC*, are interchangeable between various species of rhizobia that infect diverse hosts and are also apparently conserved in function (85-88). It has been shown that these genes are responsible for causing the increased cortical cell division in the roots during infection (89) and for the deformation of root hairs (85, 90). The host specific genes are particular to a species or biovar and are believed to be the determinants of the bacterial range of hosts. Mutations in these genes result in loss of root hair deformation ability on the homologous host and may result in the acquisition of this ability on heterologous hosts (91, 92). Examples of host specificity genes are *nodPQ* and *nodH* of *R. meliloti*, which were shown to specify nodulation of alfalfa (93, 94), and *nodE* of *R. leguminosarum* biovars *viciae* or *trifolii*, which is responsible for infection of pea or clover, respectively (95). The *nod* genes have been shown to be located on a megaplasmid called pSym which has been estimated at 15 kb (96-98).

It has been observed that most of the *nod* genes are not expressed in free-living cells rather only when in the presence of the plant host or plant host exudates or extracts (99, 100). Exceptions to this observation are the family of *nodD* genes, which are expressed constitutively. It appears that the product of the *nodD* gene is responsible for the induction of the other

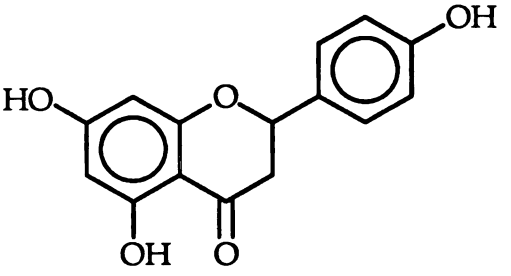
nod genes in the presence of plant exudates (101, 102). The compounds isolated from the plant exudates found to be most active on *nodD* were a family of flavonoids (or isoflavonoids). It also was found that distinct flavonoids seemed to induce the genes of a particular species or biovar of rhizobia. For example, luteolin (3',4',5,7-tetrahydroxyflavone), was shown to induce *nodD* in *R. meliloti* (103, 104) (Figure 1.4). Additionally, one of the inducer compounds from soybean, daidzein (105), has been shown to be an inhibitor of *nodD* induction in *R. trifolii*, a symbiont of clover (106).

The Nod Factor Model for Host Specificity

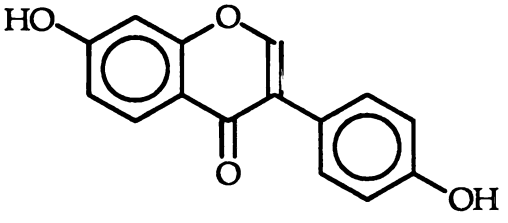
The first quantitative evidence that legume root hair deformations could be induced by incubating the roots with a cell-free culture filtrate of the appropriate rhizobial symbiont was made about sixty years ago (107). Since then, many studies were performed that implicated diffusable metabolites as the basis for nodule formation (4, 108, 109). Several different classes of molecules including bacterial oligosaccharides (124, 125), polysaccharides (121-123), nucleic acids (123) and proteins (121, 123) were implicated as the initial root hair deforming factors. None of these components, however, were isolated in quantities sufficient for a thorough structural analysis.



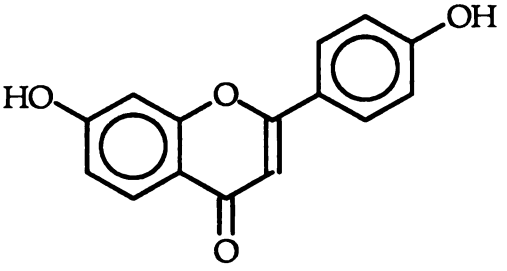
Luteolin
3',4',5,7-tetrahydroxyflavone
(*R. meliloti*) (103)



Narigenin
4',5,7-trihydroxyflavanone
(*R. viciae*) (131)



Daidzein
4',7-dihydroxyisoflavone
(*B. japonicum*) (105)



4', 7-dihydroxyflavone
R. trifolii (132)

Figure 1.4: Four *nod* gene inducing compounds in various systems.

In later studies, the involvement of the common *nodABC* genes as well as the host specific *nodPQ* and *nodH* genes in the production of the diffusable metabolite from *R. meliloti* was shown (110-112). At this point this unidentified component was termed the "Nod factor". Using wild-type *R. meliloti* strains grown in media containing the inducer luteolin, the cell-free culture supernatants exhibited root hair deformation (Had) activity on the homologous host alfalfa but not on the heterologous host vetch (110). An assay of Had activity, after application of a particular compound to the root surface, is thought to be indicative of the ability of that compound to induce nodulation. The sterile filtrate was extracted with organic solvents and analyzed by high performance liquid chromatography (HPLC). However, again the problem of insufficient material for detailed structural determination was encountered. Lerouge, *et al.*, attacked this problem by genetic overexpression; they introduced a plasmid into *R. meliloti* that contained extra copies of the *nod* genes (113). The plasmid was comprised of all of the common, specific, and regulatory *nod* genes at 5-10 copies per cell. This resulted in a greater than 100-fold increase in the Had activity of the culture filtrate of this genetically enhanced organism over that of the wild type *R. meliloti* (113). This activity also was not affected by mutations in the common *nod* genes. These workers then attempted to purify large quantities of these Nod factors by introducing the plasmid into a strain deficient for EPS

production, presumably to facilitate the filtrate extraction (113). Using reverse-phase HPLC they were able to isolate approximately 4mg of purified Nod factors out of ten liters of luteolin induced culture. The structure of the primary Nod factor was determined utilizing a combination of mass spectrometry, ^{1}H and ^{13}C-nuclear magnetic resonance spectroscopy and chemical modification. It was identified as a tetrasaccharide composed of ß-1,4-N-acetyl glucosamine with a sulfate group at the 6-OH position of the reducing sugar and a hexadecadienoic acid attached in an amide linkage at the 2-OH position of the non-reducing residue.

When this paper appeared it was considered to be a breakthrough in the field of rhizobial research not only because it showed that root deformation activity was attributable to a characterized molecular species but also because it demonstrated host specificity. When the authors applied the Nod factor in a solution (10^{-8}-10^{-11}M) to roots of the homologous host alfalfa as well as a heterologous host vetch, only the alfalfa showed Had activity (113). These authors made the claim that this particular Nod factor was the single molecular determinant of host specificity in *R. meliloti*. They did mention, however, the presence of other "minor" Nod factor components differing in fatty acid distribution. Later, one of these other components was isolated and identified as an acetylated derivative of the original Nod factor (114). In fact, it was

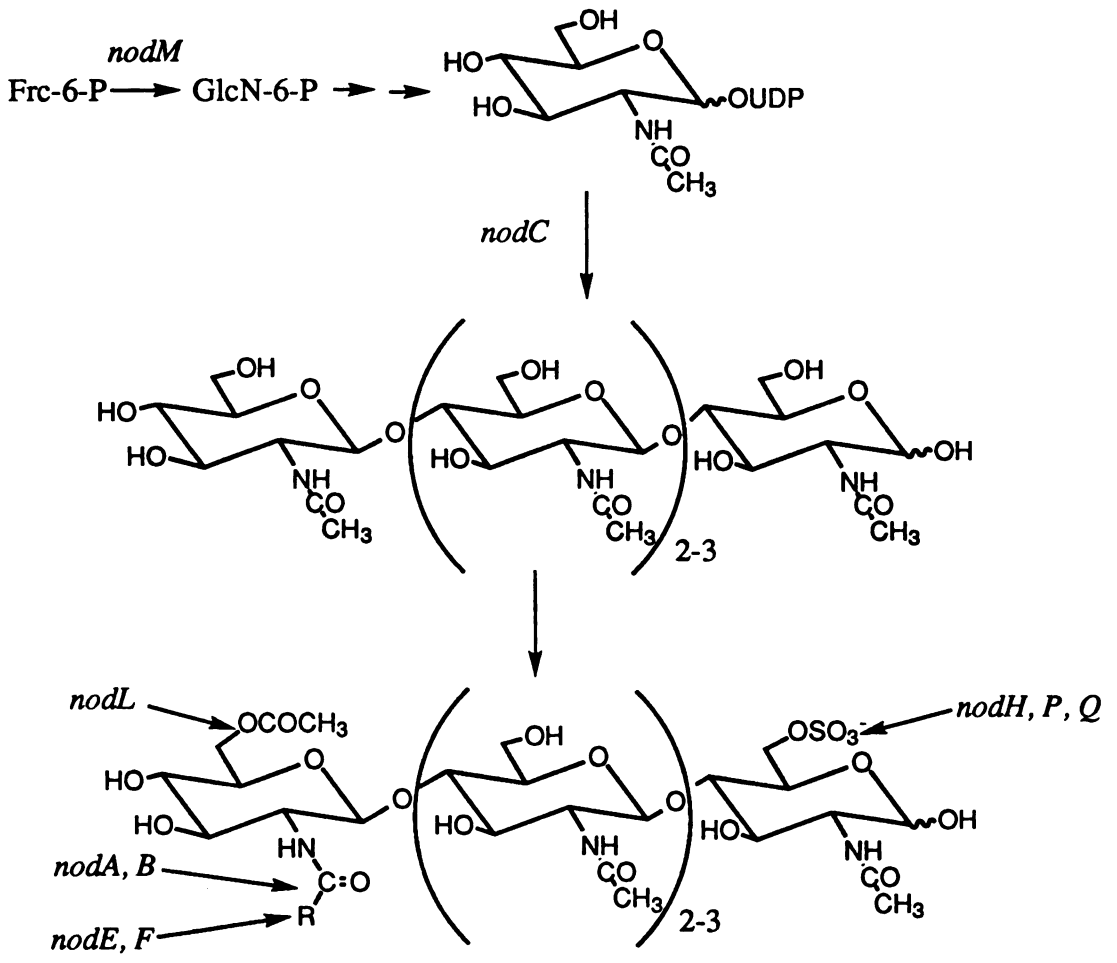
eventually determined that *R. meliloti* produces a family of sulfated chitolipooligosaccharides (CLOS) that exhibit a host specific effect on plant roots (116). These compounds range in degree of polymerization from three to five sugar residues and contain a distribution of fatty acid chain lengths and unsaturations. Therefore, the general consensus in the field is that the only constant structural element, the sulfate linkage, is the essential basis for the activity of these molecules. Indeed, it was shown that hydrolysis of the sulfate group resulted in a strong decrease in the morphogenic activity of the Nod factor (115) whereas lack of the acetate group, changes in the number of glucosamine residues or in the fatty acid distribution resulted in a less pronounced reduction in these activities (115-117). It was also demonstrated that synthetic *R. meliloti* CLOS with or without this sulfate group was active or inactive for Had activity on clover roots, respectively.

After the isolation of the Nod factors, many studies were aimed at determining the exact roles of the common and host specific *nod* genes which, as discussed above, had been shown to be required for the production of the alfalfa specific signals. A depiction of the biosynthesis of Nod factors as well as the roles of some of the more important *nod* genes is presented in Figure 1.5. It was first discovered that the host specific *nodP* and *nodQ* genes displayed a great deal of homology to the *cysD* and *cysN* genes of *E. coli* (118). These *E. coli* genes encode proteins that are

involved in the synthesis of adenosine-5'-phosphosulfate (APS) (119). This ATP sulfurylase activity is the first step to the formation of activated sulfate which, of course, would be necessary for addition of the sulfate group to Nod factors that is required for specificity. The function of the other host specificity gene, *nod H*, was then proposed by Roche, *et al.*, to be involved in the transfer of the activated sulfate moiety to the nascent Nod factors (120). These authors showed that strains with mutations in the *nodH* gene produced chitolipooligosaccharides that were nonsulfated and that these strains also lost their ability to nodulate alfalfa. They then suggested, using sequence homology with known proteins, that the gene product of *nodH* was a sulfotransferase.

The common nod genes, *nodA*, *nodB* and *nodC*, have been implicated in the assembly of the glycolipid backbone that receives the host specific modifications. It was first shown that the product of *nodC* functioned as a UDP-GlcNAc transferase based on sequence similarity to chitin synthases (126, 127). This was later supported by experiments showing that bacteria expressing the NodC protein incorporated GlcNAc from UDP-GlcNAc into chitin oligomers (128). The NodB protein was shown to de-acetylate the non-reducing terminal GlcNAc on chitin oligomers and also was shown to share sequence homology to proteins with similar functions (129). Lastly, the product of *nodA* is thought to be

Figure 1.5: Putative pathway for the biosynthesis of chitolipooligosaccharides in *Rhizobium meliloti* and the functions of some of the associated *nod* factors.



nodA	proposed N-acyl transferase (130)
nodB	evidence for deacetylase activity and sequence homology (129)
nodC	chitin synthase homology, proposed UDP-GlcNAc transferase (126-128)
nodD	family of nod gene transcriptional activators (101, 102)
nodE	proposed to synthesize fatty acyl chain, ketoacyl synthase (133)
nodF	acyl carrier protein homology (134)
nodH	proposed transfer of activated sulfate (120)
nodL	homology to acetyl transferase (95, 135)
nodM	proposed glucosamine synthase (136)
nodP	proposed ATP sulfurylase, involved with *nodQ* (118, 119)
nodQ	proposed APS kinase, involved with *nodP* (118, 119)

Figure 1.5

an N-acyltransferase. Cells expressing this protein acylate the deacetylated GlcNAc oligomers while *nodA*⁻ mutants do not (130).

CONCLUSION

For many years, the molecular mechanisms involved in host specificity and infection in the rhizobia/legume symbiosis has been a well-studied area of research in the field of nitrogen fixation. All of the major cell-surface carbohydrates and glycolipids of rhizobia have been implicated to a certain extent in ensuring a viable infection or determining host range (or both). These components have included the lipopoly-saccharides, capsular polysaccharides, extracellular polysaccharides, cyclic ß-(1,2)-glucans and most recently chitolipooligosaccharides. Regardless of the wealth of results involving all of these classes of molecules, the latter has been heralded as the single determinant of host specificity. Additionally, one family of genes (*nod*) is thought to exist for the exclusive purpose of synthesizing these glycolipids. Mutations in these genes, which have been shown to cause defects in chitolipooligosaccharide structure, eliminate the ability of the bacteria to discriminate the proper host plant. The effect of these mutations on the other cell-surface components, however, has never been assessed. Given

the proven involvement of these molecules in all stages of infection, this seems quite odd. The experiments presented in this dissertation will address this oversight as well as attempt to provide a framework for a new model in which the entire membrane structure is the critical determinant for infection and host specificity in symbiotic nitrogen fixation.

CHAPTER 2

COMMON LINKS IN THE STRUCTURE AND CELLULAR LOCALIZATION OF *RHIZOBIUM* CHITOLIPOOLIGOSACCHARIDES AND GENERAL *RHIZOBIUM* MEMBRANE PHOSPHOLIPID AND GLYCOLIPID COMPONENTS

ABSTRACT

Several common links between the structural chemistry of the chitolipooligosaccharides of *Rhizobium* and the general rhizobial membrane lipid and lipopolysaccharide chemistry of these bacteria have been uncovered. Aspects of common chemistry include sulfation, methylation and the position and extent of fatty acyl chain unsaturation. It was found that bacteria which are known to synthesize sulfated chitolipooligosaccharides (such as *R. meliloti* strains and the broad-host-range *Rhizobium* species strain NGR234) also have sulfated lipopolysaccharides. Their common origins of sulfation have been demonstrated by using mutants which are known to be impaired in sulfating their chitolipooligosaccharides. In such cases, there is a corresponding diminution or complete lack of sulfation of the lipopolysaccharides. The structural diversity of the fatty acids observed in the chitolipooligosaccharides is also observed in the other membrane lipids. For instance, the doubly unsaturated fatty acids which are known to be predominant components of *R. meliloti* chitolipooligosaccharides were also found in the usual phospholipids and glycolipids. Also, the known functionalization of the chitolipooligosaccharides of *R.* sp.

NGR234 by O- and N-methylation was also reflected in the lipopolysaccharide of this organism.

The common structural features of chitolipooligosaccharides and membrane components is consistent with a substantial degree of biosynthetic overlap and a large degree of cellular, spatial overlap between these molecules. The latter aspect is clearly demonstrated since it is shown here that the chitolipooligosaccharides are, in fact, normal rhizobial membrane components. This increases the importance of understanding the role of the bacterial cell surface chemistry in the rhizobia/legume symbiosis and developing a comprehension of the highly integrated membrane lipid and glycolipid chemistry of rhizobia.

INTRODUCTION

Nod factors are substituted chitolipooligosaccharides (CLOS) that have been isolated from the cell-free supernatants of rhizobial strains genetically engineered to overproduce them. This class of molecules has been proclaimed as the absolute determinant of host specificity in the rhizobia/legume symbiosis. When applied to roots of the homologous host of the rhizobial strain from which they were isolated, these lipid molecules effect morphological changes on the root that mimic those seen in the early stages of an authentic infection. The production of these glycolipids also seems to be enhanced by molecules made by the plant (flavones). It is therefore believed that a molecular signaling dialogue exists that accounts for host specificity (Figure 2.1). The plant produces a specific flavone that stimulates production of CLOS in the appropriate bacteria; these glycolipids are excreted from the bacterium, diffuse across the rhizosphere and are recognized by the distinct host plant root. The plant responds by beginning to prepare itself for an upcoming symbiotic event. This model was widely accepted with great enthusiasm by the majority of the scientists working in this area. However, perhaps due to the fervor associated with the discovery of the Nod factors, some very

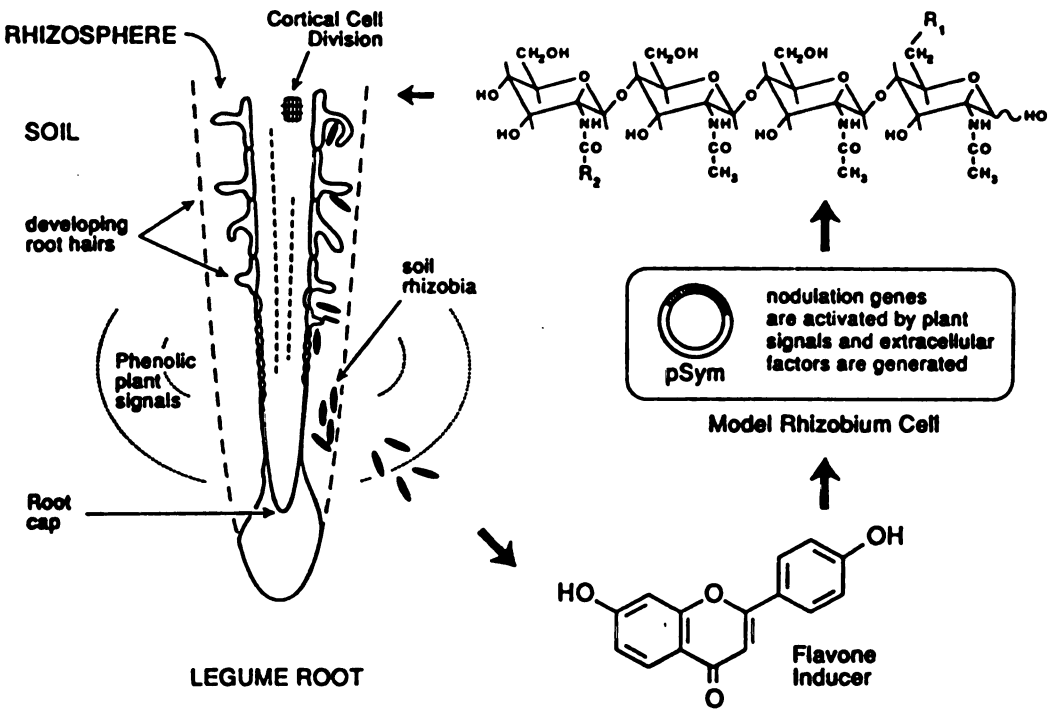


Figure 2.1: Diagrammatical representation of the putative signal exchange in the early events of the rhizobia/legume symbiosis. Flavonoids released by the plant induce the nodulation genes located on the Sym plasmid to produce specific chitolipooligosaccharides which begin the cascade of morphological events leading to a successful infection. Figure taken from reference 10.

major inconsistencies regarding this model have been overlooked. Some of these oversights are addressed by the experiments presented in this chapter.

A major conceptual problem with the Nod factor model lies in the notion of scale and the concentration of these molecules needed to bring about a reaction. The chitolipooligosaccharides have not been isolated from wild type cells in any substantial quantities, rather only through methods of genetic augmentation. Between five and ten extra copies of the entire set of nod genes along with additional regulatory genes were included in these strains (113). This resulted in more than a 100-fold increase in root hair deformation activity. It seems odd that a molecule so important to the assurance of a viable symbiosis is not produced by a wild type bacteria in an amount adequate for isolation. This is especially strange considering the fact that this molecule must be made in quantities sufficient for diffusion throughout the rhizosphere in the soil in order to signal the plant from a distance. The original manuscript on the isolation of CLOS reported a yield of four milligrams of purified glycolipid from ten liters of induced culture (113). This gives a concentration in the high nanomolar range; this is the same range that these molecules have been shown to exhibit their mitogenic effect on the root cortex (115). It seems ridiculous, therefore, to envision the same density of cells in the vicinity of a plant root in the wild as would be found in a liquid culture. In addition,

remembering that the CLOS has to diffuse through the soil and of course that the lab strains are genetically supercharged makes this proposal all the more absurd.

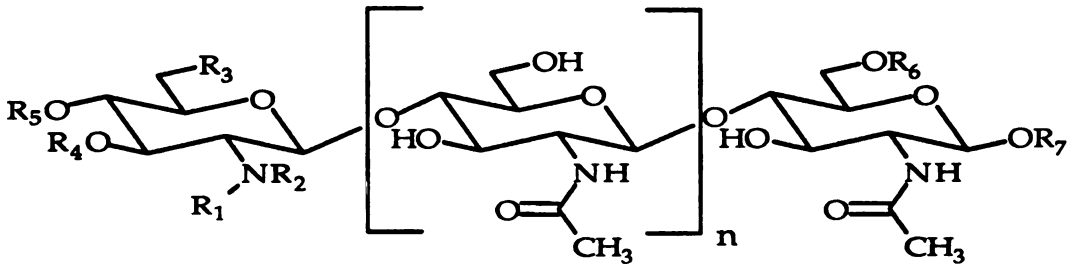
A definitive experiment to perform in order to prove that CLOS is indeed the exclusive determinant for host specificity would be to exogenously add the appropriate chitolipooligosaccharide to a Nod^-/Fix^- strain, i.e., one which fails to produce the correct CLOS and hence fails to nodulate the proper host. If the theory were correct, one would expect that the presence of the exogenous CLOS would restore the ability of these bacterial mutants to infect. This experiment has been tried in various laboratories using innumerable methodologies and to date has not been successful. This raises serious questions as to the strict exclusivity of the chitolipooligosaccharides in host specificity.

Another confounding aspect of the Nod factor model is the tremendous variability in chitolipooligosaccharide structure, even in the same strain. The first studies on *R. meliloti* CLOS promoted the idea that each rhizobial species synthesized, almost exclusively, only one molecule with no variation in carbohydrate or fatty acid chain length, degree of sulfation or acetylation, or position or extent of alkyl chain unsaturation (113). Subsequent studies, however, have demonstrated that any given bacterial species, including *R. meliloti*, can produce CLOS containing a variety of different fatty acids. These glycolipids may also contain

between zero and four double bonds in their acyl chains, differing degrees of sulfation, varying numbers of glucosaminyl residues and various substituents ranging from methyl and carbamoyl to entire glycosyl groups (Figure 2.2). In one case, 18 distinct chitolipooligosaccharides were identified or characterized in one bacterial strain (138). The structural diversity of CLOS is such that the relatively sparse number of *nod* genes cannot account for it. The only location in the cell, known to contain the vast repository of enzymatic activities capable of producing such a myriad of structures is the membrane. In order to use this machinery during their biosynthesis, chitolipooligosaccharides would have to be membrane localized. If this were true, the structural modifications one finds in CLOS should also be found in the other membrane components of that strain. If these lipids *were* membrane localized, the more complete picture that emerges would have the bacterial cell surface and membrane chemistry as the critical factors in determining host range and the outcome of a potential symbiotic event between rhizobia and legumes.

The importance of rhizobial cell-surface chemistry to various stages of the symbiotic nitrogen-fixing process between these bacteria and legumes was delineated in Chapter 1. The lipopolysaccharides (LPS) of rhizobia have been implicated in various stages of the symbiotic nitrogen-fixing process between these bacteria and legumes. These stages have

Figure 2.2: The major reported structures of chitolipooligosaccharides produced by various rhizobial strains. Upper: The N-acetyl glucosamine oligosaccharide backbone. Lower: Various substituents found in different strains.



Strain	R_1	R_2	R_3	R_4/R_5	R_6	R_7	n	Ref.
R. meliloti								
"wild type"	16:2	H	H/O-acetyl	H	SO_3H	H	2,3	113
nodPQ⁻	16:2	H	H/O-acetyl	H	H/SO_3H	H	2,3	"
nodH⁻	16:2	H	H/O-acetyl	H	H	H	2,3	"
AK41	16:3	H	H/O-acetyl	H	SO_3H	H	1-3	116
Rhizobium sp. strain NGR234	16:0/18:1	Me	H/carb	H/carb	2-O-Me-()-Fuc (3-O-acetyl) (4-O-sulfatyl)	H	3	138
R. viciae								
"wild type"	18:4/18:1	H	O-acetyl	H	H	H	2,3	95
nodL⁻	18:4/18:1	H	H	H	H	H	2,3	"
nodE⁻	18:1	H	O-acetyl	H	H	H	2,3	"
B. japonicum								
USDA 110	18:1	H	H	H	2-O-Me-Fuc	H	3	139
USDA 135	16:0/16:1 /18:1	H	H/O-acetyl	H	2-O-Me-Fuc	H	3	140
USDA 61	18:1	H/ Me	H/O-acetyl	H/carb	2-O-Me-Fuc/ Fuc	gly	2,3	140

Me = methyl; carb = carbamoyl; Fuc = fucosyl; gly = glycerol

Figure 2.2

included competitiveness for nodulation (76), infection thread development (77), bacterial release from the infection thread (27), avoidance of the plant defense mechanisms (44) and the development of complete nitrogen fixing nodules (22, 26, 29, 78). A definitive involvement of capsular polysaccharide biosynthesis and structure is also well established. Specific mutations of bacterial genes involved in the various stages of capsular polysaccharide biosynthesis show a high correlation with symbiotic deficiencies in nodulation (33). The general consensus on the role of the EPS in infection is that it ensures bacterial release from the infection thread and nodule invasion (45, 79, 80, 81). The cell surface components of rhizobia, then, have always been considered as essential in the very specific interaction of these bacteria with legumes. This interaction is known to be controlled from the bacterial side by the host specific *nod* genes. These genes, along with the common *nod* genes which are essential for nodulation in all rhizobial species, have been shown to be involved in the synthesis of CLOS. In the case of *R. meliloti* and *Rhizobium* species strain NGR234 these molecules contain a sulfate group that has been shown to be critical to host specificity (113, 114, 138). In *R. meliloti*, two of the more important host specific genes, *nodPQ* and *nodH*, are known to be involved in the sulfation of the CLOS (118, 120). The function of *nodPQ* in the biosynthesis of the activated sulfate donor has been clearly demonstrated (118, 141). The *nodH* gene has been shown to

be critical in determining host specificity and has also been implicated in transfer of the activated sulfate to the Nod factor in *R. meliloti* (120).

The experiments presented in this chapter were aimed at determining what, if any, structural relationship might exist between rhizobial chitolipooligosaccharides and other more general membrane components. The main structural feature that was targeted was the sulfate ester on the chitolipooligosaccharide found in *R. meliloti* since this was thought to be the crucial element in recognition and also because strains carrying mutations in the putative genes involved (*nodPQ* and *nodH*) were readily available. Another important goal in these experiments was to show that these lipids are in fact membrane localized as opposed to being specifically excreted from the cell.

MATERIALS AND METHODS

Strains and bacterial cultures.

R. meliloti 1021 mutants were obtained from Dr. Sharon Long (Stanford University, CA). All bacterial strains were grown in liquid cultures using modified Bergensen's media as previously described (142) except that ^{35}S-labelled sulfate in the form of sulfuric acid (New England Nuclear) at a level of 100 μCi/liter of culture was added before adjusting the pH of the medium to neutrality. This medium contains sulfate as the only form of sulfur.

Nuclear magnetic resonance spectroscopy and mass spectrometry.

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR-500 spectrometer operating at 500 MHz for protons. Spectra were acquired in deuterium oxide containing 10% deuterated methanol with chemical shifts being referenced relative to external TMS. Gas chromatography / mass spectrometry (GC/MS) was performed on a JEOL 505 mass spectrometer interfaced to a Hewlett Packard 5890 gas chromatograph. Spectra were recorded in the positive ion mode using electron impact with an ionization voltage of 70eV. For the analyses of carbohydrate derivatives, a 30-meter capillary column (J & W, DB225)

was used with a temperature program of 180 to 230°C at 3°C per minute and a 30 minute hold at the upper temperature. Fast atom bombardment mass spectrometry (FAB-MS) was recorded on a JEOL 110-HX-HF instrument in both the negative and positive ion modes. The matrix used for CLOS analysis (performed in the negative ion mode) was 1:1 glycerol/4-nitrobenzyl alcohol. For phospholipid analyses, the matrices were 4-nitrobenzyl alcohol/15-crown-5/camphorsulfonic acid for positive ion mode and 4-nitrobenzyl alcohol/15-crown-5/*tert*-butyl ammonium hydroxide for negative ion mode. Electrospray mass spectrometry was performed on a Fisons Platform I instrument operating in the negative ion mode. The sample was infused into the instrument in 1:1 acetonitrile water.

Lipopolysaccharide isolation.

Lipopolysaccharide (LPS) was isolated and purified by gel filtration chromatography on Sepharose 4B columns as previously described (22, 143). Column effluents were monitored for radioactivity by liquid scintillation counting and for carbohydrate content by the phenol/sulfuric acid method (149). The purity of LPS preparations was established by compositional analysis for fatty acids and carbohydrates by GC and GC/MS (see below). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 16% acrylamide gels.

