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THE ROLES OF RHIZOBIAL SURFACE AND MEMBRANE COMPONENTS IN SYMBIOSIS

By

Yin Tang

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of requirements
for the degree of

DOCTOR OF PHILOSOPHY

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1997

ABSTRACT

THE ROLES OF RHIZOBIAL SURFACE AND MEMBRANE COMPONENTS IN SYMBIOSIS

By

Yin Tang

A variety of rhizobial cell surface and membrane carbohydrate-containing molecules have been indicated to play some important roles in rhizobium/legume symbiosis. The functional roles of these molecules in symbiosis are closely related to the ability of bacterium to change the relative quantities or structures of these molecules and achieve the optimal bacterial surface property in order to survive in a changing environment. There is a large degree of functional and biosynthetic overlap among various rhizobial cell surface and membrane carbohydrate-containing molecules. A comprehensive picture of the rhizobial cell surface needs to be obtained in order to understand the roles played by the rhizobial surface or membrane components in symbiosis.

Studies on the membrane lipids of free living and the bacteroid form of *Bradyrhizobium japonicum* indicated that the later has high levels of digalactosyl diacylglycerol, a glycolipid which has rarely been found outside of the plant kingdom and photosynthetic bacteria. This result follows our earlier finding that the other predominant plant glycolipid, sulfoquinivosyl diacylglycerol, is a membrane component of fast growing *Rhizobia* and is found even when cells are cultivated in free culture. The near absence of these lipids in the membranes of bacteria outside of this special group of organisms and photosynthetic bacteria suggests that the trait could have been passed on through gene

transfer from plants to the bacteria at some point during the development of their symbiotic relationship. In the case of digalactosyl diacylglycerol, there is also the possibility that some common biosynthetic intermediates are used by both the plant and the bacteria. This is a striking parallel with some host-pathogen interactions.

Culturing *Bradyrhizobium japonicum* in the presence of glucose as a carbon source in a rich medium known to stimulate differentiation into bacteroid forms led to the synthesis of a family of di- and trisaccharide-containing glycerol lipids as the main membrane lipid components. Like digalactosyl diacylglycerol, these glycolipids are also rarely found in other bacteria. When *Bradyrhizobium japonicum* was grown in low-oxygen condition which mimics the condition inside root nodues. There was a dramatic switch of membrane lipid chemistry. Phosphatidyl inositol, a typical plant lipid rarely found in bacteria, was also synthesized. Lowering pH in free-living *Bradyrhizobium japonicum* did not result in the change of unsaturation state in the fatty acyl groups of the lipids.

Studies on the interrelationship of the biosynthetic events leading to various carbohydrate-containing molecules indicated that , when ^{14}C labeled 3-O-methyl-D-glucose was used as a probe, it was incorporated into membrane glycolipids but not into other major carbohydrate-containing molecules, including EPS, LPS, Cyclic β-glucans. It is likely that the biosynthesis of EPS, LPS and cyclic β-glucans does not use the same metabolite pool as utilized by glycolipids. It is also possible that the substrate specificity of the enzymes involved in synthesizing glycolipids is different.

To My Dear Mother and Father

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My Ph.D. work would not be finished without the help from many people around me during my five years of being here at MSU.

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I thank my guidance committee members, Dr. Zachary Burton, Dr. Thomas L. Deitz, Dr. Paul Kindel, Dr. Kenneth Keegstra and Dr. Barbara Sears. I appreciate the time and effort you spent on reading my reports and thesis, thinking about my projects and attending my meetings. Especially, I thank you for giving the valuable scientific criticism.

I can't think of how boring my life would be without many fellow students and

coworkers in the lab. Ben is the best colleague I can imagine in the world. I am fortunate to be your bench neighbor. You are not only the go-to person when I need scientific critique or need to operate a new instrument but also the one to consult about home improvement. Hussein, YW, Luc, Steve and Ben were the ones I often had lunches with (unfortunately the restaurants in the area were not enough for these people to go, 80% of the time we ended up in Thai Kitchen). I really enjoyed the time with you guys. I know I will miss this. Gang, Jie, Guijun, Guangfei were the elite organic chemists to ask for help when I needed to do some organic synthesis occasionally. It was especially nice to talk with you in my mother tongue, Chinese. Jim is our computer guru. Whenever I got stuck with a PC, I needed to find you. It is too hard to describe five years with one page of words. I am thankful to many other people, Rob, Vladmir, Carol, Du, Jung, Kim, Jeongrim, Gabriela, Birgit and Jimin. I appreciate the fellowship with all of you.

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Finally, I am grateful to my husband, JJ, for your support both in and out of the lab. I would never leave out my little sweetheart, Lanson, who just turned one and half years old

around the time of my defense, for the unspeakable joy you brought to me and your unbearable cry to push me work hard.

Above all, behind all of the above-mentioned is the Almighty One, who made everything happened happened. I thank you, my Lord and Savior for everything!

Wholeheartedly,

Yin

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LIST OF ABBREVIATIONS

BIII	modified Bergensen's media
CPS	capsular polysaccharide
DGDG	digalactosyl diacylglycerol
DQF-COSY	double quantum filtered J-correlated spectroscopy
EPS	extracellular polysaccharide
ESI-MS	electrosray ionization mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
GC	gas chromatography
GC/MS	gas chromatography / mass spectrometry
LPS	lipopolysaccharides
NMR	nuclear magnetic resonance
PC	phosphatidylcholine
PE	phophatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SEM	scanning electron microscopy

SQDG	sulfoquinovosyl diacylglycerol
TEM	transmission electron microscopy
TLC	thin-layer chromatography

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Biological nitrogen fixation has commanded the attention of scientists for more than 100 years. The ability to fix nitrogen is exhibited by a broad spectrum of microorganisms such as cyanobacteria, archaebacteria and actinomycetes. However, nitrogen fixation with the highest rates, also with the greatest importance, occurs in symbiotic associations between bacteria and plants. The *Rhizobium-legume* is the best known of the symbiotic systems. **Section I** of the literature review covers the importance of this symbiotic system in the nitrogen cycle and **Section II** introduces the process by which the symbiotic relationship between rhizobium and leguminous plants is established.

The interaction between rhizobia and their corresponding hosts is very specific. Most strains only nodulate plant species belonging to a few genera. This host specificity, as well as many other aspects of symbiosis such as infection thread development and nodule formation, is determined by a variety of rhizobial cell surface and membrane carbohydrate-containing molecules. They include extracellular polysaccharides (EPSs), capsular polysaccharides (CPSs), lipopolysaccharides (LPSs), chitolipooligosaccharides (*Nod* Factors), cyclic β-glucans and glycolipids. Their structures and locations on the rhizobial surface are covered in **Section III** of the literature review. The surface chemistry of rhizobium is highly variable and very sensitive to environmental factors which will change as the organism leaves the rhizosphere and goes inside of the plant **(Section IV).** The functions of various glycocongugates in the establishment of symbiosis are closely related

to the ability of rhizobia to change its surface chemistry in response to a changed environment.

Although numerous studies have been conducted on the involvement of various rhizobial surface molecules during various stages of symbiosis (summarized in **Section V** of the literature review), the specific roles played by each individual component are still far from clear. It is the diversity of the surface molecules involved in symbiosis that complicates such studies. These studies become even more complicated because of the large degree of coupling and overlap of biosynthetic events leading to the synthesis of the various molecules (**Section VI** of literature review).

Three chapters of this dissertation address the aspect of environmental regulation of rhizobial membrane chemistry. **Chapter 2** presents studies on membrane lipid chemistry of *B. japonicum* bacteroids living inside plant nodules. We have found that the predominant lipid in the membranes of *Bradyrhizobium* bacteroids is digalactosyl diacylglycerol, a typical plant lipid not found before in non-photosynthetic bacteria. **Chapter 3** presents studies on membrane lipid chemistry of a free-living, bacteroid-like form of *B. japonicum.* We have found that the predominant lipid is also a diglycosyl diacylglycerol. **Chapter 4** presents studies on membrane lipid chemistry of *Bradyrhizobium* grown under low oxygen. Low oxygen condition was chosen because it mimics conditions inside root nodules. We found that the low oxygen condition triggers the biosynthesis of phosphatidylinoisitol (PI), another lipid common in plants and not usually found in bacteria.

Understanding carbohydrate metabolism is central to understanding the connections among biosynthetic events leading to the various polysaccharides, glycolipids and

glycoconjugates which all appear to be so important to the symbiotic process. The work presented in **Chapter 5** was conducted to help to untangle the complex, interdigitized web of carbohydrate metabolism and biosynthesis in order to understand this complicated picture. In these experiments, 3-O-[^{14}C]methyl-D-glucose was used to probe the common intermediates shared by the biosynthetic pathway to various glycolipids and saccharides. We have found that carbohydrate components in the membrane glycolipids contain labeled 3-O-methyl glucose. The results indicate that the biosynthesis of glycolipids utilizes either a different substrate pool or a glycosyl transferase with different substrate specificity.

The last chapter summarized the results obtained from previous chapters and addressed the possible roles of these unusual membrane phospholipids and glycolipids in the adaptation and survival of rhizobia inside plants during symbiosis. It also raises some questions about the origin of the genes involved in the biosynthesis of these unusual phospholipids and glycolipids. Further studies on the biosynthesis of various saccharides and glycoconjugates are proposed.

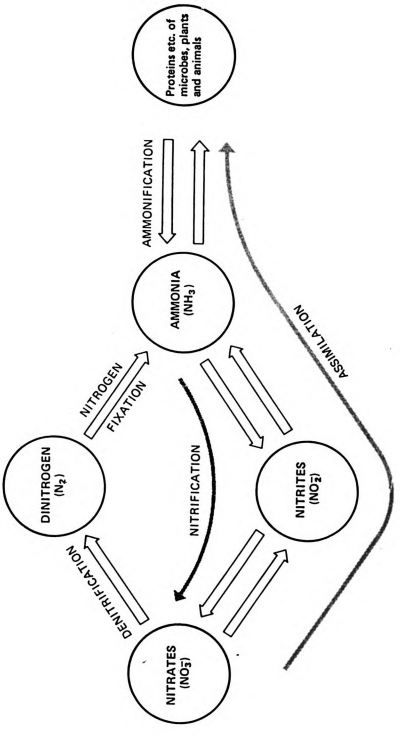
LITERATURE REVIEW

I. NITROGEN FIXATION AND THE RHIZOBIUM/LEGUME SYMBIOSIS

Living organisms continually recycle carbon, nitrogen, oxygen and sulfur from which they are composed. From both ecological and economic viewpoints, the nitrogen cycle is extremely important (1). Figure 1.1 is a simple illustration of this cycle. The lower pathway shows the synthesis of organic nitrogen compounds (principally proteins and nucleic acids) from inorganic nitrogen compounds (nitrate, nitrite and ammonium ions) during growth of plants and subsequent plant consumption by animals. Nitrogen returns to the soil as a result of decay of plant and animal materials. The upper pathway shows the loss of nitrogen to the atmosphere from nitrates, and its return to the cycle by the process known as nitrogen fixation. Biological productivity in all agricultural areas of this planet is determined by the rate at which the cycle turns, i.e. the rate of N_2 fixation.

In nature, the ability to fix N_2 is restricted to a relatively small number of prokaryotic organisms, termed diazotrophs. These organisms have the enzyme system, nitrogenase, a biochemical machinery for nitrogen fixation (1, 2). In this enzymatic process, atmospheric dinitrogen is reduced to ammonia. Diazotrophs annually add about 60% of the earth's newly fixed nitrogen, while industrially fixed nitrogen contributes only about 25%, with the remaining 15% produced by lightening, UV radiation, etc.(2). Some nitrogen-fixing bacteria like *Klebsiella pneumoniae*, *Azotobacter* and *Rhodopseudomonas* are able to reduce

Figure 1.1: The biological nitrogen cycle. Adapted from reference 1.


Proteins etc. of microbes, plants and animals
AMMONIFICATION
AMMONIA (NH_3)
ASSIMILATION
NITROGEN FIXATION
DINITROGEN (N_2)
NITRIFICATION
NITRITES (NO_2^-)
DENITRIFICATION
NITRATES (NO_3^-)

nitrogen into ammonia as free living organisms.

Other bacteria fix nitrogen mainly during symbiotic relationships with plants. Some well-known symbiotic systems are rhizobium/legume, *Frankia*/actinorhizal and cyanobacteria/*Gunnera*.

The best studied systems of symbiotic nitrogen fixation are the soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, collectively referred to as rhizobia. It has been estimated that the rhizobia/legume symbiosis accounts for over 50% of natural nitrogen fixation (2). The legumes are important in agriculture not only because they fix nitrogen and thus lower the use of nitrogen fertilizers, but also because of the high protein content of the plants and seeds. The seeds of crop legumes are important nutritionally for humans, and the forage legumes (clovers, alfalfa and lucerne) supply a high protein diet to livestock (3). The most prominent feature of rhizobial symbiosis is the formation of effective nodules on the roots of leguminous plants (4-8). Inside these nodules the bacteria are able to fix nitrogen and provide the host plant with ammonia for growth.

II. THE PROCESS OF NODULATION

The first stage of nodulation is bacteria-plant recognition. Rhizobia have a restricted host range; each strain can only infect and nodulate a limited number of legume species (1, 3). It is thought that this high degree of specificity is due to key interactions between the molecules on the surface of the partners. These molecules must be sufficiently diverse. Rhizobia have a wide range of surface saccharides and glycoconjugates that can provide sufficient diversity. Plants, on the other hand, have lectins. Lectins are proteins that can

distinguish different sugars and one theory is that they mediate the recognition process between legumes and rhizobia.

Once the proper bacteria-plant recognition occurs, the bacteria invade into the root hair. This starts, in most cases, with the morphological changes on the root hair in the presence of the bacteria. Figure 1.2 shows this process (7). The root hair curling is caused by the release of some diffusible signal molecules which now are believed to be bacterial surface saccharides or glycoconjugates. The bacteria are enclosed in a pocket of the curled hair. By hydrolyzing the local root hair cell wall, the bacteria enter the epidermal cell and are then enclosed by a newly formed wall which cuts the bacteria off from the contents of the host cell. The bacteria divide in the enclosed space and the wall enclosing them extends to grow as a tube termed the infection thread. The infection thread is actually an intracellular tunnel bounded by the plant cell wall. The infection thread proceeds to grow beyond the root hair cell and penetrates into the root cortical cells (Figure 1.3). Simultaneously, the cortical cells are stimulated to divide and give rise to the nodule primordium. Bacteria are then released from the infection thread at a point where it is free of host cell wall. They are released into the cytoplasm of the cortical cells by a process of endocytosis, surrounded by a plant-derived membrane termed peribacteroid membrane. The bacteria divide frequently and when the cytoplasm is almost full of bacteria, the bacteria enlarge and change in shape and differentiate into their endosymbiotic form, bacteroids. The nodule primordium thereupon develops into a mature nodule. There are also many biochemical changes accompanying bacteria differentiation to bacteroids. The bacteroids change their many cellular components to cope with the low oxygen conditions inside nodules and synthesize

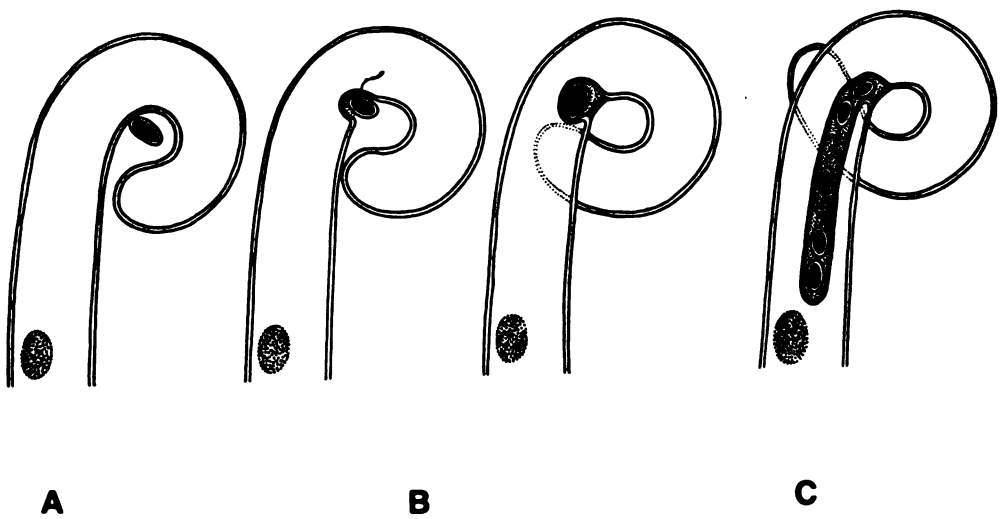
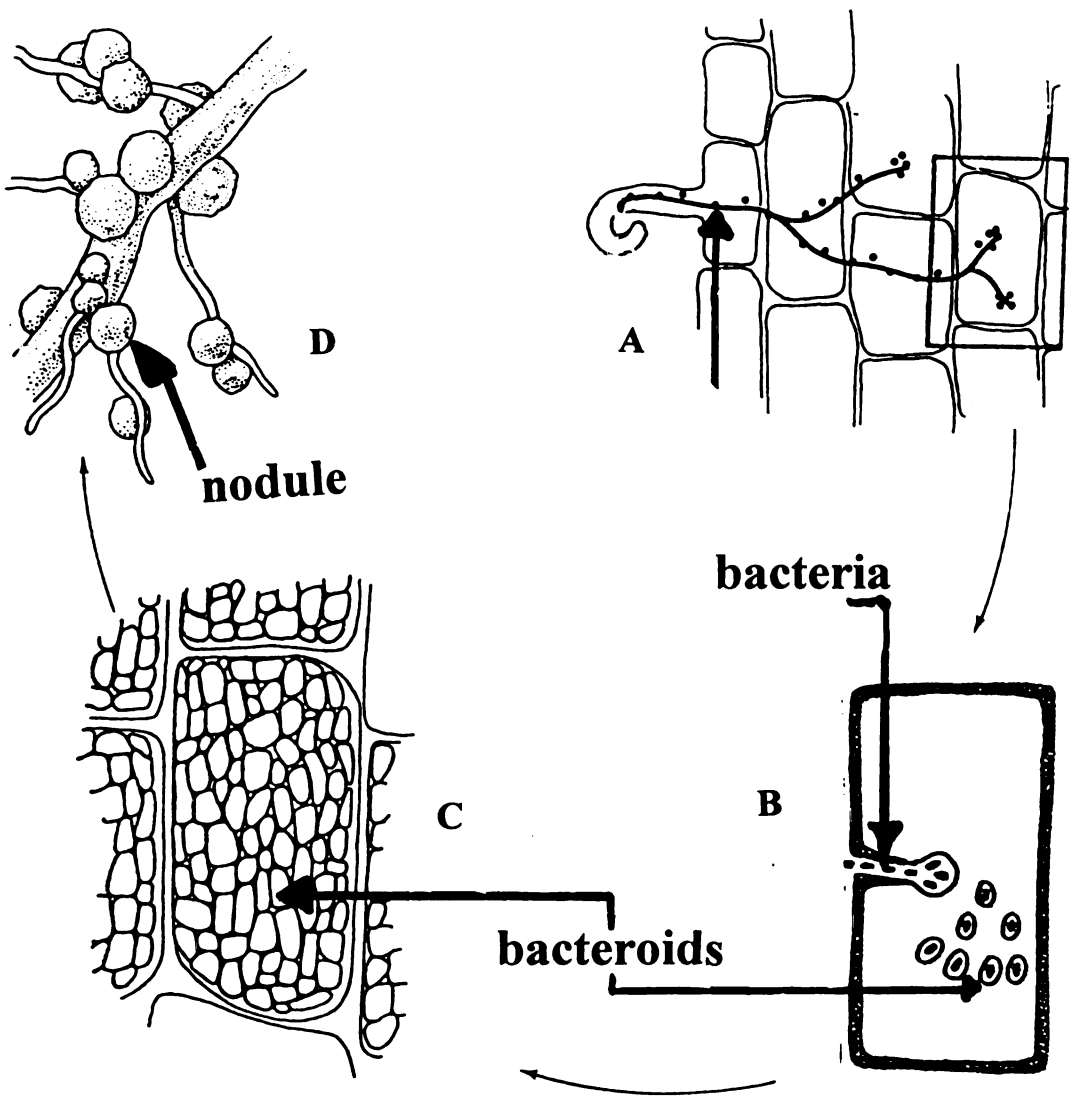


Figure 1.2: The invasion of the root hair by rhizobium. A, the bacterium becomes enclosed in the curled root hair; B, the bacterium penetrates the root hair and is surrounded by cell wall materials; C, initial infection thread growth. The bacteria divide within the infection thread.

Figure 1.3: A, spread of infection thread and division of cortical cells; B, bacteria release; C, bacteria differentiation into bacteroids; D, mature nodules on plant roots.


D
nodule
A
bacteria
C
B
bacteroids

the nitrogenase complex, the enzymatic machinery to fix nitrogen (3). The root nodules are well adapted for nitrogen fixation in air. The cortex surrounding the central infecting cells can serve both as a barrier for excess oxygen infusion and as a mediator for the active metabolite transport within nodules.

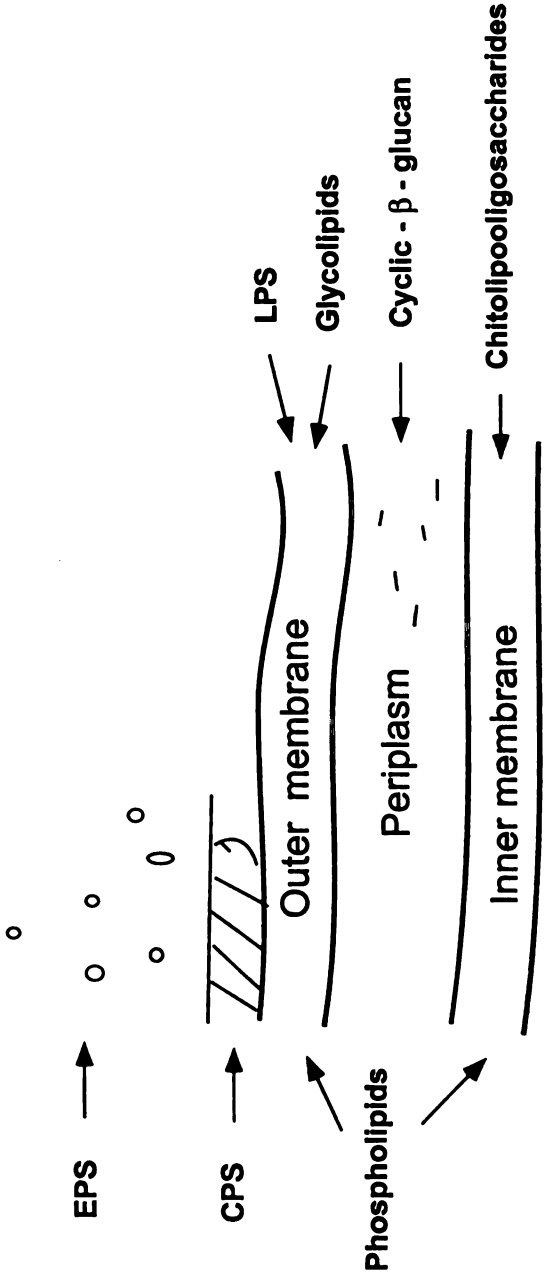
III. RHIZOBIAL CELL SURFACE AND SURFACE CARBOHYDRATE-CONTAINING MOLECULES

The process of the establishment of symbiosis was introduced in the last section. During this whole process, a variety of rhizobial cell surface and membrane carbohydrate-containing molecules are believed to be involved. They include EPSs, CPSs, LPSs, chitolipooligosaccharides (*Nod* Factors), cyclic β-glucans and glycolipids. Before giving a detailed discussion of the functions of these various surface carbohydrate-containing molecules, an introduction of their locations and the structures are needed.

Rhizobium is a Gram-negative bacterium and the envelope of rhizobium consists of an inner membrane and an outer membrane separated by a periplasmic space. Figure 1.4 is a schematic diagram of the rhizobial cell surface where the locations of various carbohydrate-containing molecules are indicated.

EPS and CPS: Like many Gram-negative bacteria, rhizobium produces a discrete capsule or capsular polysaccharide (CPS) and (or) excretes acidic extracellular polysaccharide (EPS) as a layer of slime not tightly attached to the bacterial cell surface. The structures of these two glycoconjugates are generally similar. The most studied EPS is found in *R. meliloti* and is often called succinoglycan (EPS I). It belongs to the broad class of acidic

Figure 1.4: Carbohydrate-containing molecules on the surface of Rhizobium.

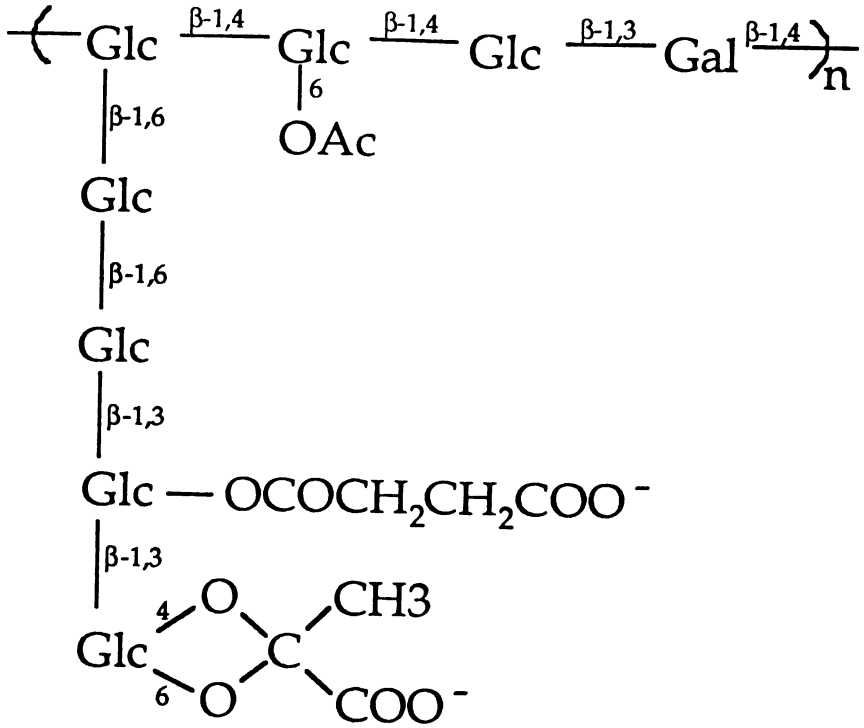

EPS
CPS
Phospholipids
Outer membrane
Periplasm
Inner membrane
LPS
Glycolipids
Cyclic - β - glucan
Chitolipooligosaccharides

heteropolysaccharides (9) and is made of repeating octasaccharides containing non-carbohydrate substitutes (a succinate, pyruvate and O-acyl group). Certain mutant strains of *R. meliloti* produce an alternate EPS structure, termed EPS II. The structures of the two *R. meliloti* EPSs is presented in Figure 1.5.