LPS on gels was detected both by silver staining and by exposure of the dried, 2,5-diphenyloxazole-impregnated gels to Kodak x-omat x-ray plates at -75°C for 4 days.

Chitolipooligosaccharide isolation and purification.

Two liters of broth culture were typically grown for CLOS isolation. Cells were harvested in late stationary phase by centrifugation and lipids in the cell pellet were extracted by stirring either with a mixture of chloroform, n-butanol, methanol and water (2:1:1:4) or chloroform, methanol, water (4:1:5). The organic layers were removed and the aqueous layers were concentrated to dryness and then redissolved in 5 ml of water and adsorbed onto a short column of C-8 or C-18 reverse phase packing (5cm X 2.5 cm). The column was eluted sequentially with 30 ml each of water, 4:1 water / methanol, 1:1 methanol / water, 4:1 methanol / water and pure methanol. Fractions were analyzed by proton NMR spectroscopy; those containing the characteristic N-acetyl methyl signals at about 2 ppm were subjected to further purification by reverse phase high performance liquid chromatography on a C-8 reverse phase column using ultraviolet (UV) detection and monitoring at 220 nm. A linear solvent gradient from 10% acetonitrile in water to 80% acetonitrile in water was used for the separation. The chitolipooligosaccharides could also be purified by gel filtration on Biogel P6 using acetonitrile/water (1:1)

as the solvent. Peaks were analyzed for glucosamine content by gas chromatography while radiolabelled samples were also analyzed by scintillation counting. The typical yield of product from 2 liters of cells was 8-12 mg and was not significantly affected by the addition of flavone (luteolin). Products were analyzed by NMR spectroscopy and electrospray mass spectrometry.

Compositional and linkage analysis of chitolipooligosaccharides.

The carbohydrate compositions of chitolipooligosaccharides were determined by hydrolysis of approximately 1 mg sample with 2M trifluoroacetic acid (1 ml) at 120°C for 2 hours followed by extraction of the free fatty acids from the monosaccharides with chloroform. The aqueous layer was then concentrated to dryness under a stream of nitrogen and the product redissolved in water and reduced to alditols using sodium borohydride (1 mg). The excess borohydride was decomposed by treatment with 30 µL of acetic acid and the resulting solution concentrated to dryness. Methanol (2 ml) containing 2% acetic acid was then added and the solution concentrated to dryness again. This process of adding acetic acid in methanol and concentrating was repeated 5 times to remove all traces of boric acid as the volatile methyl ester. The residue was finally peracetylated by treatment with pyridine (0.2 ml) and acetic anhydride (0.2 ml) at room temperature for 24 hours. The reagents

were removed under a stream of nitrogen and the residue was analyzed by GC/MS. Alternatively, both the fatty acid and the carbohydrate compositions were determined simultaneously by methanolysis of a sample of lipooligosaccharide (0.5 mg) with 3% HCl in methanol at 70°C for 24 hours, peracetylation of the mixture with acetic anhydride and pyridine and performing GC and GC/MS analyses directly on the mixture using the same column conditions as described above. Methylation analysis was performed using silver oxide as the base and methyl iodide as the methylating agent in dimethylformamide (DMF) as the solvent. A sample of lipooligosaccharide was dissolved in dry DMF (1 ml) and methyl iodide (0.2 ml) and silver oxide (50mg) were added. The mixture was stirred at room temperature for 36 hours and then diluted with chloroform (2 ml) and filtered. The filtrate was concentrated to dryness and then the residue was hydrolyzed, reduced with sodium borohydride, peracetylated and analyzed by GC and GC/MS as described above. Analyses for KDO were performed by the thiobarbituric acid assay (148).

Phospholipid isolation and characterization.

Phospholipids were separated and isolated by thin layer chromatography (TLC) on silica gel using a solvent system composed of chloroform, acetone, methanol, acetic acid and water (10:4:2:2:1, v:v).

Bands were detected by removing the edges of the plates and spraying them with 5% phosphomolybdic acid followed by heating at 120°C for 5 minutes. The bands were removed from the layers by scraping and eluting with chloroform/methanol (2:1). The components thus isolated were analyzed by FAB-MS in both the positive and negative ion modes. The fatty acids were analyzed by GC and GC/MS after methanolysis.

RESULTS AND DISCUSSION

The Sepharose 4B gel filtration chromatogram of the LPS of *R. meliloti* 2011 showed it to be labelled by ^{35}S (Figures 2.3 and 2.4) in a flavone-independent manner. The ratio of ^{35}S counts to carbohydrate absorbance units for the LPS peak (fractions 45-60) in induced and non-induced cultures is approximately the same in each case. The addition of luteolin does have some (albeit slight) effects on the size distribution of the lipopolysaccharide as well as the extent of membrane lipid synthesis and cell encapsulation. This latter point is reflected in the reduction of carbohydrate content in the fractions preceding those of the LPS. These components have been described (42) as KDO-rich capsular antigens related to the *E. coli* K antigen and are structurally distinct from LPS. The reduction in amount observed when the cells are grown in the presence of luteolin is not surprising since luteolin is, in fact, bactericidal at higher concentrations and should provoke stress responses from the bacteria even in moderate concentrations. In order to reinforce these results and verify that the label was attached to the LPS and not free, a sample of the LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in parallel with a similar preparation from *R. trifolii* ANU843. The gels were examined by silver staining and by

Figure 2.3: Sepharose 4B gel filtration profile of the crude phenol extract of ^{35}S-labelled *R. meliloti* 2011 cells grown in the absence of luteolin. Fractions were assayed for radioactivity by scintillation counting and for total carbohydrate. The second eluting peak (fractions 35-60) is due to LPS; note the presence of radiolabel. The first eluting peak (fraction 28-34) is due largely to a high molecular weight capsular polysaccharide. There is, however, a small amount of a coincident very high molecular weight LPS form. Note the presence of radioactivity in these components. The late eluting peak (fractions 68 to 82) contains free sulfate and a ß-1,2-glucan as well as low molecular weight sulfur containing metabolites.

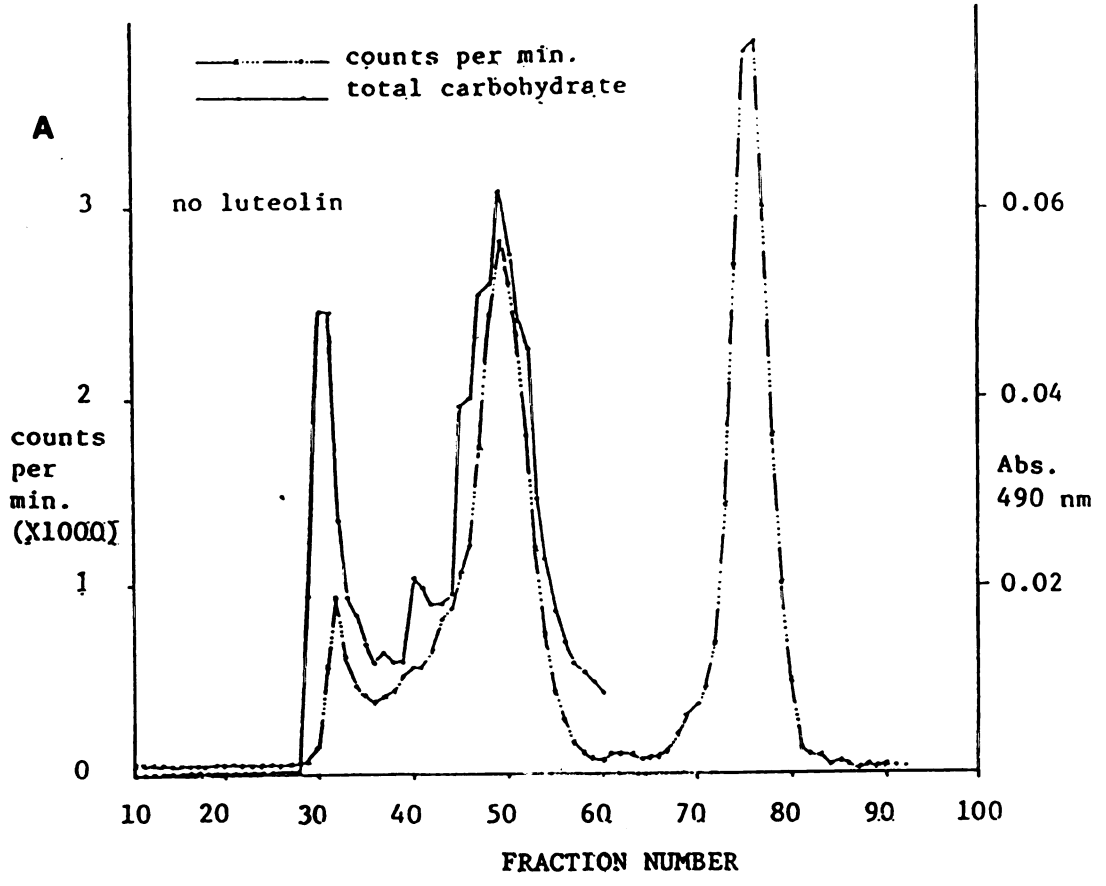

counts per min.
total carbohydrate
A
no luteolin
3
2
1
0
counts per min. (X1000)
0.06
0.04
0.02
Abs. 490 nm
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 2.3

Figure 2.4: Sepharose 4B gel filtration profile of the crude phenol extract of ^{35}S-labelled *R. meliloti* 2011 cells grown in the presence of luteolin. Note that the LPS is still radiolabelled and that the level of radiolabelling (counts/absorbance) is essentially the same as that observed in the absence of luteolin. Note that the presence of luteolin also has an effect on the extent of encapsulation as evidenced by the decrease in intensity of the first eluting peak.

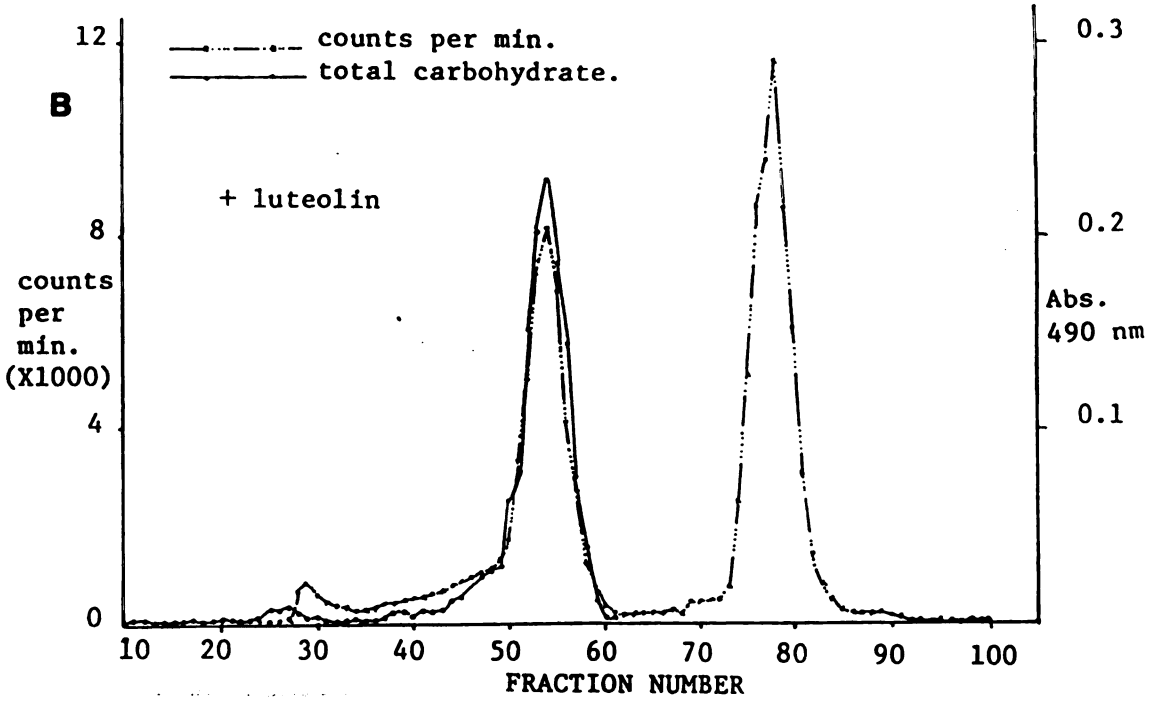

12
8
4
0
B
counts per min.
total carbohydrate.
+ luteolin
counts per min. (X1000)
0.3
0.2
0.1
Abs. 490 nm
10
20
30
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 2.4

autoradiography. These analyses also indicated that the LPS of *R. meliloti* was radiolabelled and that the level of incorporation of label was independent of flavone (Figure 2.5). In contrast, the *R. trifolii* LPS was found to contain no sulfur. These results also reflected the aforementioned changes in molecular weight distribution due to the presence of the flavone. Silver-stained gels reflected exactly what is portrayed in the autoradiograms. In SDS-PAGE, the LPS of wild type rhizobial strains generally appears as only one major band with a smaller amount of a faster moving component which is thought to be rough LPS. The form of the sulfur containing species was definitively identified as sulfate by liberating it from a Sepharose 4B purified LPS sample by mild acid hydrolysis and precipitating the free sulfate as barium sulfate. No radioactivity was detected in the solution containing the carbohydrate components. Based on the level of radioactivity and the amount of LPS isolated (assuming a molecular weight of about 30,000 daltons for a smooth LPS) a conservative estimate of 1.3 sulfate groups per LPS molecule was obtained. It should be noted that because of the extreme lability of sulfate groups, this estimate is likely to be lower than the actual value since desulfation is likely to have occurred during the isolation and purification processes. Both UV and SDS-PAGE analyses indicated that there was no protein contamination in the LPS preparations. In addition, free sulfate was not present since it would, of course, be separated from

Figure 2.5: Autoradiograph of a sodium dodecylsulfate gel showing the separation of ^{35}S-labelled LPS isolated from *R. trifolii* ANU843 and *R. meliloti* 2011 in the presence and absence of flavone inducers. The samples for each condition were applied in triplicate using decreasing amounts (3:2:1) for each lane. Note that the *R. meliloti* LPS is radiolabelled independently of flavone (luteolin). Note also that there is no incorporation of label in the *R. trifolii* LPS. Only a slight shift of one of the minor bands is observed for the *R. meliloti* LPS isolated from cells grown in the presence of flavone compared to the result obtained for cells grown in the absence of flavone.

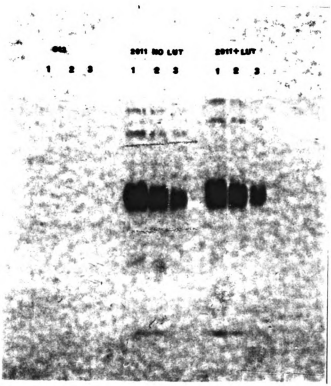


Figure 2.5

the polysaccharide during the chromatography and would also run with the ion front in the SDS gel. The LPS carbohydrate and fatty acid compositions of *R. meliloti* 2011 have been reported before (12, 143).

The effect(s) of impairment of the *nodQ1* and *nodQ2* genes on the sulfation of the LPS was next examined. The *nodQ1* and *nodQ2* mutants used in the study nodulate more slowly than the parent strain *R. meliloti* 1021 while the *nodQ1Q2* double mutant is ineffective in nodulation (141). These mutant strains were all derived from *R. meliloti* 1021 by Tn5 insertion. Assaying fractions of the Sepharose 4B gel filtration column of the parent strain clearly showed that the LPS was sulfated (Figure 2.6, fractions 10-15). A similar analysis on the *nodQ1* mutant (JSS12) showed a decrease in the level of sulfation of the LPS in comparable fractions. The same result was observed with the LPS of the *nodQ2* mutant (JSS14). However, this same LPS region in the profile of the *nodQ1Q2* double mutant (JSS16) contained no sulfate. The effect of mutation of the *nodH* gene was examined in a similar manner. This mutant displays a phenotype identical to the *nodQ1Q2* double mutant; it produces a CLOS devoid of sulfate and is ineffective in nodulation of the homologous host alfalfa. This strain was also derived as an insertion mutant of *R. meliloti* 1021. Inspection of the Sepharose 4B gel filtration profile revealed the absence of sulfate in the same LPS region as the *nodQ1Q2* mutant (Figure 2.7).

Figure 2.6: Sepharose 4B gel filtration profiles of LPS isolated from ^{35}S-labelled *R. meliloti* 1021 cells and three *nodQ* mutants assaying for total carbohydrate and radioactivity. The small, earlier eluting peak lacking radioactivity (fractions 7-10 in the first panel) has been attributed to a capsular polysaccharide related to the *E. coli* K antigen (42). Loss of sulfate groups on the LPS of all three mutants results in increased levels of synthesis of the non-sulfated protective capsular polysaccharide. (Note: A late eluting peak of totally included free radiolabelled sulfate and ß-1,2-glucan has been omitted for clarity.)

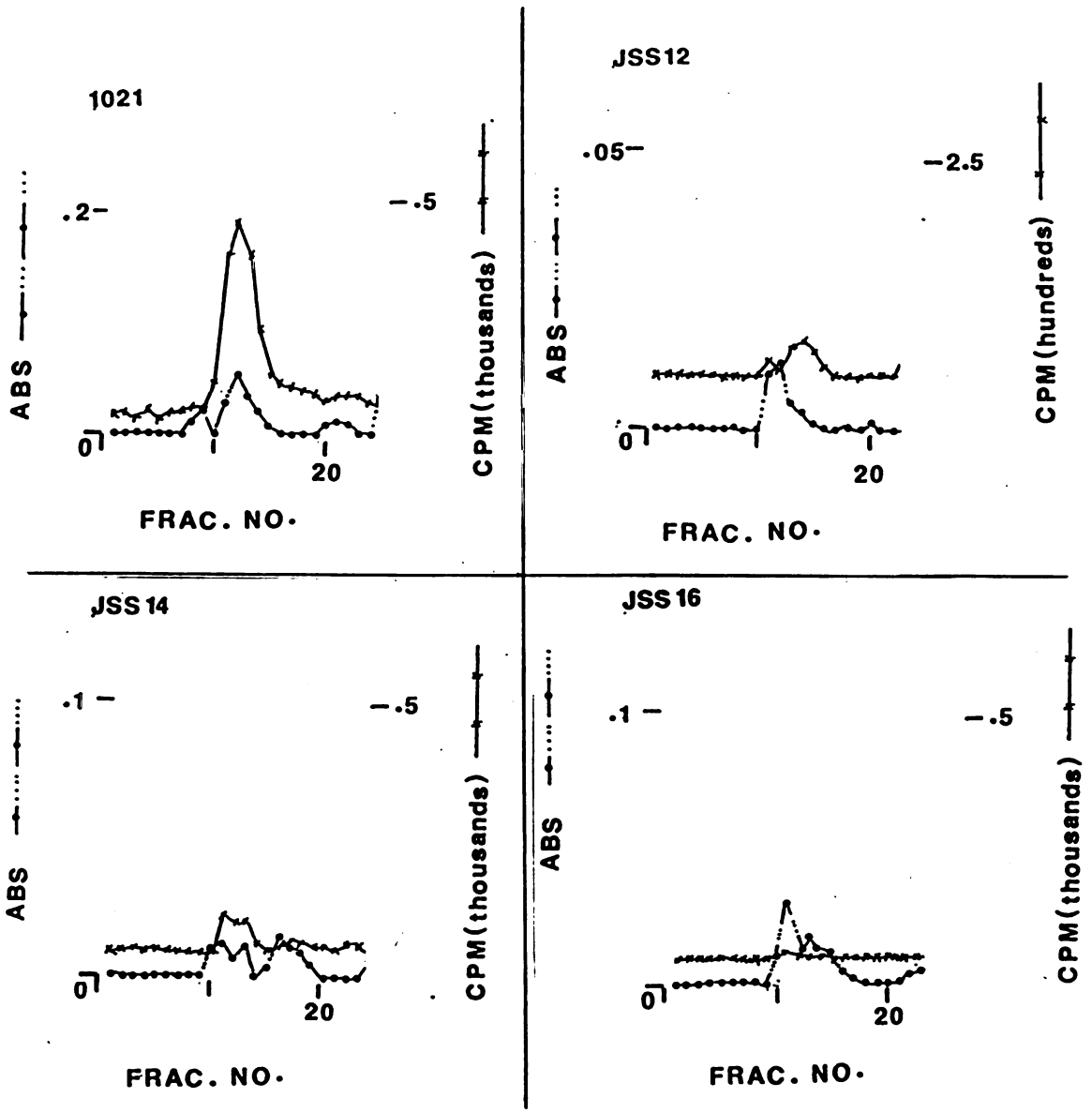

1021
.2
ABS
0
20
FRAC. NO.
.5
CPM(thousands)
JSS12
.05
ABS
0
20
FRAC. NO.
2.5
CPM(hundreds)
JSS 14
.1
ABS
0
20
FRAC. NO.
.5
CPM(thousands)
JSS 16
.1
ABS
0
20
FRAC. NO.
.5
CPM(thousands)

Figure 2.6

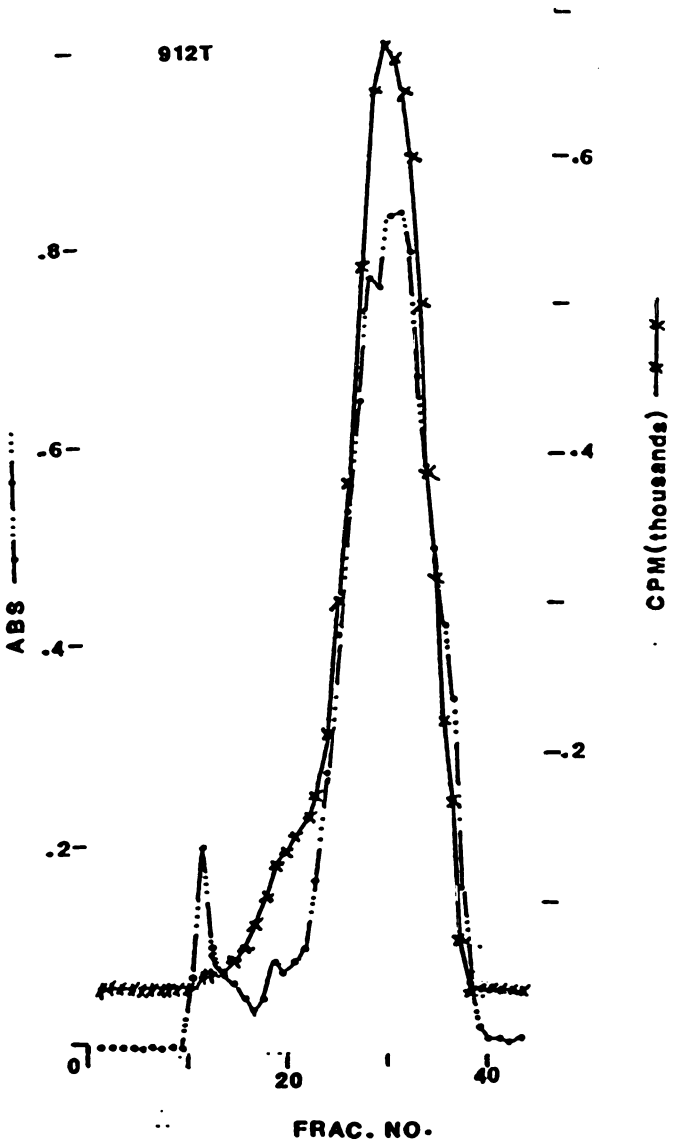


Figure 2.7: Sepharose 4B gel filtration profile of material isolated from ^{35}S-labelled *R. meliloti* 1021 cells carrying a Tn5 mutation in the *nodH* gene assayed for total carbohydrate and radioactivity.

These results indicate that the current models explaining the molecular basis of host specificity should be revised in order to factor out the role(s) of LPS from those of the glucosamine-containing lipooligosaccharides. This work shows unequivocally that the lipopolysaccharide is a substrate for enzymes encoded for by *nod* genes. The fact that the sulfation of LPS is flavone independent but the *nodQ* and *nodH* genes are (supposedly) flavone regulated requires explanation.

In order to investigate further the relationship between chitolipooligosaccharide and typical membrane lipid and glycolipid synthesis, the promiscuous *Rhizobium* species strain NGR234 was also examined. This strain nodulates at least 70 different genera of legumes and has been shown to synthesize chitolipooligosaccharides bearing a wide assortment of functionalities (See Figure 2.2). The most notable features of the NGR234 CLOS is the presence of a methylated 6-deoxyhexosyl residue on the reducing end (that also may or may not contain a sulfate moiety) and the methylation of nitrogen on glucosamine (138). *R.* sp. NGR234 was grown in the presence of ^{35}S-labelled sulfate and the LPS isolated and purified. Assays of the Sepharose 4B column eluate showed radioactivity coinciding with the marker LPS component 2-keto-3-deoxyoctulosonic acid (KDO) (Figure 2.8). This fraction was pooled and rechromatographed on Biogel P6 from which it eluted as one radioactive fraction which tested positive for LPS by the KDO assay. The

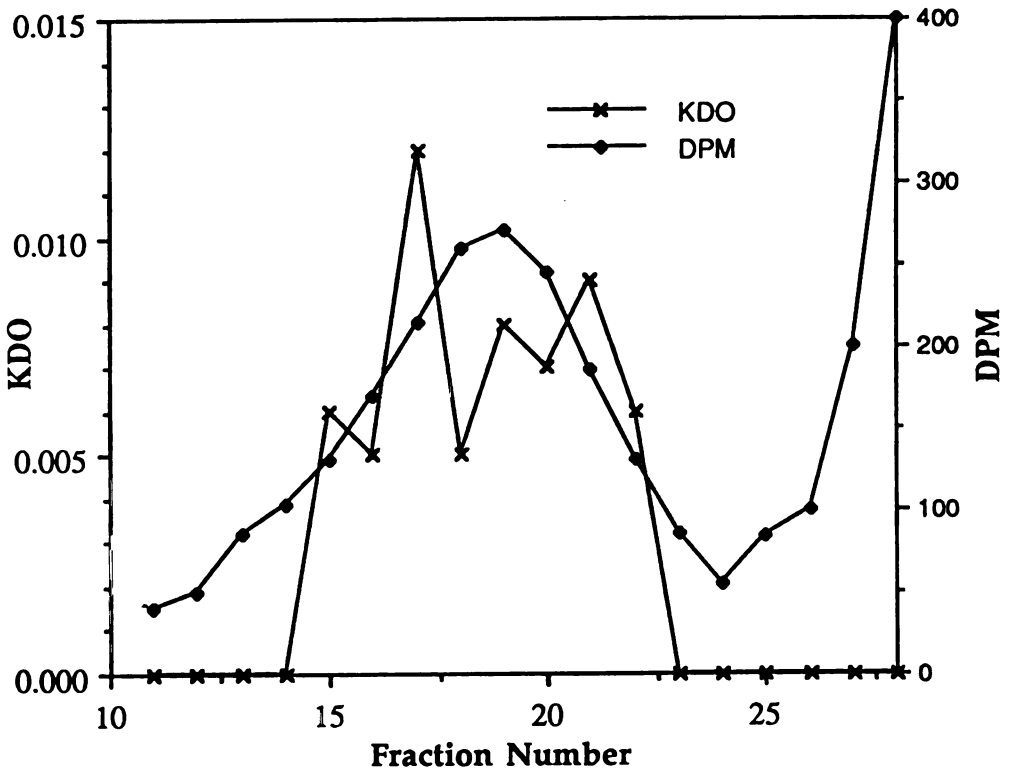


Figure 2.8: Sepharose 4B gel filtration profile of extract from ^{35}S-labelled *R.* sp NGR234 cells showing the correspondence of radioactivity to the LPS marker component KDO (measured in absorbance units). The late eluting peak of radioactivity is due to free sulfate and other small sulfur-containing substituents.

peak was analyzed by GC and GC/MS to determine its carbohydrate content. Like the chitolipooligosaccharide isolated from this strain, the lipopolysaccharide was also sulfated and both O- and N-methylated. The GC/MS analyses indicated that, like the CLOS, a 2-O-methyl-6-deoxyhexose was a component of the LPS. In addition, methylation of the nitrogen of a 2-amino-2,6-dideoxyhexose component was also observed. The predominant glycosyl component was glucose.

The fatty acid components of rhizobial CLOS are often cited as being the primary determinants of their ability to induce host specific responses in legume plants. In the case of *R. meliloti*, for instance, much importance has been placed on the presence of a 2,9-doubly unsaturated C-16 fatty acid in which one of the double bonds is conjugated to the carbonyl group and has a trans-configuration (113, 114). These unsaturated fatty acids are still thought to be critical even though it was also demonstrated that there were several other fatty acid components, including α,ß,γ,δ- chains, and saturated chains of varying lengths, in chitolipooligosaccharides from *R. meliloti* (116). The fatty acid composition of the membrane lipids and the number and stereochemistry of the unsaturated linkages therefore required special attention. The modifications of the membrane lipids were generally similar to those reported for the chitolipooligosaccharides. The presence of C18:2 fatty acids could be discerned by GC and GC/MS analyses. A total ion

chromatogram from a representative membrane lipid fatty acid methyl ester analysis of *R. meliloti* 2011 is shown in Figure 2.9 and a list of the components is given in Table 2.1. The high incidence of methoxy fatty acids can be attributed to artifacts due to the addition of methanol to unsaturations and to methanolysis of cyclopropane groups.

The NMR spectra of total membrane lipids gave definitive information on the types of fatty acids which were present in the total lipid extracts of the bacteria especially with regard to the number and types of double bonds. Hence in the case of *R. meliloti* 2011, the proton spectra (Figure 2.10A) and the ^{13}C NMR spectra (Figure 2.10B) both indicated that unsaturated fatty acids were major components of the phospholipids. Unsaturation was indicated in the proton spectrum by the presence of signals downfield of about 5 ppm and in the ^{13}C spectrum by signals between 130 and 150 ppm. One common site of unsaturation was α,ß- to the ester carbonyl group. This was indicated by the presence of a doublet (J = 12 Hz) at 6.10 ppm, attributable to the α-proton and a doublet of triplets (J = 12 + 7 Hz) at 6.88 Hz attributable to the ß-proton. The proton-^{13}C multiquantum coherence spectrum (HMQC) confirmed the assignments for the vinylic protons and carbons (Figure 2.11). Hence the signal assigned to the ß-proton of the α,ß- unsaturated system showed a connectivity to a ^{13}C resonance at the characteristic position of 149.6 ppm. The signal at 6.10 in the proton spectrum (assigned to the α-proton)

Figure 2.9: Total ion chromatogram showing the typical fatty acid species in membrane lipids from *R. meliloti* 2011 derivatized as methyl esters (see Table 2.1 for assignments). Note the high occurrence of bisunsaturated fatty acids. The large number of isomers could arise from isomerization during the derivatization. Note also the frequency of methoxylated methyl esters. These are expected to arise from addition of methanol to unsaturations and scission of cyclopropyl rings.

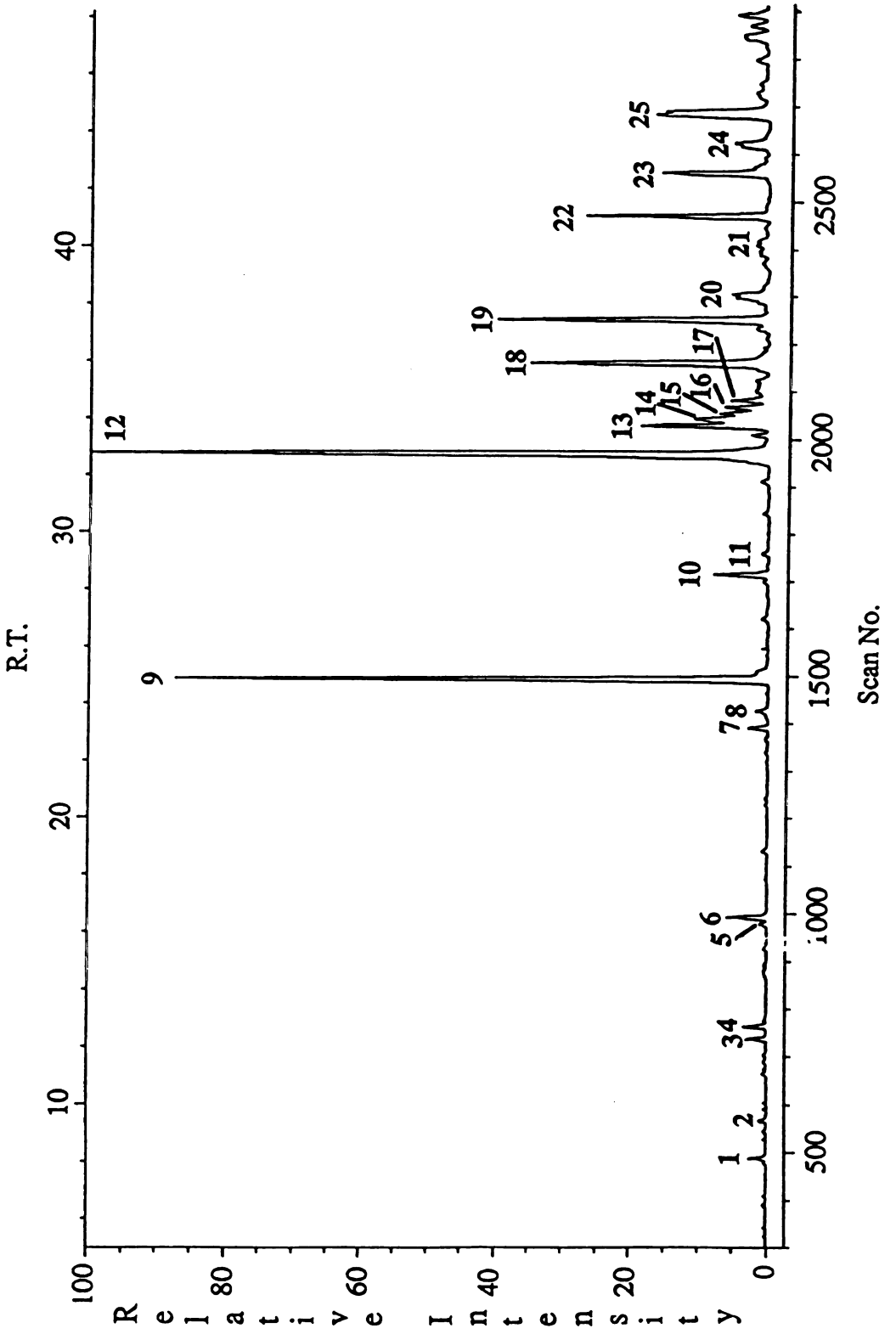
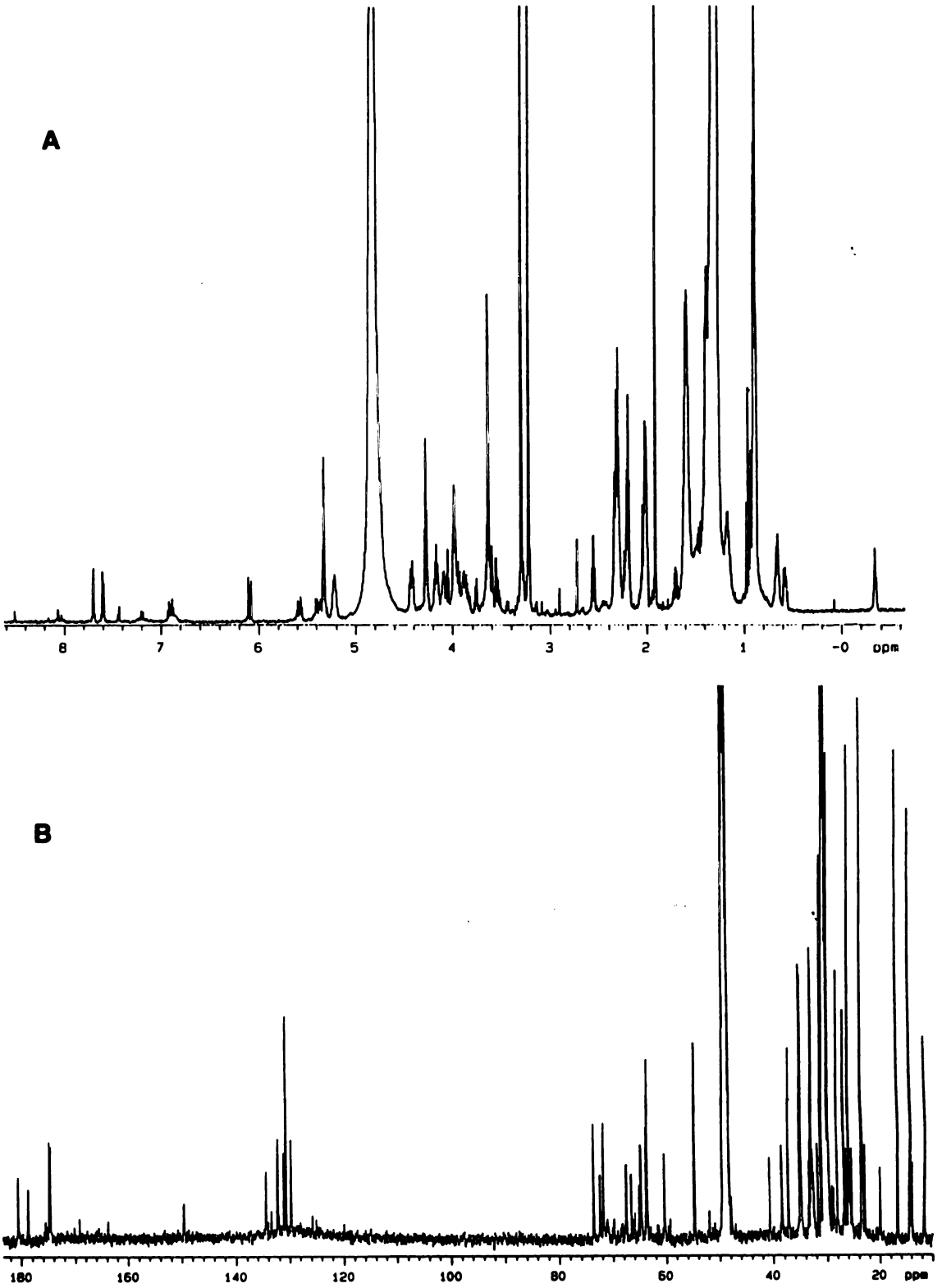

Relative Intensity
100
80
60
40
20
0
R.T.
10
20
30
40
Scan No.
500
1000
1500
2000
2500
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

Figure 2.9

Table 2.1: List of fatty acids normally present in the lipids of *R. meliloti* membranes.

Peak # (Fig. 6)	Structure	Molecular Weight
1	Unidentified	
2	$CH_3OOC(CH_2)_7COOCH_3$	216
3	$OHC(CH_2)_9COOCH_3$	214
4	$CH_3OOC(CH_2)_8COOCH_3$	230
5	$CH_3(CH_2)_{12}COOCH_3$, 14:0	242
6	$CH_3OOC(CH_2)_9COOCH_3$	244
7	Unidentified	
8	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCOOCH_3$ (x+y=12), 16:1	268
9	$CH_3(CH_2)_{14}COOCH_3$, 16:0	270
10	$CH_3(CH_2)_xCH(\diagup CH_2 \diagdown)CH(CH_2)_yCOOCH_3$ (x+y=12), 17:0Δ	282
11	$CH_3(CH_2)_{15}COOCH_3$, 17:0	284
12	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCOOCH_3$ (x+y=14), 18:1	296
13	$CH_3(CH_2)_{16}COOCH_3$, 18:0	298
14	Unidentified	310
15	$CH_3(CH_2)_xCH{=}CH(CH_2)_yCH{=}CH(CH_2)_zCOOCH_3$ (x+y+z=12), 18:2	294
16	Same as above	294
17	Same as above	294
18	Same as above	294
19	$CH_3(CH_2)_xCH(\diagup CH_2 \diagdown)CH(CH_2)_yCOOCH_3$ (x+y=14), 19:0Δ	310
20	Methoxylated FAME	
21	Methoxylated FAME	
22	Methoxylated FAME	
23	Unsaturated keto FAME	310
24	Methoxylated FAME	
25	Unidentified	

Figure 2.10: Proton NMR spectrum (A) of the total membrane extract from *R. meliloti* 2011. Note the presence of a sharp singlet at 3.22 ppm due to methyl protons on the choline headgroup. The methylene signals appear at approximately 3.61 and 4.25 ppm. ^{13}C NMR spectrum (B) of the total membrane extract from *R. meliloti* 2011.



Figures 2.10

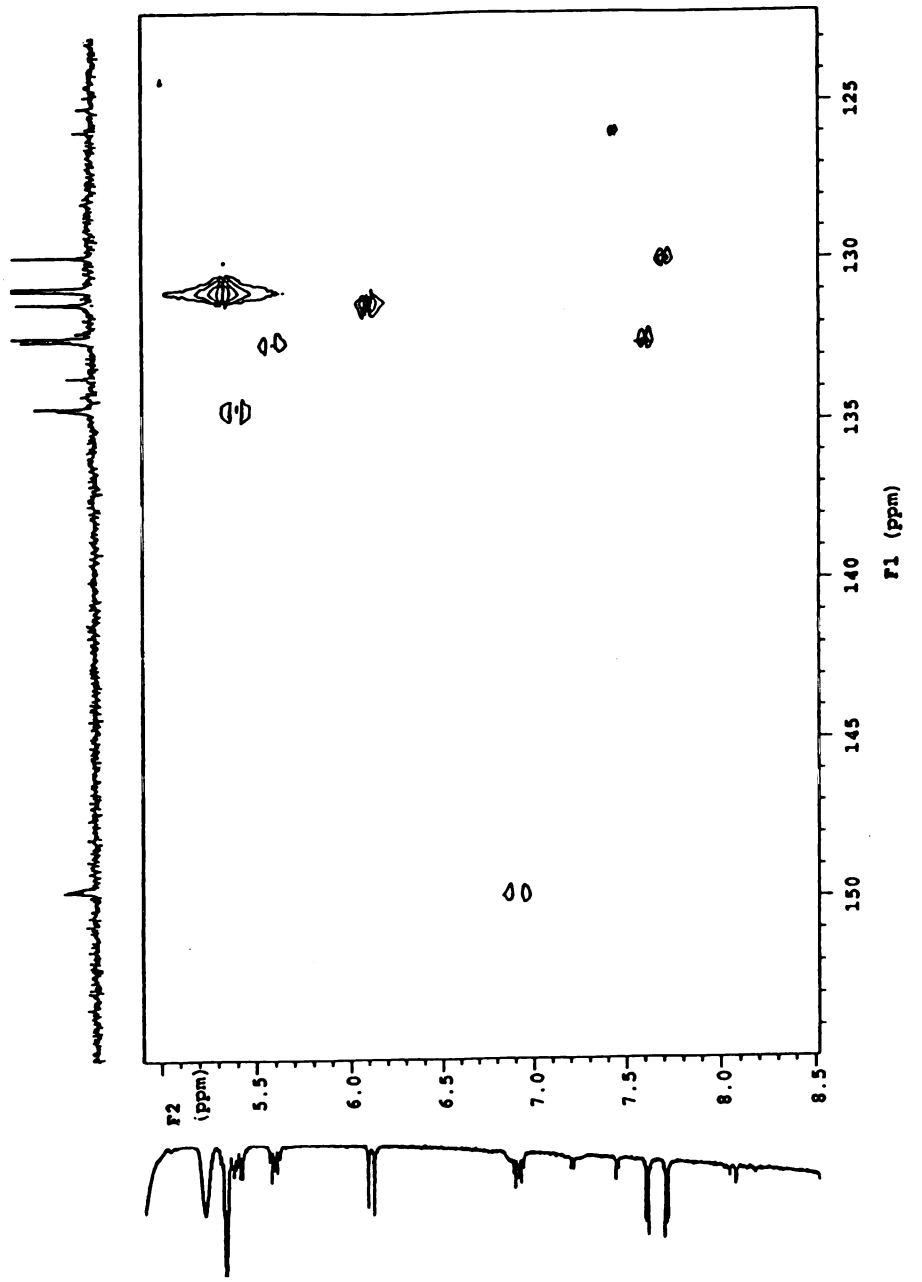


Figure 2.11: ^{1}H-^{13}C multiple quantum coherence spectrum (HMQC) of the total membrane extract from *R. meliloti* 2011. The degree and type of unsaturations is the same as reported for the CLOS of this strain. Connectivities are explained in the text.

displayed a connectivity to a signal at 131.5 in the ^{13}C spectrum. The HMQC spectrum also showed a connectivity between a signal at 5.32 ppm in the proton dimension and 130.8 ppm in the ^{13}C dimension. This was assigned to the presence of an isolated double bond. The NMR spectra confirmed the TLC and mass spectrometric results indicating that the predominant lipid component was phosphatidyl choline as evidenced by the characteristic signals for its headgroup (Figure 2.10A). The information obtained from NMR spectroscopy on the extent, position and type of unsaturation was especially important since it cannot be obtained by mass spectrometric analysis of fatty acid methyl esters. One complication in the latter type of analyses is that α,ß-unsaturated esters easily add nucleophiles such as alcohols and hydroxide ions under acid or base catalysis and a large proportion of such fatty acids is lost during their liberation.

Since LPS, CLOS and other glycolipids are likely to share similar cellular localizations (spatial overlap), the possibility that other rhizobial glycolipids might have similar types of fatty acids as were reported for the chitolipooligosaccharides was next examined. One such glycolipid is sulfoquinovosyl diacylglycerol (144). Hence the proton NMR spectrum of sulfoquinovosyl diacylglycerol from *nodQ* mutants contained signals between 6 and 8 ppm indicating that α,ß,γ,δ- unsaturated fatty acids were present (Figure 2.12).

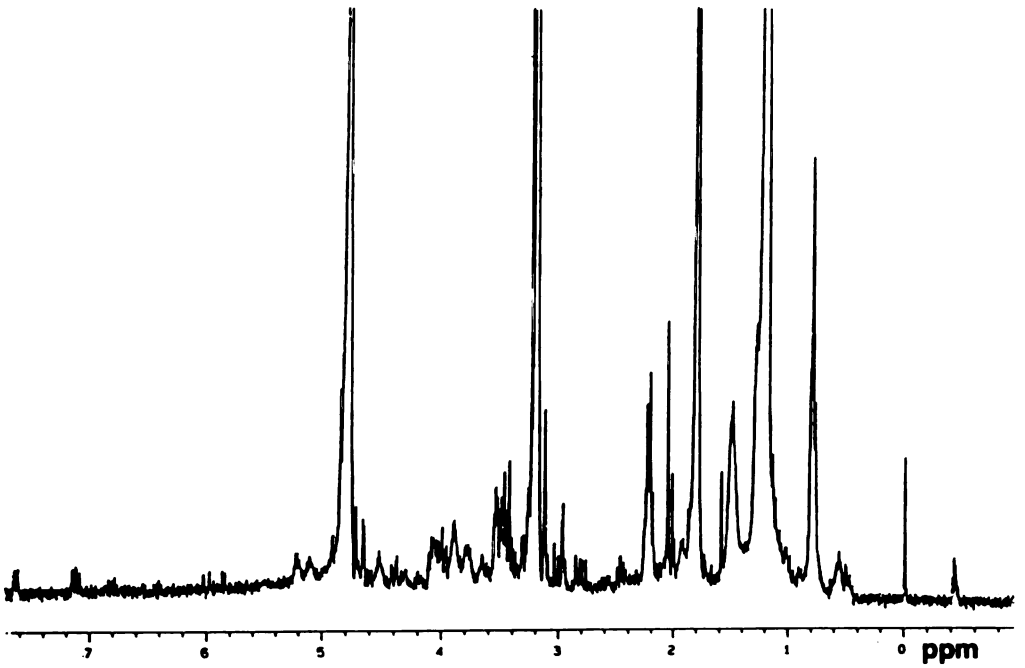


Figure 2.12: Proton NMR spectrum of sulfoquinovosyl diacylglycerol from mutant JSS16. Note the very high level of fatty acid unsaturation giving rise to the signals downfield of about 5.0 ppm. This is very unlike the situation with the parent strain again underlining the high degree of coupling and interdependence of membrane lipid chemistry.

Finally, the last goal was to obtain more direct evidence that chitolipooligosaccharides were actually membrane components as the above studies indicate, and not really excreted metabolites. If this were true, it would then be possible to obtain substantial quantities from cells after disrupting the membrane structure. This was accomplished by extracting cells with an organic solvent mixture to remove phospholipids and disrupt the membrane thus releasing the more polar lipids (such as chitolipooligosaccharides) which, along with the lipopolysaccharides, should partition into the more aqueous layer. The components of the aqueous layer were absorbed onto a C-18 reverse phase cartridge. The cartridge was then eluted with an increasingly non-polar mixture of water and methanol. Using radioactivity counts (from ^{35}S) and glucosamine content as an index, further purification yielded material which contained glucosamine as the only carbohydrate component. Methylation analysis under the mild silver oxide conditions indicated a 1,4-linkage between the glucosamine residues. There was a minor component with a different methylation pattern which was attributed to the non reducing terminal. Under these conditions, the reducing terminal is expected be methylated at the 1-position and suffer some degree of desulfation to give the same methylation pattern as the internal residues. The proton NMR spectra of the chitolipooligosaccharides obtained from *Rhizobium meliloti* strains 2011 grown in the absence of flavone is shown

in Figure 2.13. The level of unsaturation of the fatty acids in chitolipooligosaccharides from bacteria grown in the absence of flavone was generally lower than when the bacteria were grown in the presence of flavone. There was also a significant difference between the levels of unsaturation of the fatty acids of the 2011 and 1021 lipooligosaccharides. The latter tended to have a lower degree of unsaturation. The electrospray mass spectrum of the CLOS from strain 2011 grown in the absence of luteolin is shown in Figure 2.14. Trisaccharide species seem to predominate. The presence of a sulfate group was confirmed by radiolabelling with ^{35}S-labelled sulfate. Typically, several milligrams of chitolipooligosaccharides were obtained per liter of culture. Significant (though lower) quantities were obtained in the absence of flavone.

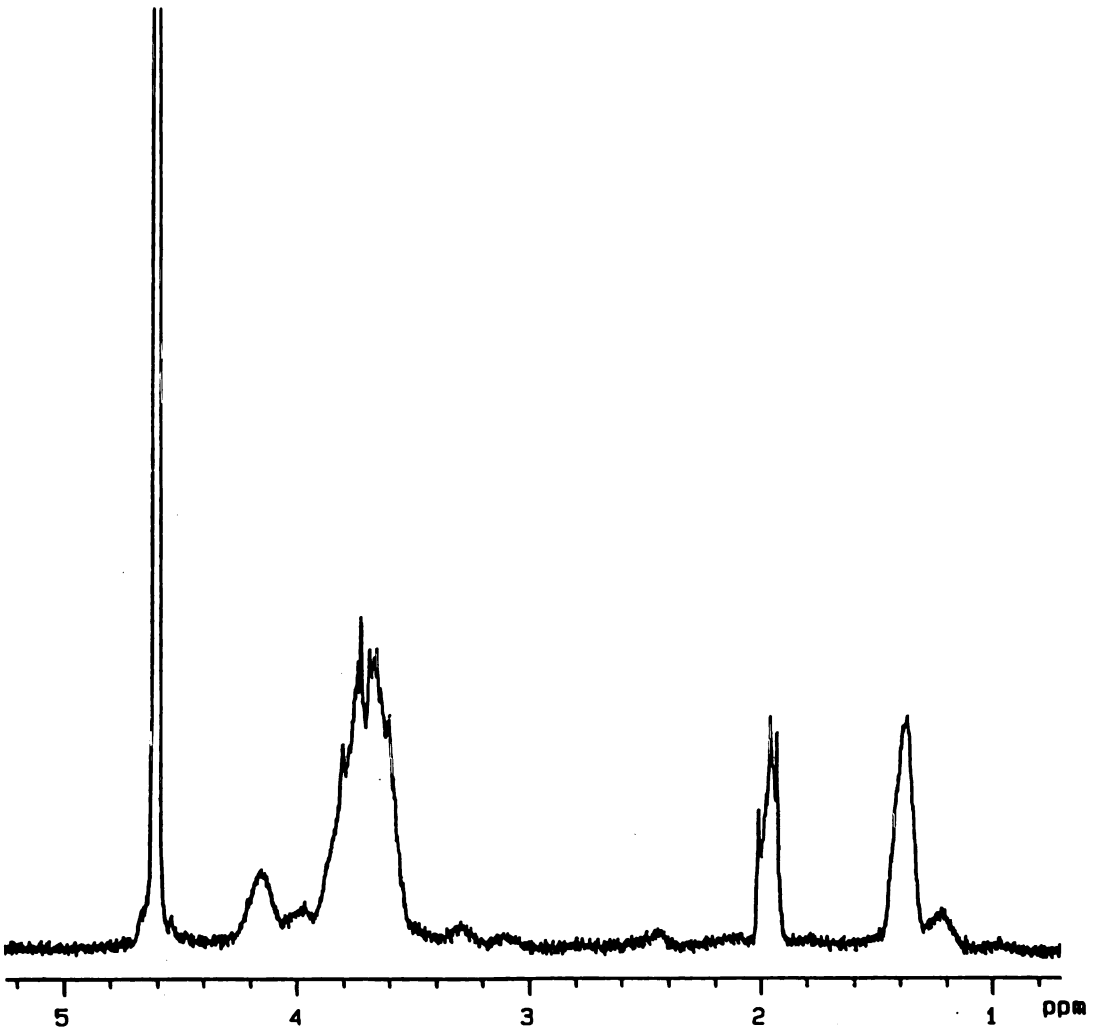


Figure 2.13: Proton NMR spectrum of chitolipooligosaccharide fraction from *R. meliloti* 2011 membranes of cells grown in the absence of flavone. The spectrum was measured in D_2O at 50°C. The anomeric protons can be seen at the edge of the residual water line at 4.60 ppm. Note the prominent acetyl group signals at ~2 ppm. The fatty acid signals appear between 1.1 and 1.6 ppm.

Figure 2.14: Electrospray (negative ion) mass spectrum of a chitolipooligosaccharide preparation from *R. meliloti* 2011. The complexity of peaks indicates a high level of heterogeneity due to differences in substitution. The peak at m/z 1197 corresponds to the M-H ion of an unacetylated tetrasaccharide species bearing a sulfate group, a octadecanoyl fatty acid chain and a sodium adduct. Lower masses correspond to species with varieties of sulfation, fatty acid type, acetylation, and carbohydrate chain length.

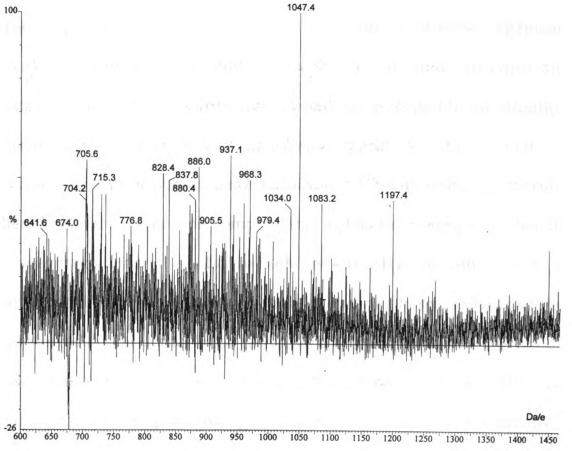

100
%
-26
1047.4
705.6
937.1
828.4
886.0
837.8
715.3
968.3
704.2
880.4
1034.0
1083.2
1197.4
641.6
674.0
776.8
905.5
979.4
Da/e
600
650
700
750
800
850
900
950
1000
1050
1100
1150
1200
1250
1300
1350
1400
1450

Figure 2.14

CONCLUSIONS

The results of this study clearly demonstrate biochemical and spatial links between the chitolipooligosaccharides of rhizobia and the other membrane lipid and glycolipid components of these organisms. There are important parallels between normal lipid structure and chitolipooligosaccharide structure especially with respect to unsaturation. The important feature of chitolipooligosaccharide sulfation is that it is coupled to lipopolysaccharide sulfation. Chitolipooligosaccharides themselves are membrane components. It has been suggested that the unsaturated fatty acids, which eventually become part of chitolipooligosaccharide structure, are first put on phospholipids and then transferred to the chitolipooligosaccharide (145). Whereas this would have been quite remarkable if chitolipooligosaccharides were synthesized and excreted as was first thought, this overlap in fatty acid structure is hardly surprising in light of the evidence presented here that these molecules are actually membrane components. The shuffling and transfer of fatty acids between membrane lipid components is a well documented phenomenon (146, 147) as are common modifications, such as hydroxylation, desaturation and methylation, of fatty acids from different lipid species in the same membrane. The *nod* genes, therefore, encode

functions that clearly affect and determine bacterial membrane and general surface chemistry. In light of this finding, it is reasonable to conclude that bacterial membrane and surface chemistry are critical determinants of the outcome of the symbiotic relationship between rhizobia and legumes.

ACKNOWLEDGMENTS

I would like to thank Dr. Sharon Long for providing the bacterial mutants used in this study. The analyses of the total lipid extracts of *R. meliloti* were performed by Jeongrim Lee. Much of the work on the *nodQ*⁻ mutants was done by Kerry Ross. The NMR studies in this chapter were performed by Rawle Hollingsworth. This work was supported by grant #DE-FG02-89ER 14029 from the U.S. Department of Energy to Rawle Hollingsworth. The GC/MS and FAB mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility which is supported in part by grant #DRR-00480 from the Biotechnology Research Technology Program, National Center for Research Resources, NIH. The NMR data were obtained on instrumentation that was purchased in part with funds from NIH grant #1-S10-RR04750, NSF grant #CHE-88-00770, and NSF grant #CHE-92-13241.

CHAPTER 3

THE "MISSING" TYPICAL *RHIZOBIUM LEGUMINOSARUM* O-ANTIGEN IS ATTACHED TO A FATTY ACYLATED GLYCEROL IN *R. TRIFOLII* 4S, A STRAIN THAT ALSO LACKS THE USUAL TETRASACCHARIDE "CORE" COMPONENT

ABSTRACT

Novel aspects of the cell surface chemistry of the unusual *Rhizobium* strain *R. leguminosarum* bv. *trifolii* 4S have been uncovered. Typically, strains of *R. trifolii* produce two distinct core regions in their lipopolysaccharide, one is a trisaccharide consisting of galacturonic acid and 2-keto-3-deoxyoctulosonic acid (KDO) while the other is a tetrasaccharide made up of galactose, mannose, galacturonic acid and KDO. Strain 4S is shown here to be devoid of the latter, galactose-containing moiety. This result, coupled with previous data showing this strain to produce an O-antigen and extracellular polysaccharide that are both lacking galactose and unique to the rest of the *R. trifolii* strains, suggested some sort of defect in galactose biosynthesis or incorporation in this strain. However, the discovery of a galactose-containing lipid-linked oligosaccharide with a composition that resembles the typical *R. trifolii* O-antigen is also presented in this chapter. This lipid contains glycerol, 2,3-di-O-methyl-6-deoxyhexose, fucose, mannose, galactose, glucose, quinovosamine, glucosamine and KDO which are all typical carbohydrate components of *R. trifolii* lipopolysaccharide. The main fatty acid constituents are stearic and oleic acids with smaller quantities of 3-hydroxy fatty acids and 27-hydroxyoctacosanoic acid. This molecule is

most likely an O-antigen-like repeating unit linked to a mono or diacylglycerol lipid anchor. There is evidence for the presence of a truncated version of the core tetrasaccharide identical to a disaccharide found in rough mutants. These findings suggest that strain 4S is an effective natural isolate that has modifications that are similar to some ineffective mutants. They also demonstrate that, contrary to previous indications, synthesis of an LPS with an intact core tetrasaccharide is not mandatory for a strain to be effective in nodulation.