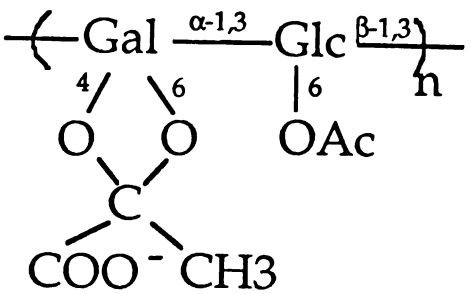
LPS: The outer leaflet of the outer membrane of rhizobium is mainly composed of lipopolysaccharide, molecules unique to the envelope of Gram- negative bacteria. The outer membrane makes the Gram-negative envelope impermeable to hydrophobic compounds and higher molecular weight hydrophilic compounds (10). This restrictive permeability properties of the outer membrane account for the resistance of Gram-negative bacteria to a wide range of antibiotics. Typical enterobacterial LPS (Figure 1.6 A) have three covalently linked domains: membrane embedded lipid A consisting of glucosamine disaccharides linked with fatty acid chains, an oligosaccharide core and an antigenic polysaccharide chain (O-antigen). The structure of the LPS Lipid A and O-antigen of *R. leguminosarum biovars* was shown in Figure 1.6B (11). This lipid A has carboxylate as the negatively charged groups.

Phospholipids and Glycolipids: The predominant rhizobial membrane phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC). While PE and PG are common phospholipids in the membrane of Gram-negative bacteria, PC is rarely found in other Gram-negative bacteria but tends to be predominant in plant cells (12). Besides this uniqueness, rhizobia also synthesize glycolipids which have not been found in other Gram-negative bacteria. It has been demonstrated that the membranes of many free-living rhizobia species contain sulfoquinovosyl diacylglycerol (SQDG), a glycolipid which was thought to occur exclusively

Figure 1.5: General structure 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 9.



Succinoglycan



EPS II

in membranes of plant plastids and photosynthetic bacteria (13). One mannose and glucose-containing diglycosyl diacylglycerol has been reported to accumulate in the membrane of *R. leguminosarum* bv. *viciae* ANU843 when the bacteria were grown under certain culture conditions (14). The occurrences of these glycolipids make the membrane chemistry of rhizobium somewhat of an enigma since it is more similar to plant membrane chemistry than to bacterial membrane chemistry. Figure 1.7 shows the structures of major rhizobial lipid molecules.

Cyclic β-glucans: Within the periplasmic space is cyclic β-glucans, molecules that are found almost exclusively in bacteria of the Rhizobiaceae family (15). Reaching concentrations as high as 5 to 20% of the total cellular dry weight under certain culture conditions, the cyclic β-glucans are major cellular constituents. In *Rhizobium* species, these contains glucose residues linked solely by β-(1,2) glycosidic bonds. In *Bradyrhizobium* species, the glucose residues are linked by both β-(1,3) and β-(1,6) glycosidic bonds (15). The glucose monomer may become substituted with nonsugar moieties such as anionic substituents. The degree of polymerization (DP) ranges from 10 to 13 in *B. japonicum* to 17 to 40 in *R. meliloti.* The structure of *B. japonicum* cyclic β-glucans is shown in Figure 1.8.

Chitolipooligosaccharides: Another class of molecules unique to rhizobia is called chitolipooligosaccharides or Nod factors (16). They are chitin-like oligosaccarides of three to five 1,4-linked *N*-acetylglucosaminosyl residues, which are N-acylated on the terminal non-reducing glucosaminosyl residue. They may be further modified by the addition of another glycosyl residue, or sulfate, carbamyl, acetyl, and/or glycerol substituents. The

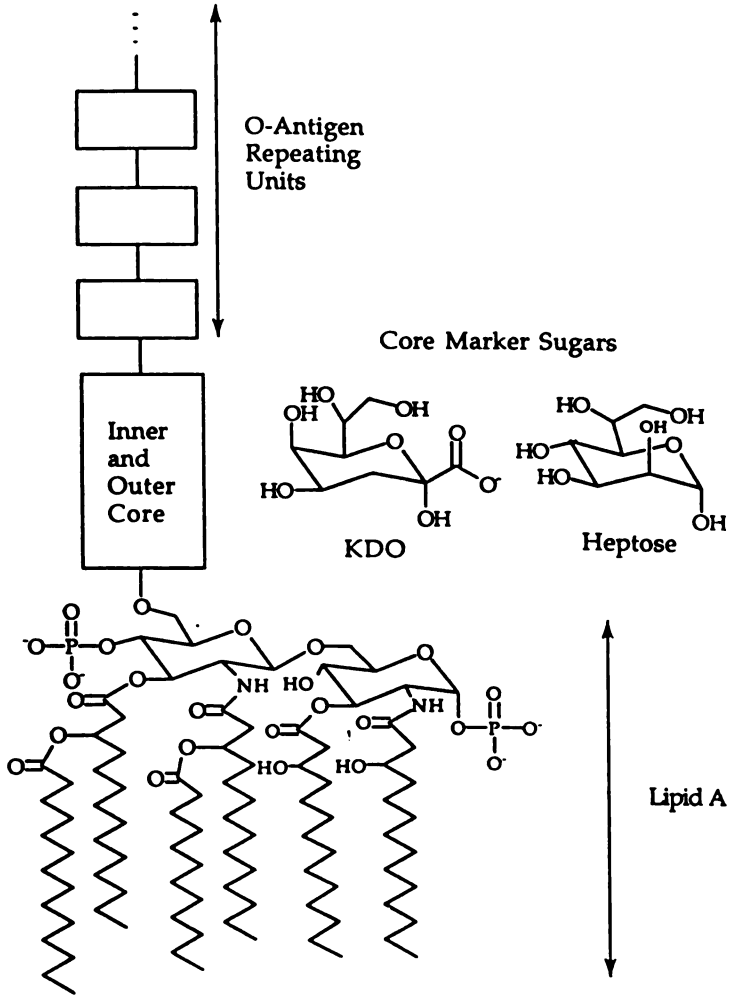
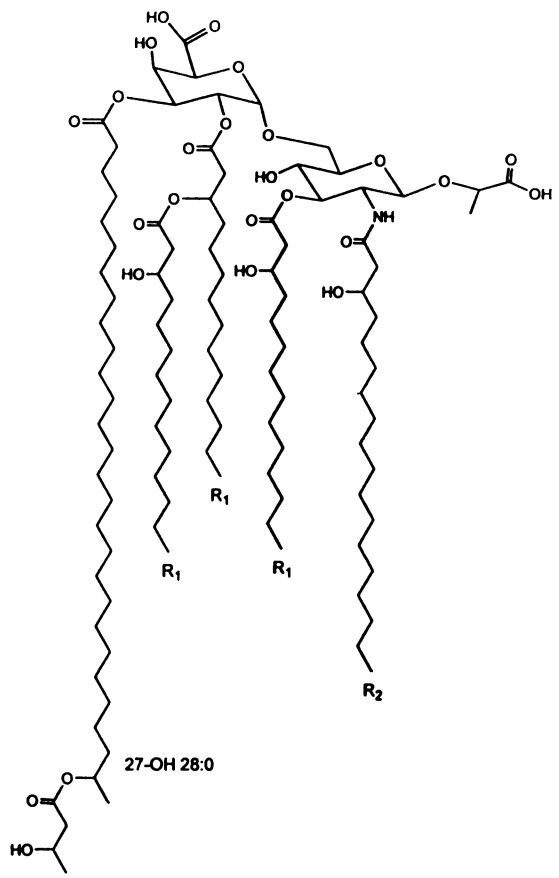


Figure 1.6A: Basic structure of typical enteric bacterial LPS. Adapted from Rietschel, E.T. et al. 1994. *FASEB J.*, **8**, 217-225.

Figure 1.6B: Structures of LPS lipid A and O-antigen of *R. leguminosarum biovars*.

lipid A



O-Antigen

α-D-ManNAc

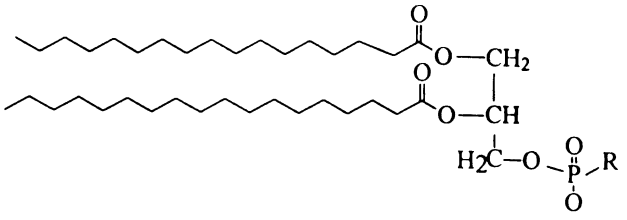
1

↓

2

⟶3)-α-L-Rha-(1→3)-α-L-Rha-(1→4)-β-D-GlcNAc-(1→3)-α-L-Rha-(1⟶

Figure 1.7: The structures of the phospholipids and glycolipids found in rhizobia.

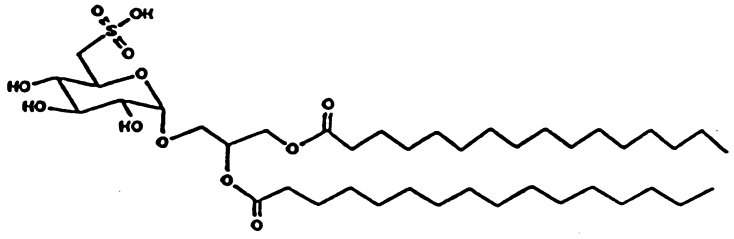


R= $-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ Phosphatidylcholine (PC)

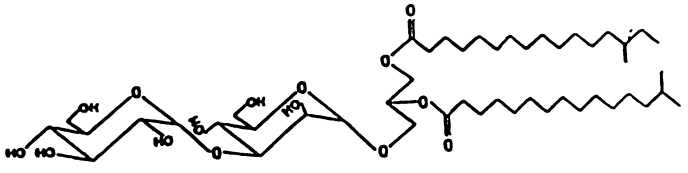
$-O-CH_2-CH_2-\overset{+}{N}H_3$ Phosphatidylethanolamine (PE)

$-O-CH_2-\underset{OH}{CH}-CH_2-OH$ Phosphatidylglycerol (PG)

Fatty acid chains vary in length and degree of unsturation



Sulfoquinovosyl diacylglycerol (SQDG)
Fatty acid chains vary in length and degree of unsturation



Mannose and glucose-containing diglycosyl diacylglycerol
Fatty acid chains vary in length and degree of unsturation

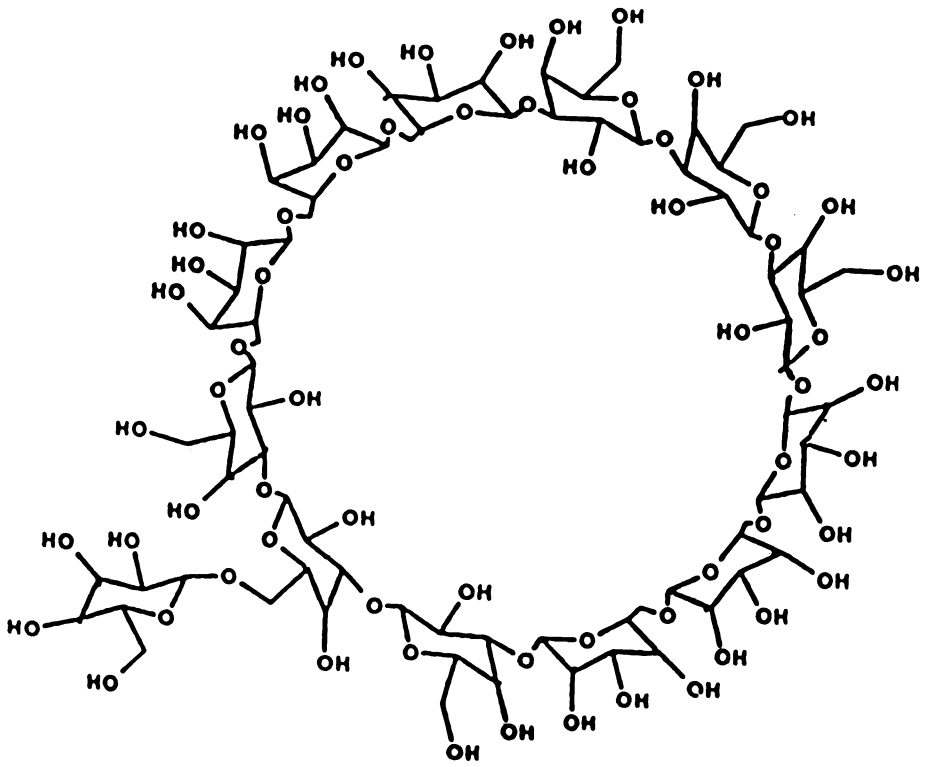
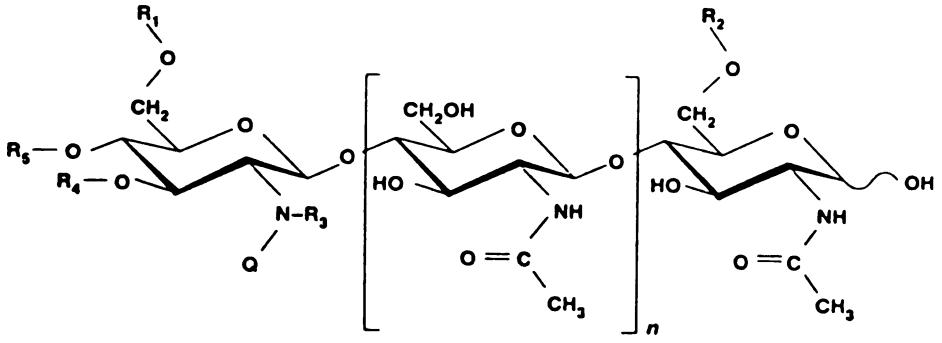


Figure 1.8: proposed structure for the unsubstituted cyclic β-(1,6)-β-(1,3)-glucan of *B. japonicum* containing 13 glucose residues. Adapted from reference 15.



	R. leguminosarum bv. *viciae*	*R. meliloti*	*R.* sp. NGR234
n	2 or 3	1, 2, or 3	3
Q	C18:1 or C18:4	C16:2	C18:1 or C16:0
R_1	CH_3CO or H	CH_3CO or H	H
R_2	H	SO_3H	2-*O*-methylfucose or substituted with either 3-*O*-CH_3CO or 4-*O*-SO_3H
R_3	H	H	CH_3
R_4	H	H	NH_2CO or H
R_5	H	H	NH_2CO or H

Figure 1.9: Structure of chitolipooligosaccharides. The chitin oligomer and the acyl moiety (Q) are present in all chitolipooligosaccharides. Adapted from reference 16.

Structure of chitolipooligosaccharides is shown in Figure 1.9. Recent studies (17, 18) suggest that these molecules are membrane-localized.

IV. THE DYNAMIC PROPERTY OF THE RHIZOBIAL SURFACE

In the previous section (section III), various rhizobial cell surface and membrane carbohydrate-containing molecules are introduced because of their importance in the establishment of symbioses between rhizobia and legumes. Before the symbiotic roles played by each class of molecules are summarized in the next section (section V), a very important property of the rhizobial cell surface needs to be addressed. It is the dynamic property of the bacterial surface chemistry. The bacterial surface is constantly regulated by environmental factors including those inside the plant. This dynamic property enables the bacterium to adapt and survive in a totally new environment such as that inside of the plant cell. The bacterium achieves its dynamic surface property mainly by changing the relative quantities or structures of its various surface molecules. The different surface and membrane carbohydrate-containing molecules present at different stages of symbiosis contributes to this dynamic property of the bacterial surface. For example, the lipids which constitute the basic structural elements of membranes are critical to bacterial adaptation (19). They are exquisitely sensitive to environmental variables, including medium constituents (carbon, nitrogen, inorganic phosphate, etc.), physical factors (temperature, oxygen tension, pH, etc.) and growth phase (19-20). The term *homeoviscous adaptation* (homeostasis) is often used to describe the ability of membrane to maintain a suitable dynamic state of the bilayer independent of temperature. The active restructuring of the membrane lipid composition in response to

environmental change is essential for an organism to adapt to and survive in an altered environment.

The dynamic properties of rhizobial surface can be observed by culturing the bacteria in different media. Studies using monoclonal antibodies as specific submolecular probes for LPS epitopes suggested that the structure of LPS was regulated by environmental factors such as ion composition, pH and oxygen tension (21-23). Symbiotic mutations leading to an impaired LPS structure in *R. leguminosarum* also affect the normal range of structural adaptations for LPS macromolecules under different physiological conditions (23).

Rhizobial surface composition changes as bacteria differentiate into bacteroid forms. The environmental conditions inside root nodules are very different from those experienced by free-living bacteria (24-25). For example, there is a large decrease in the oxygen concentration experienced by bacteroids inside nodules (as low as 20 nM) compared with that experienced by free-living bacterium outside nodules (250 μM). The peribacteroid space has a lower pH and a different carbon source because of the presence of a proton-ATPase and a dicarboxylic acid transport system on the peribacteroid membrane (26). Compared with free-living bacteria, bacteroids inside the nodules have a smaller amount or modified composition of LPS (27-31) and lower levels of EPS (32). It has even been suggested that the bacterial outer membrane may be entirely sloughed off during endocytosis by the host cell (33). Immunocytochemical studies monitoring *in planta* the variations of LPS structure point to a surprising degree of LPS epitope variations for bacteroids situated in different regions of the a given nodule (25, 28, 29). These regions represent different physiological and developmental states within the nodule. The changes in bacteroid LPS or EPS

composition can be simulated by growing free-living bacteria under culture conditions that approximate those inside root nodules. Such conditions include low oxygen (21, 24, 31, 32), low pH (21, 24, 34), and a combination of low oxygen, low pH and a different carbon source (25).

It is obvious that the changes in the various surface carbohydrate-containing molecules are related to the initiation and sustaining of infection. Some mechanisms for the modes of action of the molecules are proposed. These include roles to facilitate metabolite transport (34), to evade the plant defense systems (34,35), or to maintain certain physical properties of the bacterial surface. The next section will be devoted to discussions on the roles played by various rhizobial surface carbohydrate-containing molecules during symbiosis.

V. ROLES PLAYED BY VARIOUS RHIZOBIAL SURFACE CARBOHYDRATE-CONTAINING MOLECULES AT DIFFERENT STAGES OF SYMBIOSIS

The establishment of an effective symbiosis seems to hinge on the multiple interactions between the plant and various rhizobial cell surface carbohydrate-containing molecules such as chitolipooligosaccharides, EPSs, LPSs, cyclic β-glucans, and membrane glycolipids. In the previous sections, the structures and the cellular locations of these molecules were introduced. The dynamic nature of the biosynthesis of these molecules were covered because it confers on the organism the capability to adapt to the changing micro-environment at the different stages of symbiosis. The following is a summary of the studies on the role(s) played by each class of molecules at different stages of symbiosis.

Chitolipooligosaccharides: Rhizobia have a restricted host range. Each strain is able to interact with only one or a limited number of legume species. Chitolipooligosaccharides are considered by many to be the primary host range determinant (16, 36-37) Even though the mechanism by which they determine host specificity is largely unknown, the production of chitolipooligosaccharides is found to be specified by rhizobial *nod* genes which are located on the symbiotic plasmid. The core oligosaccharide structures of the chitolipooligosaccharides are specified by *nod* ABC genes and are common to all rhizobia. The host-specific *nod* genes control the modifications of the core molecule with side groups, which are thought to render chitolipooligosaccharides specific for their particular host. Chitolipooligosaccharides of *R. meliloti* have a sulfate group on the reducing sugar, whereas this substitution is lacking on the chitolipooligosaccharides produced by *R. leguminosarum biovars*. Normally the chitolipooligosaccharides of *R. meliloti* is not active on vetch, a host plant of *R. leguminosarum bv. viciae*, but after removal of the sulfate group it gains the ability to infect vetch and loses this ability on its own host, alfalfa (36). The involvement of chitolipooligosaccharides substitutions in host specificity is strengthened by studies on *R. Sp. NGR234*, which has a broad host range. The bacterium has the unique ability to interact with more than 70 genera of legumes as well as with *Parasponia andersonii*, the only non-legume that can form nodules with Rhizobium. *R. Sp. NGR234* produces over 18 different chitolipooligosaccharides with similar basic structure but different substitutions (38). It seems that the diversity of chitolipooligosaccharides accounts for the broad host range of this bacterial strain. *In vitro* experiments showed that purified chitolipooligosaccharides, when applied to legume seedlings at concentrations as low as 10^{-12} M, stimulate the differentiation

of epidermal cells into root hairs and elicit root hair curling and cortical cell division (3, 39-40).

EPSs: At any stage of symbiosis, EPSs are likely to be chemical signals between the bacterium and plant because of their locations on the outside of the cell and because of the diversity of EPSs structures among different bacterial species. EPS are required for the normal development of root nodules of many rhizobial species (9). Mutants that do not produce EPSs can be selected based on their lack of fluorescence on Calcofluor medium. By this method, a cluster of *exo* genes has been found to specify the production of EPSs (41). In *R. meliloti*, *exo* mutants consistently failed to nodulate the host alfalfa successfully. In these *exo* mutants, nodule invasion appeared to be blocked at the stage when the infection thread penetrates beyond the epidermal cell layer. This implies that instead of being the initial signal or the primary host range determinant, EPSs are required for nodule invasion. It has been shown that low molecular weight form of EPSs can partially suppress the symbiotic deficiencies of *exo* mutants (41).

LPS: LPSs have also been reported to play some important roles in the symbiotic infection process. Two mutants of *R. leguminosarum bv. Phaseoli* with a defective LPS O-antigens are symbiotically defective (42). When inoculated onto the host legume, *Phaseolus,* these mutants result in the initiation of nodules that become arrested in development because the infection threads which contain invading rhizobia appear to abort, usually within the root hair cell. It seems that complete LPS structures, including normal amounts of O antigen, are necessary for the development of the infection thread. In some other studies, LPSs have been reported to be required for the formation of normal infection thread or the invasion of the

root cortical cells (43-49).

Cyclic β-glucans: Cyclic β-glucans are specified by rhizobial *ndvA* and *ndvB* genes. The most compelling evidence for a role of cyclic β-glucans during plant infection comes from studies with *ndvA/ndvB* mutants of *R. meliloti* (15, 50-51). These studies have revealed that the *R. meliloti ndvA* and *ndvB* mutants form ineffective white pseudo nodules on alfalfa. Closer examination of the pseudo nodules induced by *ndv* mutants of *R. meliloti* reveals a small number of infection threads that abort at an early stage, and no nitrogen-fixing bacteroids are observed. The initiation of infection threads by these mutants suggests that the cyclic β-glucans provide functions during the later stages of alfalfa nodulation. *R. meliloti ndv* mutants resemble *exo* mutants in that they form "empty" (uninfected) nodules and differ in that they react normally with Calcofuor and curl root hairs normally (26).

Glycolipids: Possible roles for membrane lipids in the symbiotic process have been proposed in a recent study. One mannose and glucose-containing diglycosyl diacylglycerol has been reported to accumulate in the membrane of *Rhizobium leguminosarum* bv. *viciae* ANU843 when the bacteria were grown in the presence of flavonoids (14). Subnanomolar concentrations of this glycolipid could also elicit various responses typical of symbiosis such as root hair deformation and cortical cell division on the host plant. The unusual occurrence of SQDG in many rhizobial species also suggests a role for these glycolipids during symbiosis (13).

From the above mentioned studies, it is clear that a diversity of rhizobial surface carbohydrate-containing molecules are involved at different stages of symbiosis. This diversity complicates studies aimed at defining roles of each individual components. These

studies become even more complicated because of the large degree of coupling or overlap of biosythetic events leading to the synthesis of various molecules. The next section will be devoted to this aspect.

VI. FUNCTIONAL AND BIOSYNTHETIC OVERLAP AMONG VARIOUS RHIZOBIAL CELL SURFACE CARBOHYDRATE-CONTAINING MOLECULES

Most mutant studies on the involvement of certain classes of carbohydrate-containing molecules during symbiosis are carried out without checking the pleiotropic effects of the mutation(s) on other classes of molecules. However, it is the whole bacterial surface that interacts with the plant at each stage of symbiosis. Studies on the functional and biosynthetic overlap among various rhizobial cell surface components merit special attention.

The best characterized pleiotropic Exo$^-$ mutants are *exoB* mutants of *R. meliloti*. These mutants are defective in the synthesis of both LPS and EPS. Like other *Exo*$^-$ mutants of *R. meliloti*, the *exoB* mutants form ineffective nodules. It was found later that these *exoB* mutants fail to synthesize active UDP-glucose 4'-epimerase and thus fail to make galactose (52). Since galactose is a structural element of both EPS and LPS of these strains, it is not surprising both EPS and LPS are defective in these mutants (52). This raises a question about the critical factor involved in nodule formation. What accounts for the deficiency in symbiosis, EPS or LPS?

Some studies on EPS- and LPS-negative mutants of some *Rhizobium* strain (53, 54) found that these strains were still able to form nitrogen-fixing nodules, indicating that EPS or LPS are not required for nodulation. Other studies provided evidence that LPS and EPS may have the same function in the plant-bacteria interaction in some symbiotic systems

(55,56). In *R. meliloti*, the symbiotic phenotype of *exo* mutants are deficient in EPS and cannot nodulate alfalfa. Mutants carrying a *lpsZ*$^+$ gene can restore nodulation. It is found that *lpsZ*$^+$ does not restore EPS production but instead alters the composition and structure of LPS(57). The evidence that EPS and LPS can substitute each other in these strains also lead to hypotheses on the role of surface polysaccharides in plant-microbe interactions: irrespective of their exact surface structures, these carbohydrate-containing molecules work together to provide some signal(s) promoting recognition events (55-57). These signals can be oligosaccharides, polysaccharides or even the physico-chemical properties of the whole bacterial surface.

The bacterium achieves the optimum physico-chemical properties for its surface by constantly changing its composition. The latter is achieved by the biosythetic regulation of many of its surface molecules. It is not surprising, therefore, that there is a large degree of coupling of the many biosynthetic events leading to the synthesis of the various surface components. For instance, both the lipo-oligosaccharides and LPS of *Rhizobium meliloti* are known to be sulfated (58). A *nod PQ* mutant of *R.. meliloti* which is defective in nodulation makes a non-sulfated lipo-oligosaccharide. However, it was found later that this mutant also has an altered LPS (not sulfated) in addition to structural changes in membrane lipids including SQDG. In rhizobial *exo* mutants which have an altered EPS, LPS were also changed (59). *R. leguminosarum* bv. trifolii 4S has a LPS O-antigen that lacks galactose and many of the typical glycosyl components found in related strains. It has been recently found that it synthesizes an alternative glycolipid which contains galactose and the typical O-antigen glycosyl components, suggesting that in this strain, the "missing" O-antigen of LPS

is transferred to an alternative lipid acceptor (60).