INTRODUCTION

Cell surface chemistry has long been considered to be a major contributing factor in the very specific interaction between rhizobia and legumes. Acidic exopolysaccharides (EPS) (79, 80, 81, 150) capsular polysaccharides (CPS) (49, 63, 64, 70), lipopolysaccharides (LPS) (29, 73, 74, 75, 77, 151), chitolipooligosaccharides (CLOS) (113, 115, 116, 152, 153), cyclic-ß-1,2-glucans, (154) and diglycosyl diacylglycerols (155, 156) have all been implicated to varying extents in infection. The structural interrelation between these various components as well as their common genetic origins is not at all well understood although it has now been documented. For instance, it was shown in Chapter 2 that several structural links exist between the LPS and CLOS produced by *Rhizobium meliloti* and that these structural elements (e.g. sulfation) are affected by the same genes (*nodQ* and *nodH*). It has also been reported that introduction of a gene normally encoding for LPS biosynthesis in *R. meliloti* (*lpsZ*) will restore nitrogen fixing ability to a mutant that fails to produce EPS (143). Recently it has been shown that expression of this gene causes some modification of normal CPS production (157). The structural overlap between LPS and CPS has also been very well documented for many years in various Gram-negative bacteria (158-160).

Rhizobium leguminosarum biovars *trifolii* and *viciae* have been well studied with regards to EPS and LPS structure and composition. In the *Rhizobium leguminosarum* biovar *trifolii* cluster, hereafter referred to as *R. trifolii*, strain 4S has been particularly well characterized and has been found to be quite distinct from other members of the species with respect to CPS and EPS structure. It has been shown that *R. trifolii* strains typically produce EPS consisting of an octasaccharide repeating unit comprised of glucose, glucuronic acid and galactose (5 : 2 : 1 respectively) with a carboxyethylidene (pyruvate acetal) group attached to a terminal side chain galactose and another to the adjacent glucose residue (59, 61). The EPS/CPS of some strains also contain acetate groups and 3-hydroxybutyryl groups (37, 38, 58, 161). The terminal galactose residue may also bear one 3-hydroxybutyryl group and sometimes an acetate group (62). *R. trifolii* 4S, however, produces a heptasaccharide repeat unit that lacks the typical terminal galactose with the 3-hydroxybutyryl and carboxyethylidene groups (36, 162). It therefore appears that this strain somehow lacks the ability to synthesize activated galatose (UDP-galactose) or is defective in the synthesis of a specific galactosyl transferase. In the former scenario, some very important consequences would result for LPS synthesis since both the core and O-antigen components of these lipopolysaccharides typically contain galactose (19, 20, 57, 163-165).

The lack of galactose incorporation in the EPS of *R. trifolii* 4S is also evident in the O-antigen of the LPS. Recently, the complete structural characterization of the O-antigen of *R. trifolii* 4S LPS was determined (32). The structure was shown to be a pentasaccharide consisting of rhamnose, N-acetylglucosamine and N-acetylmannosamine (3:1:1). This O-antigen is vastly different in composition to that of any other *R. trifolii* strains studied to date. The "typical" *R. trifolii* lipopolysaccharides (like those of the other biovars) are rich in methyl deoxyhexoses, deoxyhexoses, mannose, galactose, glucose, 2-amino-2,6-dideoxy glucose (quinovosa-mine), uronic acids, heptose and 2-keto-3-deoxyoctulosonic acid (KDO) (57, 163-165). This total lack of galactose in the O-antigen is more than just curious and definitely supports the idea that this strain is either defective in the biosynthesis of UDP-galactose or in synthesis of a galactosyl transferase which appears to be quite general. Another fact that emerges is that there is an important overlap in LPS and EPS/CPS biosynthesis. This is completely consistent with what we know of *Rhizobium meliloti* mutants that are also impaired in integrating galactose into their EPS; some of these mutants are now known to be impaired in LPS structure as well (33).

One area of rhizobial lipopolysaccharide chemistry and biochemistry in which galactose biosynthesis and galactosyl transfer activity is critical is in the core region. There are two core

oligosaccharides in wild type *R. trifolii* LPS; one is a trisaccharide that contains two molecules of galacturonic acid linked to KDO (18) and the other is a tetrasaccharide in which galactose, mannose and galacturonic acid are coupled to KDO (19, 20). One general observation is that bacteria that are impaired in the synthesis of this galactose-containing tetrasaccharide are unable to attach an O-antigen and, as a consequence, have a "rough" phenotype (22, 78, 166). This is generally taken as an indication that the tetrasaccharide is the point at which the O-antigen is attached to the lipopolysaccharide. In the case of *R. trifolii* 4S, the fact that the O-antigen is entirely non-classical is completely consistent with the fact that the LPS might not contain the galactose-containing tetrasaccharide and that an alternative compatible core and O-antigen had evolved. Questions which then could be raised are "What is the fate of the classical O-antigen repeat? Is it not made at all or is it made but never transferred or, perhaps, transferred to some other acceptor?" It was of interest, therefore, to determine whether *R. trifolii* 4S synthesized either of these two core oligosaccharides, especially the critical galactose-containing one. If the impairment is largely an inability to incorporate galactose into these polysaccharides, then a truncated version of the tetrasaccharide, such as the mannose-KDO disaccharide found in some mutants (22, 166), should be present. It was also of interest to look for the

remnants of the classical O-antigen biosynthesis and transfer pathway, especially the fate of the classical O-antigen repeat unit.

MATERIALS AND METHODS

Bacterial cultures.

R. trifolii 4S was grown at 29°C in 4L shaken flasks containing 2L of modified Bergensen's media as previously described (142). The cultures were supplemented with 4μM 4',7-dihydroxyflavone (DHF). Cells were grown to late exponential phase and harvested by centrifugation at 10 krpm on a Sorvall RC2-B centrifuge equipped with a GSA rotor.

Lipopolysaccharide (LPS) isolation.

LPS was isolated by phenol extraction, purified by Sepharose 4B gel filtration chromatography and the lipids released by mild acid hydrolysis and extraction as described previously (14, 32). The aqueous carbohydrate-containing fraction was separated on a Biogel P2 column (120 cm X 1.5 cm) using 0.1% formic acid as the eluant. Ten milliliter fractions were collected and 100 μL aliquots removed and assayed for carbohydrate content using the phenol/sulfuric acid method (149).

Membrane lipid isolation.

The cell pellet was extracted with 200mL of 1:2:2:3 chloroform/1-propanol/methanol/water by vigorous stirring for 24 hours at 37°C. The

membrane extract was dried by flash evaporation, redissolved in 20% acetonitrile and loaded onto a LiChroprep RP-18 column (EM Separations, Gibbstown, NJ; 8 x 2 cm). Successive elutions were made with 30 ml portions of 20, 50 and 100% acetonitrile in water.

Carbohydrate compositional analysis.

Portions of the isolated fractions were methylated and reduced with $NaBD_4$. Each sample (~0.1mg) was treated with 2ml of 1% methanolic HCl, sonicated for 1 min., heated for 15 min. at 68°C and kept overnight at room temp. After drying the sample under a stream of N_2, 5mg of $NaBD_4$ in 0.4mL of 1:1 methanol/water was added and the solution kept at room temperature for 24 hours. Excess borohydride was destroyed by dropwise addition of 20% acetic acid in methanol until effervescence ceased. The sample was dried with N_2 and treated with 1mL of 2M CF_3CO_2H for 2 hours at 120°C. Reduction with $NaBH_4$ and peracetylation with acetic anhydride/pyridine was performed as previously described (32). GC analyses were performed on a Hewlett-Packard 5890 gas chromatograph using a 30m capillary column (J & W, DB225) with He as the carrier gas. A temperature program of 180°C to 220°C at 3°C/min and a final hold of 60 min was used. GC-MS analyses were performed on a JEOL 505 mass spectrometer using electron impact ionization (70eV) in the positive ion mode. The same temperature program and column as described above for

GC analyses were used. Samples were also assayed for 2-keto-3-deoxyoctulosonic acid (KDO) by using the thiobarbiturate procedure as previously described (167).

Fatty acid compositional analysis of lipid fraction.

Lipid fractions were analyzed for fatty acid content by converting them to the methyl ester derivatives. Portions of each fraction were dissolved in 1mL 5% methanolic HCl and sonicated for 15 min. To each sample, 2mL of chloroform was added and the solution kept at 70-75°C for 36 hours with intermittent sonication. After cooling to room temperature and drying with N_2, the sample was partitioned between water and $CHCl_3$. The organic layer was removed and the aqueous layer extracted twice with $CHCl_3$. These organic layers were combined, reduced to ~10μL and used for GC and GC-MS analysis. These analyses were performed as above except the temperature program used was 150°C to 300°C at 3°C/min with a final hold of 20 min. and the column used was a DB1 (J & W).

Nuclear magnetic resonance spectroscopy.

Proton nuclear magnetic resonance spectroscopy (^1H NMR) was performed on a Varian VXR500 spectrometer (500 MHz). Spectra were recorded in D_2O at 25, 30 or 70°C as indicated.

RESULTS AND DISCUSSION

The Biogel P2 chromatogram (Figure 3.1) of the water-soluble fraction of the mild acid hydrolysate of the LPS of *R. trifolii* 4S showed some major differences to that typically obtained from most wild-type *R. leguminosarum* biovars. These chromatograms typically show three very well defined peaks, the first corresponding to the O-antigen, the second to the core-tetrasaccharide and the last to the trisaccharide (22). In this case, the first peak did correspond to the O-antigen but the second peak was broad and appeared to contain more than one component. It was divided into four sections based on the general shape and each section was analyzed separately by proton NMR spectroscopy and by GC-MS of the alditol acetates. In the ^{1}H NMR spectra (Figure 3.2A), the three characteristic doublets between 4.8 and 5.4 ppm attributable to the anomeric protons of the tetrasaccharide hexosyl residues were not observed. In addition, GC-MS analyses indicated that galactose was absent from all of the fractions. The NMR spectrum of the material in the fourth section of the second peak (Figure 3.2B) indicated that it contained a previously described disaccharide composed of mannose and KDO. This was readily identifiable by the anomeric proton at 5.0 ppm and the other carbohydrate signals. This molecule has been characterized before in a

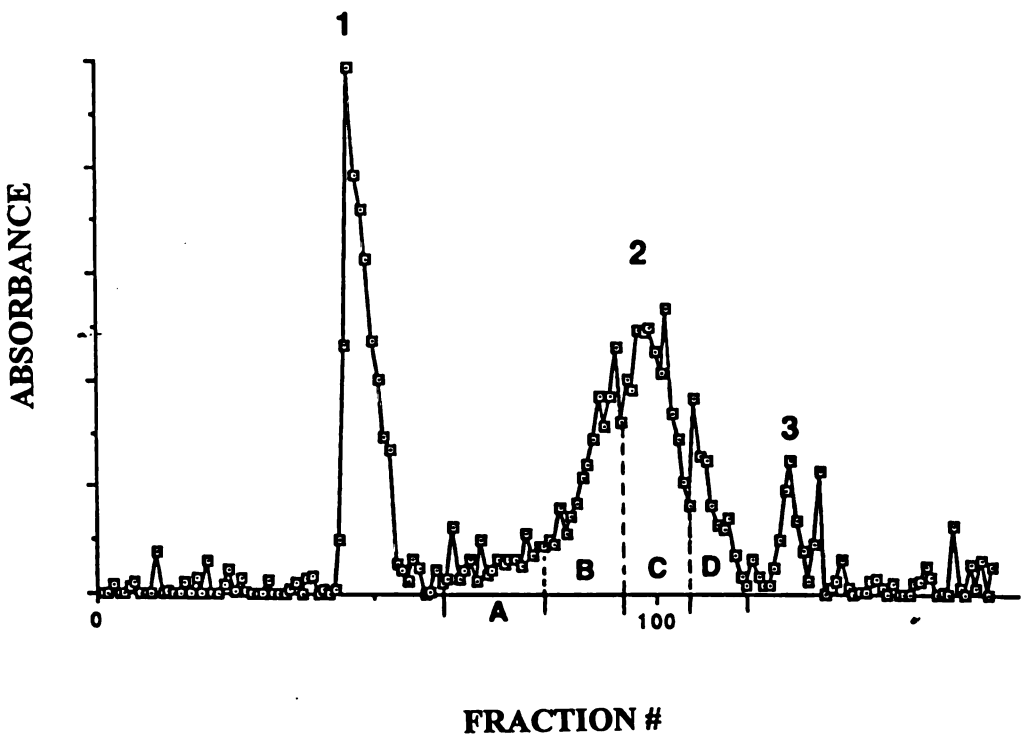
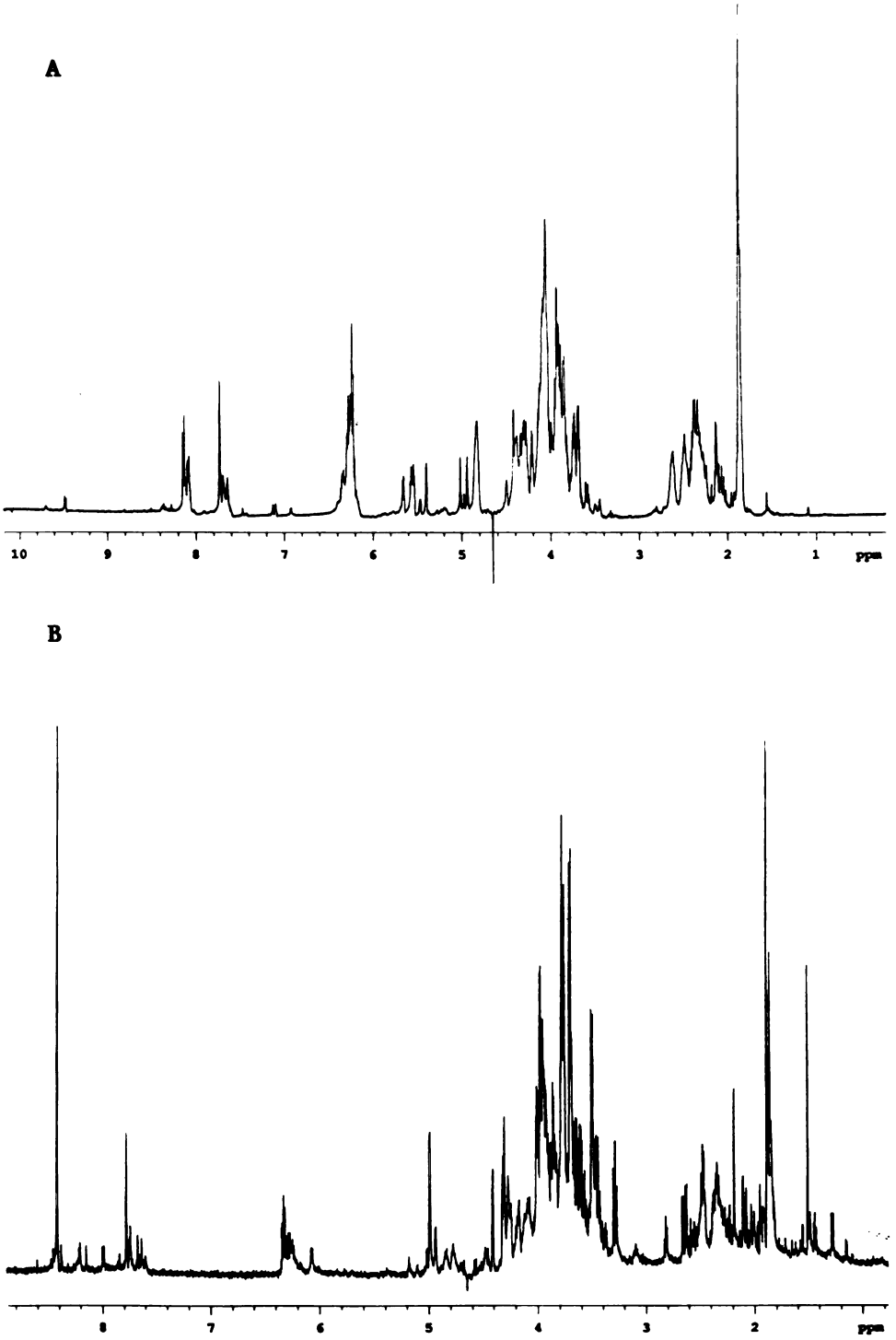


Figure 3.1: Biogel P2 chromatogram of LPS hydrolysate of *Rhizobium trifolii* 4S. The small peak after 3 was devoid of carbohydrate material.

Figure 3.2: ^1H NMR spectrum (A) of fraction C of peak 2 from Figure 3.1. This is representative of fractions A and B. The ^1H NMR spectrum (B) from fraction D. Note the mannose anomeric proton at ~5.0 ppm. Both spectra were recorded in D_2O at 25°C.



Figures 3.2

rhizobia mutant that is incapable of making the core tetrasaccharide (64). The NMR spectrum shown in that report is consistent with the one collected here. The spectrum of this fourth section contained signals indicating that smaller amounts of another component might also be present. The proton NMR spectrum of the third peak was readily identifiable as that of the trisaccharide core component which lacks galactose. These results definitively showed that the galactose-containing tetrasaccharide was absent.

The 20% acetonitrile fraction from reverse phase chromatography of the *R. trifolii* 4S membrane extract when subjected to GC and GC-MS analyses was found to be composed exclusively of glucose. The proton NMR spectrum compared favorably to published spectra of cyclic-ß-1,2-glucan (168). This fraction was not analyzed further. The ^{1}H NMR spectra of the other two fractions, eluting with 50 and 100% acetonitrile, although very similar to each other were both broad and ill-defined. Spectra of the aqueous fractions from the mild acid hydrolysis were, however, much more informative (Figure 3.3). There were resonances that indicated the presence of methoxy, deoxy and amino sugars. These were, specifically, sharp singlets between 3.2 and 3.4 ppm that indicated a relatively large amounts of O-methylation while the signal at ~2.0 ppm indicated the presence of an N-acetyl group. Additionally, signals at ~2.6 ppm indicated the presence of KDO. There were also signals for fatty acid

Figure 3.3: ^{1}H NMR spectrum of mild acid hydrolysis products of combined *R. trifolii* 4S fractions eluted from C18 column with 50 and 100% acetonitrile in water. The spectrum was measured in D_2O at 70°C. Note the presence of intense peaks between 3.2 and 3.4 ppm indicative of a high degree of methylation. Multiple signals due to anomeric protons can be seen between 4.7 and 5.2 ppm. The 6-deoxy group signals are at ~1.1 ppm and the N-acetyl group signals at ~2.0 ppm. The intense peak at ~1.83 ppm is from residual acetic acid. The intense signals at ~3.5 ppm are from a low molecular weight contaminant that is released from the reverse phase packing.

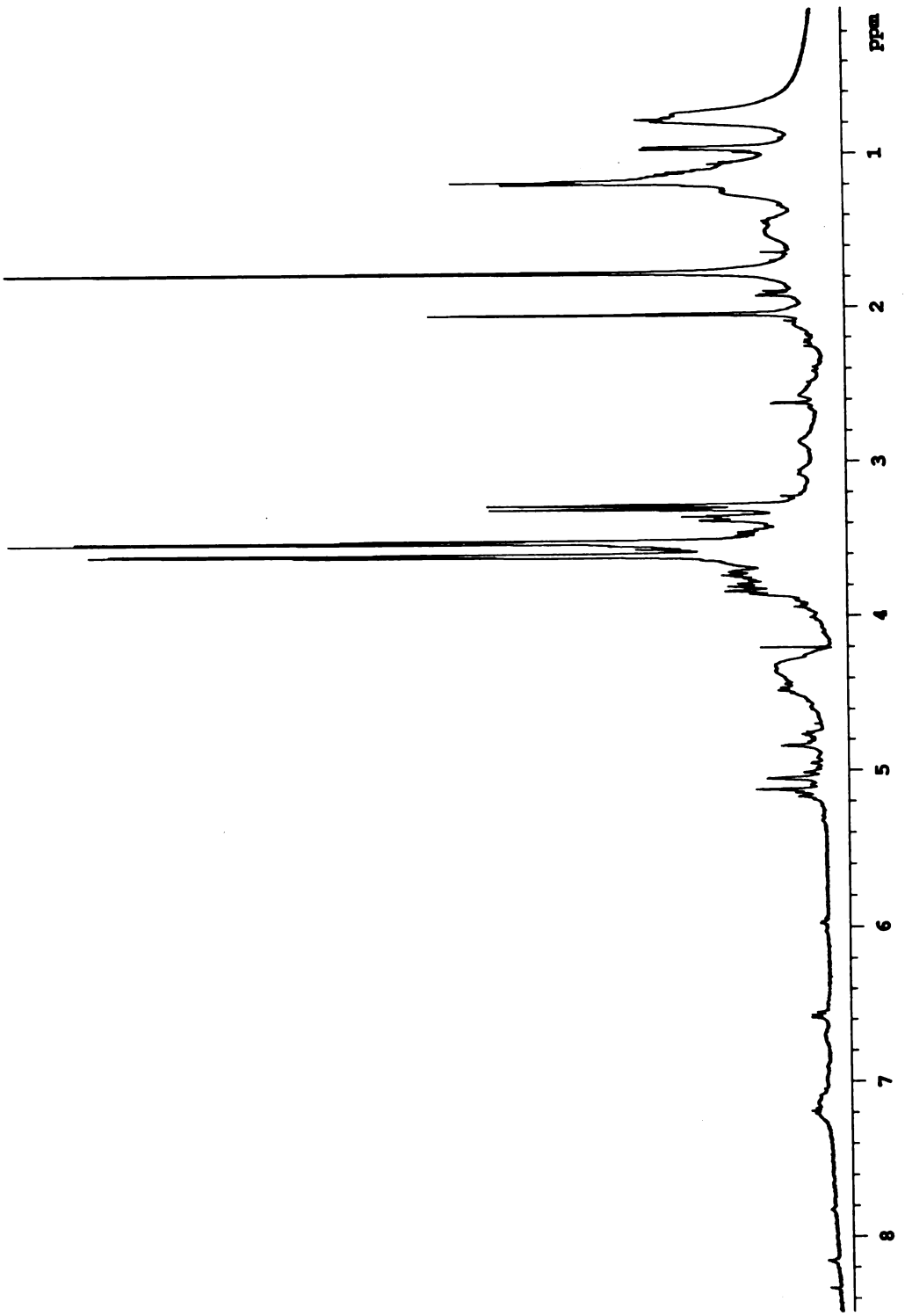


Figure 3.3

groups between 0.9 and 2 ppm. The carbohydrate composition was determined definitively by GC-MS. The composition of the two fractions were virtually identical and the total ion chromatogram of the last eluting fraction is shown in Figure 3.4. The major components were glycerol, 2,3-di-O-methyl-6-deoxyhexose, fucose, mannose, galactose, glucose, quinovosamine and glucosamine. The thiobarbituric acid assay also indicated the presence of KDO in these samples. These are the typical glycosyl components of rhizobial LPS O-antigens and have been described in the various *R. leguminosarum* biovars that have been analyzed over the years. The data in Table 3.1 summarizes some of these publications and illustrates the great similarity of the glycosyl composition of the LPS of these strains to that of this newly discovered lipid. As noted earlier, most of these glycosyl components, especially the methylated deoxyhexoses and quinovosamine, are missing in the LPS of strain 4S (32). GC-MS analyses of the methyl ester derivatives of these fractions revealed that the predominant fatty acids were typical phospholipid fatty acids, namely stearic and oleic acids (Figure 3.5). However, other fatty acids which are characteristic of lipopolysaccharides, such as the 3-hydroxy fatty acids and 27-hydroxyoctacosanoic acid (11, 12, 14) were also present. The fatty acids in the anchor, therefore, are a curious mix of LPS and other membrane lipid fatty acids. The question then arose as to what is the lipid anchor for these oligosaccharides. The possibility that it was the typical

Figure 3.4: Total ion chromatogram of alditol acetates of the *R. trifolii* 4S extract eluted from a C18 column with 100% acetonitrile. The identity of the major peaks were determined by co-elution with standards and analysis of mass spectra.

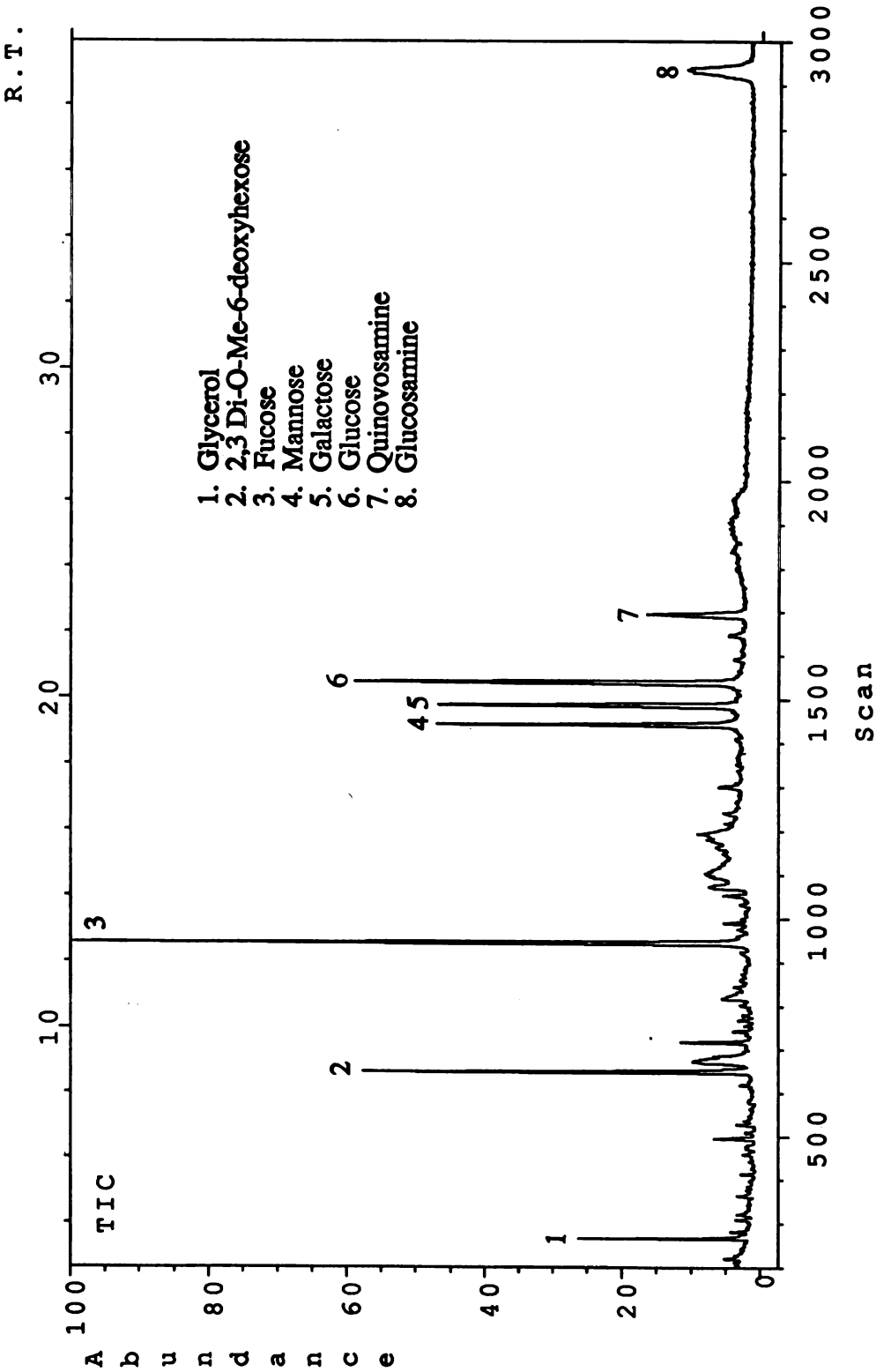

R.T.
1.0
2.0
3.0
TIC
Abundance
100
80
60
40
20
0
500
1000
1500
2000
2500
3000
Scan
1
2
3
4 5
6
7
8
1. Glycerol
2. 2,3 Di-O-Me-6-deoxyhexose
3. Fucose
4. Mannose
5. Galactose
6. Glucose
7. Quinovosamine
8. Glucosamine

Figure 3.4

Table 3.1: Reported carbohydrate composition of LPS of various *Rhizobium trifolii* strains.

Component	*R. trifolii* strain					
	0403[a]	Ar-3[b]	Coryn KL[b]	K-8[b]	162S7[c]	2S[c]
3-O-methylhexose	nr	nr	nr	nr	0	1.1
2-O-methyl-6-deoxyhexose	8	1.4	2.1	6.4	4.5	0
3-O-methyl-6-deoxyhexose	nr	nr	nr	nr	0	3.6
3,4-di-O-methyl-6-deoxyhexose	nr	nr	nr	nr	0.7	0
3-N-methyl-3-amino-3,6-dideoxyhexose	15	nr	nr	nr	0	0
Rhamnose	4	40.3	0.2	1.9	0.6	7.5
Fucose	13	20.2	0.8	7.2	4.0	7.5
Mannose	7	0.9	7.9	3.2	1.9	1.7
Galactose	tr	0	4.9	2.8	1.1	2.2
Glucose	4	26.6	28.4	7.1	0.9	1.0
Heptose	21	0	1.0	0	0	0
Hexosamines	nr	0.8	2.1	1.2	1.5	1.6
Uronic acids	27	2.2	11.7	5.3	2.2	7.0
2-Keto-3-deoxyoctonoic acid	nr	1.3	2.9	4.1	7.2	2.7

Taken from references 164[a], 165[b], and 163[c]; results are expressed as percent of dry weight of LPS.
nr = none reported, tr = trace.

Figure 3.5: Total ion chromatogram of fatty acid methyl esters of the same fraction as in Figure 3.4. The identity of the major peaks were determined by co-elution with standards as well as analyses of mass spectra.

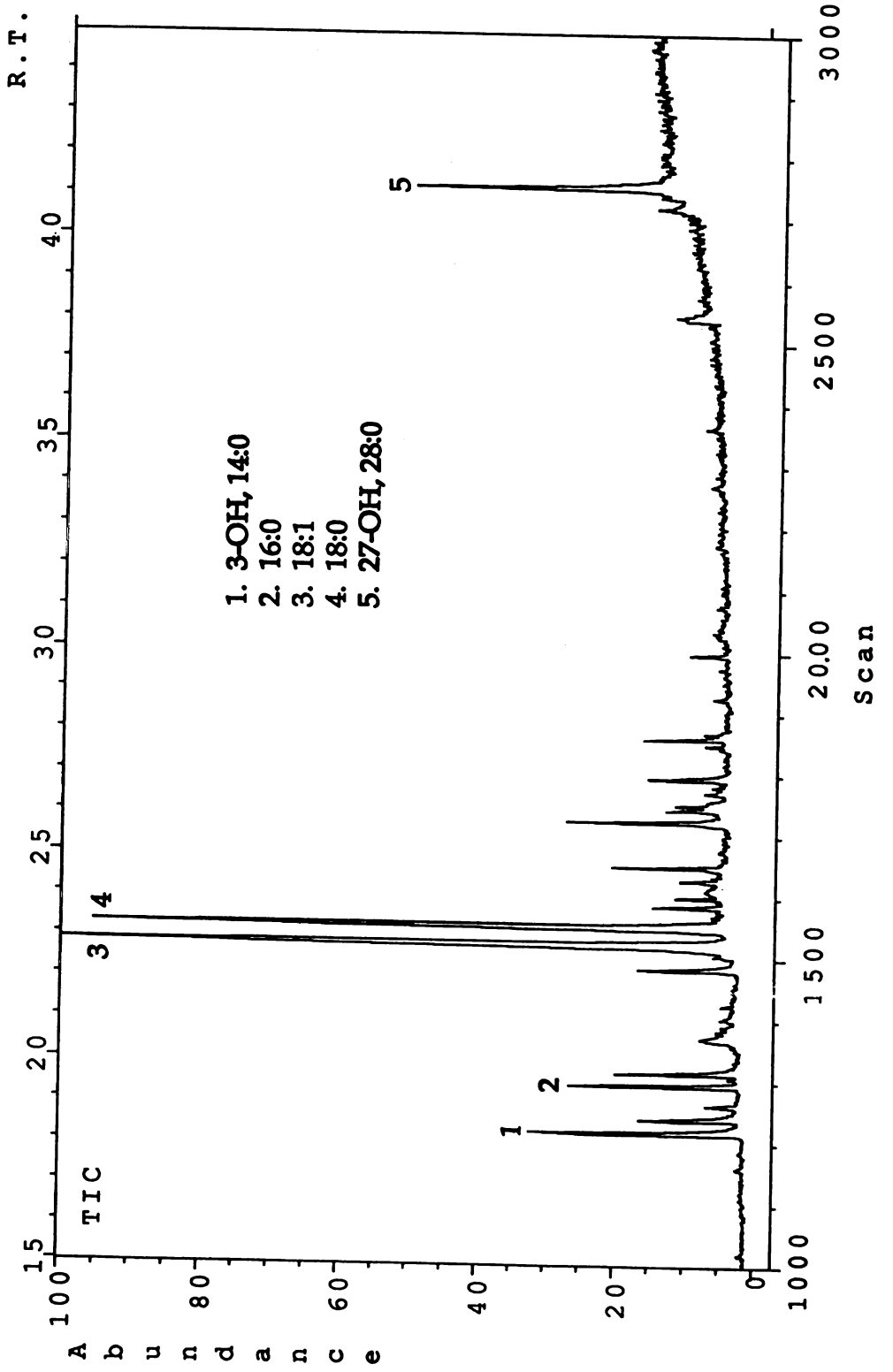

R.T.
1.5
2.0
2.5
3.0
3.5
4.0
TIC
Abundance
100
80
60
40
20
0
1000
1500
2000
2500
3000
Scan
1
2
3
4
5
1. 3-OH, 14:0
2. 16:0
3. 18:1
4. 18:0
5. 27-OH, 28:0

Figure 3.5

lipid A was easily ruled out by two major facts. First, there was the relatively low abundance of hydroxy fatty acids compared to the other fatty acids. The fatty acids of *R. trifolii* LPS are almost exclusively hydroxy fatty acids. The second and most important fact is that all GC and GC-MS analyses clearly indicated that galacturonic acid was absent from the samples. Galacturonic acid is a component of the lipid A (15). The presence of glycerol could indicate that the glycolipid was contaminated with phospholipids. This was not likely, however, because phospholipids normally do not elute from a C-18 reverse phase column with 50% acetonitrile in water. In any event, the absence of phospholipids was definitively proven by thin layer chromatography analysis using a total lipid extract from the same strain and another as references (Figure 3.6). From this it could be seen that there were no contaminating phospholipids. Since glycerol is a component of the glycolipid, and could be freed by acid hydrolysis, it follows that it has to be linked glycosidically to the reducing terminus of the oligosaccharide chain where it could serve as a membrane anchor through acylation by fatty acids. This is the usual configuration of glycerol-containing glycolipids.

A structure that is consistent with the data is shown in Figure 3.7. It could arise by the simple transfer of the activated LPS O-antigen repeat from the carrier lipid to diacylglycerol as indicated. This could happen since the usual acceptor core oligosaccharide is not present and the

Figure 3.6: Orcinol/sulfuric acid stained TLC plate of (A) a total lipid extract of *R. trifolii* ANU843, (B) a total lipid extract of *R. trifolii* 4S, and (C) the combined *R. trifolii* 4S fractions eluted from the C18 column with 50 and 100% acetonitrile in water. Note that the fastest moving component in each total lipid extract is diacylglycerol (arrow). Solvent system used was chloroform, acetone, methanol, acetic acid, water (10:4:2:2:1, v:v). Total lipid mixtures were obtained by extraction with chloroform, methanol, water (4:1:5, v:v).

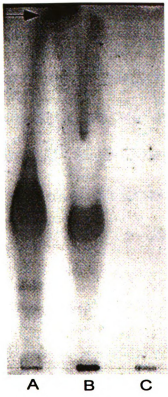


Figure 3.6

Figure 3.7: Possible mechanism of transfer of LPS O-antigen repeat unit from the carrier lipid to diacylglycerol. The product is a chimeric lipid. The O-antigen repeat might be polymerized by successive transfers to a hydroxyl group on the end sugar residue.

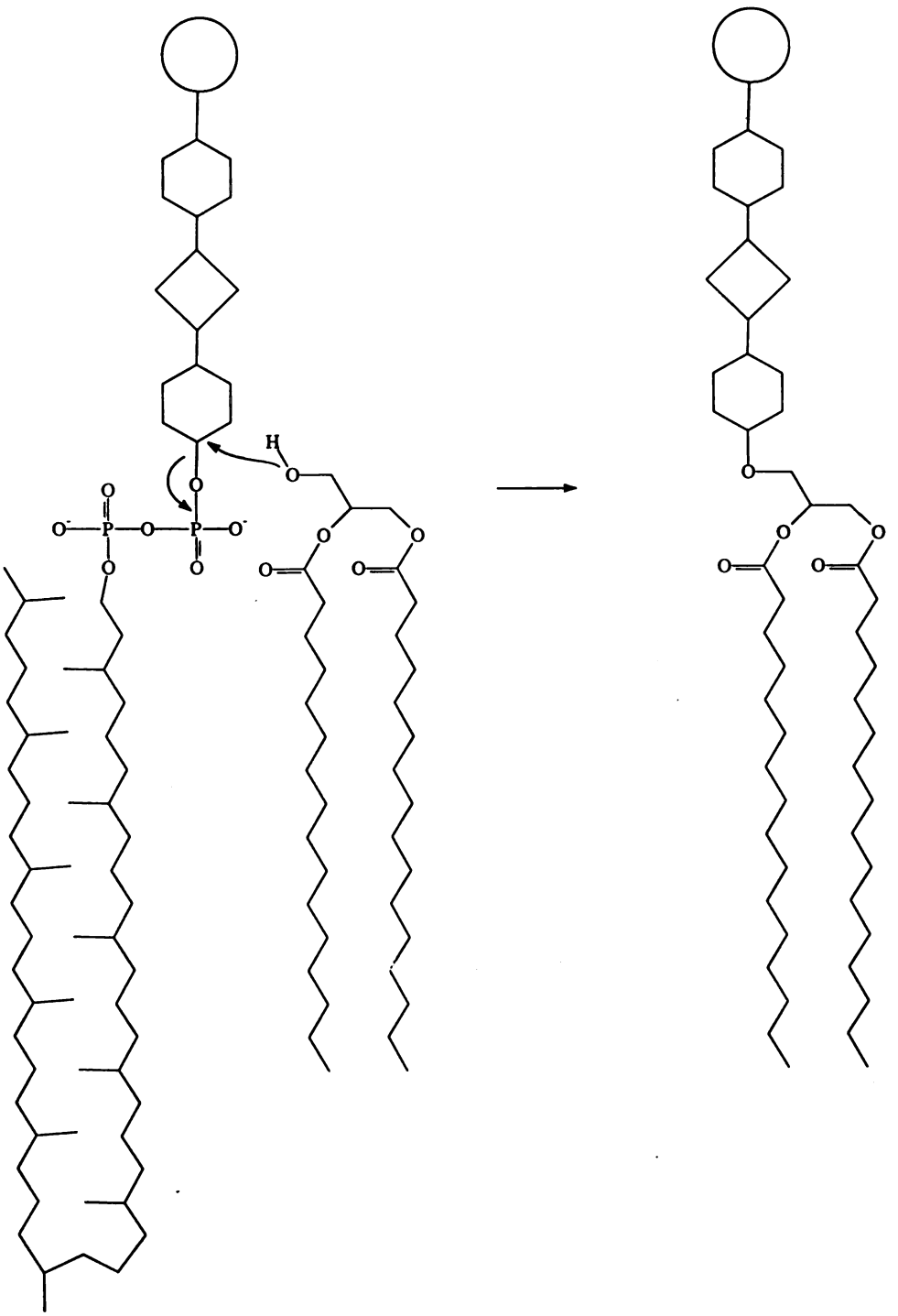


Figure 3.7

membrane of this strain has a high proportion of diacylglycerol (See Figure 3.6). The transfer of activated carbohydrates to diacylglycerol is very common in *Rhizobium* since two classes of glycolipids, sulfoquinovosyl diacylglycerols and diglycosyl diacylglycerols are both synthesized by these organisms (144, 155, 156) and both involve the transfer of activated sugars to diacylglycerol. It is quite reasonable that in the event of aberrant LPS and EPS biosynthesis, as is present in *R. trifolii* 4S, there could be some surrogate function for other glycolipids and glycosyl acceptor molecules. It is also quite possible that a fair amount of this hybridization of molecular structure occurs in normal strains. In the event that the carbohydrate chain in the glycolipid becomes further polymerized by the addition of more O-antigen chains, the resulting polymer would have the usual carbohydrate composition of lipopolysaccharides (including the presence of KDO) as well as some of the characteristic fatty acids. Such a hybrid LPS could restore the proper functionality of LPS/EPS defective strains. The "LPS" molecule whose production is controlled by the *lpsZ* gene in *R. meliloti* (143, 169) fits the profile of such a hybrid molecule.

The discovery of this class of LPS-like lipid-linked oligosaccharides in *R. trifolii* 4S has other significant implications. *R. trifolii* 4S has been considered to be an unusual but important member of the clover nodulation group. The recent discovery that the LPS O-antigen of *R.*

trifolii 4S has a vastly different structure to the rest of the *R. trifolii* strains raised an interesting question about the role of LPS in infection of this strain. It is well documented that LPS plays an important role in various stages in the nodulation process. As reviewed in Chapter 1, these stages have been demonstrated to include infection, bacterial release from the infection threads, and bacteroid transformation and maturation (26, 27, 29, 75, 77, 143). It seemed puzzling then that 4S could have such a different LPS O-antigen structure and still belong to the same clover nodulation group. The results presented in this report, however, resolves this apparent conflict. If LPS is required to play a direct role, it is quite possible that this new lipid somehow performs the same function in the nodulation process for 4S that the "typical" LPS would in the other strains of *trifolii*. It is also clear that the synthesis of a core tetrasaccharide is not a prerequisite for a strain to be effective in nodulation, contrary to earlier observations (22, 166).

Another salient point that emerges from this study is whether or not *R. trifolii* 4S possesses the capability of synthesizing galactose. There are two possibilities, the first one is that *R. trifolii* 4S is incapable of synthesizing UDP-galactose and the galactose in this glycolipid is made by the action of an epimerase on glucose after its incorporation. The second is that UDP-galactose is synthesized but there is a specific galactosyl transferase for this glycolipid that is functional and the others (or some

other general transferase) are defective. Attempts to detect the presence of UDP-galactose in this strain were unsuccessful.

ACKNOWLEDGMENTS

The experiments on the *R. trifolii* 4S lipopolysaccharide core region were performed by Ying Wang. This work was supported by grant #DE-FG02-89ER 14029 from the U.S. Department of Energy to RIH. The GC/MS data were obtained at the Michigan State University Mass Spectrometry Facility which is supported in part by grant #DRR-00480 from the Biotechnology Research Technology Program, National Center for Research Resources, NIH. The NMR data were obtained on instrumentation that was purchased in part with funds from NIH grant #1-S10-RR04750, NSF grant #CHE-88-00770 and NSF grant #CHE-92-13241.

CHAPTER 4

OCCURRENCE OF SULFOQUINOVOSYL DIACYLGLYCEROL IN SOME MEMBERS OF THE FAMILY *RHIZOBIACEAE*

ABSTRACT

A radiolabelled component of a membrane extract of *Rhizobium meliloti* 2011 cells grown in the presence of ^{35}S-labelled sulfate was isolated by silica flash chromatography and purified by HPLC. Based on 1- and 2-dimensional nuclear magnetic resonance (NMR) spectroscopic and mass spectrometric analyses, the structure of the compound was determined to be sulfoquinovosyl diacylglycerol (SQDG). NMR analyses indicated substantial heterogeneity in the fatty acid composition and that an important group was the cyclopropyl fatty acids. This first report of the occurrence of SQDG outside of the plant kingdom, photosynthetic bacteria or diatoms deserves special attention since, in this case, the bacterium is one which can fix nitrogen in symbiosis with plants. The origins of the ability of the bacteria to synthesize this class of membrane lipids is an important question. Membrane extracts of other strains of the family *Rhizobiaceae* were screened for the presence of SQDG. The occurrence of SQDG in the symbiotic organisms was confirmed while no SQDG was detected in either the *Agrobacterium tumefaciens* or the *Escherichia coli* strains tested. The current function of these lipids in symbiosis and the commonality of the ability of bacteria which function as

plant symbionts to synthesize such molecules are all germane to studies of the *Rhizobium*/legume symbiosis.

INTRODUCTION

The rhizobial cell surface has always been considered to be an essential factor in our attempts at understanding the interactions between these bacterial symbionts and their legume partners. The tremendous wealth of information pertaining to the role of membrane components in this relationship is reviewed extensively in Chapter 1. Much debate and, admittedly much controversy, has revolved around the roles of capsular polysaccharides (36, 58-61). The roles of lipopolysaccharides in ensuring a successful infection process is much more generally accepted. It is well established that bacterial mutants having defective lipopolysaccharide structures are incapable of infections which progress into viable, occupied nodules which are capable of supporting nitrogen fixation (22, 29, 73, 166). The bacterial cell envelope, in which of course the lipopolysaccharide resides, has still not been rigorously characterized, despite many years of study. For instance, it was only relatively recently shown that 27-hydroxyoctacosanoic acid, a 28-carbon fatty acid, was found to be a major component of the lipopolysaccharide of virtually all rhizobia (11, 12). There have been several studies attempting to elucidate the total lipid composition of the membranes of some of the members of the family *Rhizobiaceae*. A major consistent finding in these studies is the presence

of phosphatidylcholine (PC), a typical plant lipid, in the membrane of all of the species investigated thus far (170-172). This finding may suggest either a role for this lipid in the infection process or the possibility of gene exchange between the bacterium and the plant in the distant past. However, although PC is not common amongst Gram-negative bacteria, it is also not exclusive to the *Rhizobiaceae* (172).

As described in detail in Chapters 1 and 2, the Nod factors have been advanced as the sole determinants of host specificity in the *Rhizobium*/legume symbiosis. The Nod factors are a class of closely related mono-N-acylated chitin oligomers substituted with various side groups. The type of fatty acid and the nature of the side group have been thought to account for the host specificity (173). In the case of *R. meliloti* it has been shown that the critical feature that determines host specificity is the sulfate group on the reducing terminal glucosamine residue (115, 120, 141). The emergence of sulfated lipid-linked carbohydrates as mediators of the infection process in the *R. meliloti*/alfalfa symbiosis raised the question of a possible link of this phenomenon to the bacterial surface chemistry. Therefore, a systematic study of the bacterial membrane was performed with a focus on finding sulfur-containing lipid components.

Bacteria were grown in the presence of ^{35}S-labelled sulfate and labelled membrane components were purified chromatographically and characterized. The isolation, purification and characterization of a

sulfur-containing lipid, whose occurrence was thought to be restricted to plants, algae and photosynthetic bacteria, is presented in the first part of this work (174). Some questions that arise due to this finding are: "Was this a trait that was captured from plants but has no symbiotic significance?" or, "Is the ability of the bacteria to make plant-like lipids a prerequisite for the close interaction (possibly membrane fusion) between bacteria and plants during symbiosis?" In the second part of this study these questions were begun to be addressed by screening various members of the *Rhizobiaceae* for the presence of this sulfolipid. These experiments were conducted in an attempt to probe the relatedness of the various genera and to, perhaps, provide a clue to the possibility of a requisite membrane compatibility in rhizobial infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The various bacterial strains which were used are as follows: *Rhizobium meliloti* 2011 (from Ethan Signer, Massachusetts Institute of Technology), *R.* sp. strain NGR234 (from Frank Dazzo, Michigan State University), *R. leguminosarum* bv. *trifolii* ANU843, *R. leguminosarum* bv. *trifolii* ANU845 (from Barry Rolfe, Australian National University), *Bradyrhizobium japonicum* USDA110 (from Frank Dazzo, Michigan State University), *Agrobacterium tumefaciens* C58 (from Frans DeBruijn, Michigan State University), and *Escherichia coli* TG-1 (from Gregory Zeikus, Michigan State University). All strains of bacteria (with the exception of *E. coli*) were grown at 30°C in liquid cultures containing modified Bergensen's (BIII) media as previously described (142) except that in addition, ^{35}S-labelled sulfate at a level of 100μCi/liter of culture was added in the form of carrier free sulfuric acid (New England Nuclear). The *E. coli* strain was grown at 37°C in MSX4 media with ^{35}S-labelled sulfate added as above.

Lipid isolation and purification.

Lipids were extracted by stirring the bacterial cells (after harvesting by centrifugation) with 100 ml of a mixture of chloroform, methanol, n-butanol and water in the ratio of 2:1:1:4 at room temperature for 24 hours. The cell debris was removed by centrifugation and the supernatant allowed to partition into two layers. The lower organic layer was recovered and the aqueous layer was recombined with the cell debris and the mixture re-extracted with 200 ml of a 2:1:1 mixture of chloroform, methanol and n-butanol, respectively. The organic layer was again recovered and combined with the one from the first extraction. The combined organic layer was concentrated to dryness and chromatographed on a silica column (4 cm x 2 cm) with a mixture of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:2:1, respectively. Fractions of 4 ml were collected and a 100 µl aliquot of each subjected to analysis for radiolabel by scintillation counting. One major fraction (from a plot of fraction number vs. d.p.m.) was recovered and subjected to further purification by high performance liquid chromatography (HPLC) on an ALTEX ultrasphere C-8 reverse phase column (1 cm x 25 cm). A linear gradient was employed starting with a combination of 1:1 acetonitrile/water (40%) and n-propanol (60%). This ratio was maintained for 10 minutes at a flow rate of 1ml/min. The flow rate was then increased to 1.2ml/min and the gradient was run to a

final composition of the two solvent systems of 10 and 90%, respectively. The column effluent was monitored at 230 nm and the peaks were collected and counted. The entire isolation and purification was repeated using unlabelled sulfate to obtain material for use in structural characterization. Purity of the product was assessed by thin layer chromatography (TLC) on silica layers using a mixture composed of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:2:1, respectively.

Chemical characterization.

Fatty acids were released as methyl esters from the purified lipid by methanolysis of a sample (~50 μg) at 75°C with 1 ml of methanol containing 2% HCl. The methanolysate was concentrated to dryness and partitioned between 2 ml of hexane and 1 ml of water. The upper hexane layer was recovered and subjected to gas chromatography and gas chromatography/mass spectrometry.

The glycosyl component of the purified lipid was obtained by treating a sample (3 mg) of the component with ethanolic potassium hydroxide (0.5 M) at 100°C for 30 minutes. The mixture was treated with 10 μl of acetic acid and then concentrated to dryness. The fatty acids were removed by partitioning the residue between 4 ml of a 1:1 mixture of water and hexane. The aqueous layer was subjected to gel filtration

chromatography on a Biogel P2 column (20 cm x 0.5 cm) in pure water. One milliliter fractions were collected and assayed for the presence of carbohydrates by spotting on silica plates, spraying with orcinol/sulfuric acid and heating at 120°C for 5 minutes. The fractions giving a positive reaction were pooled and lyophilized. The optical rotation was measured in water at the sodium D line on a Perkin Elmer Model 141 polarimeter.

Nuclear magnetic resonance (NMR) spectroscopy.

All NMR spectra were measured in CD_3OD solution at 500 MHz for the ^1H or 125 MHz for the ^{13}C nucleus. The ^{13}C-DEPT spectrum (175) was measured at 75 MHz for ^{13}C. Double quantum filtered J-correlated 2-dimensional spectra (phase sensitive mode) (176) were obtained using a total of 512 data sets (32 transients) over a spectral width of 3000 Hz. The total number of points used was 2048. The FIDs were multiplied by a gaussian function in the f_2 dimension and by a shifted gaussian in f_1. The final data set was symmetrized by triangular folding. Data for the HOHAHA experiments (177) were obtained using similar acquisition and processing conditions. A mixing time of 80 msecs was used. Spectra were not symmetrized. For the HMQC experiment (178), a spectral width of 19,000 was employed for the ^{13}C dimension. A total of 80 transients were acquired for each of a total of 512 data sets. A total of 1024 points was used in these analyses.

Mass spectrometry.

Fast atom bombardment mass spectra were recorded on a JEOL HX-110 HF mass spectrometer using Xenon neutrals (6 keV) as the primary beam. Triethanolamine was used as matrix and spectra were obtained in the negative ion mode. Gas chromatography/mass spectrometry was performed on a JEOL 505 mass spectrometer using a DB1 capillary column. The gas chromatography program used was: 150° to 300° at 3°/minute with a 10 minute hold at 300°C.

Screening for sulfoquinovosyl diacylglycerol (SQDG).

Cells were harvested by centrifugation and lipids were extracted using the procedure described above. The crude organic extracts were subjected to thin layer chromatography (TLC) on silica gel using isolated lanes to prevent cross-contamination and employing the solvent system described above. Sample extracts were chromatographed along with a standard of SQDG, the isolation and characterization of which is presented in this chapter. Bands containing glycosyl components were visualized by spraying with an orcinol/sulfuric acid mixture and heating at 120°C for approximately 10 min. Radioactive bands were detected by exposure of the 2,5-diphenyloxazole-impregnated TLC plate to a Kodak x-omat x-ray plate at -80°C for three weeks. Additionally, TLC plates were developed in which the radioactive glycosyl-containing bands were

scraped and the lipids extracted from the silica with chloroform, methanol (1:1, v:v). Verification of the presence of SQDG was obtained by FTIR spectroscopy using a Nicolet 710 Fourier Transform Infrared Spectrometer. The samples were analyzed as thin films on a NaCl substrate.

RESULTS

Analysis of purified lipid.

TLC analysis of the membrane lipids of *Rhizobium meliloti* 2011 on silica layers followed by autoradiography revealed the presence of two major radiolabelled components (R_f=0.22 and 0.18). These components were eventually isolated using a combination of flash chromatography on silica and reverse phase chromatography. The ^{1}H-NMR spectra of the two components were very similar except for slight differences due to fatty acid distribution. The spectrum of the slower migrating component on TLC (first eluting on reverse phase HPLC) is shown in Figure 4.1. It contained signals between 2.8 and 4.7 characteristic of carbohydrate groups as well as signals between 0.8 and 2.4 ppm characteristic of fatty acid groups. A triplet at 5.35 ppm was assigned to the vinyl protons of an unsaturated fatty acid. A multiplet at 2.02 ppm was assigned to the methylene groups adjacent to the unsaturation. Signals for the methylene groups adjacent to the carbonyl carbons of fatty acid groups appeared as a multiplet at 2.32 ppm. The signals for the terminal methyl groups of fatty acid chains appeared at 0.89 ppm. One interesting feature was the presence of two sets of very upfield signals. These were assigned to signals for protons on cyclopropyl groups. The most upfield signals, at

Figure 4.1: ^{1}H-NMR spectrum of the purified ^{35}S-labelled major component (R_f=0.22) from the membrane extract of *R. meliloti* 2011. Signals between 2.8 and 4.8 ppm are due to a carbohydrate and a glyceryl component. The other signals are due to fatty acids. Note the presence of a high degree of unsaturation (vinyl proton resonances at 5.35 ppm) and the presence of cyclopropyl group (most upfield signals). Assignments are made in the text. The strong resonances at 3.30 and ~4.8 ppm are due to residual $C\underline{H}D_2^-$ and $O\underline{H}$ signals, respectively, of the solvent. The signals between 0.5 and 2.4 ppm are due to resonances in the fatty acids.

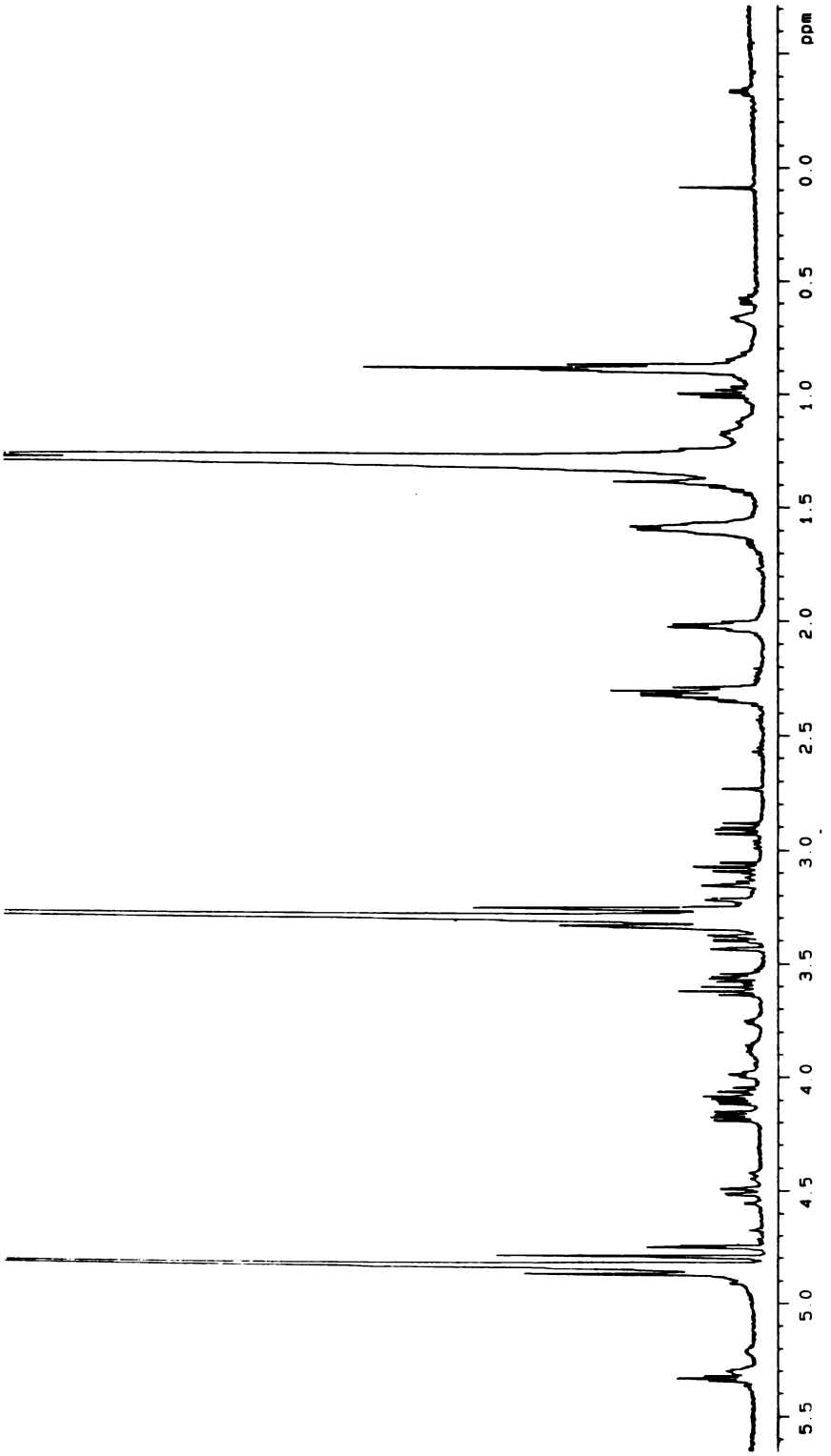


Figure 4.1

-0.33 ppm, were assigned to the methylene group of the cyclopropyl function. A broad multiplet at 5.32 ppm indicated the presence of a glyceryl moiety. This signal was assigned to H-2 of the glyceryl moiety. The other signals between 2.8 and 4.8 ppm were generally too complex to assign directly. The ^{13}C-DEPT spectrum of the same slower migrating component (Figure 4.2) contained signals between 10 ppm and 40 ppm, consistent with the aliphatic resonances of fatty acid chains. The presence of unsaturations was confirmed by resonances at 130 ppm. A resonance at 99.2 ppm was assigned to an anomeric proton of a glycosyl residue. Based on the chemical shift of a proton (assignable to an anomeric proton) at 4.76 in the ^{1}H-NMR spectrum, the α-configuration was assigned. This proton displayed a coupling constant of 2-3 Hz indicating that the neighboring proton was axial. Signals between 50 and 110 ppm were sufficient for only 1 glycosyl unit. The ^{13}C-DEPT spectrum indicated that the molecule contained three primary carbons bearing heteroatoms. One of these at 53.5 ppm was assigned to carbon linked to sulfur. The other two were assigned to a glyceryl moiety. Five carbons bearing oxygen atoms gave rise to signals 62 and 76 ppm, giving a total of 9 carbon attached to heteroatoms.