It is obvious that there is functional and biosynthetic overlap among various classes of rhizobial cell surface components. Without studies on the pleiotropic effects of a particular mutation, the classical approach of mutating-then-evaluating is not applicable to the studies on the roles played by each individual bacterial surface component. It is clear that in studies on the mechanism of plant-microbe interaction, importance should be put on the following aspects: 1, to obtain a comprehensive picture of the rhizobial cell surface with respect to the structures of various surface and membrane molecules; 2, to grasp the changes in this picture when rhizobia live in different environments, especially the environment inside of the plant; 3, to understand the carbohydrate metabolism underlining the biosynthesis of various carbohydrate-containing molecules which are so central to symbiosis.

CONCLUSION

The first two sections of the literature review introduced the importance rhizobium/legume symbiotic system and the process by which the symbiotic relationship between rhizobia and leguminous plants is established. At each stages of symbiosis, a variety of rhizobial cell surface and membrane carbohydrate-containing molecules have been indicated to play some important roles (section V). The structures and cellular localizations of these molecules were covered in section III. The functions of various glycoconjugates in the establishment of symbiosis are closely related to the ability of rhizobia to change its surface chemistry in response to a changed environment (section IV). There is a large degree of functional and biosynthetic overlap among various classes of rhizobial cell surface components (section VI).

It is the whole bacterial surface that interacts with plants. A comprehensive picture of the rhizobial cell surface is needed in order to understand bacteria-plant interactions. There are two important parts of the big picture: the environmental regulation of the bacterial surface and the coupling of the biosynthetic events leading to various surface molecules.

The work presented in this dissertation is based on these two parts. Three chapters of this dissertation address the aspect of environmental regulation of rhizobial membrane chemistry. Chapter 2 and 3 respectively presents studies on the membrane lipid chemistry of *B. japonicum* living as bacteroids inside plant nodules and living as bacteroid-like form in bacteroid-inducing media. Chapter 4 presents studies on membrane lipid chemistry of

Bradyrhizobium grown under low oxygen and low pH conditions which mimic condition inside root nodules. Chapter 5 describes an investigation on the interrelationship of the biosynthetic pathways leading to various rhizobial surface carbohydrate-containing molecules.

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CHAPTER 2

THE LIPID CHEMISTRY OF BACTEROIDS ISOLATED FROM PLANT NODULES IS DISTINCTLY DIFFERENT FROM THAT OF FREE-LIVING RHIZOBIA

ABSTRACT

The membrane lipids of free-living and bacteroid forms of *Bradyrhizobium japonicum*, obtained from nodules occupied by both typed and untyped bacteria, were isolated and characterized by a combination of NMR spectrometry, mass spectrometry and other chemical and physical methods. These studies indicated that both the free-living and bacteroid forms of this organism contain glycolipids almost exclusively of the type found in plant cells. In the bacteroid forms, there was a dramatic shift towards the synthesis of digalactosyl diacylglycerol as the major lipid. This glycolipid has rarely been found outside of the plant kingdom and photosynthetic bacteria and its occurrence in the bacteroid form of a plant symbiont is therefore remarkable. The presence of significant plant cell and organelle contamination in the bacteroid preparations was ruled out by scanning electron microscopy, southern blot analysis for plant DNA using specific gene probes and chemical analysis for plant marker steroids, steroid glycosides and glycosyl ceramides. DGDG is not found in the plasma membrane of plant cells (of which the peribacteroid membrane is an extension) but is thought to be restricted to plastids. This result follows our earlier finding that the other predominant plant glycolipid, sulfoquinivosyl diacylglycerol, is a membrane component of fast growing *Rhizobia* and is found even when cells are cultivated in free culture where there is no question of plant contamination. The absence of these lipids in the membranes of bacteria outside of this special group of organisms and photosynthetic bacteria suggests that the trait could have been passed on through gene transfer from plants

to the bacteria at some point during the development of their symbiotic relationship. In the case of digalactosyl diacylglycerol, there is also the possibility that some common biosynthetic intermediates are used by both the plant and the bacteria. This is a striking parallel with some host-pathogen interactions.

INTRODUCTION

The chemistry of bacterial cell surfaces is critical to the physiology and hence the adaptability of these organisms. When adaptation involves a close symbiotic relationship with another organism, the relative chemistry of the surfaces of the two species becomes a major issue. Among the biological systems in which two vastly different species become involved in a symbiotic relationship which benefits both partners, none (perhaps) has received as much attention as the *Rhizobiu*-legume symbiosis. Membrane chemistry is very closely tied to surface chemistry. This is because many of the predominant cellular surface antigens of both eucaryotes and procaryotes cells are, in fact, often structural components of the membrane. For instance, lipopolysaccharides which contain the O-somatic antigen are actually integral bacterial outer membrane components. Various rhizobial surface and membrane carbohydarate-containing molecules have been implicated to function in the process of symbiosis as covered in Chapter 1.

In an earlier study (1) we demonstrated that the membranes of members of the family *Rhizobiaceae* contained SQDG, a glycolipid which was thought to occur almost exclusively in membranes of plant plastids and photosynthetic bacteria although its presence has been demonstrated in a few other bacterial species (2). This unusual occurrence prompted questions about the developmental and evolutionary relationship between *Rhizobium* and plants. Since the membrane is a critical structural feature that determines the ability of an organism to occupy a particular ecological niche, it is not very surprising that two organisms

whose survival revolves around shared ecological pressures of temperature, pH, and water and nutrient availability and whose development and metabolism must be synchronized during the symbiotic period, have common membrane components. SQDG is not the only common component between the membrane lipids of *Rhizobium* and legumes. One of the predominant rhizobial membrane lipids is PC, a lipid that is not common in bacteria but typically occurs in plant cells (3).

In previous studies, we examined vegetative, free-living organisms. It was therefore important to examine *in situ* bacteroid forms of *Rhizobium* to see whether they contained predominantly large amounts of SQDG, DGDG or any of the common plant glycolipids. Bacteroids are the *in planta* nitrogen-fixing form of *Rhizobium.* For this study, we chose to investigate the *Bradyrhizobium* / soya bean system since we had detected small, but measurable, quantities of SQDG in the membranes of this species and could, therefore, readily determine whether substantially higher levels were present in their bacteroids. Soya bean nodules of two types, one inoculated with a known typed strain (USDA 110) and the other one infected by some untyped indigenous strain from the soil, were used. Soya bean nodules were used because of their large size which would ensure a high yield of bacteroids to allow fairly rigorous chemical characterization.

MATERIALS AND METHODS

Cultures of Free-living Bacteria and Preparations of Bacteroids

Soya bean nodules were provided by Dr. Thomas Deits, Michigan State University. *Rhizobium japonicum* USDA 110 was grown at 30°C in B-III broth medium and harvested as previously described (4). The pellet was collected and used for membrane lipid extraction. Two different sets of soybean root nodules were used in the experiments. One was obtained by inoculating the host plant (soya bean) with free-living *Bradyrhizobium japonicum* USDA 110. Prior to inoculation, plants were grown in sterilized soil and were maintained under green house conditions. The other set of nodules was obtained from field grown plants in which indigenous, untyped rhizobium strain(s) resided. Nodules were crushed in 0.3 M sucrose at 4°C and the bacteroids were separated from plant debris by filtering through glass wool filter. The filtrate was centrifuged at 100g for 30 min. The supernatant was then centrifuged at 5000g for 1 h. The two pellets were examined separately by scanning electron microscopy, found to be the same and therefore combined and used for DNA isolation and membrane lipid extraction. The plant debris and the final supernatant fraction were also used for lipid extraction.

Electron Microscopy

Scanning SEM was performed on a JEOL JMS-35CF SEM instrument. Samples were fixed at 4°C for 1-2 hours in 4% glutaraldehyde and dehydrated in an ethanol series (25%, 50%, 75%, 95%, 100%). After this, samples were coated with gold (20 nm thickness)

in an Emscope Sputter Coater model SC 500, purged with argon gas and then examined by SEM.

Isolation and Electrophoresis of DNA and Southern Hybridization

Roots and leaves were excised from soyabean plants grown in the fields and treated separately. One gram of material was cut into small pieces and ground in liquid nitrogen. A pellet of *B. japonicum* USDA 110 bacteroids was also ground in liquid nitrogen. 3 mL of cetyltrimethylammonium bromide buffer (pH 8.0, 20 gram/mL) was added to each sample. The mixtures were incubated at 60°C for one hour, cooled down to room temperature, extracted three times with equal volume of chloroform and centrifuged at 8500g for 10 minutes. The final aqueous extractions were transferred to sterile plastic tubes , 5M NaCl was added to each tube to achieve a final concentration of 2M and then 0.75 volume isopropanol was added to precipitate the DNA at -20°C for 2 h. The precipitated DNA was recovered by centrifugation at 6000g for 5 min, redissolved in water, precipitated again at -20°C in 3M sodium acetate and two volume of ethanol, washed with 70% ethanol and resuspended in 500 µL triethanolamine buffer at pH 8.0. Comparable amount of DNA samples were loaded in 1% nondenaturing agarose gel. Electrophoresis was conducted, bands visualized (ethidium bromide) and DNA transferred to nitrocellulose membranes and hybridized to probes according to standard procedures (5). The DNA probes used were from clone pOj116 containing the chloroplast *pet A* gene and from clone pRS2 containing the rhizobial *nif* genes. These were obtained from Dr. Barb Sears and Dr. Frans de Bruijn, respectively.

Lipid Extraction and Thin Layer Chromatography

Standard PS, PC and DGDG were purchased from Sigma Chemical Co. (St Louis, Missouri). Standard PG was purchased from Avanti Polar Lipids (Alabaster, Alabama). Lipids were extracted, analyzed by TLC on silica plates using a mixture of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:1:1 (13) and the components located as previously described (1).

Gas chromatography and Mass Spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) and gas chromatography / mass spectrometry (GC-MS) were performed and recorded as previously described (1) except that, for FAB-MS, glycerol was used as the matrix and the ionization voltage was 10 kV. Gas chromatographic separations for fatty acids were accomplished on a 30 meter DB-1 capillary column (J & W Scientific, Folsom, California). The program used was: 100° to 150°C at 25°C/minute and 150° to 300°C at 3°C/min with a 10-min hold at 300°C. For the separation of alditol acetate derivatives, a J & W Scientific DB-225 column was used. The program used was: 100° to 180°C at 40°C/min and 180° to 220°C at 4°C/min with a 50-min hold at 220°C.

Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton NMR analyses were performed on a Varian VXR-500 spectrometer (500 MHz for protons) at 25°C in deuterated chloroform or perdeuterated methanol. Chemical shifts are referenced relative to the residual chloroform line at 7.24 ppm or the residual methanol line at 3.30 ppm.

Chemical Characterization of Lipids

Fatty acids from lipids of bacteroid, plant debris and supernatant were obtained as methyl esters as previously described (1) except that the methanolysate was partitioned between 2 mL of chloroform and 1mL of water. The lower chloroform layer was recovered and subjected to GC and GC/MS. The glycosyl components of the lipids of these three samples were identified with GC / MS after converting them to alditol acetate derivatives as described before (6).

Methylation Analysis

The linkage pattern within the carbohydrate moieties of the lipids was determined by methylation analysis (7) with some modifications. A sample of the total lipid extract (~ 100 μg) was treated with 100 μL of a 0.2 M solution of sodium methylsulfinyl ion at room temperature for 12 hours. Iodomethane (100 μL) was then added and the mixture stirred for another 12 hours and then diluted with 2 mL of water and extracted with 3 mL of chloroform. The chloroform layer was recovered and the contents of the aqueous layer adsorbed onto a short C-18 reverse phase cartridge attached to a syringe. The cartridge was washed with water (2 mL) and the adsorbed material was then released by eluting with methanol (5 mL). The methanol wash was combined with the first chloroform extract and the solvent removed under a stream of nitrogen. The residue was hydrolyzed by treatment with 2 M trifluoroacetic acid for 3 hours at 100°C. The glycosyl components thus released were then reduced with sodium borohydride and the alditols so formed converted to peracetates as described earlier (1). The methylated, acetylated products were analyzed by GC and GC / MS.

RESULTS

The method used for the isolation of bacteroids from plant nodules has been widely used and is known to result in negligible contamination by plant material (8). Most plant organelles cannot be pelleted with the bacteroids at the centrifugation speeds used, especially in a medium with the buoyancy of the sucrose solution used. Prior filtration removes plant debris and associated plant-derived lipids. Several experiments aimed at addressing whether trace levels of plant contaminants were present were conducted even though gross contamination could not have led to the levels of glycolipids in the bacteroids. Scanning electron microscopy examination of the bacteroid suspension was an attractive proposition because of the large differences in the morphology and sizes of bacteria and bacteroids on one hand and plant cells and organelles on the other. Plant cells and many organelles are much larger than bacteroids and vegetative rhizobial cells. The latter cells are readily recognized by their characteristic cigar shapes. These may be spherical at certain stages of bacteroid development. SEM micrographs (Figure 2.1) of bacteroids isolated from soybean nodules in this study showed that there were two cell types, one rod shaped and the other, though of comparable size, spherical. The cell morphology observed in the SEM were identical to those in published literature (9). There was no detectable contamination by plant cells. Southern hybridization using probes for chloroplast marker genes were performed to look for the presence of plant root plastids. DNA from soybean leaves, soybean roots and bacteroids of *B. japonicum* USDA 110 were loaded on to electrophoresis gels. The DNA

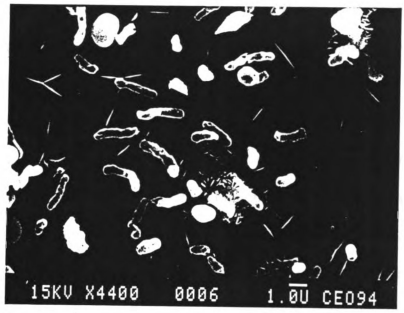


Figure 2.1: Scanning electron micrograph showing a typical field in the bacteroid preparation from soya bean root nodules. The sharp needle-shaped structures are sucrose crystals. Note the typical elongated, cigar and spherical shapes of bacteroid forms of *Bradyrhizobium japonicum.*

isolated from both soybean roots and leaves gave positive signals with chloroplast *pet A* gene probe (Figure 2.2, panel A, lane 1 and lane 2). In contrast, no signal was detected in the DNA isolated from USDA 110 bacteroids (Figure 2.2, panel A, lane 3). When DNA samples were hybridized with the probe for the rhizobial *nif* gene, only the DNA from bacteroids gave a positive response (Figure 2.2, panel B, lane 3). Since the *pet A* gene is highly conserved in different plastids (including chloroplasts) among various plant species, the conclusion could be made that no intact plastids were present.

To exclude the possibility that the bacteroids were contaminated with plant lipid vesicles from broken plastids, a proton NMR (Figure 2.3A) spectrum of the lipids was compared to that (Figure 2.3B) of an extract of the supernatant which contained plant lipid vesicles that could not be pelleted at the centrifugation speeds used for bacteroid isolation. Some major difference were observed in the two spectra. One was the presence of several signals between 6 and 8 ppm (attributable to vinyl protons) in the spectra of the bacteroid lipid extracts (Figure 2.3A) but were absent from the supernatant extract (Figure 2.3B). Other major differences were observed in the region between 2.8 and 5 ppm. Signals corresponding to carbohydrate headgroup of lipids in this region were predominant in the bacteroid lipid spectra. However, some other signals in this region were present in the supernatant extract but were absent from that of the bacteroid extract. These included (Figure 2.3B) a doublet at 4.1ppm, another doublet at 5.4 ppm and some signals between 3 to 4 ppm. All these results conclusively ruled out the contamination of plant lipid vesicles which contained lipids of broken plastids.

The glycosyl components of the lipids of bacteroid samples were identified by GC

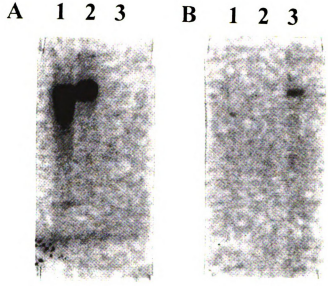
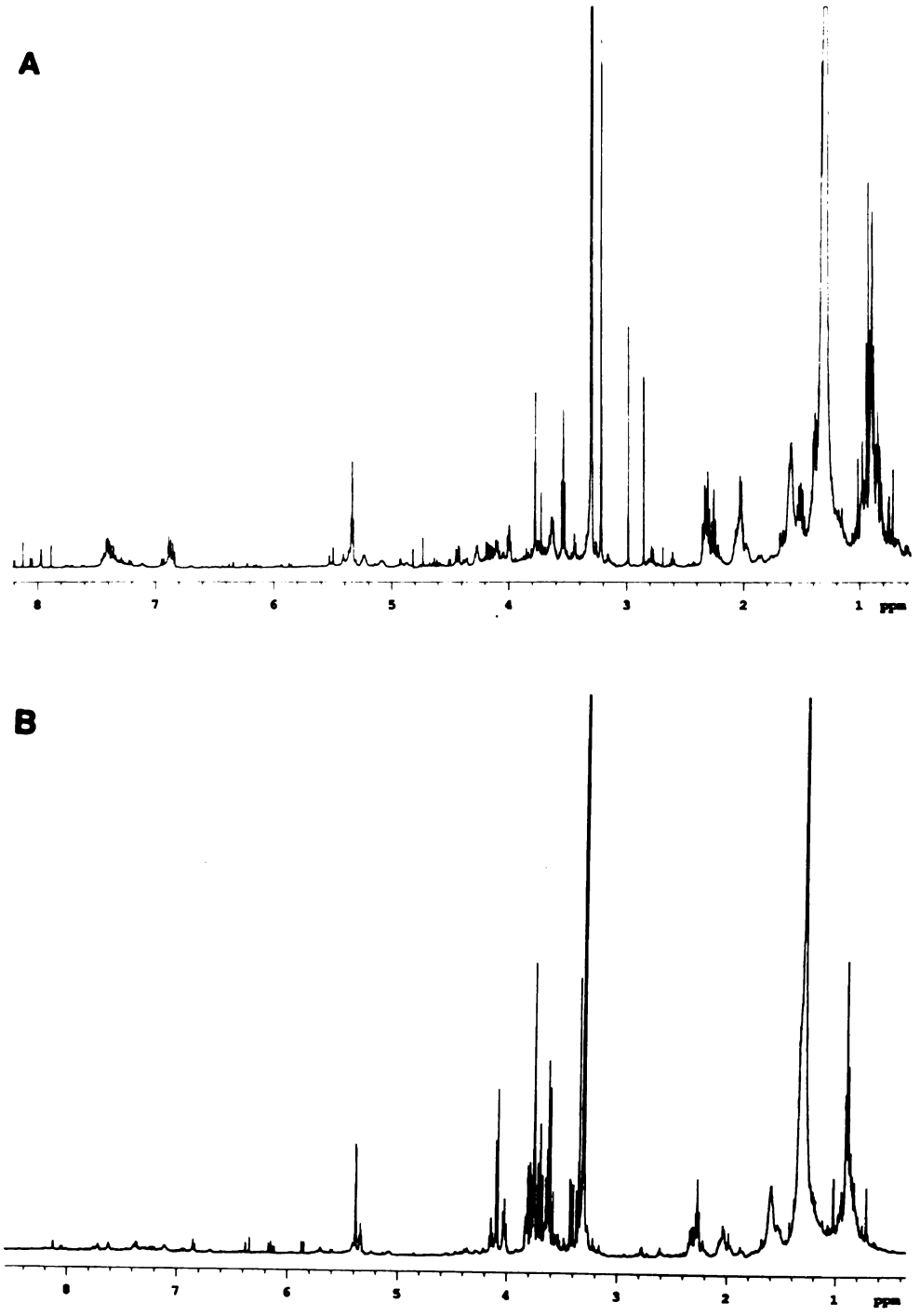


Figure 2.2: In both panels, Lane 1: Soybean leaf DNA; Lane 2: Soybean root DNA; Lane 3: USDA 110 Bacteroid DNA. Panel A: Hybridized with a probe from a clone pOj116 containing the chloroplast *pet A* gene which is highly conserved in different plastids (including chloroplasts) among various plant species. B: Hybridized with a probe from clone pRS2 containing rhizobial *nif* gene.

Figure 2.3: Proton NMR spectrum of lipids from bacteroids (A) and from extract of the supernatant which contained plant lipid vesicles (B).


A
8
7
6
5
4
3
2
1
ppm
B
8
7
6
5
4
3
2
1
ppm

/ MS after converting them to alditol acetate derivatives. Glycerol and galactose were the primary components of these lipid preparations (Figure 2.4). Gas chromatography of the alditol acetate derivatives from chloroform extracts of plant debris and the supernatant indicated that no glycolipids were recovered from the debris (Figure 2.5A) and that glycerol and glucose were the primary components in the supernatant (Figure 2.5B). Glucose is a typical component of glycosyl ceramides, the predominant sphingolipid in plant tissue. These results demonstrated that the galactose in the bacteroid sample did not come from plant material and rule out the possibility of contamination of the bacteroid sample by plant lipids. Gas chromatography of fatty acid methyl ester derivatives from the same 3 samples (Figure 2.6A-C) also supported this conclusion. The chromatogram of the bacteroids sample (Figure 2.6 C) indicated the presence of only fatty acid methyl esters and there were no peaks for any of the long retention time components (presumably steroids and sphingosine component of glycosyl ceramides) that the chromatograms from the debris (Figure 2.6A) and supernatant (Figure 2.6B) contained.

The bacteroid total lipid extracts were analyzed by fast atom bombardment mass spectrometry (FAB-MS). The spectra of the lipids from the USDA110 bacteroids (Figure 2.7A) and untyped bacteroids (Figure 2.7B) contained quite intense signals clustered at m/z 941 and 947 respectively. These masses are uncharacteristic of any phospholipids but corresponded to diglycosyl diacylglycerols containing two octadecanoyl chains in the case of the 947 ion and a similar lipid but with a total of 3 double bonds between the two fatty acids in the case of the 941 ion. Since galactose was essentially the only significant hexose component of these lipid preparations, it followed that such a glycolipid would have to be

Figure 2.4: Gas chromatogram of alditol acetate derivatives of the headgroup components from the total membrane lipid preparation of *Bradyrhizobium japonicum* bacteroids. The peak labeled A is from glycerol and the one labeled B is from galactose. Note that glucose, a typical component of glycosyl ceramides (predominant sphigolipid in plant tissue) is absent. The elution position of glucose is indicated by arrow.

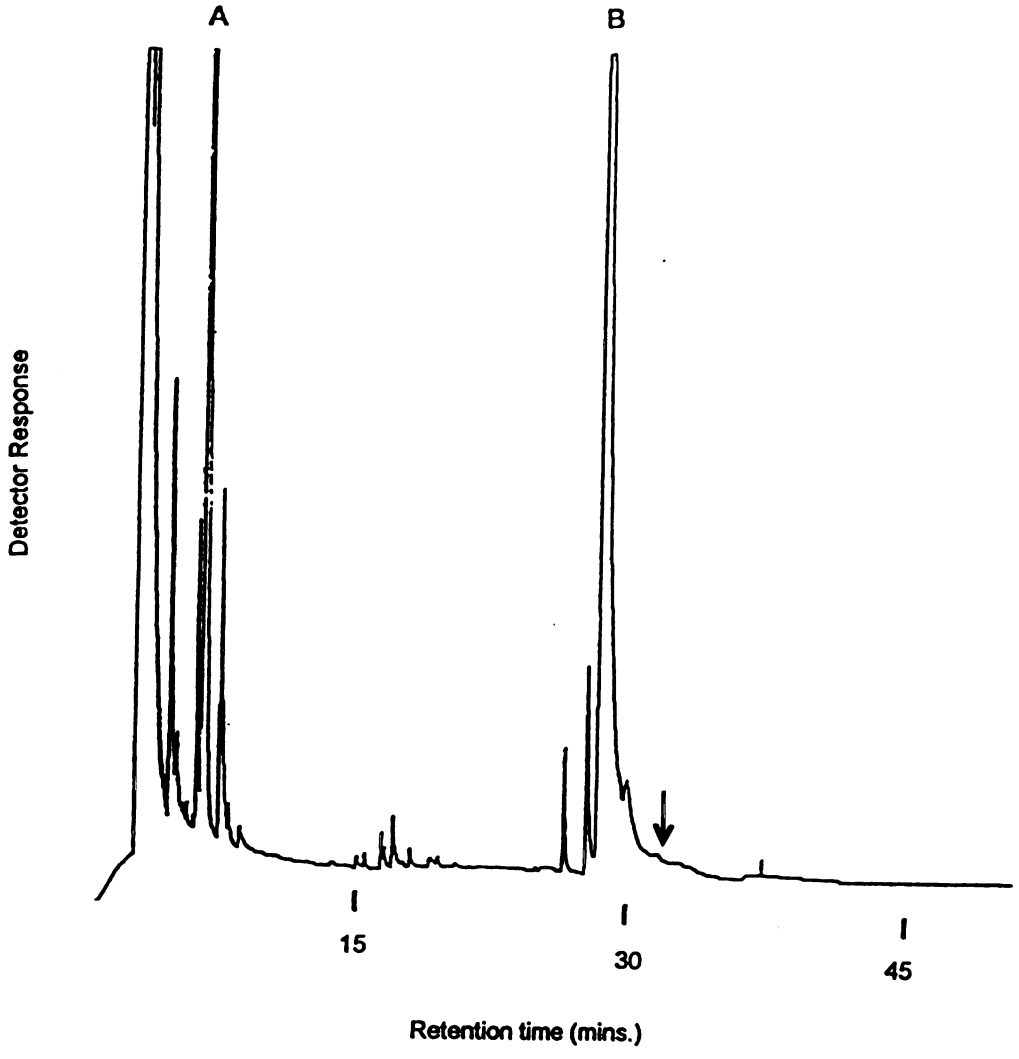

A
B
Detector Response
15
30
45
Retention time (mins.)

Figure 2.5: Gas chromatogram of the alditol acetate derivatives of the residue (A) and of the supernatant from the bacteroid extraction procedure. It was found that glycerol and glucose were the primary components in the supernatant.