The HOHAHA spectrum of the slower migrating major lipid component (Figure 4.3) conveniently broke down the spectrum into spin systems for the glyceryl, carbohydrate and fatty acid components. Based

Figure 4.2: ^{13}C-DEPT-NMR spectrum of the purified ^{35}S-labelled component (R_f=0.22). The anomeric carbon of a glycosyl residue appears at 99.2 ppm. The number of signals in the range of 50-110 ppm and the number of anomeric carbons are consistent with the presence of only one glycosyl unit. The signal at 130 ppm is due to vinyl carbons. Note the very upfield primary carbon resonance at 53.5 ppm in the carbohydrate region of the spectrum, indicative of a carbon-sulfur linkage.

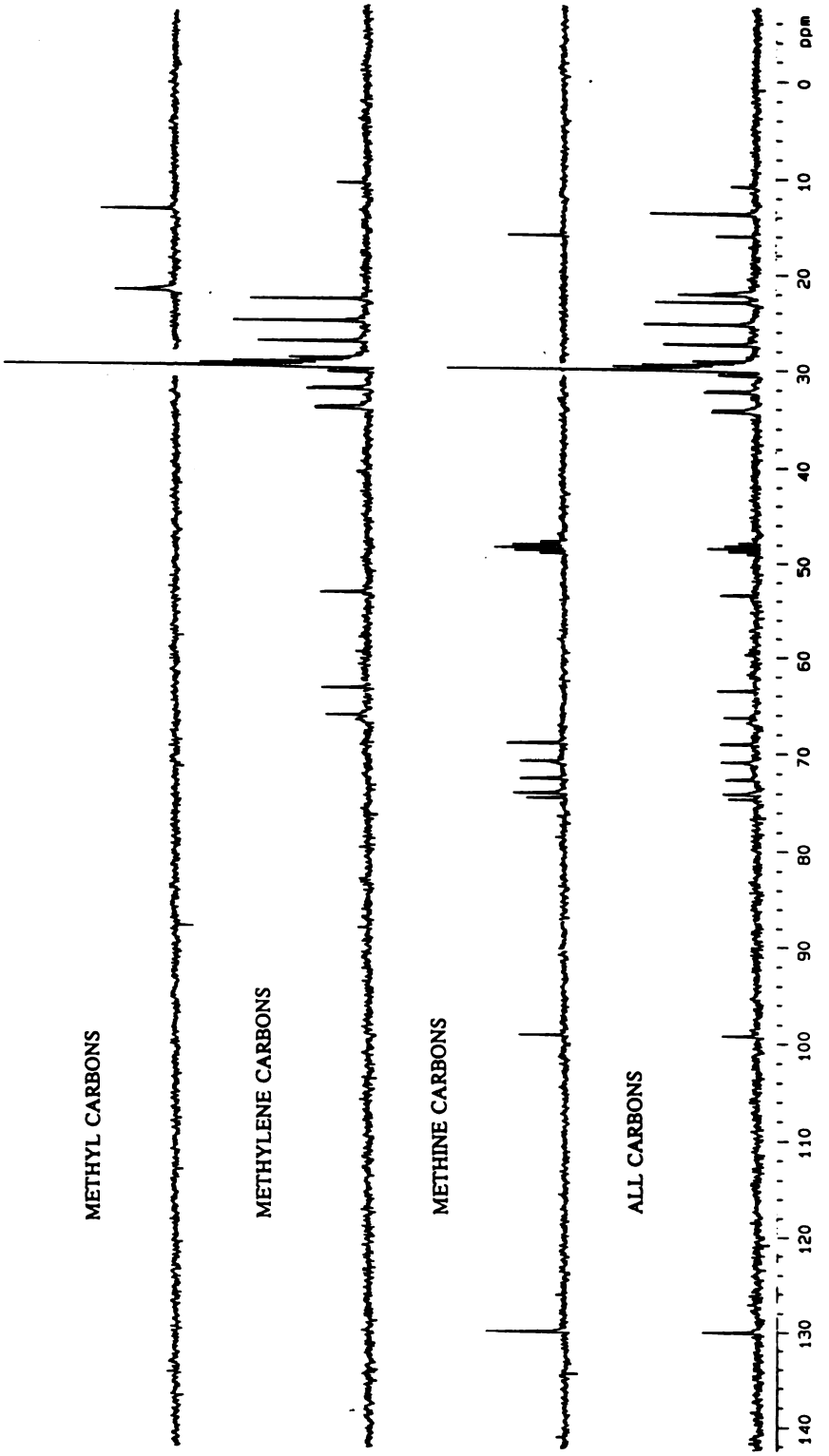

METHYL CARBONS
METHYLENE CARBONS
METHINE CARBONS
ALL CARBONS
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
ppm

Figure 4.2

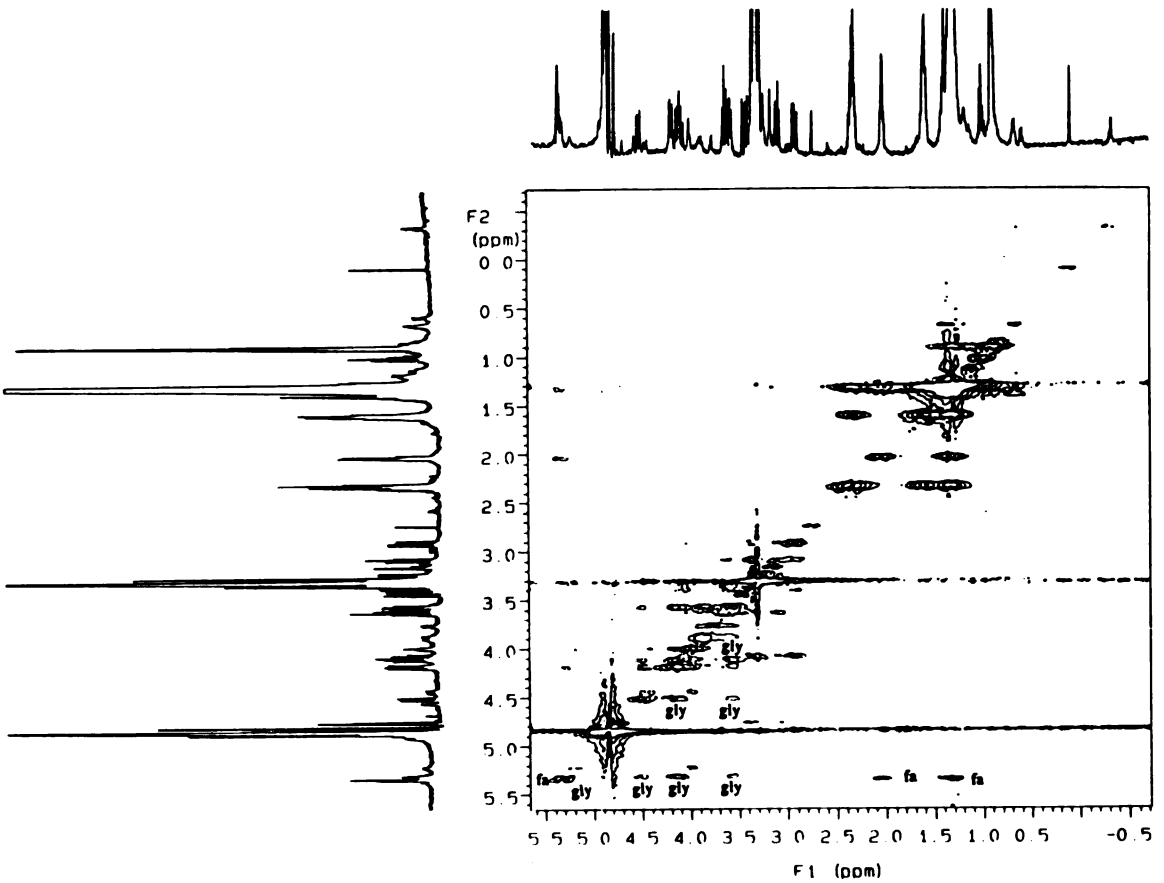


Figure 4.3: ^{1}H-HOHAHA NMR spectrum of the purified ^{35}S-labelled component (R_f=0.22). Note the connectivities for glycerol (gly) and for the fatty acid components (fa). The carbohydrate connectivities are not marked.

on this spectrum, the signals at 5.32 ppm, 4.50 ppm, 4.17 ppm, and 3.58 ppm were assigned to the glyceryl moiety. The other signals between 2.8 and 4.8 ppm were assigned to the glycosyl residue. The DQF-COSY spectrum (Figure 4.4) allowed the assignments of the spin connectivities in the spectrum. Hence, the signal at 3.39 ppm was assigned to H-2 (a double of doublets with J=7.3 Hz and 2.0 Hz). Based on these coupling constants, it was determined that the glycosyl H-3 signal at 3.62 ppm (triplet with J=7.3 Hz) was axial and that the hydroxyl group at this position was therefore equatorial. The large coupling with both H-4 and H-2 indicated the *gluco* configuration. H-4 appeared further upfield (3.08 ppm) as a triplet (J=7.3 Hz). The signal for one of the H-6 protons (dd, J=13.5 + 7.9 Hz) appeared at 2.90 ppm, indicating that it was not attached to a carbon atom bearing oxygen. It was concluded that this carbon was attached directly to sulfur. Based on the chemical shift a sulfonic acid moiety was proposed. The proton assignments agreed well with the ^{13}C-NMR assignments. These were confirmed by an HMQC experiment (Figure 4.5). The carbon attached to sulfur appeared at 53.5 ppm as expected. The glyceryl methylene carbons appeared between 62 and 67 ppm while the remaining glyceryl resonance, as well as those for the carbohydrate carbons, was further downfield. Assignments for the shifts in the fatty acid region also appear as expected. The complete proton assignments are given in an expanded ^{1}H-NMR carbohydrate region in

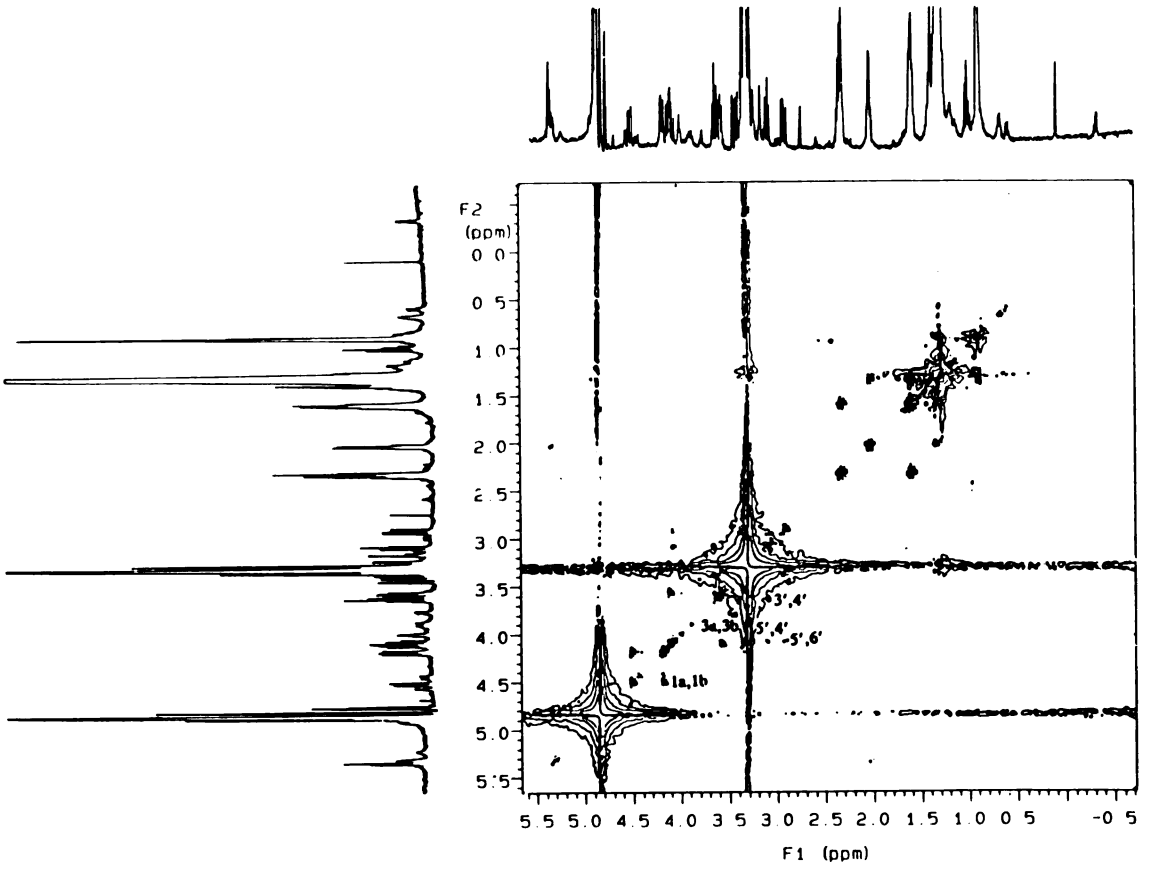


Figure 4.4: ^{1}H-DQF-COSY spectrum of the purified ^{35}S-labelled component (R_f=0.22). Note the assignments for the carbohydrate and glycerol components. Primed numbers refer to the carbohydrate component.

Figure 4.5: $^{1}H/^{13}C$-HMQC spectrum of purified ^{35}S-labelled lipid component (R_f=0.22). All cross peaks due to the carbohydrate and glycerol components are assigned. Note the proton and carbon chemical shifts at the 6-position, the site of sulfonylation. Primed numbers refer to the carbohydrate components.

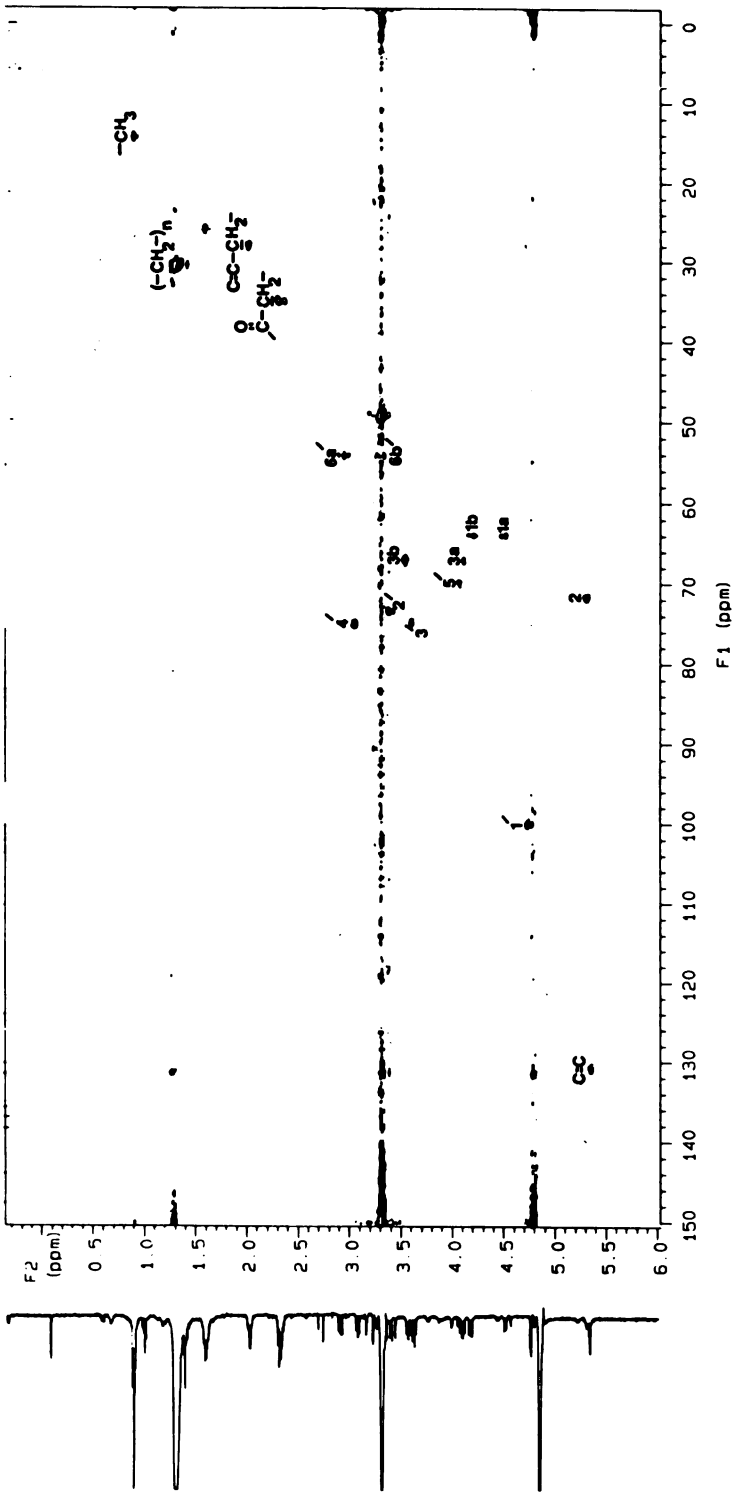


Figure 4.5

Figure 4.6. Based on the NMR analyses, the molecule was determined to be a diacylglyceryl derivative of a 6-deoxy-α-glucopyranose-6-sulfonic acid. These assignments agree with previously published results (204). According to integration of NMR signals in analyses of crude lipid extract, SQDG comprises approximately 5-10% of the total lipid in *Rhizobium meliloti*.

The ^{1}H-NMR spectrum of the faster migrating component (R_f=0.22) had essentially the same features as that of the slower migrating other major component (R_f=0.18). The fatty acid distribution was, however, different since signals for multiple unsaturation were more dominant. This splitting of TLC components for these lipids is quite common and has been demonstrated and studied by other workers who also attribute differences in mobility to fatty acid class (179). These workers also observed differences in the orcinol response for the two TLC components.

The fatty acids were determined by gas chromatography/mass spectrometry (GC/MS) to be predominantly hexadecanoic acid, octadecenoic acid and a methyleneoctadecanoic acid. There were other minor peaks assignable to a bis-unsaturated C-18 fatty acid, tetradecanoic acid and tetradecenoic acid.

The proposed structure was confirmed by performing fast atom bombardment mass spectrometry on the slower migrating component (Figure 4.7). The major ion in the pseudo-molecular ion region, i.e.,

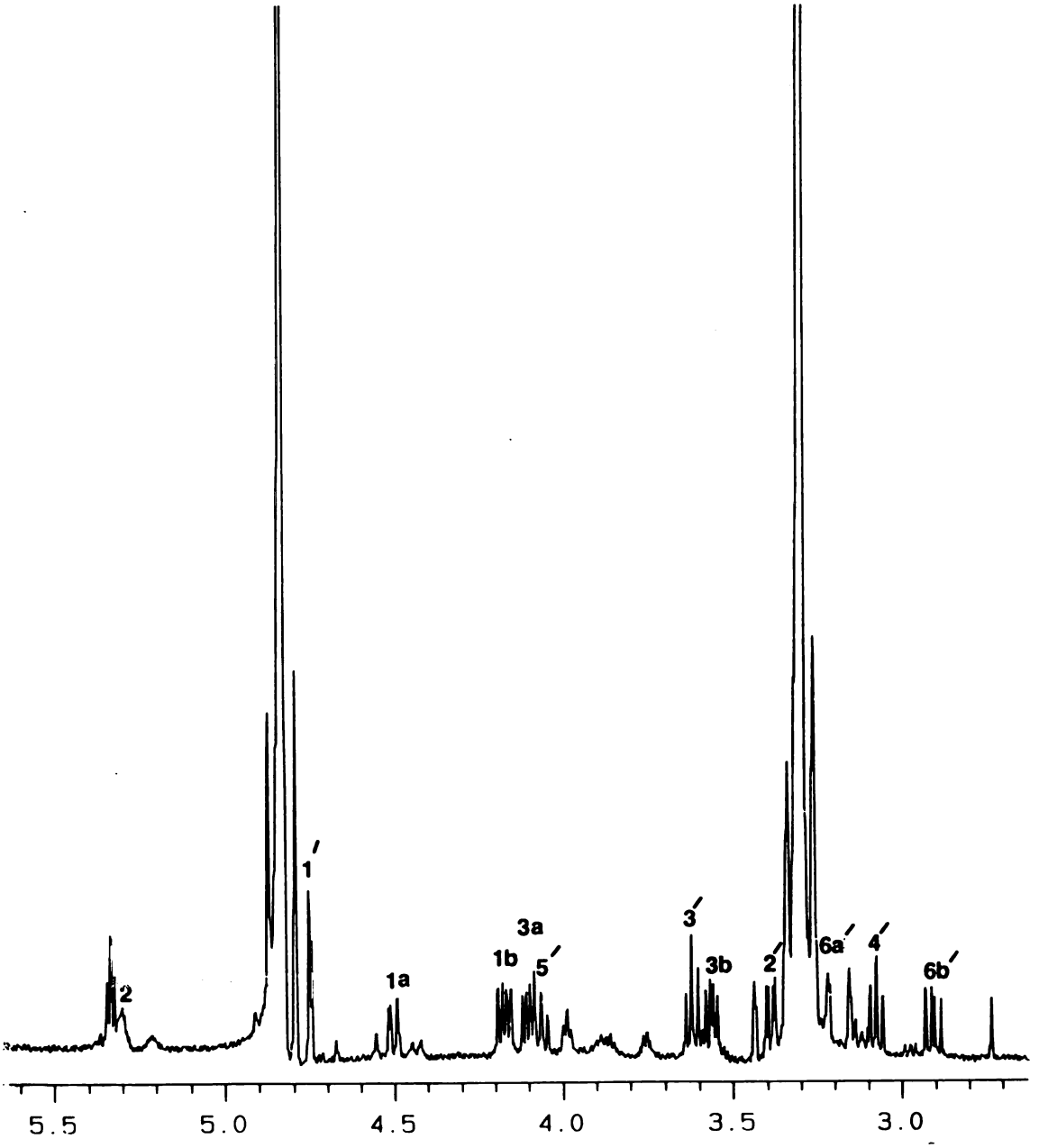


Figure 4.6: ^{1}H-NMR spectrum of purified ^{35}S-labelled lipid component (R_f=0.22). Complete proton assignments for sulfoquinovosyl diacylglycerol are shown. Primed numbers refer to the carbohydrate protons.

Figure 4.7: Negative ion fast atom bombardment mass spectrum of purified lipid component. The cluster of ions between 700 and 900u are $(M-H)^-$ pseudo-molecular ions for the different species. The ion at m/z 793 is due to the molecule containing two hexadecanoyl residues (Structure 4.1). This is the predominant component. The other ions are due to heterogeneity in the fatty acyl components. The assignments are made in the text. Signals between 250 and 300 are due to carboxylate ions of the fatty acyl components formed by primary cleavage. The assignments are: m/z 255 (C_{16}) the predominant component, 281 ($C_{18:1}$), 295 (C_{18} with one cyclopropyl group) and 279 ($C_{18:2}$).

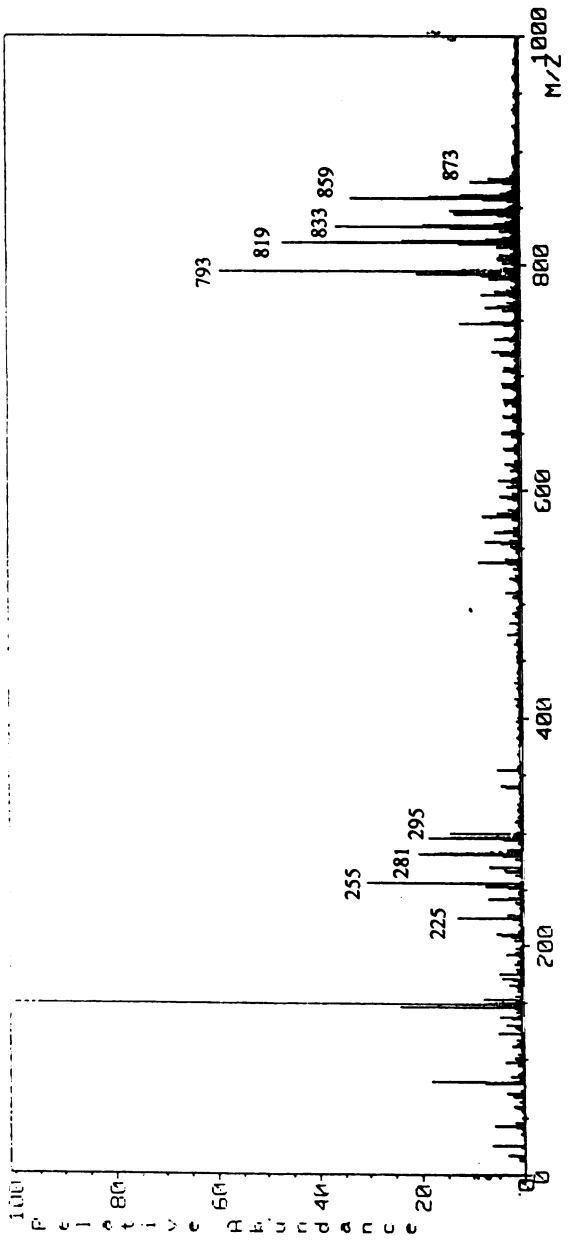
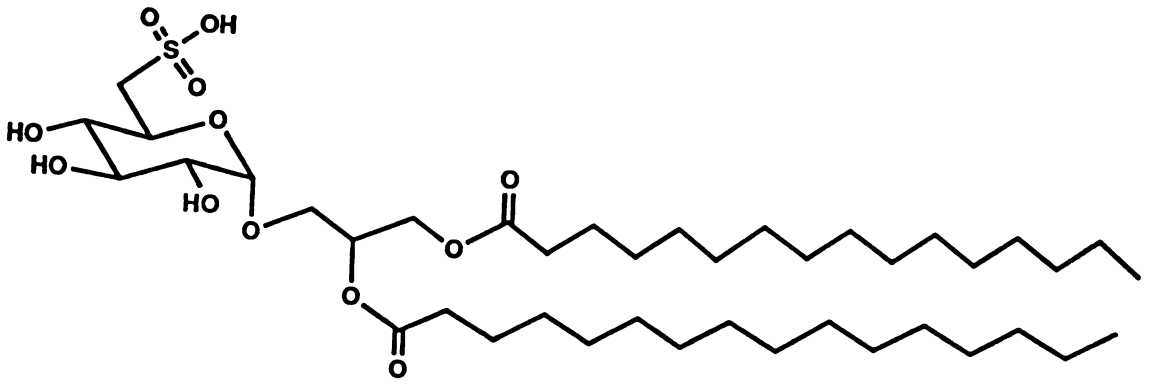

100
80
60
40
20
0
0
200
400
600
800
1000
m/z
225
255
281
295
793
819
833
859
873

Figure 4.7

($(M-H)^-$=793u) was assigned to sulfoquinovosyl dihexadecanoyl glycerol (Structure 4.1). The next major ion at m/z 819 corresponded to a form in which the fatty acids were a hexadecanoyl component and an octadecenoyl component. The next major peak at m/z 833 corresponded to the presence of a methyleneoctadecenoyl and a hexadecanoyl fatty acid residue. The next most intense peak appeared at m/z 859 and corresponded to the presence of octadecenoic acid and a methyleneoctadecenoic acid. Other minor peaks corresponding to different combinations of the fatty acids present were also observed. Signals for the carboxylate ions of the fatty acids appear between 250 and 300u. The peaks corresponding to C_{16} and $C_{18:1}$ are the most abundant (m/z 255 and 281, respectively). The peak at m/z 225 is most likely due to the dehydrosulfoglycosyl anion corresponding to a minor sulfoglycosyl ion at m/z 243. The stereochemistry of the glycerol to carbohydrate linkage was confirmed by removing the fatty acid by base treatment and subjecting the carbohydrate component to polarimetry. Not enough material was obtained to allow an accurate measurement of the optical rotation of the glycoside but the sign could be determined. The rotation of the glyceryl glycoside in water was small and positive, consistent with the proposed D-configuration (180).



Structure 4.1: Structure of the major component in the family of sulfoquinovosyl diacylglycerols elaborated by *R. meliloti* 2011. The most prominent fatty acyl species are C_{16}, $C_{18:1}$ and C_{18} cyclopropyl species. The 1-position of glycerol is acylated.

Occurrence of sulfoquinovosyl diacylglycerol (SQDG) in symbiotic species of the *Rhizobiaceae*.