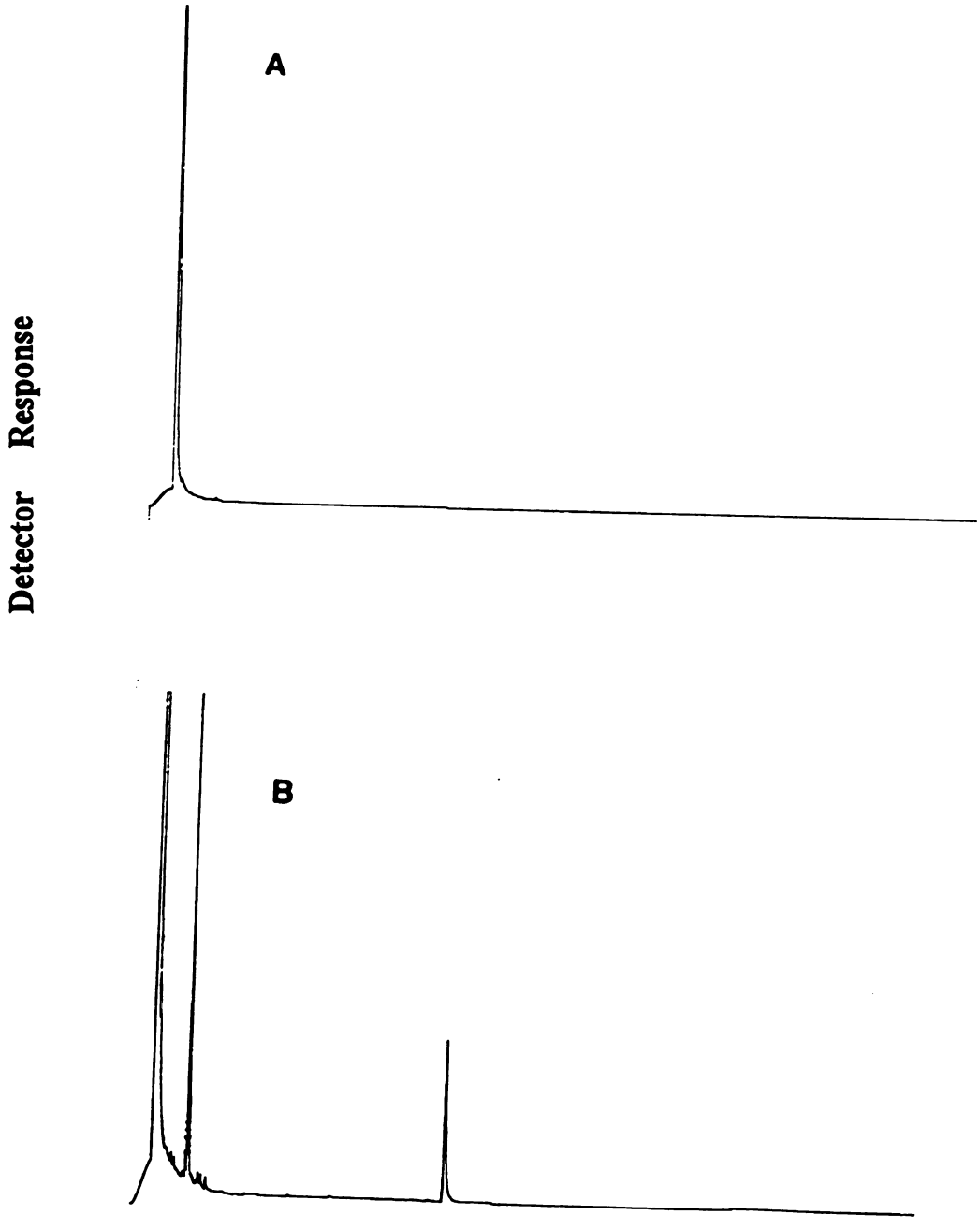

Detector Response
A
B

Figure 2.6: Gas chromatogram of the chloroform-soluble fraction (A) after acid-catalyzed methanolysis of the residue, the supernatant (B) and the bacteroid preparations (C). Note that the bacteroids do not contain any of the long retention time components (presumably steroids and sphingosine component of glycosyl ceramides) that the residue and supernatant contain. Only fatty acid methyl esters were present in the bacteroid sample. The major fatty acids were labeled in all three figures.

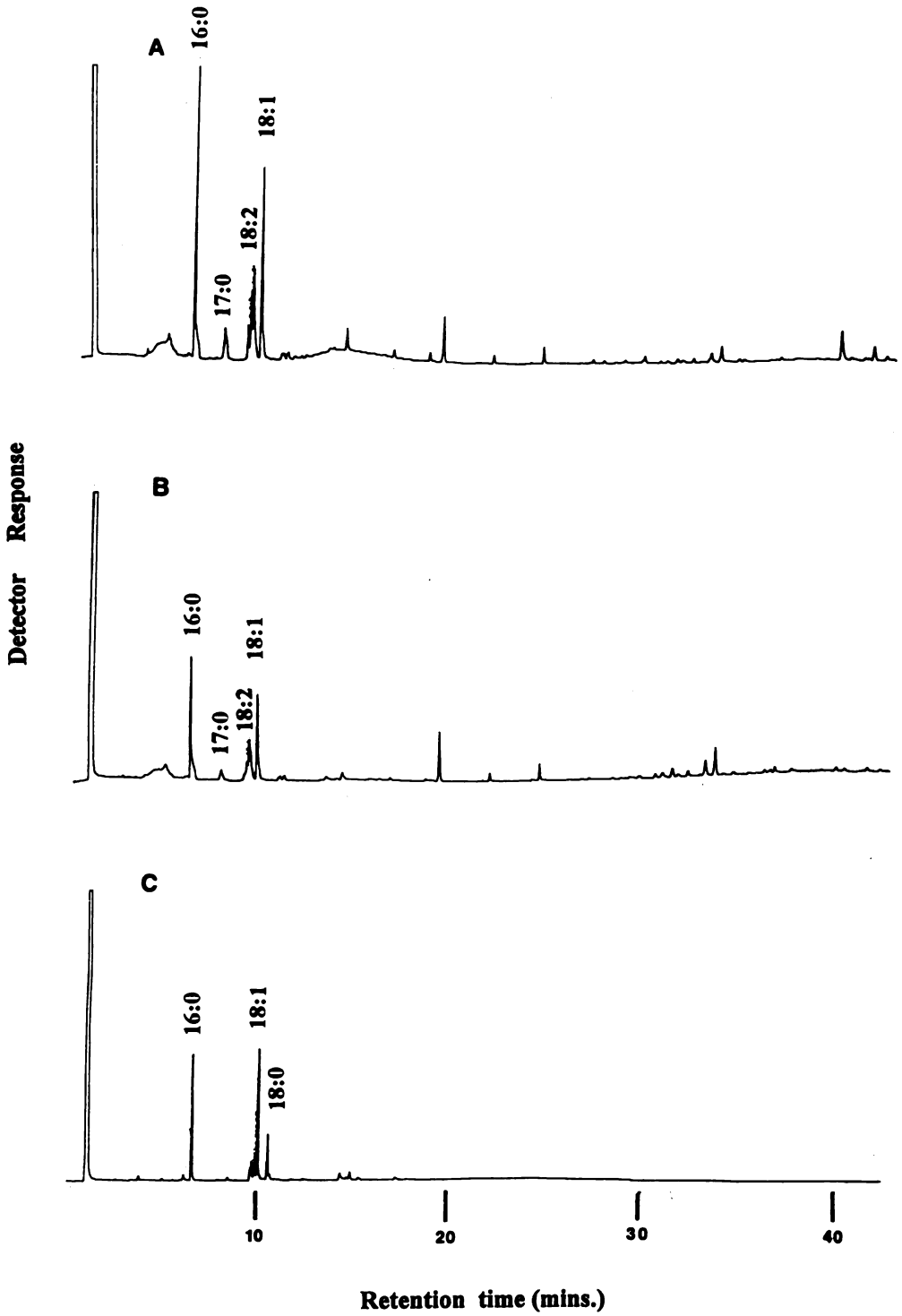
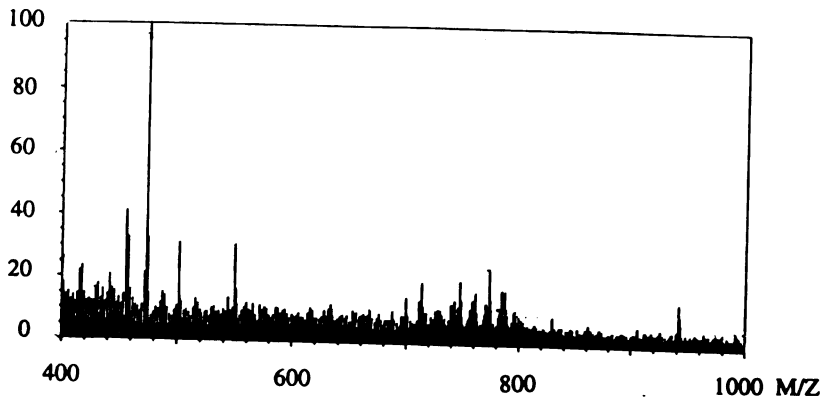
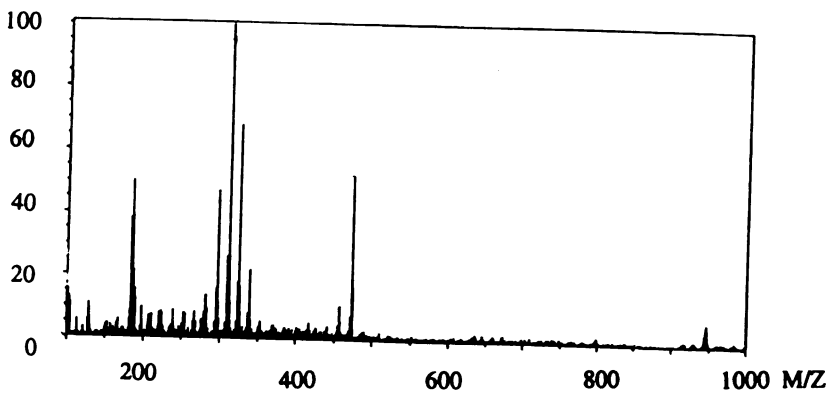

A
16:0
18:1
18:2
17:0
B
16:0
18:1
17:0
18:2
C
16:0
18:1
18:0
Detector Response
10
20
30
40
Retention time (mins.)

Figure 2.7: Negative-ion FAB-MS of the total membrane lipid extracts from *Bradyrhizobium japonicum* USDA 110 bacteroids (A) (matrix: glycerol), untyped bacteroids (B) (matrix: Glycerol) and untyped bacteroids C (matrix: triethanolamine).

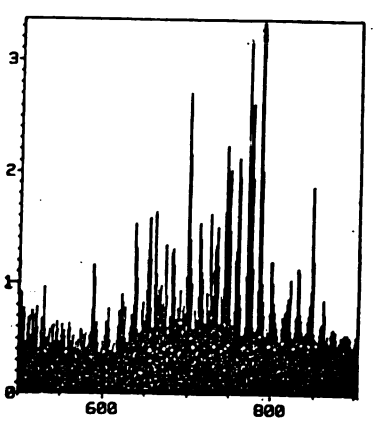
A


100
80
60
40
20
0
400
600
800
1000 M/Z

B


100
80
60
40
20
0
200
400
600
800
1000 M/Z

C


3
2
1
0
600
800

a DGDG. Signals for phospholipids were completely suppressed in the MS spectrum of the untyped bacteroid lipids but could be observed in those from the *Bradyrhizobium japonicum* USDA 110 bacteroid lipids. In both spectra, signals for SQDG were not very prevalent in the positive ion FAB spectra but were major ions in negative ion spectra when triethanolamine was used as the matrix (Figure 2.7C). Previous studies (10-11) demonstrated that the predominant membrane phospholipids of free-living *Bradyrhizobium japonicum* were PC, PE and PG. TLC analysis of the total lipid extracts from two bacteroids (untyped and USDA 110) and the vegetative, free living bacteria showed that there were major compositional differences among the three extracts (Figure 2.8). Spots corresponding to PC, PE and PG are observed in all three extracts. The striking difference between the two bacteroid extracts and free-living bacterial extract was the presence of a very slow migrating component in the extracts from the bacteroids. The R_f values were very similar to that of a commercial standard of DGDG (Figure 2.9). There was considerable streaking indicating a fair degree of heterogeneity in the fatty acid chains. This was also noticed for the commercial standard. In both bacteroid extracts, there was a fast moving component (which also appeared as a streak in USDA110 bacteroid extracts) which was identified as poly 3-hydroxybutyric acid by NMR spectroscopy. The slow moving component on TLC of the untyped bacteroid sample was isolated by using both preparative silica gel TLC and reverse phase adsorption chromatography employing a step gradient. The FAB mass spectra of the isolated component contained the cluster of signals at m/z 947 which was assigned to diglycosyl diacylglycerol. Compositional analysis confirmed the presence of glycerol and galactose as the sole polar components.

Figure. 2.8: Orcinol-sprayed TLC plate of membrane lipids extracts. DGDG is the authentic standard of digalactosyl diacylglycerol. The last three lanes contain membrane extracts from untyped bacteroids, USDA 110 bacteroids and free living USDA 110, respectively.

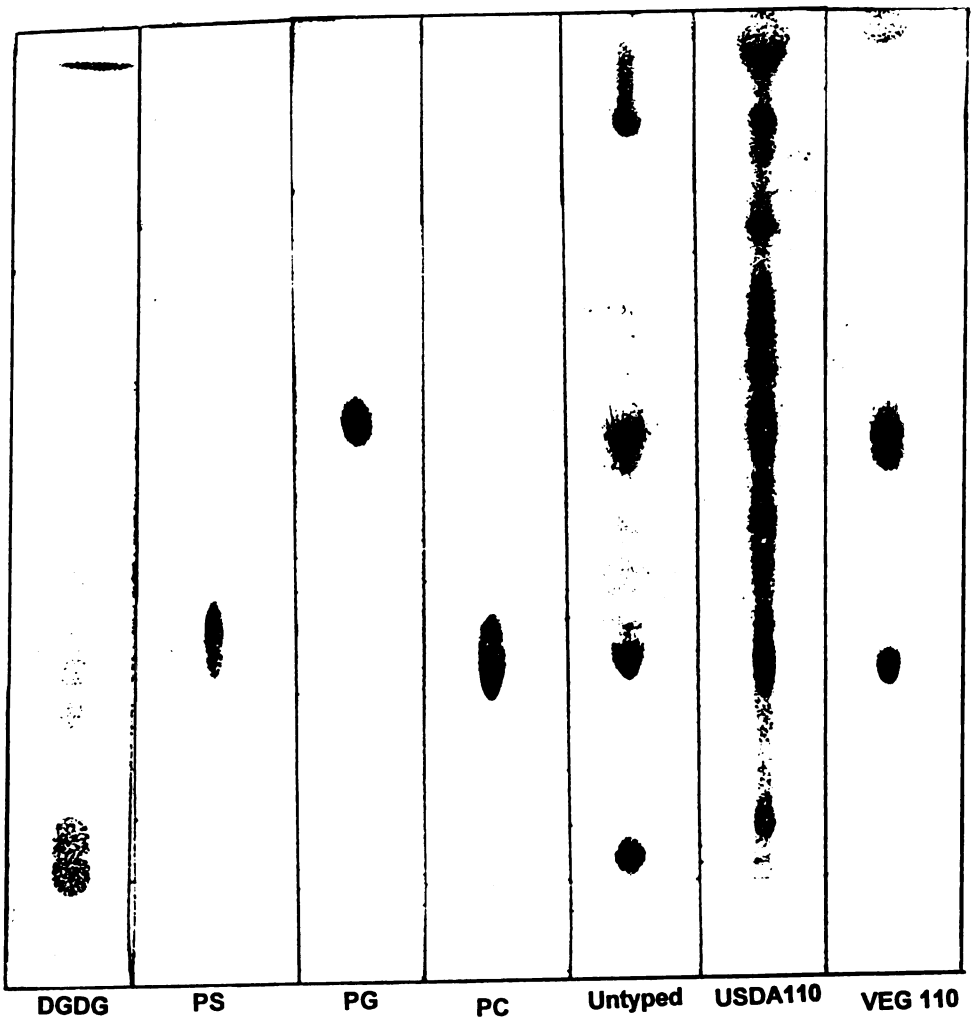
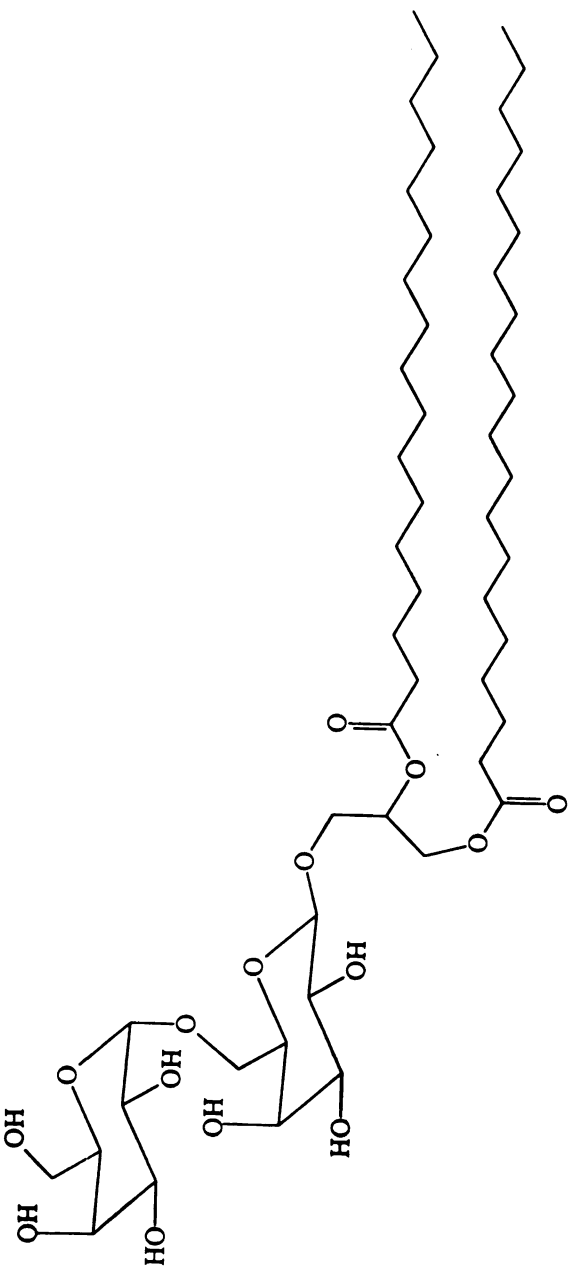

DGDG
PS
PG
PC
Untyped
USDA110
VEG 110

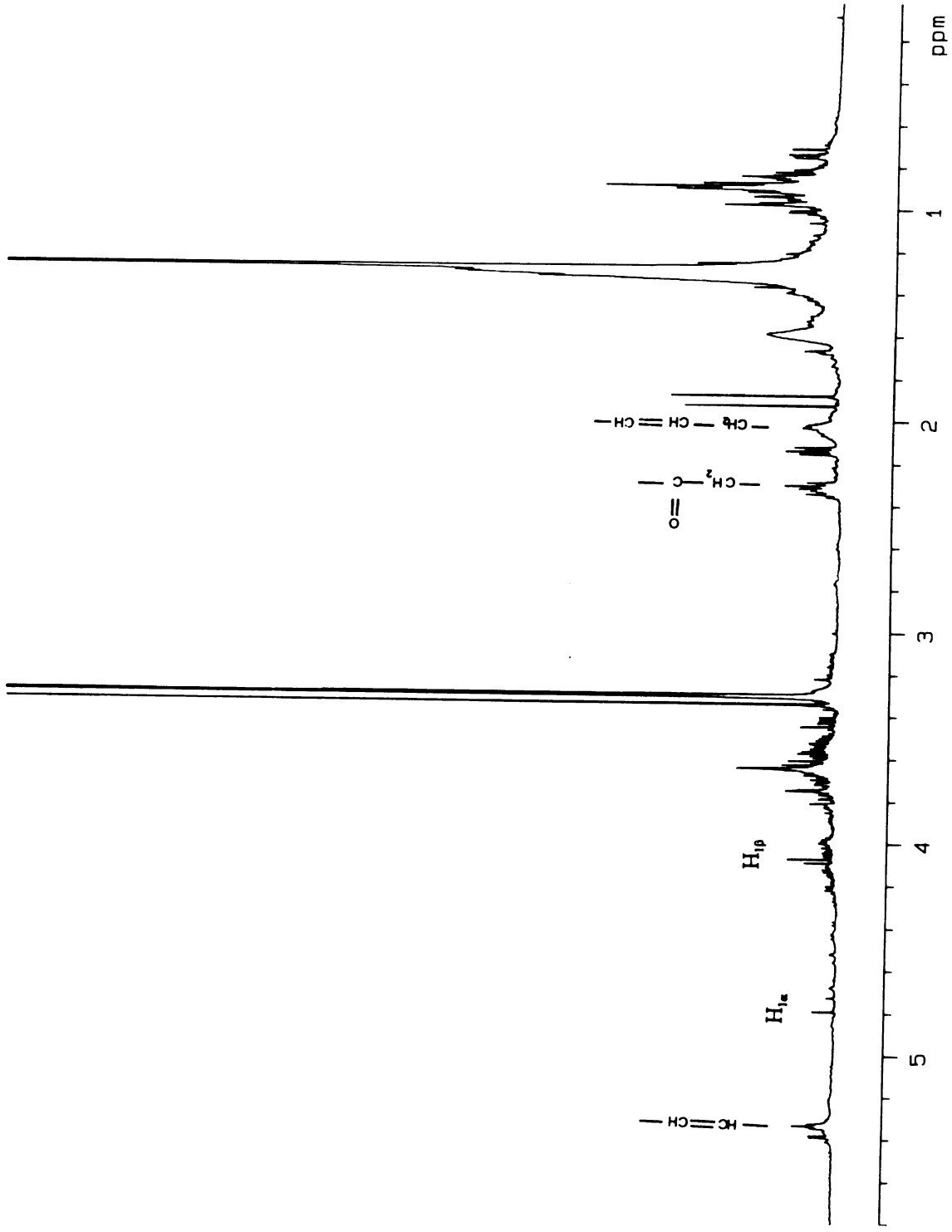
Figure 2.9: The structure of isolated DGDG.



The linkage of the two galactosyl residues in the galactose-containing glycolipids was determined by methylation analysis according to published procedures (7). This analysis clearly indicated the presence of one non-reducing terminal and one 6-linked galactose residue. This is the usual linkage of galactose in plant-derived DGDG. To confirm the anomericity (α vs β) of the glycosyl linkages and the site of acylation, a proton NMR spectrum (Figure 2.10) of the isolated DGDG from strain 110 was obtained. One signal, a doublet at 4.80 ppm with J ~ 2 Hz, was assignable to an α anomeric proton. Another doublet at 4.11 ppm with J = 8 Hz was assignable to the β anomeric proton. These were in reasonable agreement with the positions for these signals in a standard spectrum of DGDG in which the α proton signal was at 4.85 ppm and the β proton signal was at 4.23 ppm. The fatty acid compositions of the standard and the isolated sample are very different with the former being rich in C18:3 fatty acids. This might give rise to slightly different conformations of the head group and to slightly different chemical shifts of the anomeric protons. This result, however supportive of the notion, is not a definitive proof of which galactose unit has the α configuration and which has the β. Such a study would necessitate degradative (chemical) or NMR spectroscopy n.O.e studies. These were precluded because of the very limited amount of material that can be isolated and the very small amounts that remain after purification. The information we can extract from the data and TLC comparisons with a standard, however, strongly indicates that the configurations are the same as that found in the typical plant glycolipids.

Since all of the lipids present in the membrane of *Bradyrhizobium* contain one molecule of glycerol (except for phosphatidyl glycerol which has two) it was possible to

Figure. 2.10: Proton NMR spectrum of the isolated digalactosyl diacylglycerol.


ppm
1
2
3
4
5
$H_{1\beta}$
$H_{1\alpha}$
HC≡CH—
—CH₂—C(=O)—
—CH₂—CH=CH—

make an estimate of the proportion of DGDG that was present from the relative amounts of galactose and glycerol (Figure 2.4). This comparison gave a result of 25-30% DGDG. This is a minimum value. The actual value should be even higher since one molecule of PG contains two molecules of glycerol.

DISCUSSION

A dramatic change in chemistry in the transition from vegetative cells to bacteroid forms has been suggested in several chemical and immunochemical studies. In one study, attempts to isolate LPS from the bacteroid forms of cells were unsuccessful, indications being that only low molecular weight glycolipids were present (12). Several groups have now defined antigenic changes in rhizobial cell surface chemistry concurrent with the transformation to bacteroid forms (13-18). The finding that SQDG and DGDG become major membrane components in bacteroid forms of *Bradyrhizobium* (and probably other rhizobia) is very consistent with the apparently seamless interfacing of plant and bacterial physiology during the *Rhizobium* / legume symbiosis. Important questions now arise as to how critical to symbiosis is the ability of *Rhizobium* to synthesize these glycolipids. Questions also arise as to the degree of coupling (common pathways) between the biosynthetic routes for these molecules and other bacterial carbohydrate-containing molecules such as EPS and LPS. It should be pointed out that, barring gene redundancy, mutants such as an *R. meliloti exo* B mutant (19) and some *Rhizobium* LPS mutants (20-21), which appear to be impaired in synthesizing galactose, should also be defective in synthesizing DGDG. Which then is the critical deficiency, the impairment of polysaccharide synthesis or of DGDG synthesis?

DGDG and SQDG are characteristic plastid lipids. Plastids, according to endosymbiotic theory, are originated from a symbiotic photosynthetic bacterium. *Rhizobium* has similar membrane chemistry to plant organelles and might actually be a (usually

extracellular) nitrogen-fixing counterpart to the carbon dioxide fixing chloroplast. The lipid composition of bacteroids might be a necessary prerequisite for reaching or maintaining a symbiotic state without triggering plant defense responses. These findings might also explain why some rhizobial symbiotic mutants which are impaired in LPS or EPS biosynthesis (and then maybe also DGDG or SQDG) are incapable of being released into nodules to become bacteroids.

The results of this study are also directly relevant to certain evolutionary questions. There has been much debate on whether horizontal (lateral) gene transfer occurs between distinctly different organisms (22). *Rhizobium* is a case in point since it was discovered that the sequence of one of the glutamine synthase genes of *Bradyrhizobium japonicum* was consistent with it being transferred directly from the plant to the bacterium at some stage during their co-development (23). This possibility has been discussed from several perspectives but the case for gene acquisition by horizontal gene transfer, is still quite strong (16). The ability of *Rhizobium* to somehow synthesize the plant lipids described here would seem to represent another very strong case for invoking horizontal gene transfer. This should not be too surprising considering how ecologically and developmentally close the two organisms in question are.

There is one other hypothesis that can be advanced to explain the presence of DGDG in bacteroid membranes. One can invoke parallels with well studied host - pathogen systems in which there is direct transfer of lipids from the host to the pathogen or parasite. This phenomenon has been demonstrated in several host-pathogen (parasite) systems such as rat - *Hymenolepis diminuta* (24), human-*Plasmodium falciparum* (25), human-*Schistosoma*

mansoni (26) and snake-*Spirometra erinacei* (27). In the case of *H. diminuta*, the abundant lipids of the adult parasite contain predominantly unsaturated fatty acids which were obtained from the host intestinal cells. In this case, the parasite has, apparently, lost its capacity for synthesizing these unsaturated fatty acids, transferring these lipids from its host is essential for the biochemical adaptations of the adult parasite to its anaerobic existence in the rat intestine. In the human-*Plasmodium falciparum* interaction (malarial infections), the invading pathogen is incapable of synthesizing its own fatty acids and cholesterol and relies on the host for these. This requires that phospholipase activities in the cell of the invading organism be capable of degrading the host cell erythrocyte membrane making the lipid components available. Another human parasite, *S. erinacei*, must undergo both morphological and biochemical changes to adapt to the host environment (c.f. bacteroid formation in *Rhizobium*). Developmental stage- dependent utilization (uptake, processing and incorporating) of host lipids by these parasites have been indicated to play a role in their evasion of host immunity and to be a requirement for their survival in the new environment. In this scenario the only troubling aspect is that DGDG is not found in the plasma membrane of plant cells (of which the peribacteroid membrane is an extension) but is thought to be restricted to plastids. In view of the large proportion of DGDG present, (over 25% of total lipids) direct lipid transfer from the plant peribacteroid membrane to the bacteria is not very likely but cannot be ruled out. The acquisition of glycosyl transferases by the bacterium from the plant is unlikely since they would have to be transported into the bacterial cell to the inner membrane surfaces where the activated sugars and lipid carrier are.

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CHAPTER 3
THE LIPID CHEMISTRY OF FREE-LIVING AND BACTEROID-LIKE FORMS OF RHIZOBIA

ABSTRACT

The membrane chemistry of vegetative and artificially-induced bacteroid forms of *Bradyrhizobium japonicum* was studied by NMR spectroscopy and mass spectrometry. Culturing *Bradyrhizobium japonicum* in the presence of glucose as a carbon source in a rich medium known to stimulate differentiation into bacteroid forms led to the synthesis of a family of di- and trisaccharide-containing glycerolipids as the main membrane lipid components. This high level of glycolipid synthesis in induced bacteroid-like forms of free-living *Bradyrhizobium* is also of interest since glycolipids are the predominant membrane lipids in plants. These results underline the close developmental tie between these bacteria and their plant host and suggest a real possibility of gene transfer or common gene usage between these plants and their bacterial symbiont at some stage of their co-development.

INTRODUCTION

Establishment of an effective symbiosis between *Rhizobium* and legume plants is critical to the available nitrogen balance for living systems. It is a process of cellular interaction leading to compatibility and shared resources between these organisms. The development of a new plant organ (nodule) and maturation of bacteria to bacteroids inside nodules seem to be regulated at all stages by a diversity of bacterial factors. These include bacterial surface glycoconjugates (including integral membrane components such as LPS and glycolipids), EPS and nod factors (Chapter 1).

The cell surface of *Rhizobium* is a dynamic entity whose chemistry and antigenicity is dependent on environmental factors such as oxygen tension and pH. This has been demonstrated by both chemical and immunochemical analysis (1,2). The environmental conditions inside root nodules are very different from those experienced by free-living bacteria (3,4). The process of differentiation from bacteria to nitrogen-fixing bacteroids is associated with many morphological, biochemical and presumably, cell surface compositional changes. Bacteroids are more sensitive to detergents and lysozymes, have a smaller amount and/or a changed composition of LPS (5, 6-8) and usually have lower levels of EPS (9). Some of the changes, in LPS and EPS, for example, can be simulated by growing free-living bacteria under appropriate culture conditions, such as low oxygen , low pH, a special carbon source, or combinations of these conditions (Chapter 1). The rationale for such experiments is that these conditions are very similar to those experienced by

bacteroids inside the nodule although the latter environment is sure to be much more complicated.

Results from studies described in Chapter 2 showed that true bacteroids of rhizobia, isolated from plant root nodules, contained DGDG as the predominant membrane lipids. This raised an important question about the common links between membrane chemistry of *Rhizobium* in its infective or its bacteroid stages and that of its plant host. Since bacteroids are found intracellularly in the plant and since these two symbiotic organisms share ecological pressures such as oxygen availability, pH, temperature, water and nutrient availability, it is not surprising that they share some common membrane and surface features in order for the bacteria to survive the host defense mechanisms. Characterizing the cell surface and membrane chemistry of bacteroids will enable us to obtain a better understanding of what biosynthetic functions are necessary for bacteroid transformation and to make a big step forward in understanding this important and critical cellular differentiation event. *Bradyrhizobium japonicum* is a good system to study because we have investigated the membrane lipid chemistry of the true bacteroids isolated from nodules and *Bradyrhizobium japonicum* can be transformed to bacteroid-like forms by changing environmental conditions (10,11).

At least three previous studies (12-14) have been conducted on the membrane lipid composition of vegetative, free-living *Bradyrhizobium japonicum*. Although there was agreement on the presence of PC and PE, there were still some conflicts with respect to the presence of PG, PI and PS. No glycolipids were reported in these studies. Our studies described in Chapter 2 confirmed the existence of PG, PE and PC. Since SQDG has been

found in the membrane of free-living rhizobia and DGDG has been found to be predominant in the membrane of symbiotic bacteroids, we propose that the proportion of membrane glycolipids in bacteroids is much higher than that in vegetative cells. This makes the membrane chemistry of bacteroids a mimicry of that of the host plant and may be necessary for the bacteroid's transformation and survival. In the experiments described here, we will address this hypothesis by studying the membrane lipid chemistry of induced, bacteroid-like forms of rhizobia.

MATERIALS AND METHODS

Bacterial Culture

B. japonicum USDA 110 was grown at 30°C in modified Bergensen's medium as previously described (15). Type I nutrient enrichment medium was created by supplementing the normal medium with a mixture of 0.1% Casamino Acids, 0.45% sodium succinate, 0.1% yeast extract and 0.4% D-glucose during exponential growth as described earlier (10,11) . Type II enriched medium was created by supplementing the normal medium with all the nutrients when starting the culture. All cultures were harvested by centrifugation at late exponential phase of growth. The pellets were collected and used for membrane lipid extraction.

Lipid Extraction

Lipids were extracted from cells by stirring the pellets with a mixture of chloroform, methanol, butanol and water in the ratio of 2:1:1:4 at room temperature for 24 h. The cell debris was removed by centrifugation and the supernatant was partitioned into two layers. The lower organic layer was recovered and the cell debris was re-extracted with a mixture of chloroform, methanol and butanol in the ration of 2:1:1. The organic layer was again recovered and combined with the one from the first extraction. The combined organic layers were concentrated to dryness on a rotary evaporator at a bath temperature of ~40°C and then dissolved in 1 mL of a 1:1 mixture of chloroform and methanol.

Thin Layer Chromatography

Standard PC and PE were purchased from SIGMA Chemical Co. (St. Louis, MO). The extracted lipids were analyzed by TLC on silica plates using a mixture of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:1:1. After development, the plates were sprayed with orcinol and heated at 120°C. For the detection of glycolipids, the orcinol-treated plates were heated for only 3-4 min over which time only the spots corresponding to glycolipids were visible. These appeared as bright purple against a white background. Prolonged heating for 15 min led to the development of brown spots for the phospholipids. Components that moved too slowly on straight-phase chromatography to be eluted efficiently in preparative separations were isolated by preparative C-18 reverse-phase chromatography using H_2O/methanol (4:1) as an eluent.

Nuclear Magnetic Resonance Spectroscopy

NMR analyses were performed on a Varian VXR-500 spectrometer (500 MHZ for protons) at 25°C in perdeuterated methanol. Chemical shifts are referenced relative to the residual methanol line at 3.30 ppm. Double quantum filtered J-correlated spectroscopy (DQF-COSY) spectrum was recorded using a spectral width of 4748.9. Generally, 32 scans were accumulated in the f2 dimension and a total of 512 spectra transformed in the f1 dimension.

Electrospray Mass Spectrometry

ES-MS spectra was recorded on a Fisons Platform mass spectrometer. The spectra were recorded in the negative ion mode using a capillary voltage of 3 kv. Samples were infused into the instrument in methanol.

Chemical Characterization

The glycosyl components of the lipids were identified by GC after converting them to alditol acetate derivatives. Samples (300 μg) were hydrolyzed with 2M TFA at 100°C for 2 h. After removing the free fatty acids by extraction with chloroform, the aqueous acid was removed under a stream of nitrogen and the free glycosyl components were reduced with sodium borohydride. The alditols thus formed were peracetylated with 0.1 mL of a 1:1 mixture of acetic anhydride and pyridine at room temperature for 24 h. The acetylating reagents were removed under a stream of nitrogen and the residue partitioned between 1 mL of water and 1 mL of chloroform. The lower chloroform layer was recovered and subjected to GC and GC/MS analysis.

GC and GC/MS

Gas chromatography was performed on a Hewlett Packard 5890 gas chromatograph using helium as a carrier and a flame ionization detector. For the separation of alditol acetate derivatives, gas chromatography was performed on a J and W Scientific DB-225 column. The initial temperature was 180°C, the temperature was raised at the rate of 2°C/min to a final temperature of 220°C. The column was held at 220°C for 50 min. GC/MS was performed on a JEOL 505 mass spectrometer using an accelerating voltage of 70 eV.

RESULTS

In our studies, we used bacteroid-inducing media as demonstrated by others (10, 11). Electron micrographs of *Bradyrhizobium japonicum* USDA 110 grown in regular B-III media (Figure 3.1A) and in bacteroid-inducing media (Figure 3.1B) showed that under the same magnification of the microscope, bacteroid-like forms were longer than those of the control. Pleomorphism was also observed in bacteroid-like forms. These are the characteristic morphological changes the bacteria undergo in the bacteroid-inducing media (10,11).

Culturing *B. japonicum* USDA 110 in bacteroid-inducing media had a dramatic effect on the spectrum of membrane lipids synthesized. Figure 3.2 is a TLC chromatogram of the total lipids from the various bacterial cultures. In panel A are the lipids from the type I and II bacteroid inducing media. Spots with the characteristic purple color of glycolipids were visible after heating the plate for 30 seconds after spraying with orcinol. Bacteria grown in both type I and type II bacteroid-inducing media synthesized the same two new components. Lanes 1 and 2 of panel B had the same material as in panel A but the plate was heated for a longer period so that all of the lipid components (including phospholipids) were detected. Lane 3 was a lipid preparation from vegetative cells. Lanes 4-5 are phospholipid standards. The spots indicated by arrows were due to glycolipids. The lower one was barely visible when the plate was heated for 30 seconds (panel A). These glycolipids are not synthesized in un-induced cells but are major lipids in the bacteroid forms.

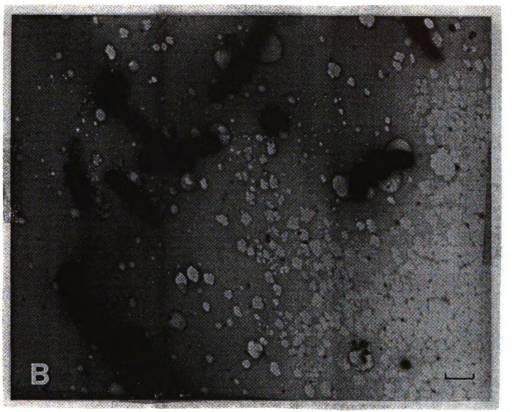


Figure 3.1A: *Bradyrhizobium japonicum* USDA 110 grown in regular BIII medium. The sample was negatively stained with 1.5% potassium phosphotungstate. Bar=1.8 μm.

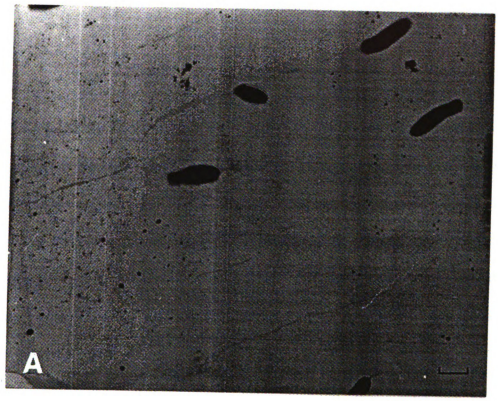
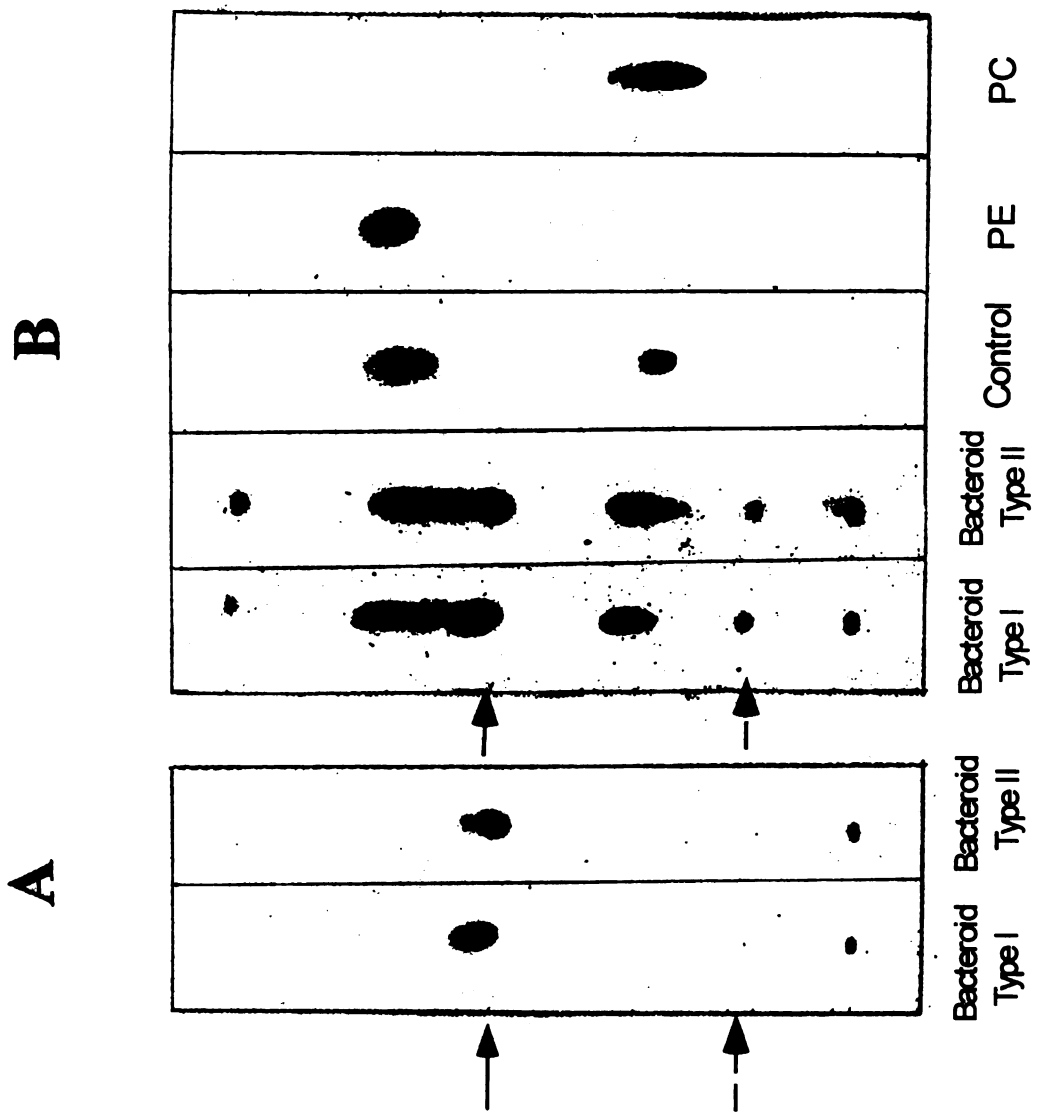


Figure 3.1B: *Bradyrhizobium japonicum* USDA 110 grown in bacteroid-inducing medium. The sample was negatively stained with 2% ammonium molybdate. Bar=1.8 μm.

Figure 3.2: Thin layer chromatograms of lipid extract from cell grown under nutrient enrichment conditions. In panel A the plate was heated for 2 min to reveal only glycolipids. In panel B the plate was heated for 20 min. Panel A lanes 1 and 2 shows the membrane lipids extracts from *Bradyrhizobium japonicum* USDA 110 grown in type I and II enriched media respectively. The first two lanes in panel B correspond to panel A. Arrows indicate the glycolipids. The next lane is a membrane preparation from un-induced cells and the last two lanes are standards.


A
B
Bacteroid Type I
Bacteroid Type II
Bacteroid Type I
Bacteroid Type II
Control
PE
PC

Analysis of the glycosyl components of the total crude lipids extracts from bacteria grown in type I and type II media using alditol acetate derivatives showed that glucose was a major polar component of the total lipids (Fig. 3.3). There was also a peak due to the alditol acetate of ribose indicating the presence of a ribosylated glycolipid. The faster-moving glycolipid components in lanes 1 and 2 of panel A were isolated by preparative thin layer chromatography on silica. Gas chromatography and GC/MS analysis of alditol acetate derivatives revealed that glucose and glycerol were the only polar components present. Proton NMR spectroscopy analysis (Fig. 3.4) indicated that the component was the same for both lipid preparations. Two signals (doublets at 4.30 and 4.38 ppm with 8 Hz coupling constants) were readily assignable to β-anomeric protons. Also readily assignable were signals due to a glyceryl moiety. These were a multiplet at 5.28 ppm due to H2 and a doublet of doublet at 4.43 ppm, with 14 Hz and 3 Hz coupling, due to one of the two H1 glyceryl protons. The other H1 glyceryl proton appeared at 4.20 ppm overlapping with some other signals but its connectivity could be traced through the DQF-COSY spectra (Fig. 3.5). The other signals could also be readily assigned from the ^{1}H-NMR DQF-COSY spectra. The presence of two signals for β-anomeric protons in the NMR spectrum and glucose and glycerol as the sole polar components of this lipid (by GC-MS analysis) indicated that it was a diglucosyl diacyl glycerol. This was readily confirmed by negative ion electrospray mass spectrometry (Fig. 3.6). In the high mass region of the spectrum, a cluster of ions with individual signals at m/z 887, 901, 915 and 929 corresponding to M-H parent species was detected. These differed by 14 mass units indicating that they are a homologous series of ions and varied only in the lengths of the fatty acyl groups in the different species. The ions

Figure 3.3: Gas chromatography spectrum of alditol acetate derivatives of the total lipid extract from *Bradyrhizobium japonicum* USDA 110 grown under nutrients enriched conditions. Peak A, Glycerol; Peak B, Ribose; Peak C, Glucose.

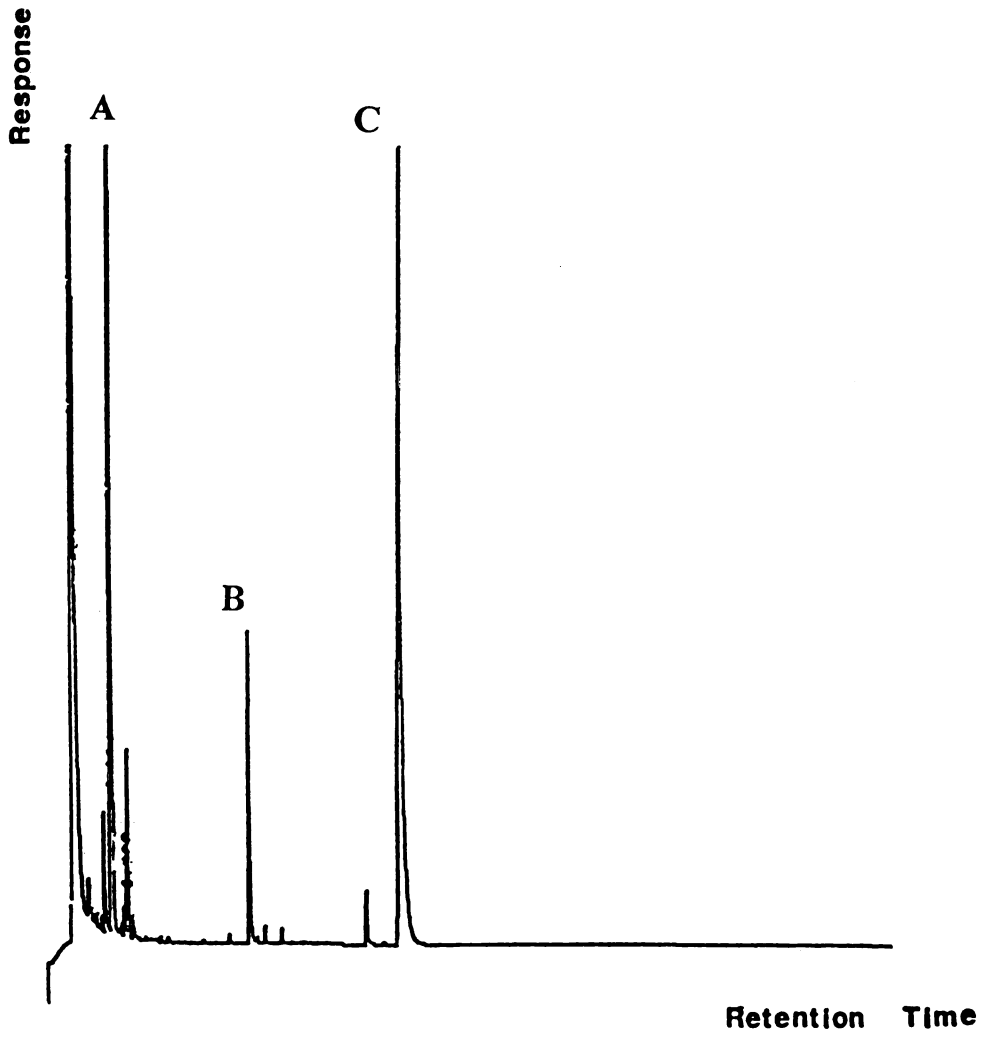

Response
A
B
C
Retention Time

Figure 3.4: Proton NMR spectroscopy analysis of the fast moving component in Panel A of Figure 3.1 indicated that two signals (doublets with 8 Hz coupling constants at 4.30 and 4.38 ppm) were readily assignable to β-anomeric protons. The multiplet at 5.28 ppm was due to H2 and a doublet of doublet at 4.43 ppm, with coupling constants of 14 Hz and 3 Hz, was due to one of the two H1 glyceryl protons.

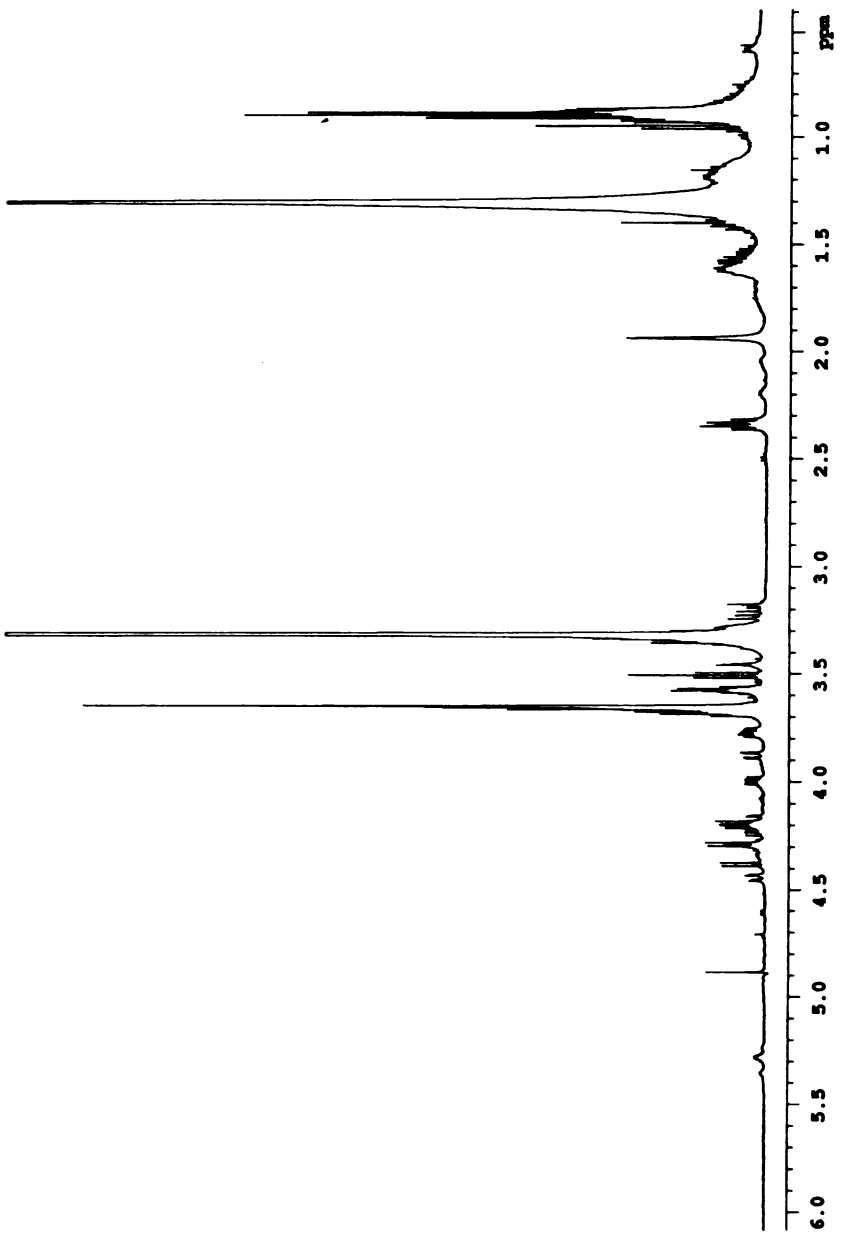

6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

Figure 3.5: The connectivity of H1 glyceryl proton appeared at 4.20 ppm overlapping with some other signals could be traced through the DQF-COSY spectra. Some other signals could also be assigned from this spectra.