Organic extracts of several strains belonging to *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* were screened for the presence of SQDG by TLC followed by visualization with orcinol. The results are shown in Figure 4.8. Glycosyl-containing bands (R_f=0.22 and 0.18) with similar mobilities to the standard SQDG bands seemed to be present in all members of the rhizobia species tested. No such bands occurred in the *Escherichia coli* control. The *A. tumefaciens* sample showed what appeared to be a faint band in this region also. However, when the TLC plates were subjected to autoradiography (Figure 4.9), the *Rhizobium* bands were found to be radiolabelled while the same region of the *A. tumefaciens* lane was not. These results were verified by scraping the bands from the plate, extracting with a chloroform:methanol mixture (1:1, v:v), and checking radioactivity by scintillation counting. In addition, a third faint radioactive band with slower mobility than SQDG appeared in the autoradiogram in the lanes of the rhizobia species. This band has not yet been identified. The *Bradyrhizobium* strain tested also showed a band with a similar mobility to the standard and when extracted showed about twice baseline radioactivity (results not shown). In a previous study, a TLC of the total lipid extract from *R. meliloti* failed to show the presence of SQDG (181). This was most likely due to the different methods of

Figures 4.8: Orcinol stained TLC plate of membrane extracts from members of the *Rhizobiaceae* (Strains are identified in text). Two bands can be seen with similar mobilities to the two SQDG standards in all strains except the *E. coli* control and the *A. tumefaciens* sample. Some major lipids are identified as a reference in the *Rhizobium* wild type (2011) strain. They are phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and diphosphatidylglycerol (DPG).

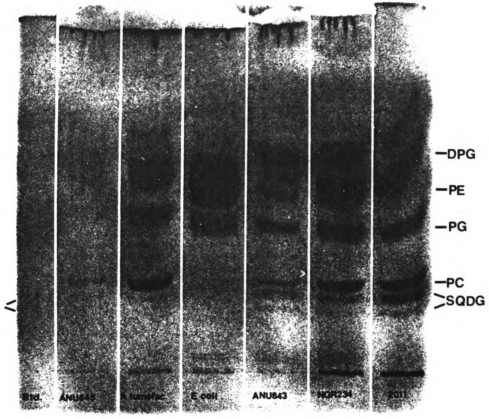


Figure 4.8

Figure 4.9: Autoradiogram corresponding to TLC presented in Figure 4.8 (not same scale) of membrane extracts from members of the *Rhizobiaceae* (Strains are identified in text). Two radiolabelled bands can be seen with similar mobilities to the SQDG standard in all strains except the *E. coli* control and the *A. tumefaciens* sample. The minor radiolabelled component (X) has yet to be identified. The standard was not radiolabelled.

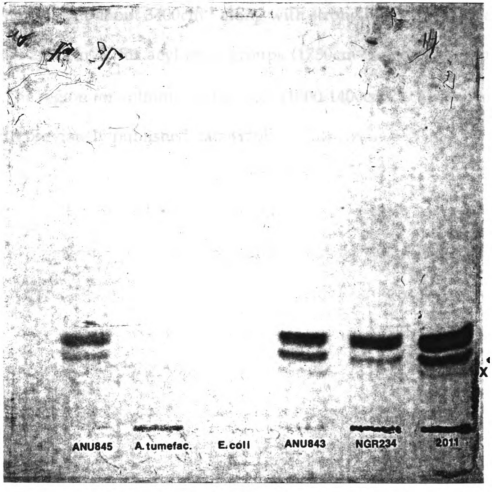


Figure 4.9

extraction. The other major bands, however, had similar mobilities to those observed here. Extracted radioactive TLC bands were also analyzed by Fourier transform infrared spectroscopy (Figure 4.10). All samples as well as the standard showed broad absorption bands for the hydroxyl groups at about 3400cm^{-1} along with strong bands for CH_2 and CH_3 (2800-2950cm^{-1}), for acyl ester groups (1750cm^{-1}) and overlapping bands in the region for sulfonic acid groups (1000-1400cm^{-1}). These values agree with previously published data (179).

Figure 4.10: Fourier transform infrared spectra of (A) SQDG standard and (B) extract from putative SQDG TLC band of the *R. meliloti* 2011 sample (spectra from the other samples were identical). The major absorption bands from the standard can also be seen in the sample and are identified in the text. The bands at 1400 and 1600 cm^{-1} of the sample spectrum are due to a contaminant.

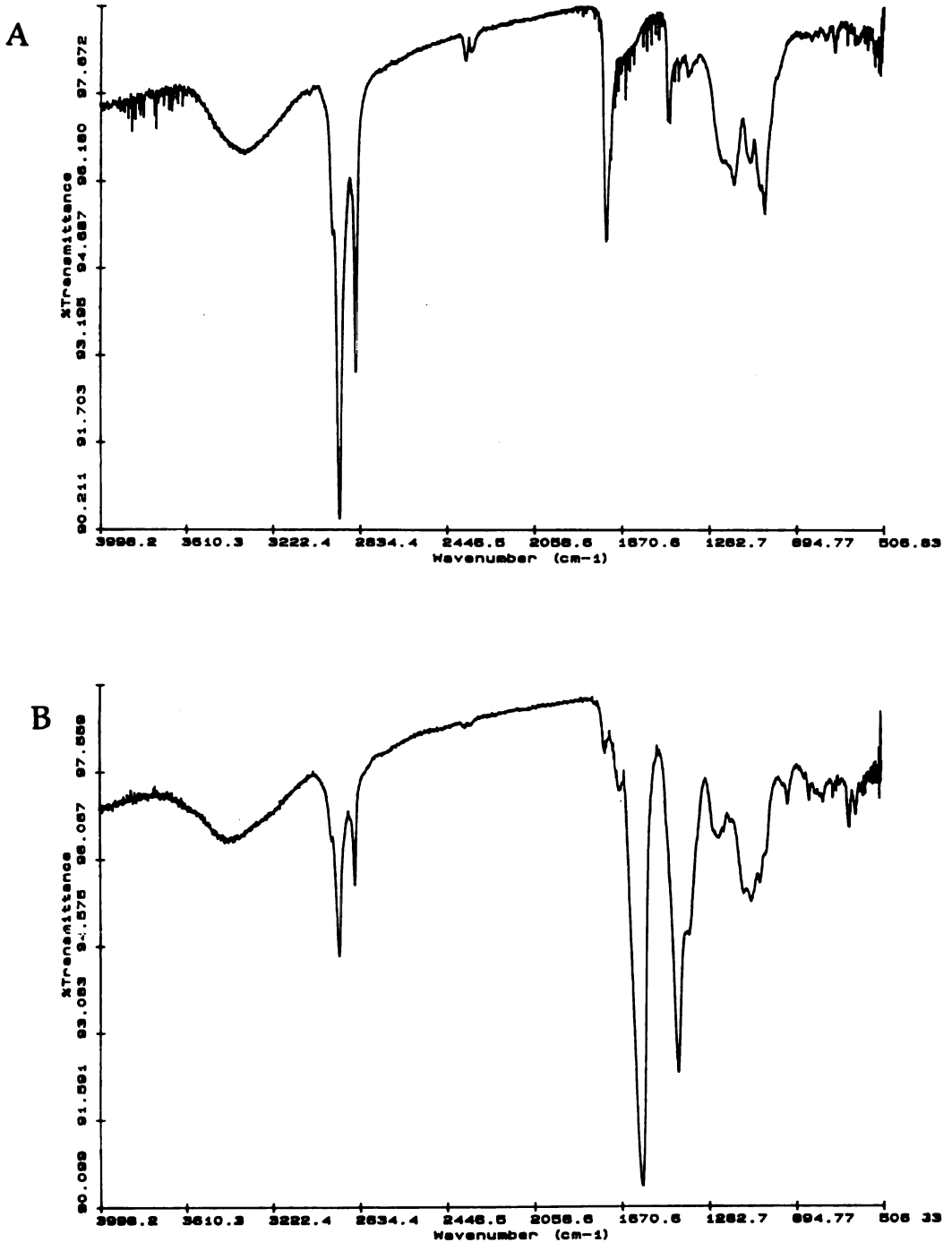

A
%Transmittance
97.672
96.180
94.687
93.195
91.703
90.211
3998.2
3610.3
3222.4
2834.4
2446.5
2058.6
1670.6
1282.7
894.77
506.83
Wavenumber (cm-1)
B
%Transmittance
97.558
96.067
94.575
93.083
91.591
90.099
3998.2
3610.3
3222.4
2834.4
2446.5
2058.6
1670.6
1282.7
894.77
506.33
Wavenumber (cm-1)

Figure 4.10

DISCUSSION

It has been shown in these studies that sulfoquinovosyl diacylglycerol (SQDG) is a membrane lipid in certain cultured rhizobial species. The occurrence of SQDG outside of plants or photosynthetic bacteria has virtually never been reported before. A strict exclusivity of this lipid to photosynthetic systems has generally been assumed. It is, therefore, remarkable that this lipid component is found in a Gram-negative bacteria. It is at once puzzling and understandable that it is found in a plant symbiont. Several questions immediately arise in connection with this finding. The first one is: Do these lipids have any functional role in symbiosis? It is tempting to propose that, perhaps, the presence of this lipid might be necessary in order to ensure some compatibility between the plant and bacterial membranes during symbiosis. It has been known for many years that another typical plant lipid, phosphatidylcholine (PC), exists in the membrane of members of the *Rhizobiaceae*. However, a role for PC in symbiosis and/or pathogenesis has not yet been elucidated. The second question is: What is the origin of the genes responsible for biosynthesis of SQDG; were they transferred from the plant or were they developed in the bacterium? Based on zero evidence in either direction, the former scenario seems much more

palatable since the rhizobia and legumes have shared a long association and there has been sufficient chance for this to have occurred. Another alternative is that these bacteria were, at one stage, plant organelles.

With an aim of determining the generality of SQDG amongst legume bacterial symbionts, included in this study were a representative sampling of the family *Rhizobiaceae*. *R. leguminosarum* bv. *trifolii* ANU843 and *R.* sp. strain NGR234 are wild type strains that represent a variety in the range of *Rhizobium* host specificity. The finding of SQDG in these organisms increases the consequence of the previous finding and strengthens the possibility of the widespread importance of this lipid in symbiosis. *R. leguminosarum* bv. *trifolii* ANU845, which lacks the "symbiotic plasmid", was studied in an effort to determine whether the pSym genes are involved in the biosynthesis of SQDG thus indicating a possible role in nodulation as opposed to a later stage in infection. The presence of SQDG in this mutant suggests that this lipid may have a role (if any) during or after bacteroid formation, possibly by ensuring membrane compatibility between the bacterium and the plant thus allowing for release from the infection thread. More studies will need to be performed on other mutant strains that lack this ability. *B. japonicum* was studied because of its genetic dissimilarity to *R. meliloti* yet similarity in the function of symbiosis. The lower amounts of SQDG found in *B. japonicum* does not disallow the possibility of its importance to symbiosis

in this organism. The possibility exists that SQDG is a major component only of the bacteroid form and that the levels of expression observed here are background. Further studies on the presence of this lipid in bacteroid membranes are ongoing. Finally, *A. tumefaciens* was investigated to probe the possibility that SQDG is a key determinant in the distinction of symbiosis from pathogenesis. Although there appeared to be an orcinol positive band corresponding to the faster migrating SQDG component in the TLC analysis for the *Agrobacterium* species, this band did not show any detectable levels of radioactivity either by autoradiography or scintillation counting. Despite the obvious difference in function, *Agrobacterium* and *Rhizobium* species have been shown to be indistinguishable phenotypically (182) and genetically by DNA-rRNA hybridization experiments (183) and 16S rRNA sequence analysis (184). Since this species is genetically quite similar to *R. meliloti*, the lack of SQDG suggests that this lipid plays a role in symbiosis. Although this study was not exhaustive in the strains tested, it does reinforce the possibilities of a role for sulfoquinovosyl diacylglycerol in the *Rhizobium*/legume relationship.

ACKNOWLEDGMENTS

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CHAPTER 5

INVESTIGATION OF THE BIOSYNTHESIS OF SULFOQUINOVOSYL DIACYLGLYCEROL IN *RHIZOBIUM MELILOTI*

ABSTRACT

Investigations into the biosynthetic pathway of sulfoquinovosyl diacylglycerol in *Rhizobium meliloti* were performed. One and two-dimensional chromatography were used to analyze ^{35}S-labelled extracts of *R. meliloti* and other related organisms. These experiments revealed major differences in the labelled metabolites of these various strains attributable to the variety of their sulfur-containing glycolipids (i.e., lipopolysaccharide, chitolipooligosaccharide and sulfoquinovosyl diacylglycerol). The identity of these components in *R. meliloti* was probed by qualitative tests on the extract followed by one-dimensional chromatography. Two compounds were identified that showed resistance to acid and base hydrolyses, cleavage by periodate treatment, oxidation by a bromine/water solution and no affinity for anion or cation exchange resins. Based on results of these tests coupled with comparisons to standards, these two compounds were proposed to be thiolactaldehyde and thioquinovose. Although these molecules have not previously been identified in relation to sulfoquinovosyl diacylglycerol synthesis, it is entirely possible that they would fit into one of the many proposed pathways. Attempts were made to verify the identity of these compounds by comparison to synthetic standards but proved unsuccessful.

INTRODUCTION

Using ^{35}S-labelled extracts of some photosynthetic microorganisms and higher plants, Benson and coworkers in 1959 were able to isolate significant amounts of a sulfur-containing lipid and characterize it as 1'-O-(6'-deoxy-α-D-glucosyl-6'-sulfonic acid)-3-O-diacyl glycerol (Structure 4.1) (185). This lipid, sulfoquinovosyl diacylglycerol (SQDG), has since been detected in all plants, various algae (including green, blue-green, red and brown algae), purple bacteria, some photosynthetic bacteria and a non-photosynthetic marine diatom (186, 187). Although SQDG has been found to be mainly associated with photosynthetic membranes, it has also been shown to occur in such diverse non-photosynthetic tissues such as roots (188), potato tubers (189) and apples (190). Significant amounts have also been found in etiolated tissue (191). Sulfoquinovosyl diacylglycerol represents a major portion of the ether-extractable lipids in the organisms in which it has been identified. For example, it can account for up to 40% of marine red algae glycolipids (206). The amount of SQDG in the leaves of higher plants is generally only about 5%, however, the lipid is confined to the chloroplast lamellar membranes. This confinement of SQDG to plastids has led to the assumption of a role for the lipid in photosynthesis (for review see 207). Various functions of the lipid that have been

proposed include chlorophyll binding, involvement in electron transport and association with certain enzymatic activities (207).

Since the discovery of the plant sulfolipid, relatively little progress has been made towards the elucidation of its biosynthesis. Numerous theories have been proposed based on analogy to classical pathways but have not been supported by conclusive evidence. A compilation of some of these various hypotheses is represented in Figure 5.1. An early study by Shibuya *et al.*, used radiochromatography and radioelectrophoresis to analyze ^{35}S-labelled extracts of the green algae *Chlorella* for the presence of possible intermediates in SQDG metabolism (192). These workers found over 50 sulfur-containing compounds, of which at least twenty contained a sulfonic acid group. Most notable of the identified compounds were sulfolactate, sulfolactaldehyde, sulfopropanediol, sulfoquinovose and a nucleoside diphosphate sulfoquinovose. These results led Benson to propose that the activated sulfoquinovose might react with diacylglycerol (DAG) to give SQDG (193). It was also suggested that the sulfoquinovose precursor was synthesized in a "sulfoglycolytic" pathway; i.e., the aldol condensation of a 3-carbon sulfonic acid-containing moiety with dihydroxyacetone phosphate (DHAP). This idea was expanded and outlined in more detail by Davies and coworkers (194). These researchers found that cysteic acid could act as a precursor for sulfolipid biosynthesis. They also suggested the possible

Figure 5.1: Compilation of some of the proposed pathways for the biosynthesis of sulfoquinovosyl diacylglycerol. Enzymes are indicated where the particular reactions have been demonstrated. Compounds that are underlined have been used as precursors. Adapted from ref. 207.

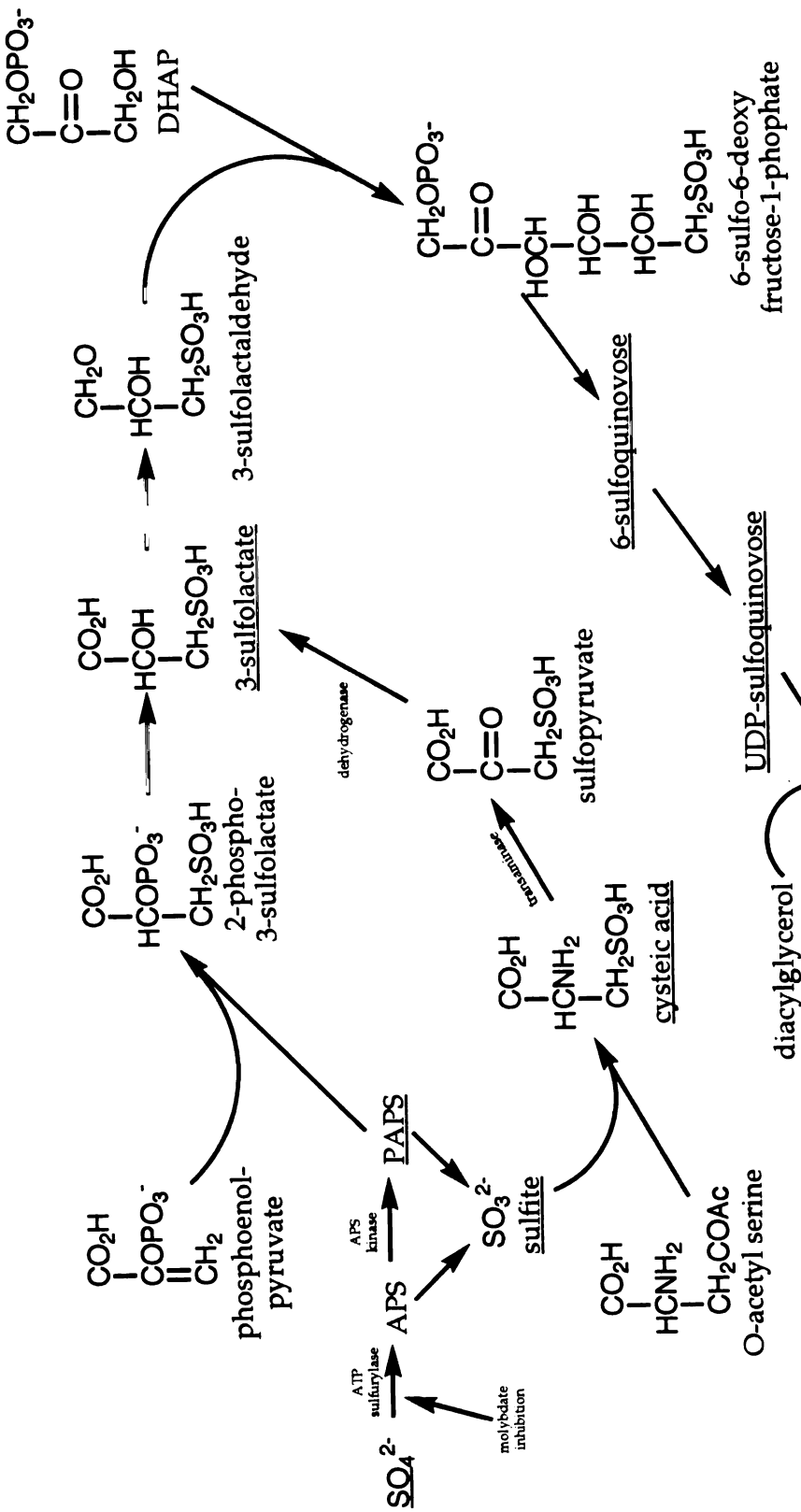
SO_4^{2-}
ATP sulfurylase
molybdate inhibition
APS
APS kinase
PAPS
SO_3^{2-}
sulfite
phosphoenol-pyruvate
2-phospho-3-sulfolactate
dehydrogenase
3-sulfolactate
3-sulfolactaldehyde
DHAP
transaminase
sulfopyruvate
cysteic acid
O-acetyl serine
6-sulfoquinovose
6-sulfo-6-deoxy fructose-1-phophate
UDP-sulfoquinovose
SQDG synthase
diacylglycerol
sulfoquinovosyl diacylglycerol

Figure 5.1

involvement of ATP-sulfurylase due to an inhibitory effect of molybdate on $^{35}SO_4^{2-}$ incorporation. This implicated either adenosine-5'-phosphosulfate (APS) or 3'-phospho-adenosine-5'-phosphosulfate (PAPS) as intermediates. Accordingly, the proposal of Davies *et al.* begins with a transfer of the sulfur from the high energy intermediate, PAPS, to phosphoenolpyruvate followed by various modifications of the 3-carbon moiety and finally the aldolase reaction suggested by Benson. As mentioned earlier, however, this theory is supported by very little concrete evidence.

A second possible pathway in the biosynthesis of 6-sulfoquinovose centers around the controversial involvement of cysteic acid. The conclusion of the aforementioned study by Davies *et al.* was that incorporation of cysteic acid into SQDG was due to a side reaction or to degradation products (194). In subsequent work, Haines suggested a more direct involvement of cysteic acid as an important intermediate and modified the existing theory (Figure 5.1) (186). This pathway involved the displacement of an O-acetyl group from O-acetyl serine by a sulfite moiety made by reduction of the activated sulfate. This theory was based on 1) results from Hodgson *et al.* which showed that PAPS was directly involved in the formation of a sulfite intermediate in *Chlorella* (195) and 2) a study showing the availability of O-acetyl serine as an intermediate in cysteine biosynthesis in *Salmonella typhimurium* (196). Once again, no

direct evidence supported this pathway other than the possible association of cysteic acid which itself has been controversial. One subsequent study showed incorporation of labeled cysteic acid into SQDG in alfalfa (197) while another found no evidence for incorporation in spinach (198).

A novel proposed pathway for the biosynthesis of 6-sulfoquinovose involves the addition of sulfite to a double bond to yield the sulfonic acid group (Figure 5.2). Lehmann and Benson were able to synthesize 6-sulfoquinovose from sulfite and methyl-α-D-glucoseenide in 5 minutes at room temperature in aqueous solution at pH 6.4 to 7.0 and suggested a similar reaction could be possible *in vivo* (199, 200). Recent studies by Benning and Sommerville have identified four genes in the SQDG biosynthetic pathway, one of which displayed sequence similarity to a UDP-glucose epimerase (203, 204). The presence of this enzyme is consistent with the proposed scheme in Figure 5.2. A recent study has also supplied new biochemical data to support this pathway (205). The evidence included stimulation of SQDG production by UTP and the lack of inhibition by compounds likely to repress the "sulfoglycolytic" pathway. In addition, these authors demonstrated that UDP-[^{14}C]-glucose could be incorporated into SQDG and that this incorporation was augmented by the addition of methyl-α-D-glucoseenide. As referred to above, several studies have shown direct evidence for the involvement of sulfite in

Figure 5.2: Proposed pathway for the biosynthesis of sulfoquinovosyl diacylglycerol. These workers suggested that the sulfite group could be added either to UDP-4-ketoglucose-5-ene or to UDP-glucose-5-ene This figure was taken from reference 205.

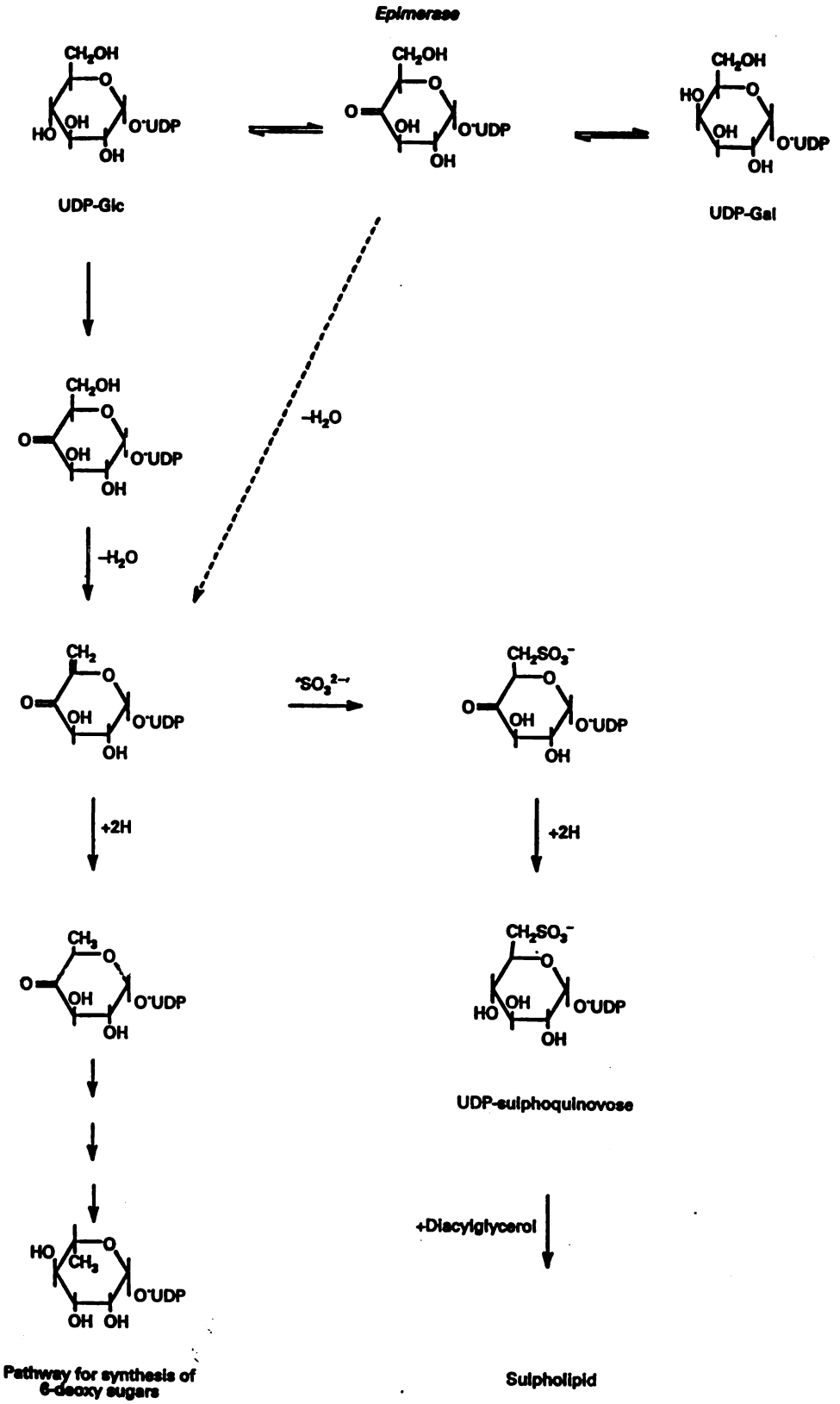
Epimerase
UDP-Glc
UDP-Gal
-H2O
-H2O
+2H
+2H
UDP-sulphoquinovose
+Diacylglycerol
Pathway for synthesis of 6-deoxy sugars
Sulpholipid

Figure 5.2

various systems (197, 201, 202). However, the role of sulfite was called into question in a study by Hoppe and Schwenn on cell-free extracts of *Chlamydomonas reinhardtii*. While studying the incorporation of possible precursors into SQDG, these workers found PAPS to follow a normal substrate saturation curve while sulfite was not saturable and therefore possibly due to a non-enzymatic artifact (201).

The only steps in the pathway for the biosynthesis of SQDG that have sufficient evidence to support them are the first one, activation of sulfate to either APS or PAPS, and the last one, reaction between the sulfoquinovose nucleoside diphosphate and diacylglycerol. Several results from various labs have supported the first step and these are mentioned in detail above. A significant experiment by Heinz *et al.* seems to have also definitively proven the final step. Using ^{14}C-labelled diacylglycerol, these authors found that addition of UDP-sulfoquinovose increased the amount of label in SQDG while the GDP derivative had only half the activity and the ADP and CDP derivatives were inactive (208).

The experiments presented in Chapter 4 proved sulfoquinovosyl diacylglycerol to be a major component of various rhizobial strains. In addition, *Agrobacterium tumefaciens*, a genetically closely related plant pathogen, was found to be devoid of the lipid. The significance of these discoveries lies not only in the implications of a role for this lipid in

symbiosis, but in the possibilities for clarifying its biosynthetic pathway. Since it was clear that various rhizobia (and *A. tumefaciens*) synthesized different sulfur-containing membrane components, it followed that intermediates unique to one molecular species may be isolable. In other words, the results presented in Chapters 2 and 4 show that *Rhizobium meliloti* makes SQDG and sulfated lipopolysaccharide and chitolipooligosaccharide, *R. trifolii* synthesizes only SQDG while *A. tumefaciens* and *E. coli* have not been found to contain these sulfated species. It may be possible, therefore, to gain some insight into the biosynthesis of SQDG by comparing the sulfur-containing metabolites in *R. meliloti* and *A. tumefaciens*, two otherwise closely related bacteria. In addition, since this problem has generally only been studied in plants, it may be easier to analyze in a less complex system.

The aforementioned study by Shibuya, *et al.* used two-dimensional paper chromatography to identify sulfur-containing biosynthetic intermediates in algae (192). It was supposed that this type of analysis would yield a "fingerprint" for each organism that could then be compared to the others. The compounds that differed between the strains could also be identified by cutting the bands, eluting the sample from the paper and identifying the component. The information gleaned from these studies would possibly resolve some of the uncertainties related to the biosynthesis of SQDG and either confirm or reject the theorized pathways.

MATERIALS AND METHODS

Strains and bacterial cultures.

The various bacterial strains which were used are as follows: *Rhizobium meliloti* 2011 (from Ethan Signer, Massachusetts Institute of Technology), *R. leguminosarum* bv. *trifolii* ANU843 (from Barry Rolfe, Australian National University), *Agrobacterium tumefaciens* C58 (from Frans DeBruijn, Michigan State University), and *Escherichia coli* TG-1 (from Gregory Zeikus, Michigan State University). All strains of bacteria (with the exception of *E. coli*) were grown at 30°C in liquid cultures containing modified Bergensen's (BIII) media as previously described (142) except that in addition, ^{35}S-labelled sulfate at a level of 100μCi/liter of culture was added in the form of carrier free sulfuric acid (New England Nuclear). The *E. coli* strain was grown at 37°C in MSX4 media with ^{35}S-labelled sulfate added as above. Alfalfa seeds were from the laboratory of Frank Dazzo, Michigan State University.