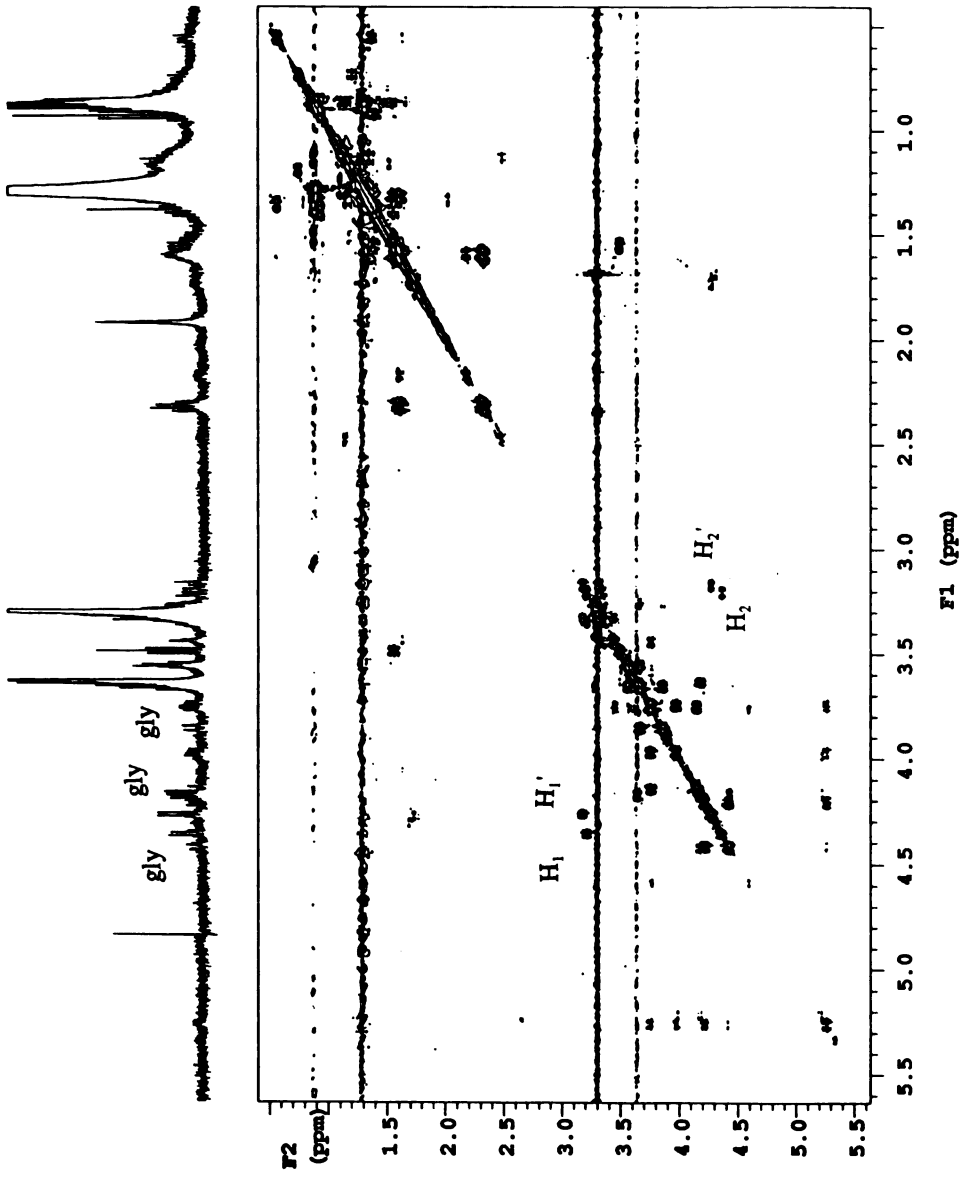
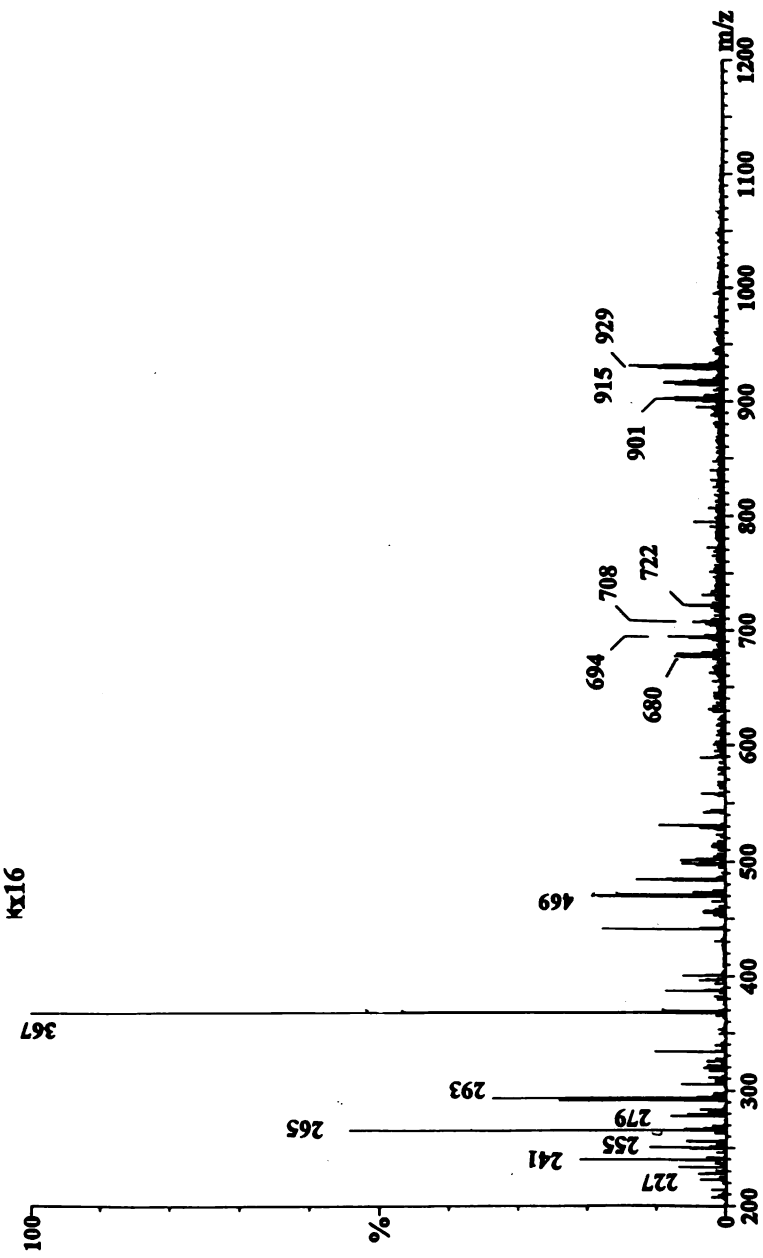

gly
gly
gly
H1
H1'
H2
H2'
F2 (ppm)
F1 (ppm)

Figure 3.6: Negative ion ES-MS confirmed the fast moving component in Panel A of Figure 1 was diglucosyl diacyl glycerol. In the high mass region of the spectrum, a cluster of ions with individual signals at m/z 887, 901, 915 and 929 corresponding to M-H parent species was detected. The ions corresponded to fatty acid chains ranging from 14 to 18 carbons with a maximum of two double bond equivalents due to unsaturation or cyclopropyl groups. In the middle mass region, another cluster of ions with peaks at m/z 680, 694, 708 and 722 were due to loss of acyloxy groups from the parent species. Low intensity fragment ions at m/z 721 and 749, corresponding to loss of one glucose residue from the parent species at m/z 901 and 929 respectively. Most of the ions appeared as doublets. Signals corresponding to fatty acyloxy ions were prominent in the low mass region of the spectrum. These appeared at m/z 227 (14:0), 241 (15:0), 255 (16:0), 253 (16:1), 251 (16:2 or 16:1 cyclopropyl), 265 (17:2 or 17:1 cyclopropyl), 279 (18:2 or 17:1 cyclopropyl), 281 (18:1) 279 (18:2) and 293 (18:1 cyclopropyl).


100
%
0
200
300
400
500
600
700
800
900
1000
1100
1200
m/z
227
241
255
265
279
293
367
469
680
694
708
722
901
915
929
x16

corresponded to fatty acid chains ranging from 14 to 18 carbons with a maximum of two double bond equivalents due to unsaturation or cyclopropyl groups. In the middle mass region, another cluster of ions with peaks at m/z 680, 694, 708 and 722 was also detected. These were due to loss of acyloxy groups from the parent species. Low intensity fragment ions at m/z 721 and 749, corresponding to loss of one glucose residue from the parent species at m/z 901 and 929 respectively, were also observed. Most of the ions appeared as doublets since both saturated and unsaturated forms of most fatty acids were present. Signals corresponding to fatty acyloxy ions were prominent in the low mass region of the spectrum. These appeared at m/z 227 (14:0), 241 (15:0), 255 (16:0), 253 (16:1), 251 (16:2 or 16:1 cyclopropyl), 265 (17:2 or 17:1 cyclopropyl), 279 (18:2 or 17:1 cyclopropyl), 281 (18:1) 279 (18:2) and 293 (18:1 cyclopropyl). These were consistent with the fatty acid analysis. The linkage between the two glycosyl units was determined to be 1,6 from the characteristic fragments at m/z 251 and 281. These are due to the $^{0,3}A_2$ and the cleavage and $^{0,2}A_2$ cleavages respectively (16). The positive mode MS (data not shown) also gave confirmation of the molecular weight.

Both GC-MS analysis and the electrospray mass spectrum (Fig. 3.7) of the slower moving component (isolated by C-18 reverse phase chromatography and by far the minor component) indicated that it was a ribosylated version of the diglucosyl diacyl glycerol. In the high mass region of this glycolipid clusters of ions at 993, 1007, 1021 and 1035 were detected. This sequence corresponded to the diglucosyl diacyl glycerol series plus a ribosyl residue. In this series, the typical number of double bond equivalents in the fatty acid chains was 1. The ribosyl residue was attached to the diglucosyl core. This was apparent since,

except for the parent ions, the other fragment ions corresponded to those from the diglucosyl compound indicating that the 5-carbon sugar was the first to be lost leaving the diglucosyl compound which then further fragments by the known route. For instance, in the middle mass region, the same sequence of ions at m/z 680, 694, 708 and 722 seen in the fragmentation of the diglucosyl compound was also detected. The quantities of this triglycosyl diacylglycerol that were obtained precluded a thorough analysis of the linkage position. The structures of the two glycolipid components are shown as **1** and **2** in Figure 3.8.

Figure 3.7: Negative ion EI-MS of the slower moving component in Panel A of Figure 3.1 confirmed GC-MS analysis that it was a ribosylated version of the diglucosyl diacylglycerol. In the high mass region, a cluster of ions at 993, 1007, 1021 and 1035 were detected, corresponding to the diglucosyl diacylglycerol series plus a ribosyl residue. In the middle mass region, the same sequence of ions at m/z 680, 694, 708 and 722 seen in the fragmentation of the diglucosyl compound was also detected.

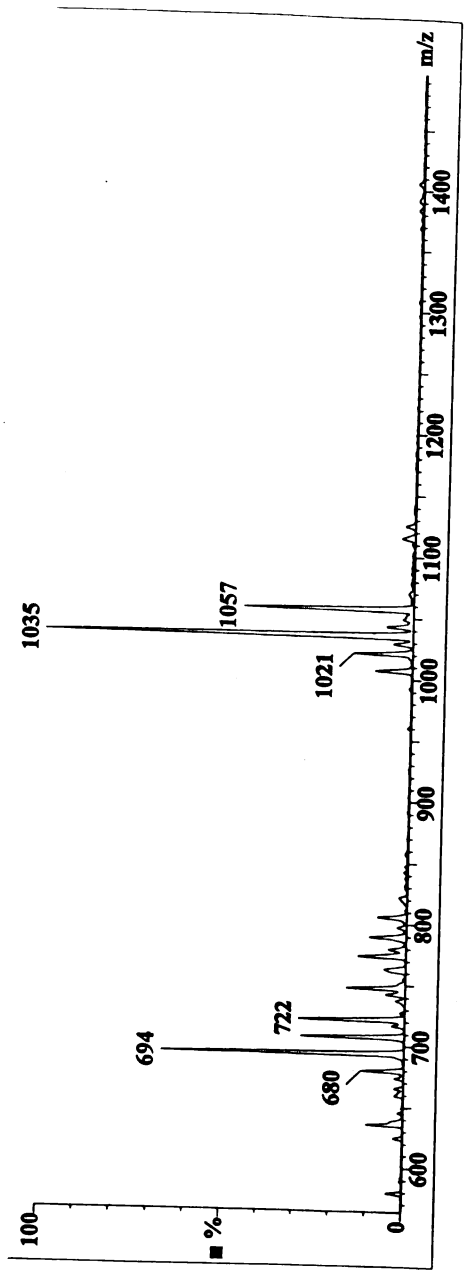
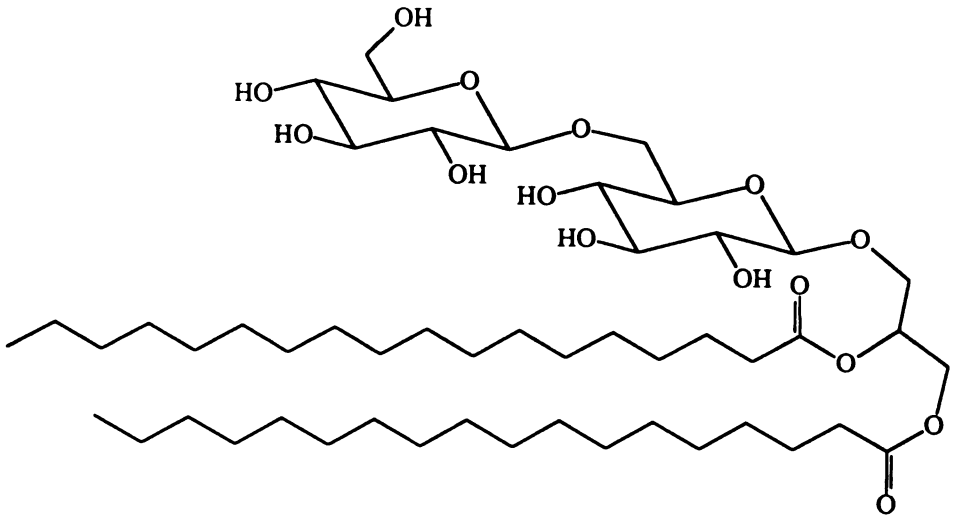
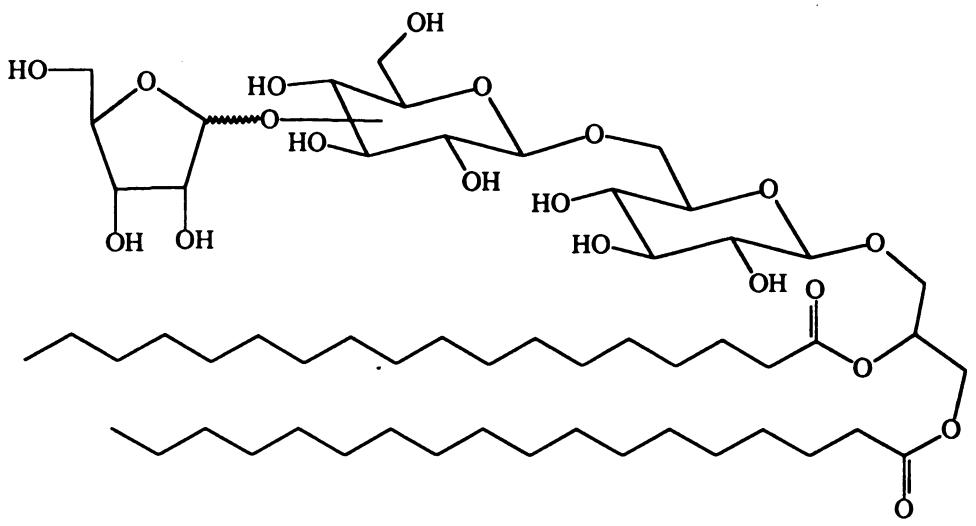

100
%
0
600
680
694
700
722
800
900
1000
1021
1035
1057
1100
1200
1300
1400
m/z

Figure 3.8: The structures of di- and trisaccharide-containing lipids.



1



2

DISCUSSION

The bacteroid models we use here resembled true bacteroids found in nodules since they share certain undefined carbohydrate cell surface antigens with the latter (4). They have similar morphology as true bacteroids and are even capable of fixing nitrogen in their free-living state. The switch by *Bradyrhizobium japonicum* to synthesizing glycolipids during bacteroid differentiation is quite noteworthy especially since the disaccharide is the *gluco*-analog of the common galactose-containing plant glycolipid DGDG and these glycolipids are rarely found outside the plant kingdom (Chapter 1)

The results become even more interesting when considering the finding (described in Chapter 2) that DGDG has also been found to be the predominant membrane lipid of true bacteroids isolated from nodules. It seems that the synthesis of these plant glycolipids are critical to the formation of bacteroids and *ex-planta* bacteroid-like forms of rhizobia. Why the bacteroid-like forms synthesize *gluco*- containing glycolipids instead of galactose-containing ones as synthesized in true bacteroids? One possible explanation was that the glycosyl transferases typically do not have absolute specificity. If UDP-galactose were provided (say from a plant) it is quite possible that this organism could synthesize the *galacto*-lipid instead.

Questions now arise as to whether it is a coincidence that *Bradyrhizobium japonicum* synthesizes large amounts of plant-like lipids in bacteroid forms. Several groups have now defined antigenic changes in rhizobial cell surface chemistry concurrent with transformation

to bacteroid forms (1-4, 8). There is every indication of shared epitopes between bacteroids and plant cell surfaces. This notion and the results of this study are consistent with the apparently seamless interfacing of plant and bacterial physiology during the *Rhizobium*/ legume symbiosis.

ACKNOWLEDGMENT

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CHAPTER 4
THE LIPID CHEMISTRY OF RHIZOBIA UNDER LOW OXYGEN AND LOW pH CONDITIONS

ABSTRACT

Lowering oxygen tension in free-living *Bradyrhizobium japonicum* resulted in a dramatic switch in membrane chemistry in which PC, the predominant lipid in aerated cultures, was no longer synthesized and PE became the major lipid. Besides this change, PI, a typical plant lipid rarely found in bacteria, was also synthesized. Lowering pH in free-living *Bradyrhizobium japonicum* did not result in any change in the lipid headgroups but resulted in a change of the unsaturation state of the fatty acyl groups of the lipids.

INTRODUCTION

Bacteroids live in a microoxic environment. The oxygen diffusion barrier of nodules, in conjunction with the plant leghemoglobin and active bacteroid respiration, creates a low oxygen concentration zone in the infected zone of the nodule. Compared with dissolved oxygen concentration at 250 μm outside the nodule, the oxygen concentration inside nodules is below 30 nm. This microoxic environment is critical for the operations of nitrogen reducing enzyme nitrogenase (1). Besides, the bacteroids live in an acidic environment because of the acidity of peribacteroid fluid (Chapter 1).

Like any other environmental factor, oxygen tension and pH regulate membrane and cell surface chemistry. Both chemical and immunochemical analyses support this (2, 3). In Chapter 2 and Chapter 3, I described the effects of two environments, one inside root nodules and the other bacteroid-inducing medium, on membrane lipid chemistry of rhizobium. Experiments described here were conducted to study the effects of oxygen tension and pH on glycolipid biosynthesis in *Rhizobium*. These are factors a bacterium experiences once it enters the plant and are critical to the adaptability and survival of the bacterium once it is inside of the plant. Low oxygen concentrations and low pH in the media are known to trigger bacteroid differentiation in free-living cultures (2, 3 and Chapter 1). Bacteroids are the nitrogen-fixing form of the bacterium when it resides inside of the plant cell.

MATERIALS AND METHODS

Bacterial Culture

Rhizobium japonicum USDA 110 was grown at 30°C in Modified Bergensen's medium as previously described (4). Low oxygen conditions were obtained by stoppering the four liter Erlenmeyer flasks containing two liter culture media with butyl rubber stoppers at the time of inoculation to restrict oxygen exchange with the atmosphere. The concentration at the time of harvesting the cells was typically between 500 and 1000 ppm as measured by an O_2 electrode. The cultures were harvested by centrifugation at late exponential phase of growth. The pellets were collected and used for membrane lipid extraction. .

Lipid Extraction

Lipids were extracted from cells by stirring the pellets with 160 mL of a mixture of chloroform, methanol, 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 was partitioned into two layers. The lower organic layer was recovered and the cell debris was re-extracted with 240 mL of a mixture of chloroform, methanol and butanol in the ratio of 2:1:1. The organic layer was again recovered and combined with the one from the first extraction. The combined organic layers were concentrated to dryness on a rotary evaporator at a bath temperature of 40°C and then dissolved in 1mL of 1:1 mixture of chloroform and methanol.

Thin Layer Chromatography

Standard PC and PE were purchased from SIGMA Chemical Co. (St Louis,

Missouri). Standard PI and PG were purchased from AVANTI Polar Lipids (Alabaster, Alabama). The extracted lipids were analyzed by TLC on silica plates using a mixture of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:1:1. After development, the plates were sprayed with orcinol or phosphomolybdic acid reagent and heated at 100°C for 15 minutes.

Nuclear Magnetic Resonance Spectroscopy

Proton NMR analyses were performed on a Varian VXR-500 spectrometer (500 MHz for protons) at 25°C in perdeuterated methanol. Chemical shifts are referenced relative to the residual methanol line at 3.30 ppm.

Chemical Characterization

The glycosyl components of the lipids were identified by GC after converting them to alditol acetate derivatives. Samples (300μg) were hydrolyzed with 2M TFA at 100°C for two hours. After removing the free fatty acids by extraction with chloroform, the aqueous acid was removed under a stream of nitrogen and the free glycosyl components were reduced with sodium borohydride. The alditols thus formed were peracetylated with 0.1 mL of a 1:1 mixture of acetic anhydride and pyridine at room temperature for 24 h. The acetylating reagents were removed under a stream of nitrogen and the residue partitioned between 1 mL of water and 1 mL of chloroform. The lower chloroform layer was recovered and subjected to GC analysis.

GC and GC/MS

For the separation of alditol acetate derivatives, gas chromatography was performed on a J and W Scientific DB-225 column. The initial temperature was 180°C, the temperature

was raised at the rate of 2° C/min to a final temperature of 220 °C. The column was held at 220°C for 50 min. GC/MS was performed on a JEOL 505 mass spectrometer interfaced with a Hewlett Packard 5890 gas chromatograph. Spectra were obtained in the positive mode by using electron impact ionization at an acceleration voltage of 70 eV.

RESULTS

Fig. 4.1 was the TLC analyses of the lipids from *Bradyrhizobium japonicum* USDA 110 grown under low oxygen (microanaerobic) conditions and grown under normal aeration. There were major compositional differences between the two. Under the low oxygen conditions, there was a large increase in PG and PE and the amount of PC synthesized was greatly reduced. The most striking difference between the TLC analysis of the two different lipid extracts was the presence of a slow moving component in the extracts from the bacteria grown under microanaerobic conditions. The retention time was very similar to that of the PI standard.

Proton NMR spectroscopy of the total lipid extract from bacteria grown under normal aeration was shown in Fig. 4.2A. It indicated that the major lipid species under normal aeration was PC. This was clear from the presence of strong signals at 3.23 ppm (the N-methyl groups). Also present in the spectrum were signals for PE and the other N-methyl derivatives of PE. Signals for these other N-methyl groups appeared as sharp singlets between 2.7 and 3.3 ppm. The proton NMR spectrum of the total lipid extract from the bacteria grown under microanaerobic conditions was quite different (Fig. 4.2B). Changes observed in the latter were: disappearance of signals corresponding to PC, a large decrease in the peak intensity for the N-methyl groups of mono and dimethyl PE, a large increase in peaks corresponding to PE at 3.18 and 4.07 ppm and appearance of new signals between 3.5 and 4.0 ppm. There were new signals between 3.5 to 4.0 ppm characteristic of a carbohydrate

Figure 4.1: Orcinol-sprayed TLC plate of membrane lipids extracts from the lipid extract of USDA 110 grown both in normal and low oxygen conditions. Lane 1, PG; Lane 2, PC; Lane 3, lipid extract of USDA 110 grown in normal conditions; Lane 4, lipid extract of *B. japonicum* USDA 110 grown in low oxygen conditions. Lane 5, PI; Lane 6, PE. Note that under the low oxygen conditions, PC was no longer synthesized, but there was a significant increase in the amount of PE. The most striking difference between the two different lipid extracts was the presence of slow moving component in the extracts from the bacteria grown under low oxygen conditions. The retention time of this slow moving component was very similar to that of an authentic standard PI.

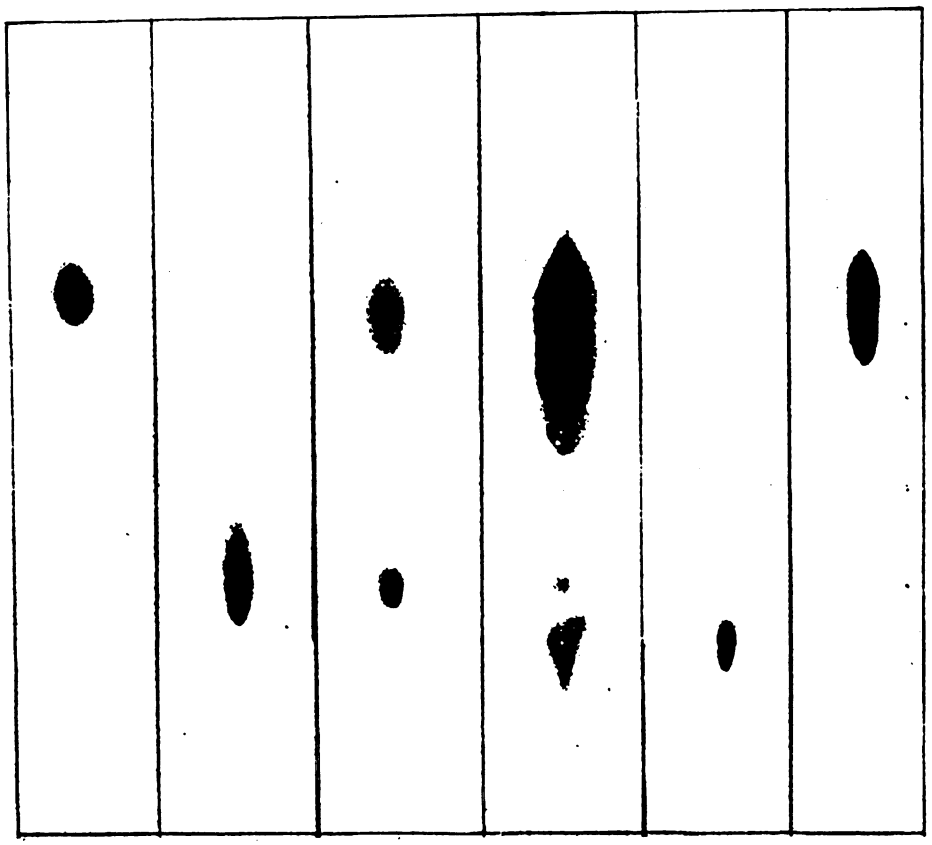
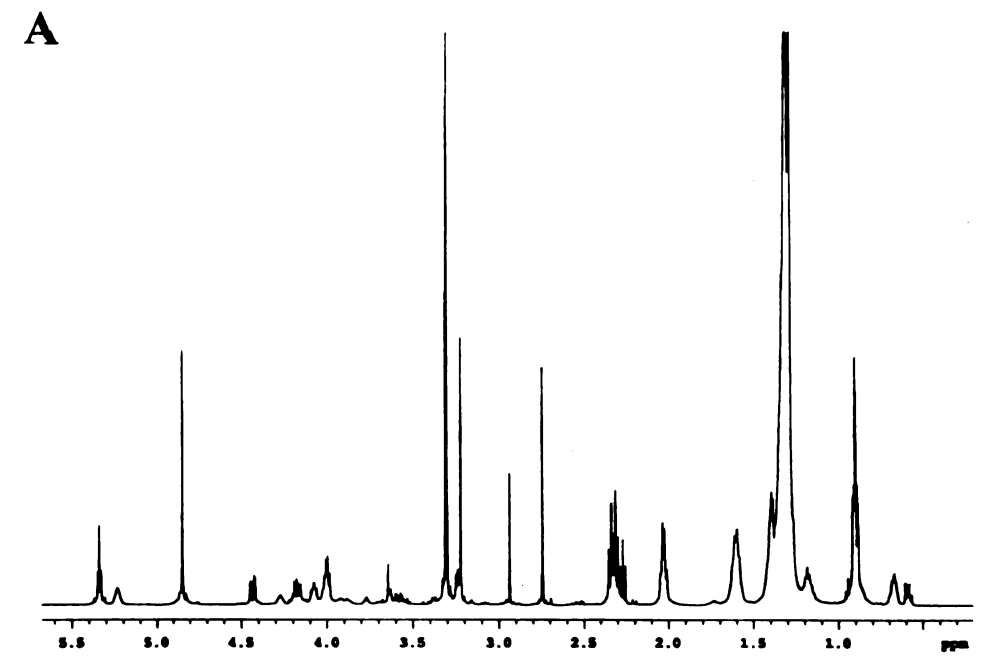
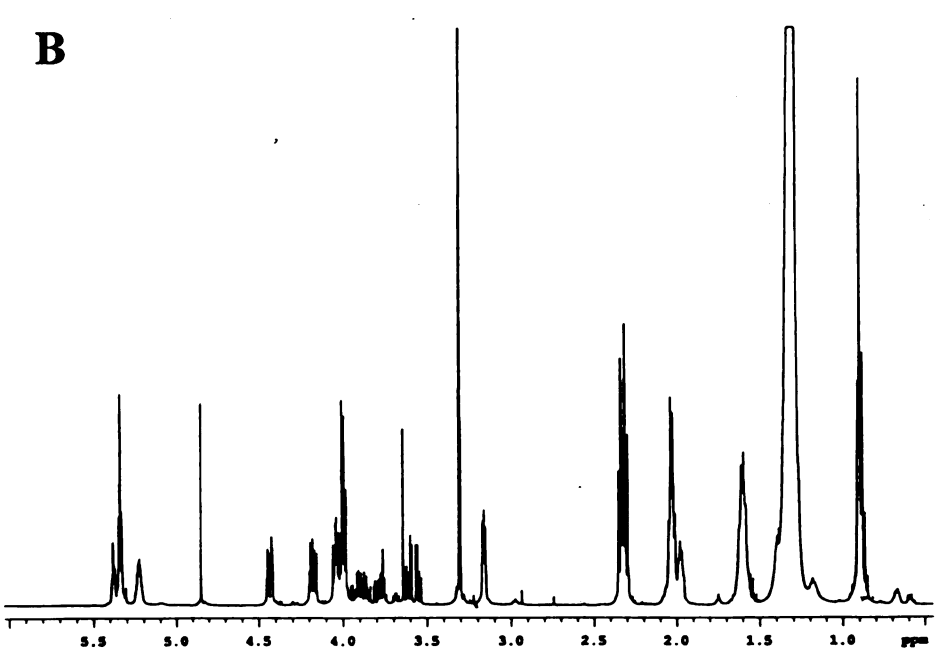

PG
PC
Control
Low Oxygen
PI
PE

Figure 4.2: Proton NMR spectrum of the extracted membrane lipids of free living *B. japonicum* USDA 110 (A) and of the membrane lipids of free-living *B. japonicum* USDA 110 grown under low oxygen conditions (B). In A, signals between 2.8 and 4.8 ppm are due to carbohydrates and glyceryl components. The signals between 0.5 and 2.4 ppm are due to resonances in the fatty acids. The strong resonances at 3.30 and 4.86 ppm are from residual CHD_2OD and OH signals, respectively, of the solvent. In B, the signals at 3.23 ppm and 4.27 ppm which correspond to PC are gone. There is also a decrease in the peak intensity at 2.7 ppm and 3.3 ppm (other N-methyl groups) and an increase in peak intensity at 3.18 ppm and 4.07 ppm (PE). Note the appearance of new signals between 3.5 and 4.0 ppm.


A
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm


B
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

residue; however, there was no signal that was assignable to an anomeric proton indicating that an inositol group was present as indicated by the TLC analysis.

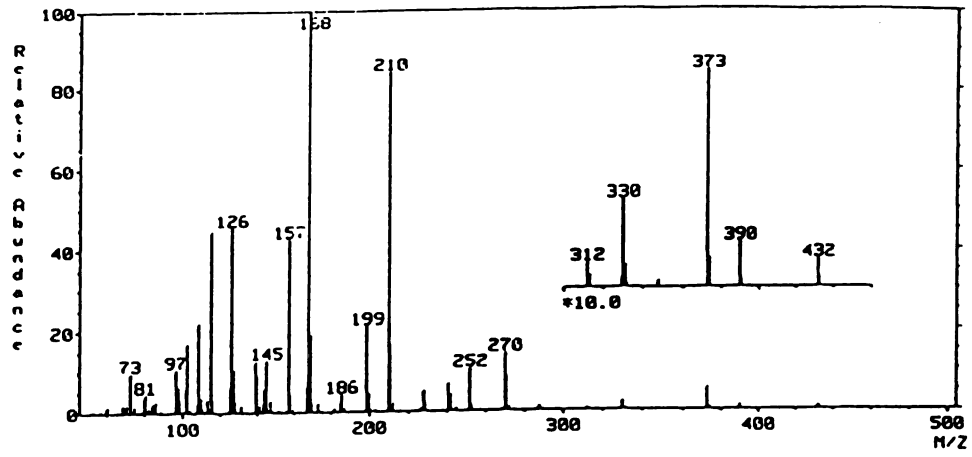
Inositol was conclusively identified by GC and GC-MS analysis of the lipid headgroup after hydrolysis and peracetylation. Gas chromatography analysis showed that the predominant glycosyl component of membrane lipid from bacteria grown under microanaerobic conditions (Figure 4.3B) had the same retention time as the peracetate of standard inositol (Figure 4.3A). The mass spectrum of this component (Figure 4.4B) showed the same fragmentation pathway as that of the standard PI (Figure 4.4A).

Compared with that of bacteria grown under normal conditions, the NMR spectrum of the total lipid extract of *Bradyrhizobium japonicum* USDA 110 grown under low pH conditions showed no change in the region below 6.0 ppm. This indicated that there were no changes in the head groups of the lipids. Some changes in spectrum from 6.0 to 8.0 ppm were observed for a total lipid extract of *Bradyrhizobium japonicum* USDA 110 grown under low pH conditions (Figure 4.5B). Signals corresponding to the unsaturation state of the fatty acyl groups in the lipids became stronger compared with those in the spectrum of bacteria grown under normal conditions (Figure 4.5A).

Figure 4.3: Gas chromatogram of alditol acetate derivatives of lipid extract from *B. Japonicum* USDA 110 grown under normal conditions (B) and under low oxygen conditions (B). The predominant peak in B has the same retention time as that of standard myo-inositol in A.

Figure 4.4: GC/MS spectrum of alditol acetate derivatives of standard myo-inositol (A) and of lipid extract from USDA 110 (B). Note that the predominant component in GC (Figure 4.3B) has the same fragmentation pathway as standard inositol derivatives.

A


Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
M/Z
168
210
373
330
126
157
390
432
312
*10.0
199
270
252
145
73
97
81
186

B

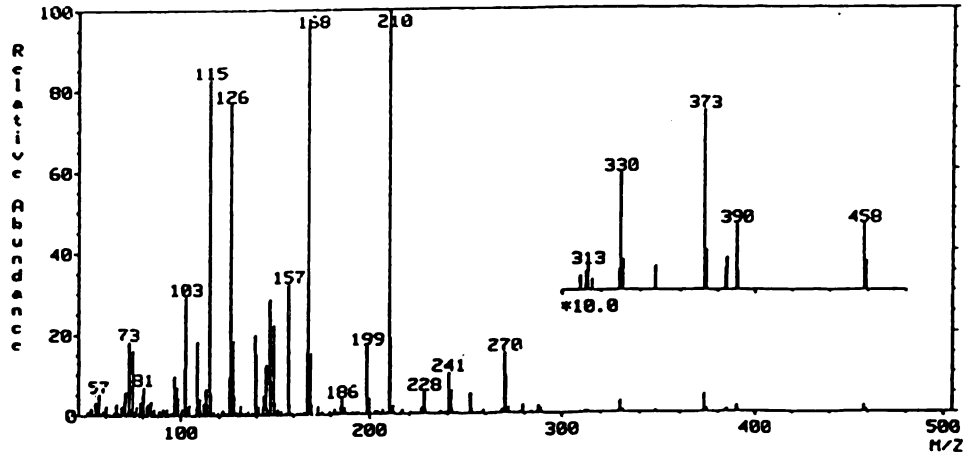
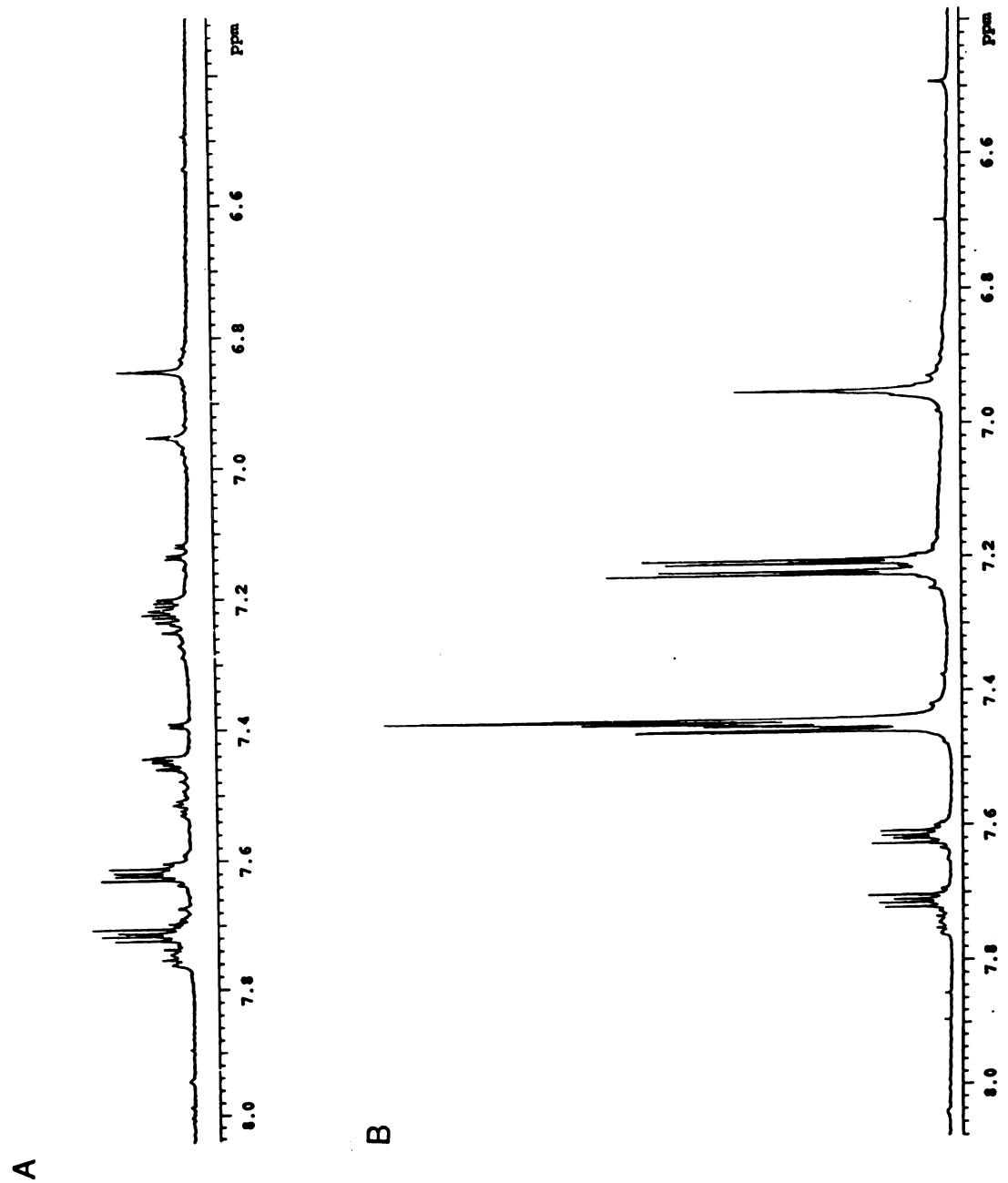

Relative Abundance
100
80
60
40
20
0
100
200
300
400
500
M/Z
168
210
115
126
373
330
390
458
313
*10.0
157
103
73
199
270
241
228
57
81
186

Figure 4.5: Proton NMR spectrum of the extracted membrane lipids of free-living *B. japonicum* USDA 110 grown under normal conditions (A) and low pH conditions (B). Changes were observed in signals corresponding to the unsaturation in the fatty acyl groups of the lipids.


A
B
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
ppm

DISCUSSION

At least three previous studies (5,6,7) have been conducted on the membrane lipid composition of free-living *Bradyrhizobium japonicum*. Although there was much agreement on the presence of PC and PE , there were still some conflicts with respect to the presence or absence of PG, PI and PS. These discrepancies could, however, arise from differences in growth medium (nutrients) and/or aeration (oxygen tension) and therefore could be reflecting the sensitivity of the bacteria to these. In liquid and unsparged culture the extent of aeration is determined by the geometry of the culture flask and the speed of shaking, conditions that may not be controlled by laboratory routines. N-Methyl derivatives of PE and PG are not easily resolved on TLC although they can be easily identified by NMR or MS. This may explain why in the previous studies (5,6) PG or N-methyl derivatives of PE were not detected.

It is of interest that *Bradyrhizobium japonicium* synthesize large amounts of inositol-containing lipids in low-oxygen growth media because PI is rarely found in Gram negative bacteria (8). It is one of the major phospholipids of plant membranes and may comprise up to 19% of the total phospholipid of the plasma membrane (9). Its presence here is certainly noteworthy since it further underlines the uniqueness of rhizobial membrane chemistry among bacteria and its close similarity to plant membrane chemistry.

ACKNOWLEDGMENT

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9. Hetherington, A. M. and B. K. Drobak. 1992. *Prog. Lipid Res.* **21,** 53-63.

CHAPTER 5
AN INVESTIGATION ON THE INTERRELATIONSHIP OF RHIZOBIAL POLYSACCHARIDE AND GLYCOLIPID BIOSYNTHESIS

ABSTRACT

There is functional and biosynthetic overlap among the various classes of rhizobial cell surface components. Understanding carbohydrate metabolism is central to understanding the connections among the biosynthetic events leading to the various polysaccharides, glycolipids and glycoconjugates. 3-O-[^{14}C]methyl-D-glucose was used as a probe to see whether it can be taken up, intact, into carbohydrate-containing molecules. 3-O-methyl-D-glucose is used extensively in glucose uptake studies. Our results clearly demonstrated that 3-O-[^{14}C]-methyl-D-glucose was incorporated into membrane glycolipids but not into other major carbohydrate-containing molecules, including EPS, LPS and cyclic β-glucans. It is likely that the biosynthesis of EPS, LPS and cyclic β-glucans do not use a common pool of monosaccharide with membrane glycolipids but rather utilizes locally generated pools. Alternatively, the substrate specificity of the glycosyltransferase(s) synthesizing glycolipids may be different from enzymes synthesizing EPSs, LPSs, and cyclic β-glucans. Biosynthesis of glycolipids in this strain was shown to be independent of those synthesizing other carbohydrate-containing molecules.

INTRODUCTION

In section III of Chapter 1 we introduced a variety of rhizobial cell surface carbohydrate-containing molecules such as EPS, CPS, LPS, lipooligosaccharides, cyclic β-glucans and glycolipids (1-6). In section V of Chapter 1 we discussed that all these molecules are believed to function in the establishment of symbiosis between rhizobia and legumes (7-20)

As described in section VI of Chapter 1, the diversity of bacterial cell surface carbohydrates involved in symbiosis make it difficult for studies aiming to define the roles of each individual component. The existence of a large degree of coupling of many biosynthetic events leading to the synthesis of various surface components further complicates these studies. Examples for such coupling go from the common structural (and genetic) links between chitolipooligosaccharides, LPSs and general membrane lipids (21), the transfer of the "missing" O antigen of LPS to an alternative glycolipid (22), to structural links between EPSs and LPSs (12-25). In addition to this, there also exist overlaps in the symbiotic functions of these molecules (26-29).

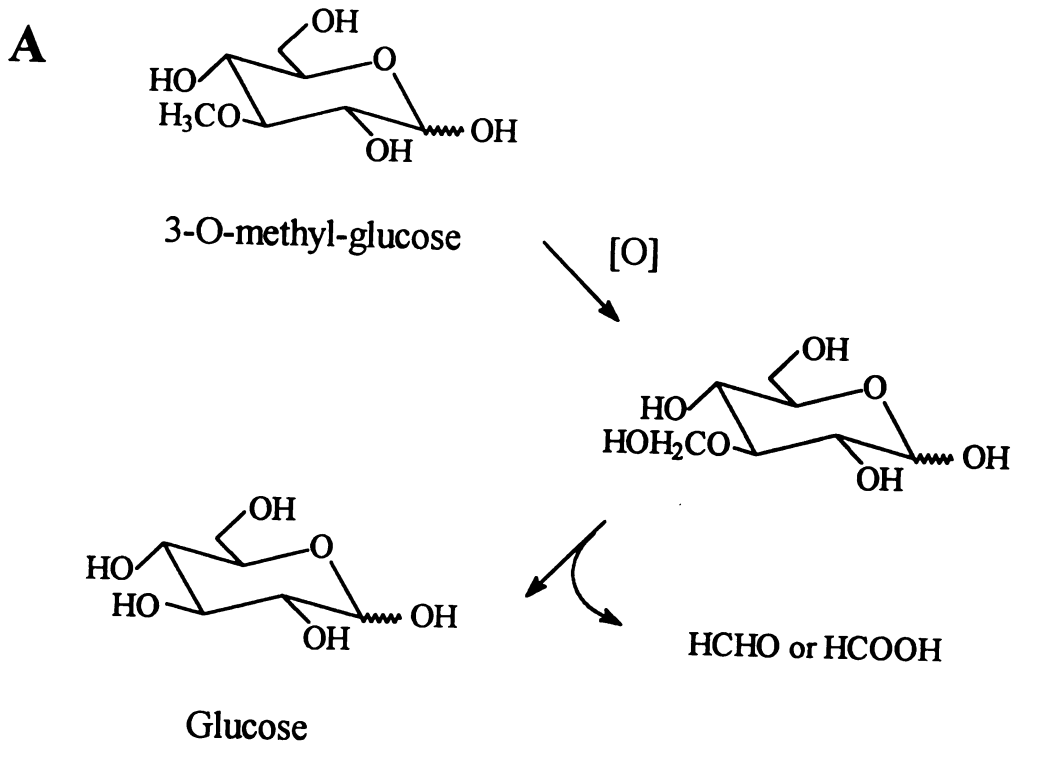
Understanding carbohydrate metabolism is central to understanding the connections among the biosynthetic events leading to the various polysaccharides, glycolipids and glycoconjugates. We are concerned with probing the biosynthesis of various molecules by using a monosaccharide analog to see whether the analog can be taken up, intact, into a carbohydrate-containing molecule. This can give us information about the common

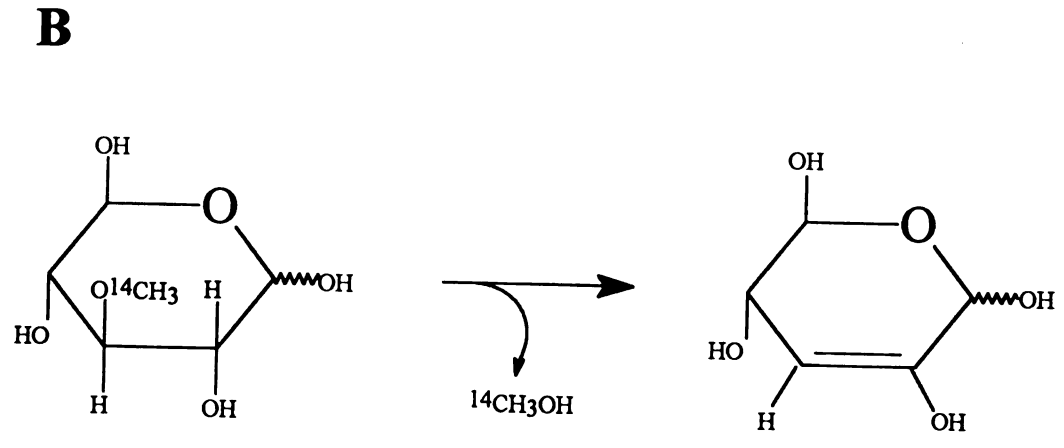
intermediates among the biosynthetic pathways leading to different molecules.

In experiments described here, 3-O-[^{14}C]-methyl-D-glucose was used a probe. 3-O-methyl-D-glucose is used extensively in glucose uptake studies (30-32). It is generally known to be taken up via the same facilitated diffusion uptake pathway as glucose (33). It has been shown that 3-O-methyl-glucose can be phosphorylated both in yeast and in mammalian systems (34-37). In our experiments, 3-O-methyl-D-glucose was chosen because it is a close analogue of glucose, a component of almost all of the rhizobial cell surface carbohydrate-containing molecules. The ^{14}C label will be lost if demethylation occurs to give glucose. Oxidative demethylation will yield labeled formaldehyde of formate (Figure 5.1A). Elimination gives labeled methanol (Figure 5.1B). Both are C 1 units that do not enter into normal metabolism. Hence, any radiolabeled polysaccharides or glycolipids would contain 3-O-methyl-glucose.

There are other aspects of biosynthesis that such experiments will allow us to test. If, for example, the biosynthesis of a particular polysaccharide or glycolipid does not use a common pool of the monosaccharide but utilizes instead a locally generated pool, then we might expect the polysaccharide or glycolipid not to contain the label. This will address a very important point. The biosynthesis of that particular glycolipid or polysaccharide will not be affected by mutations affecting the common pool. We therefore can determine to what extent the biosynthesis of certain carbohydrate-containing molecules utilizes common pathways.

Figure 5.1: A, Oxidative demethylation of 3-O-methyl-D-glucose yields formaldehyde of formate; **B,** Elimination of 3-O-methyl-D-glucose gives methanol.


A
OH
HO
H3CO
O
OH
OH
3-O-methyl-glucose
[O]
OH
HO
HOH2CO
O
OH
OH
OH
HO
HO
O
OH
OH
Glucose
HCHO or HCOOH


B
OH
O
O14CH3
H
OH
HO
H
OH
14CH3OH
OH
O
OH
HO
H
OH
3-O-methyl glucose
Enol

In a series of experiments described in this chapter, *Rhizobium leguminosarum* bv. *viciae* ANU843 was grown in a medium with 3-O-[^{14}C]-methyl-D-glucose as the only carbohydrate component. We reasoned that if it were used as the sole carbohydrate component in a culture medium, it would perhaps be activated for glycosyl transfer and compete with glucose at several levels of biosynthesis of polysaccharides, oligosaccharides and glycolipids. This will allow us to separate the carbohydrate-containing molecules that are synthesized from the common pool of available glucose from those that need locally-generated pools of *de novo* synthesized or activated glucose.

MATERIALS AND METHODS

Cell Culture

3-O-methyl-glucose was purchased from SIGMA Chemical Co. (St Louis, Missouri) and 3-O-[^{14}C]-methyl-D-glucose was purchased from Amersham International plc (Buckinghamshire, England). *Rhizobium leguminosarum* bv. *viciae* ANU843 was grown at 30°C in modified Bergensen's medium with mannitol omitted and 3-O-methyl glucose (10mM) added. 15 υCi 3-O-[^{14}C]methyl-D-glucose was added to two liters of medium at the time of inoculation. The bacteria grown on regular modified Bergensen's medium was used as control. To cultures which were used for tracing S-containing molecules, 0.3 mCi of ^{35}S-sulfate was added. The cultures were harvested by centrifugation at late exponential phase of growth. The supernatant was used for EPS extraction and the pellet was used for the extraction of membrane lipids, LPSs, cyclic B-glucans and cell metabolites.

EPS Extraction

The supernatant from the bacterial culture was concentrated to one-forth of its original volume on a rotary evaporator under reduced pressure at a bath temperature of ~ 40°C. Equal volume of ethanol was added and the mixture was left at room temperature for half an hour. EPS was recovered by centrifugation at 6,000g for 20 min and dialyzed against distilled water. After five to six water change, EPS solutes were lyophilized to dryness. A small amount was redissoved in water by sonication and subjected to scintillation counting.

Lipid Extraction

Lipids were extracted from cells by stirring the pellets of bacteria with 160 mL of 2:1:1:4 chloroform: methanol: butanol: water at room temperature for 24 h. The bacterial cell debris was removed by centrifugation at 6,000g for 20 min at room temperature. The supernatant was separated into two layers. The lower organic layer was recovered after centrifugation was concentrated to dryness on a rotary evaporator under reduced pressure at a bath temperature of ~ 40°C. The lipids were redissolved in 1 mL of 1:1 chloroform: methanol.

Separation of LPS and cyclic β-glucans

The upper aqueous layer from the lipid extract mixture was centrifuged and the supernatant was concentrated to a syrup and chromatographed on a Sepharose-4B column (76 cm x 6 cm) in 0.05 M formic acid (pH%5.5). Fractions of 1 mL were analyzed for carbohydrate by using the phenol-sulfuric acid assay (21) and for radioactivity by using scintillation counting.