One and two dimensional chromatographic analyses of cell extracts.

Cells were lysed with a combination of freeze/thaw cycles, probe sonication and lysozyme treatment and then extracted twice each with hot 80% ethanol, absolute ethanol and 50% ethanol. Extracts were condensed

to dryness and redissolved in 50% ethanol for chromatography. Alfalfa was grown on a Fahraeus agar medium (142) containing ^{35}S-labelled sulfate at a level of 200 μCi/liter of culture. Plants were harvested after about six days growth. Roots were separated from stems and both ground and extracted as above. Two-dimensional paper chromatography was performed using a modified method of Shibuya, *et al.* (192). Whatman #1 filter paper (20 x 20 cm) was developed in the first direction with phenol / water (5:2 w/w) and in the second direction with butanol / propionic acid / water (142:71:100 w/w). Radioactivity was visualized using a Molecular Dynamics Phosphorimager after exposure of the phosphor screen for approximately ten days. One-dimensional paper chromatography was run using the mobile phase containing butanol.

Qualitative analyses of cell extracts.

The ethanol extract mentioned above was subjected to various treatments for qualitative analysis by one-dimensional TLC. Acid and base hydrolyses were performed using 1% HCl and 0.1% NaOH, respectively, and heating the sample for 20 minutes at 70°C. Periodate oxidation was achieved using a 0.1% sodium meta-periodate solution at room temperature for 15 minutes. The bromine / water reaction was performed using a 5% solution in the dark at room temperature for 15 minutes. Anion and cation exchange chromatography were performed

using TEAE cellulose and CM cellulose, respectively. Samples were eluted with water and the flow-through collected. Trifluoroacetic acid (TFA) hydrolysis was accomplished with a 2M acid solution that was heated for two hours at 120°C. Radiolabelled SQDG was acquired from cells grown in ^{35}S-labelled culture. The cells were extracted with chloroform / methanol / 1-butanol / water (2:1:1:4) and the organic layer, containing the sulfolipid, was condensed. Sulfoquinovose was prepared by hydrolyzing a portion of this SQDG-containing organic fraction with TFA as above. Radiolabelled sulfate was obtained carrier free from DuPont (Wilmington, DE). Thin-layer chromatography was performed using ethyl reversed phase layers with a mobile phase consisting of 2-propanol / ammonium hydroxide (3:2). Carbohydrate spots were visualized using an orcinol / sulfuric acid solution. Methionine and cysteine were visualized using a 0.25% ninhydrin in butanol spray.

RESULTS AND DISCUSSION

Radiolabelled cultures of *Rhizobium meliloti* 2011 and *R. trifolii* ANU843 were analyzed using a combination of treatments intended to lyse the cells followed by extraction of the cell debris with ethanol. This extraction method has previously been shown to be effective in allowing an analysis of ^{35}S-labelled components by two-dimensional paper chromatography (192). Figures 5.3 and 5.4 show the phosphorimager scans of paper chromatograms of the *R. meliloti* and *R. trifolii* extracts, respectively. The chromatogram of the *R. meliloti* extract is fairly complex and appears to consist of at least a dozen distinct radiolabelled components. This is not surprising since *R. meliloti* would be expected to have various sets of biosynthetic pathways and intermediates for its diverse sulfur-containing components (i.e., LPS, CLOS and SQDG).

In contrast to the *R. meliloti* results, the chromatograph of the *R. trifolii* extract shows relatively few distinct labelled components. It is entirely reasonable that the pattern of sulfur-containing components is simpler in the *R. trifolii* extract due to absence of sulfate in the LPS and CLOS of this organism. Regarding SQDG synthesis, however, these results may be indicative of two possibilities; either the pathway involves relatively few sulfur-containing intermediates or the steady-state

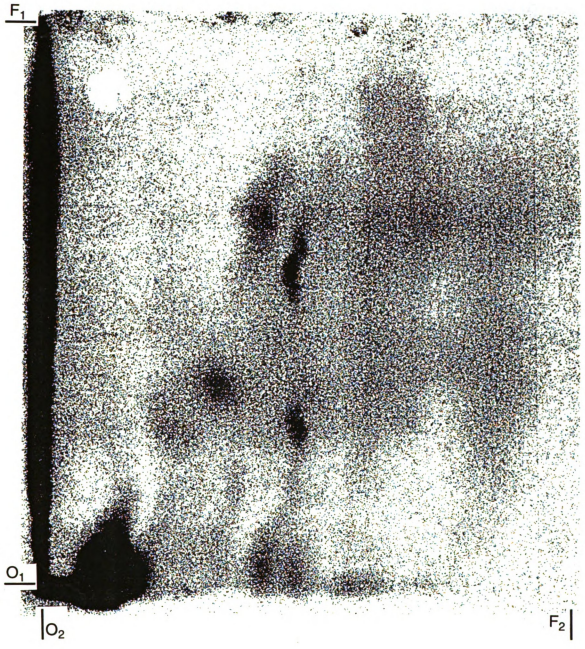

Figure 5.3: Phosphorimager scan of paper chromatogram of *R. meliloti* extract. Paper was developed first in the y-direction with the phenol mobile phase. O = origin, F = solvent front.

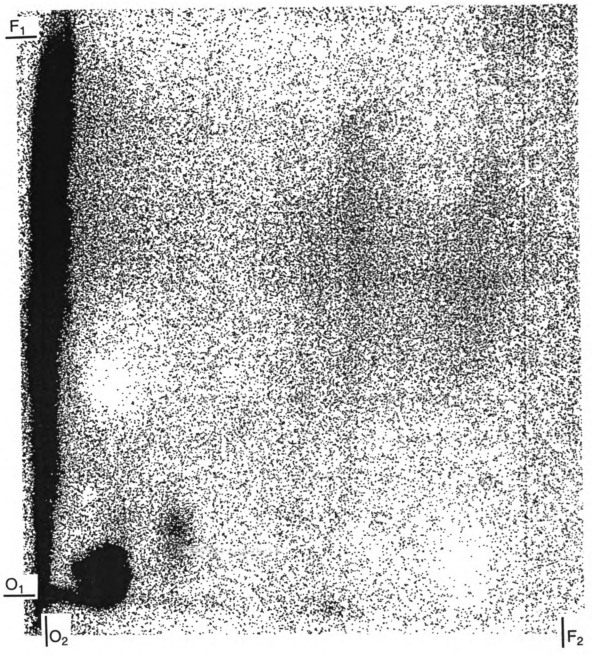

Figure 5.4: Phosphorimager scan of paper chromatogram of *R. trifolii* extract. Paper was developed first in the y-direction with the phenol mobile phase. O = origin, F = solvent front.

concentrations of these intermediates are below the level of detection. This latter point illustrates an important caveat about these types of experiments. Although these results show few radiolabelled components, the bacteria obviously makes many other compounds bearing sulfur in order to survive (i.e., cysteine, methionine, coenzyme A, etc.). The steady-state concentrations of such molecules or their intermediates, however, must be sufficiently low as to not be detected by the methods employed here. Accordingly, we must always keep in mind that the intermediates of SQDG may also be present at levels which are not readily observable.

In order to verify these results, the entire experiment was repeated. Suprisingly, the pattern of radiolabelled components on the chromatogram of the *R. meliloti* extract was vastly different from that seen in the first experiment. The chromatogram of the *R. trifolii* extract appeared again, however, to be virtually identical to the that in the original. Since this method proved to be too irreproducible for a direct comparison of the various extracts, a one-dimensional system was employed. This method was more reproducible and allowed a direct comparison of the various bacterial and plant extracts.

An example of a one-dimensional paper chromatographic analysis is shown in Figure 5.5. The extracts from various organisms including *R. meliloti*, *R. trifolii*, *Agrobacterium tumefaciens*, *Escherichia coli*, and alfalfa were chromatographed concurrently on paper using the 1-butanol/

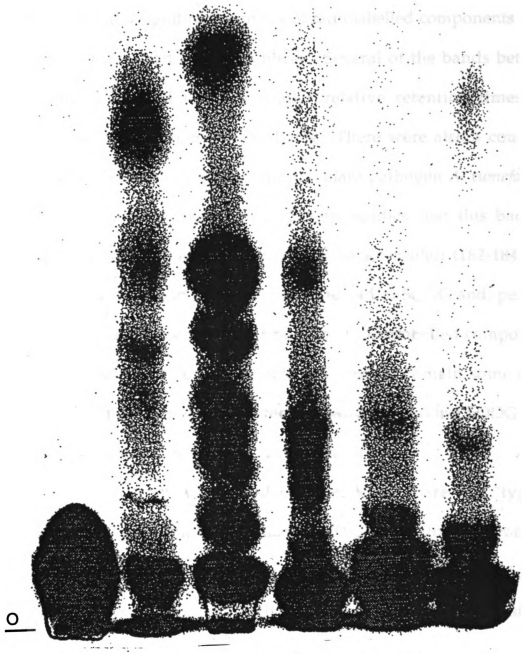

Figure 5.5: Phosphorimage of one-dimensional paper chromatogram of ethanol extracts of various organisms.

propionic acid / water (142:71:100) mobile phases from the previous experiment. As seen in the two-dimensional study, the extract from *R. meliloti* contained a significant number of radiolabelled components while that from *R. trifolii* had relatively fewer. Several of the bands between these samples seemed to have similar relative retention times and therefore may be common intermediates. There were also a couple of commonalties between *R. meliloti* and the plant pathogen *A. tumefaciens*. It has been shown in several phylogenetic studies that this bacterial species is genetically very closely related to *R. meliloti* (182-184). *A. tumefaciens* was shown in Chapter 4 to be devoid of SQDG and, perhaps, as a result displays a much simpler pattern of radiolabelled components. It is quite possible, however, that this strain could contain some of the same intermediates if not part of the pathway for making SQDG as *R. meliloti*, but does not produce the sulfolipid itself.

The *E. coli* strain was tested in order to compare this "typical" Gram-negative bacterium to the rhizobia. The chromatogram from this sample showed a single broad band near the origin that is most likely composed of free sulfate and perhaps other components with similar mobilities. This seems to agree with results from Chapter 4 that demonstrated the absence of sulfur-containing lipids in this strain. As mentioned above, however, these results may not reflect the true level of

sulfur-containing components due to the transience of biosynthetic intermediates.

The alfalfa was tested in order to see how related the pattern of radiolabelled components in its extract compared to that of its symbiont, *R. meliloti*. The root and leaf were segregated to determine what difference, if any, existed between them especially since the latter would be expected to harbor most of the SQDG. Each extract, however, showed a relatively simple pattern of bands and had some overlap to those in the *R. meliloti* sample. These results are encouraging since one possibility for the appearance of SQDG in rhizobia, as discussed in Chapter 4, could be because of horizontal gene transfer. If this were true, then the same intermediates would exist in both the bacteria and its host.

In order to begin to characterize some of these sulfur-containing metabolites, qualitative analytical tests were performed on the extract of *R. meliloti*. The treatments, to which the ^{35}S-labelled extract was subjected, included mild acid and base hydrolyses, periodate cleavage, bromine oxidation, anion and cation exchange chromatography, and triflouroacetic acid hydrolysis. The mild acid and base hydrolyses were intended to reveal the presence of any molecules containing sulfate groups since they would be expected to be cleaved under these conditions. The resulting disappearance of a band in a particular lane of the paper chromatograph compared to the untreated extract would indicate that

that component contained a sulfate group. The periodate oxidation reaction was used to reveal the possible presence of carbohydrates or polyalcohols. The outcome of this reaction is the cleavage of the bond between carbon atoms bearing hydroxyl groups and subsequent oxidation of the hydroxyls to aldehydes. Any molecule containing vicinal hydroxyls will be cleaved and therefore have its chromatographic properties altered. A bromine solution will oxidize aldoses nearly quantitatively by converting them to the corresponding lactone while leaving ketoses unaffected. This test was meant to provide some insight into what particular types of radiolabelled sugars were present in the extract. Small amounts of the extract were also passed through anion and cation exchange columns in order to detect what types of charged molecules might be present. This would help define the oxidation state of the sulfur in the radiolabelled bands. Most of the sulfur-bearing components involved in SQDG biosynthesis would likely remain on the anion exchange column since they would be expected to be in the form of the initial state, sulfate, or the final one, sulfonic acid. A compound that passed through the anion exchange would either contain an uncharged sulfur-containing group, such as a thiol or thioether, or have an overall positive charge. The triflouroacetic acid treatment, in addition to indicating the presence of sulfate groups as with the mild hydrolysis, would be expected to cleave any glycosidic linkages that might be present.

Figure 5.6: Phosphorimage of one-dimensional paper chromatogram of ethanol extract of *R. meliloti* after various treatments (See Materials and Methods for complete description). The glucose and methionine were run in a different experiment than the samples using the identical method.

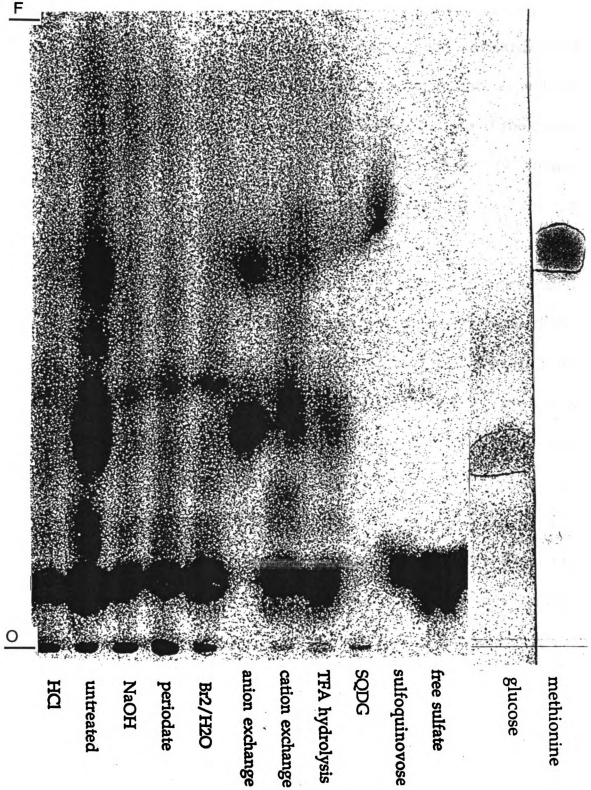
F
O
methionine
glucose
free sulfate
sulfoquinovose
SQDG
TFA hydrolysis
cation exchange
anion exchange
Br2/H2O
periodate
NaOH
untreated
HCl

Figure 5.6

The products of each treatment were chromatographed on paper one-dimensionally as above along with standards. The standards used included ^{35}S-labelled SQDG, sulfoquinovose, and free sulfate as well as unlabelled methionine, cysteine, and glucose. An example of the paper chromatography is shown in Figure 5.6. As expected, the phosphorimage of the mild hydrolysis lanes appeared similar, however, the resolution of the various bands was generally poor. It appears as though all of the bands in these sample lanes lost intensity compared to the untreated sample except, of course, for the band corresponding to the free sulfate. Suprisingly, the results were similar for the samples treated with periodate and bromine which were intended to show the presence of vicinal hydroxyl groups and reducing sugars, respectively. Although these tests would not be expected to produce the same effect on the extract, the results were generally reproducible.

More interesting results were obtained for the samples subjected to ion exchange chromatography. In the case of the extract passed through the anion exchange column, only two distinct bands appeared in the phosphorimage of the paper chromatogram. Interestingly, these two bands were also detected, albeit slightly less resolved, in the sample exposed to the cation exchange column. This would imply that these components are uncharged and that the sulfur might be in the form of a thiol group. These two compounds were also found to be stable to

triflouroacetic acid hydrolysis which would also be consistent with the presence of thiol moieties. Neither of the components had relative retention times matching the standard SQDG, sulfoquinovose, sulfate, methonine or cysteine. The slower moving compound, however, did align fairly well with the glucose standard. Based on this it seems that a likely candidate for the slower moving component could be thioquinovose (Figure 5.7). This compound would definitely have a very similar mobility on the chromatogram as glucose and fit very well with the additional data presented above (i.e., resistant to acid hydrolysis, flow-through ion exchange columns, and possible susceptibility to periodate cleavage and bromine oxidation). Furthermore, based on an analogy to the proposed "sulfoglycolytic" pathway (Figure 5.1) it can then hypothesized that the other molecular species in question could be thiolactaldehyde. This compound should combine easily with dihydroxyacetone phosphate to form thioquinovose (Figure 5.7). In order to verify this, attempts were directed at synthesizing these compounds in order to compare them directly on the chromatogram but these efforts proved unsuccessful. In order to increase resolution and make sample retrieval easier, a thin layer chromatography (TLC) system was developed. A fresh bacterial culture was grown and extracted identically as above. The TLC analysis, however, showed vastly different results than before in that only two radiolabelled bands were detected. The slower moving component tested

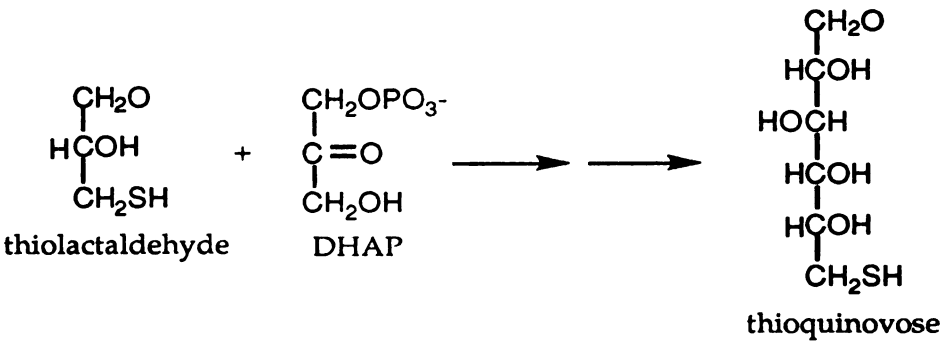

Figure 5.7: Proposed scheme of thioquinovose formation from thiolactaldehyde condensation with DHAP.

positive for the presence of carbohydrate by staining pink after reaction with orcinol. It was unresolved, however, from other carbohydrate components with similar mobilities and therefore not easily isolable. The faster moving radiolabelled component was most likely SQDG due to its mobility (very near the solvent front) along with its reaction to orcinol (stained a more brownish color indicative of a glycolipid).

CONCLUSIONS

Based on the data obtained in this study a hypothesis was made that thiolactaldehyde and thioquinovose could be possible intermediates in SQDG synthesis. This not only leaves the question of SQDG biosynthesis unresolved, but it may even increase the confusion since these types of

intermediates (i.e., containing the reduced form of sulfur) have not yet been proposed. It is, however, entirely possible for incorporation of sulfur into the pathway at the thiol level followed by a final oxidation step from thioquinovose to sulfoquinovose. In fact, unpublished results from the Hollingsworth lab have indicated the incorporation of ^{35}S into SQDG using radiolabelled cysteine as the only form of sulfur in the media. This would be consistent with a pathway analogous to the one presented in Figure 5.1 with cysteine replacing the cysteic acid. The possibility also remains that there exists more than one pathway for the biosynthesis of SQDG. Given the evidence for such disparate scenarios as those shown in Figures 5.1 and 5.2, this concept seems more than likely.

The results presented in this chapter are perhaps reflective of why the question of SQDG biosynthesis has remained a mystery for so long; the transience of biosynthetic intermediates makes them very difficult to isolate or reproduce from culture to culture using wild type strains. This is demonstrated in the results from the extract of the *E. coli* strain which show the lack of any significant amounts of sulfur-containing compounds. Generally, strains carrying mutations that allow intermediates to accumulate are used in these types of studies. Perhaps the recent discovery of four genes related to SQDG biosynthesis (203, 204) will lead to such mutants and a solution to this long-studied question.

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CHAPTER 6

RETROSPECT

Over the years, the cell-surface carbohydrates of *Rhizobium* have been the central focus of our attempts to understand the important symbiotic interactions of these bacteria with their host leguminous plants. Previous ideas regarding the roles of rhizobial polysaccharides and glycolipids had these molecules each providing a specific function to the infection process. For instance, it has been generally believed that the role of the extracellular polysaccharides in infection is to assure bacterial release from the infection thread and nodule invasion. It has also been suggested that the role of the capsular polysaccharides is to provide a recognition component to the lectins which reside on the root surface. A belief even more passionately held than these is that the chitolipooligosaccharides are the sole determinants of host specificity and that the genes involved in their synthesis are exclusive to them. The studies presented in this dissertation begin to dispel these ideas and provide the basis for a comprehensive model in which the key determinant of host specificity and infection is the entire rhizobial membrane distribution and not individual molecular species.

Since their discovery, the chitolipooligosaccharides (i.e., CLOS or Nod factors) have garnered a large proportion of the attention in the area of rhizobial research. These molecules are oligomers of N-acetylglucosamine with a fatty acid in place of the acetyl group on the

non-reducing terminus. Various modifications to this basic structure, including sulfation and fatty acid unsaturations, are presumed to be the absolute determinants of the bacteria's ability to infect the proper host. This assertion was made on the lone observation that exogenous addition of CLOS to plant roots causes morphological changes that mimic those seen in the early stage of an authentic infection. This hardly reflects the actual situation of a bacteria/root interaction since it is well known that the bacterium will bind to a root hair *before* it actually begins to curl (209). Why, then, would rhizobia need to synthesize a "diffusable metabolite" such as CLOS if it is already in close contact with the root? This fact, coupled with the basic structure of CLOS and other points raised in Chapter 2, would intuitively lead one to believe that these molecules are membrane localized. In these studies, we verified this by isolating ten-fold more Nod factor from the membrane of a wild-type rhizobial strain than other researchers have been able to extract from the culture medium of genetically engineered induced bacteria. The CLOS from these latter results was undoubtedly the result of normal blebbing of the membrane during cell divisions. Small amounts of both inner and outer membrane components are extractable from culture supernatants due to this phenomenon (214).

The exclusivity of the *nod* genes and Nod factors to host range selection has always been a questionable hypothesis in our minds. The

definitive reason for our skepticism is the inability of anyone to show that a non-infective Nod$^-$ mutant could be "rescued" by the addition of purified Nod factors. The only explanation for the constant failure of this experiment is that the process of host selection is more complex than is generally thought. Either CLOS is not the sole determinant of host range or the *nod* genes are not responsible for only CLOS synthesis. Results presented here prove that the former scenario is highly probable and the latter is definite. The CLOS produced by *Rhizobium meliloti* has a sulfate group on the reducing sugar and it is this substituent that is believed to be responsible for the ability of this bacteria to infect its proper host, alfalfa. Strains of this bacteria with mutations in the *nod* genes responsible for the attachment of this sulfate group to the Nod factor have a concomitant loss in their ability to nodulate alfalfa. It has been shown here that these strains also have the identical impairment in their ability to attach a sulfate group to their lipopolysaccharide (LPS). This is irrefutable evidence that directly links structure, function and genetic control of the chitolipooligosaccharides with another family of cell-surface molecules.

An interesting, albeit challenging avenue to pursue would be the total characterization of the O-antigen of *R. meliloti* 2011. This is not a trivial undertaking as reflected by the fact that only one other rhizobial LPS has been characterized. The challenge is magnified by the lability of the sulfate linkage. It would be extremely difficult to pinpoint its location

in the molecule due to losses during the isolation process. Nevertheless, interesting questions remain regarding the location of the sulfate ester. For instance, does the O-antigen repeat contain a reducing terminal glucosamine residue and if so, is the sulfate attached as in the Nod factor? Also, is the sulfate attached to the diglucosamine in the lipid A and if this is the case, what functionality provides the negative charge to take its place in the case of the *nodQ* and *nodH* mutants?

To prove the universality of the concept of membrane structural overlap in rhizobia, it was demonstrated that another strain that synthesizes a sulfated CLOS, *R.* sp. strain NGR234, also makes LPS that contains sulfate. Furthermore, the sulfate group is not the only functionality involved in the structural overlap. It was also shown that there is an interrelation in the fatty acid distribution and carbohydrate substituents between the CLOS and other membrane components in these strains. These types of commonalties in the distribution of fatty acids between lipid components of the same membrane is a well documented phenomenon (146, 147). It is ridiculous, therefore, to propose that specific fatty acids of one molecular species, the chitolipooligosaccharides, are responsible for directing host range.

In order to probe further this flexibility in fatty acid distribution, *R. trifolii* 4S cells were grown in a culture medium containing parinaric acid. This fatty acid is a conjugated polyene which could be detected easily due

to its spectroscopic properties. It was thought that the cells would take up the fatty acid and incorporate it into its membrane components as was shown in an earlier study using *E. coli* (215). Unfortunately, using several different methodologies, chitolipooligosaccharides were not successfully isolated from this strain. Perhaps in the future other strains from which we have already isolated CLOS, e.g. *R. meliloti* 2011 or *R. trifolii* ANU843, might be a better choice for such a study.

The results presented in Chapter 2 paint a picture that depicts the membrane as a highly integrated web of biosynthesis. Altering one specific gene will not necessarily result in the perturbation of one molecular species but will have a cascade effect throughout the membrane. This hypothesis was tested by comparing the total membrane extracts of the various *nodQ* mutants that were used in Chapter 2 with the wild type strain. The putative function of the *nodQ* gene is to control sulfation of the chitolipooligosaccharide by making the activated sulfate transfer molecule. When the total membrane extracts of these mutant strains were compared, however, important differences seemingly unrelated to sulfation were observed (Figure 6.1)[1]. Primarily, the changes seen were in the relative intensities of the various bands, although at least one component was detected in the mutants that was not seen in the wild

[1] For information regarding the strains used in this experiment, see Chapter 2. For the extraction procedure and chromatographic conditions, see Chapter 4.

Figure 6.1: Thin layer chromatogram of membrane extracts of three *nodQ* mutant strains along with the parent wild type strain. Note the differences in relative intensities of various bands and presence of a novel band in the mutants (arrow). Weak bands which did not show up when the plate was copied are indicated. Bands were visualized with orcinol.

type bacteria. The effects of these mutations, therefore, are much more far-reaching than is generally thought. Not only are the levels of the various lipids affected, but the machinery to make molecules undetected in the wild type is suddenly turned on. It is rather absurd, then, to propose that a single gene and the associated CLOS structural modification are solely responsible for host specificity and not take into account the dynamic nature of the membrane. Studies such as this may be expanded in the future in order to possibly identify the novel components in the mutants and quantitate more carefully the changes in the various lipid species produced.

Another example of structural overlap of membrane components important to rhizobia is presented in Chapter 3. These studies focused on an unusual strain, *R. trifolii* 4S, that has a membrane composition very distinct from closely related bacteria which have the same host range. In particular, this strain lacks one of the two typical LPS core regions produced in its species. It also produces an LPS O-antigen and extracellular polysaccharide that are very unique. Questions therefore arose as to the molecular basis for the infectability of this strain. The isolation and partial characterization of a novel glycolipid is presented here which may answer these questions. The molecule contains a polysaccharide with a composition that mimics the "typical" LPS O-antigen of this species. This polysaccharide appears to be linked to the

membrane by an assortment of fatty acids usually found in various membrane components. It therefore seems that although the membrane has an inconsistency in its "normal" cell-surface carbohydrate biosynthesis, it has found a way to circumvent this problem and make a new glycolipid that apparently corrects for the putative defect. This example demonstrates the power that the membrane affords the bacteria in its ability to adapt. It also suggests again the importance of general membrane distribution versus the specific structure of any one component.

A further important aspect of the rhizobia/legume symbiosis, besides host specificity, is the ability of the invading organism to avoid the plant's defense mechanisms. In some way the bacterium must be able to disguise itself in order for the plant to disregard it as a hostile agent. A substantial step was taken in this thesis regarding a possible molecular mechanism to explain this phenomenon. Sulfoquinovosyl diacylglycerol (SQDG) was shown to be a membrane component of several rhizobial species. The significance of these findings lies in the fact that this lipid was previously thought to have been restricted to photosynthetic organisms. The occurrence of a plant lipid in *Rhizobium* may play a critical role in ensuring an avoidance of plant defenses. These implications are magnified when considering the recent discovery in the Hollingsworth lab of other classes of plant lipids in a rhizobial species; the mono- and digalactosyl diacylglycerols as well as phosphatidyl inositol. In addition,

another typical plant lipid, phosphatidylcholine, has been known to exist in rhizobia for years. Consequently, the rhizobial membrane may appear to the host plant to look more like one of its own organelles than an invading bacterium. It is interesting to note that in the case of the mono- and digalactosyl diacylglycerols and phosphatidyl inositol, the synthesis of these lipids are augmented once the bacteria are subjected to low oxygen or other conditions that would simulate the environment of the bacteroid state. This is an important detail since it is at this point, after the bacteria have been released from the infection thread into the cell, in which they should be most vulnerable to host defenses. We see once again, therefore, the adaptability of the membrane and the criticality of the entire spectrum of lipids in the rhizobial membrane in determining a viable symbiotic interaction.

The final experiments in this dissertation were directed at elucidating the unresolved biosynthetic pathway of the plant sulfolipid, SQDG. Based on these studies, two compounds, thioquinovose and thiolactaldehyde, were proposed as possible intermediates in a variation of the long-standing "sulfoglycolytic" pathway. The intention of these experiments were to delineate the pathway in order to eventually surmise the origin of the genes involved. The complexity of SQDG biosynthesis dictates that a simple scenario of molecular mimicry would not be sufficient. Multiple genes in the bacterium most likely would have had to

be transferred from the plant at some point in their evolutionary coexistence. The likelihood of horizontal gene transfer has, in fact, been demonstrated in the *Rhizobiaceae* (210) as well as in other systems where there is close contact between eukaryotes and prokaryotes (211-213). The possibility also exists that rhizobia were at one point plant organelles and subsequently developed the ability to become independent of the plant. This concept is becoming increasingly plausible as we are discovering more about the rhizobial membrane and its similarities to that of the plant. These possibilities can only be proven by the isolation and comparison of plant and bacterial genes responsible for common links such as SQDG. Therefore, future studies need to be aimed at investigating plant lipid biosynthesis as well as the rhizobial system.

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