Periodate Oxidative Cleavage of Lipid

The lipid extraction was concentrated to dryness and suspended in 0.1 mL of methanol. To this suspension 0.1mL of 0.1% sodium periodate was added. The reaction mixture was sonicated , left at room temperature overnight and concentrated to dryness. A solvent of water: chloroform: methanol in the ratio of 6:5:1 was added to the reaction mixture and the lipid was recovered in the lower organic layer.

Thin Layer Chromatography

Standard PC was purchased from SIGMA Chemical Co. (St Louis, Missouri). The lipids extracted were analyzed by TLC on silica plates by using a mixture of chloroform, acetone, methanol, acetic acid and water in the ratio of 10:4:2:1:1. The cyclic β-glucans were analyzed by TLC on silica plates by using a mixture of propanol, ammonium hydroxide and water in the ratio of 60:40:10. After development, the plates were sprayed with orcinol reagent, heated at 100°C for 15 min and exposed to a phosphoimager.

RESULTS

EPSs

EPSs extracted from cells grown in the 3-O-[^{14}C]methyl-D-glucose media were analyzed for radiolabel by scintillation counting. No label was detected.

LPSs

The upper aqueous layer from the lipid extraction mixture of bacteria grown on 3-O-[14C]methyl-D-glucose was chromatographed on Sephrose-4B column. Phenol-sulfuric acid assay revealed a few carbohydrate-containing peaks (Figure 5.2A). The first three small peaks (a,b,c) corresponded to LPSs. The large, late-eluting peak (d) corresponded to the normal elution volume of cyclic β-glucans. Scintillation counting (Figure 5.2B) revealed that the label was located only in this latter peak. There was no detectable radioactivity associated with the first three LPSs peaks, indicating that 3-O-[^{14}C]-methyl-D-glucose was not incorporated into LPSs.

Cyclic β-glucans

To separate cyclic b-glucans from free 3-O-methyl glucose, the late eluting carbohydrate peak was subjected to thin layer chromatography. Figure 5.3 was the photocopy of the orcinol-sprayed plate. 10 μL of sample from the two fractions with the highest radioactivity were applied onto the TLC plates and each was resolved into two components. The fast-moving component was free 3-O-methyl glucose as shown by the same retention time as the standard whereas the one at the origin was cyclic β-glucans. All the fractions in

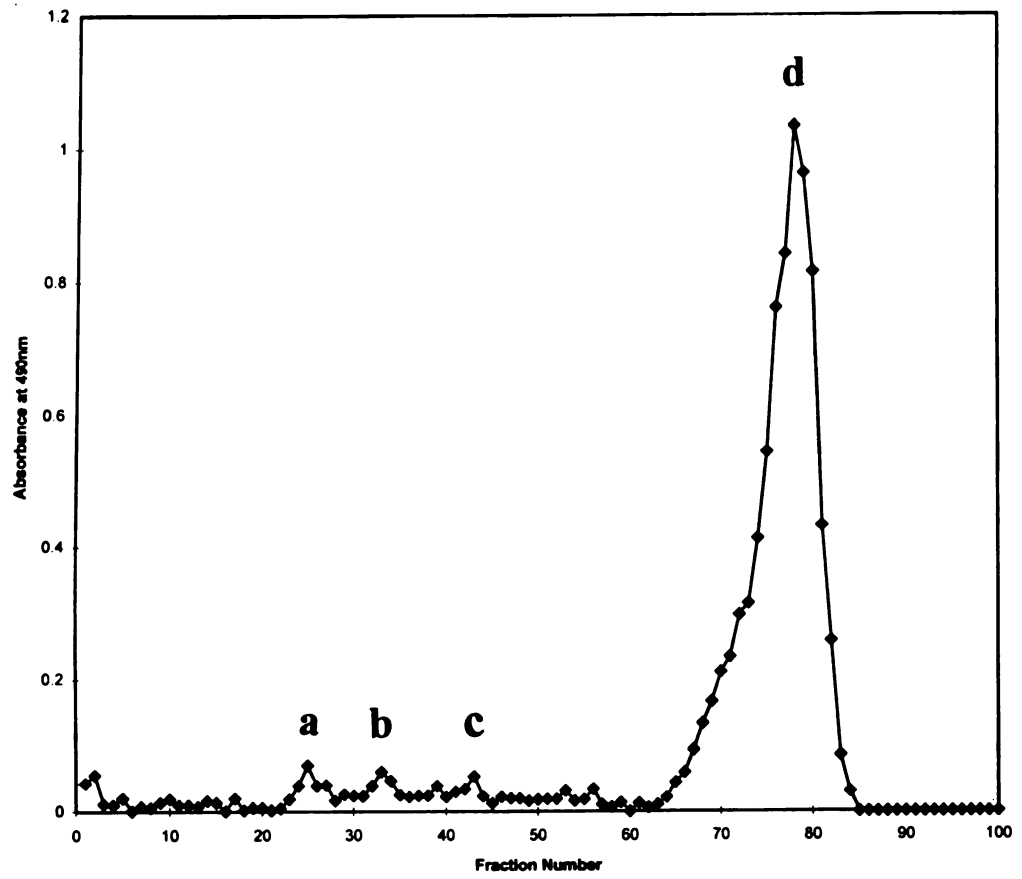


Figure 5.2A: Phenol-sulfuric acid assay revealed a few carbohydrate-containing peaks. The first three small peaks (a,b,c) corresponded to LPSs. The large late-eluting peak (d) corresponded to the normal elution volume of cyclic b-glucans and free 3-O-methyl glucose.

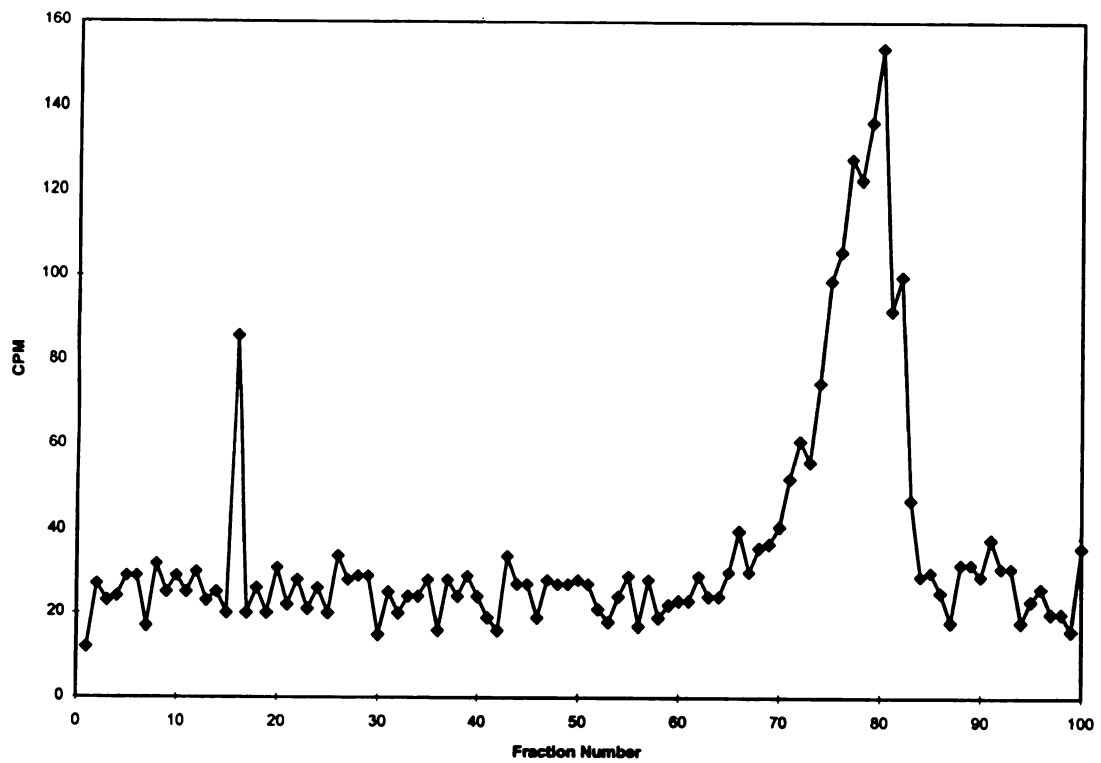
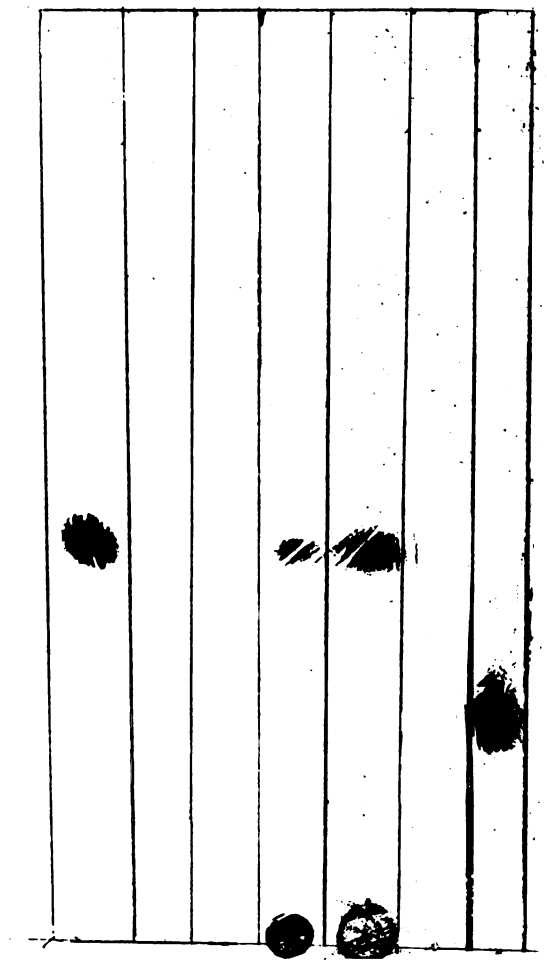


Figure 5.2 B, Scintillation counting revealed that the radioactivity was associated with the latter peak (d) in 5.2A.

Figure 5.3: TLC analysis of two fractions with the highest radioactivity (in peak d from Figure 2). Each was resolved into two components respectively. The fast-moving component was free 3-O-methyl glucose as shown by the same retention time as the standard whereas the one at the origin was cyclic β-glucans.

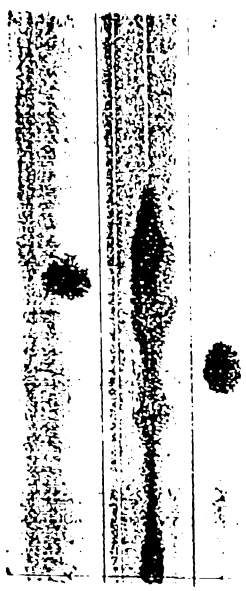

3-O-methyl glucose
Fra79
Fra 80
Glucose

Membrane Lipids

Total lipid extract of bacteria grown on 3-O-[14C]methyl-D-glucose was analyzed by TLC (Figure 5.5A) and the chromatogram analysed by autoradiography (5.5B). Two groups of lipids were radiolabeled, indicating that they were glycolipids containing 3-O-methyl glucose. There was a possibility that 3-O-methyl group was incorporated into SQDG, a common glycolipid in rhizobial species containing sulfoquinovose as the head group (38). The biosynthetic pathway of SQDG is largely unknown. One hypothesis about the biosynthesis of SQDG was that SQDG came directly from glucose. If there was the case, 3-O-methyl glucose could be changed to 3-O-methyl sulfoquinovose and ^{14}C labeled 3-O-methyl SQDG could be detected on the TLC plate. To investigate the possibility that radio-labeled glycolipids are SQDG, the same strain of bacteria was grown on both regular media and media with non-radioactive3-O-methyl glucose as the only carbohydrate source. ^{35}S labeled sulfate was added to both media to trace SQDG (38) by analyzing the membrane lipid extract on TLC. Figure 5.6A was the photocopy of the ocinol sprayed plate showing the lipid extracts from bacteria grown on three different media: CM: 3-O-methyl glucose with 3-O-[^{14}C] methyl-D-glucose ; SM: 3-O-methyl glucose with [^{35}S] sulfate; SR: regular media with [^{35}S] sulfate. Figure 5.6 B was the autoradiograph of the same plate. Two major ^{35}S-containing lipids were found respectively in the lipid extracts of bacteria grown on two

Figure 5.4: A. TLC analysis of the pooled, concentrated radioactive fractions (Peak d from Figure 2). B. Autoradiograph of the same plate. All of the radioactivity was associated with the fast-moving carbohydrate component corresponding to free 3-O-methyl glucose.

A



M-Glc Glucan Glc

B

M-Glc Glucan Glc

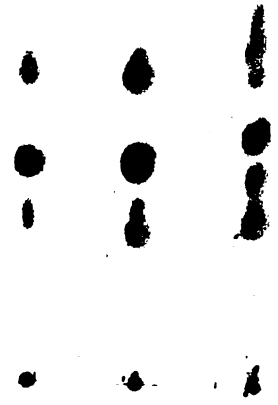
Figure 5.5: A. TLC analysis of the total lipid extract of bacteria grown on 3-O-methyl glucose; B. Autoradiograph of the same plate. Two groups of lipids contained 3-O-methyl glucose.

A

B

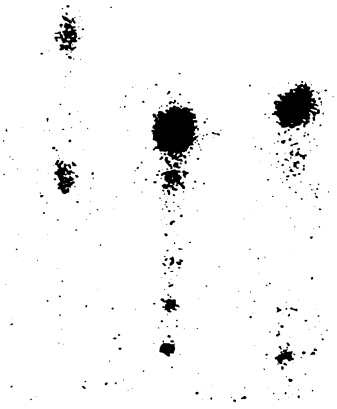
Figure 5.6: A.TLC analysis of the lipid extracts from bacteria grown on three different media: CM: ^{14}C labeled 3-O-methyl glucose medium; SM: ^{35}S labeled 3-O-methyl glucose medium; SR: ^{35}S labeled regular B-III medium; B. Autoradiograph of the same plate.

A



CM SM SR

B



CM SM SR

kinds of ^{35}S- labled media (SM and SR). These ^{35}S- labeled lipids had the same retention time as that of SQDG (2) and their retention time was different from those of glycolipids containing 3-O-methyl glucose.

Periodate Oxidative Cleavage of Lipid

To confirm the glycolipids containing 3-O-methyl glucose was not the same as 3-O-methyl SQDG, periodate oxidative degradation was used. The mechanism of the reaction is shown in Figure 5.7. In this reaction, vicinal glycols are cleaved to yield a dialdehyde and iodate via a cyclic intermediate. This reaction has tremendous analytical significance since it can be used to ascertain what proportion of glucosyl residues might be blocked at the O-2 or O-3 position. Substitution of either renders the ring insensitive to periodate oxidation. The three aforementioned lipid extracts before and after oxidation were analyzed by TLC. Changes were observed in all three lipid extracts (SR, CM, SM) after oxidation (Figure 5.8A). Figure 5.8 B was the corresponding autoradiograph. The major ^{35}S labeled lipids corresponding to SQDG in both SR and SM disappeared after oxidation, indicating that both were ^{35}S labeled SQDG instead of 3-O-methyl SQDG. The glycolipids containing 3-O-methyl glucose in CM remained after oxidation.

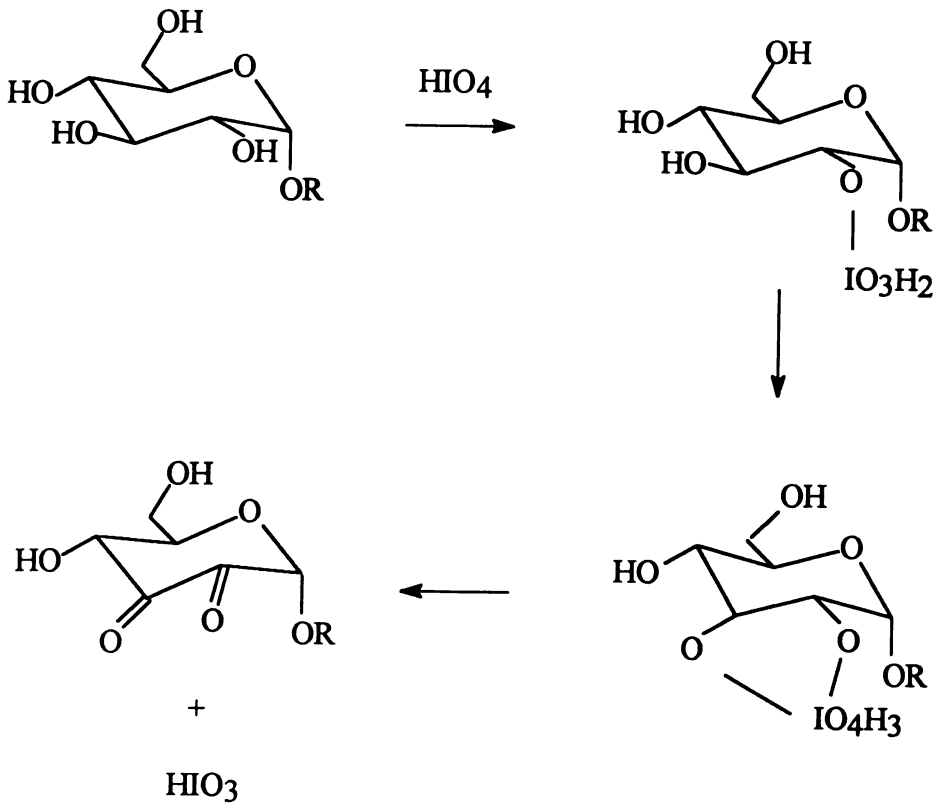


Figure 5.7: The mechanism of periodate oxidative degradation of glucosyl rings to form dialdehydes. In the reaction, vicinal glycols are cleaved to yield a dialdehyde and iodate via a cyclic intermediate.

Figure 5.8A: TLC analysis of three lipid extracts before (SR, CM, SM) and after oxidation (SRO, CMO, SMO).

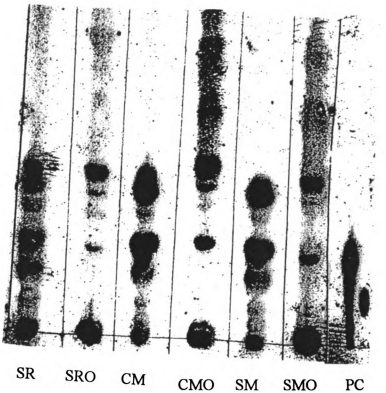
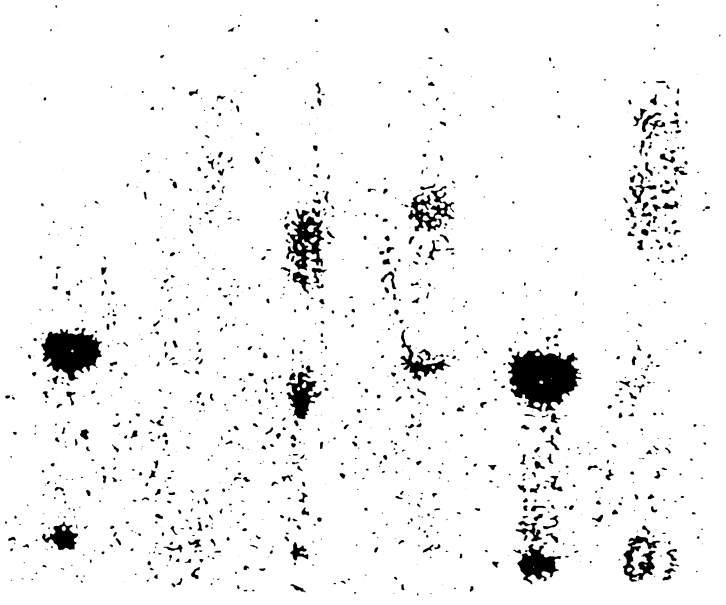

SR
SRO
CM
CMO
SM
SMO
PC

Figure 5.8B: Autoradiograph of the same plate. Note that the major ^{35}S labeled lipids corresponding to SQDG in both SR and SM disappeared after oxidation while the glycolipid containing 3-O-methyl glucose in CM remained after oxidation.


SR
SRO
CM
CMO
SM
SMO
PC

DISCUSSION

Our results clearly indicated that 3-O-[^{14}C]-methyl-D-glucose was incorporated into membrane glycolipids but not into other major carbohydrate-containing molecules, including EPS, LPS and cyclic β-glucans. This gives us some information about the biosynthetic pathways for the synthesis of these molecules. It is likely that EPS, LPS and cyclic β-glucans are not generated from a common pool of the monosaccharides with membrane glycolipids but utilizes locally generated pools. In this local pool, normal polysaccharide molecules may be synthesized from glucose synthesized *de novo*. We can conclude that the biosynthesis of glycolipids in this strain was independent, to some degree, from those synthesizing other carbohydrate-containing molecules. There was another possible explanation to our results. Previous studies (1) have indicated the similarities of glycosyltransferases involved in the biosynthesis of EPS, glycogen, cellulose and the O-antigen of LPS. Our results may indicate the existence of a substrate specificity difference between the glycosyltransferase synthesizing glycolipids and those synthesizing EPSs, LPSs, and cyclic β-glucans.

In these experiments, we did not further characterize the structure of the 3-O-methyl-D-glucose containing glycolipid. In the membrane of same bacterial strain,, one mannose- and glucose-containing diglycosyl diacylglycerols (Figure 1.7 in Chapter 1) has been identified (12). These molecules had a 1,3 glycosidic linkage between the two sugars. It is very likely that the glycolipids containing 3-O-methyl glucose was the same as DGDG except for the terminal sugar which is 3-O-methyl glucose instead of glucose. The 1,3 linkage between the two sugars will render the whole molecule resistant to periodate oxidation.

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CHAPTER 6
SUMMARY AND PERSPECTIVE

SUMMARY AND PERSPECTIVE

At each stages of rhizobium/legume symbiosis, a variety of rhizobial cell surface and membrane carbohydrate-containing molecules have been indicated to play some important roles. The functions of these molecules in the establishment of symbiosis are closely related to the ability of rhizobia to change its surface chemistry in response to a changed environment. It is the whole bacterial surface that interacts with plants and there is a large degree of functional and biosynthetic overlap among various rhizobial cell surface and membrane carbohydrate-containing molecules. A comprehensive picture of the rhizobial cell surface needs to be obtained in order to understand the roles played by the rhizobial surface or membrane components in symbiosis. There are two important parts of the big picture: the environmental regulation of the bacterial surface and the coupling of the biosynthetic events leading to various surface and membrane molecules.

Chapter 2 to Chapter 4 of this dissertation address the aspect of environmental regulation of rhizobial membrane chemistry. In the studies described in Chapter 2, the membrane lipids of free living and bacteroid forms of *Bradyrhizobium japonicum*, obtained from nodules occupied by both typed and untyped bacteria, were isolated and characterized by a combination of NMR spectroscopy, mass spectrometry and other chemical and physical methods. These studies indicated that in bacteroid forms of *Bradyrhizobium japonicum*, there was a dramatic shift towards the synthesis of DGDG, the glycolipid which has rarely been found outside of the plant kingdom and photosynthetic bacteria. This result follows our

earlier finding that the other predominant plant glycolipid, SQDG, is a membrane component of fast growing *Rhizobia* and is found even when cells are cultivated in free culture. In the studies described in Chapter 3, culturing *Bradyrhizobium japonicum* in the presence of glucose as a carbon source in a rich medium known to stimulate differentiation into bacteroid forms led to the synthesis of a family of di- and trisaccharide-containing glycerolipids as the main membrane lipid components. Like DGDG, these glycolipid are also rarely found in other bacteria. In the studies described in Chapter 4, *Bradyrhizobium japonicum* was grown in a low-oxygen condition which mimics the condition inside root nodues. There was a dramatic switch of membrane chemistry in which PC, the predominant lipid in aerated cultures, was no longer synthesized and PE became the major lipid. Besides this change, PI, a typical plant lipid rarely found in bacteria was also synthesized. Lowering pH in free living *Bradyrhizobium japonicum* did not result in any change in the lipid headgroups but resulted in the change of unsaturation state in the fatty acyl groups of the lipids.

The possible functional role played by these unusual lipid molecules in symbiosis deserves some further discussion. When the bacterium is living inside of the plant, the plant cell in which the bacterium is growing provides the growth medium for the bacterium. The bacteria have to use many intermediates in this growth medium to modify its own membrane and surface chemistry in order to support its division and differentiation. If the cellular chemistry of the bacterium is modified to such a degree that it approximates that of its host, the change in the cellular chemistry of the host will be minimal and the defense system of the host may be avoided. There is another way to look at this. Membrane is a critical structural feature that determines the ability of an organism to occupy a particular ecological

niche. It is, therefore, not too surprising that rhizobium and its plant host have developed similar membrane chemistry because the two organisms share ecological pressures of temperature, pH, water and nutrient availability and their metabolism have to be synchronized. This is a striking parallel with some host-pathogen interactions.

The results presented in this dissertation not only suggest a close developmental tie between rhizobia and its host plant but also raise questions about the origin of the genes involved in the biosynthesis of the membrane lipids of *rhizobium*. Were these genes transferred from the plant or were they developed in the bacterium by parallel evolution? It is very possible that these genes be passed on through gene transfer from plants to the bacteria at some point during their long symbiotic relationship. Although it is still a conjecture, the close relationship between the two organisms could provide many opportunities for gene transfer..

Future work can be conducted on the characterization of various membrane and surface saccharides from different species to establish the generality of our findings. Besides, the changes in the bacterial surface and membrane chemistry in different environments should also be investigated.

There is functional and biosynthetic overlap among various classes of rhizobial cell surface components. In the studies described in Chapter 5, ^{14}C labeled 3-O-methyl-D-glucose was used as a probe to investigate the interrelationship of the biosynthetic events leading to various carbohydrate-containing molecules which are important to symbiosis. Our results clearly indicated that ^{14}C labeled 3-O-methyl-D-glucose was incorporated into membrane glycolipids but not into other major carbohydrate-containing molecules, including

EPS, LPS and cyclic β-glucans. It is likely that the biosynthesis of EPS, LPS and cyclic β-glucans do not use the same metabolite pool as glycolipids. It is also possible that the substrate specificity of the glycosyltransferase(s) synthesizing glycolipids is (are) different. Our conclusion is that the biosynthesis of glycolipids in this strain was independent, to some degree, from those synthesizing other carbohydrate-containing molecules. Future studies on the interconnections among the biosynthetic pathways leading to various saccharides can be conducted by using both radiolabeled and functional labeled analogs of proposed substrates or intermediates, following their incorporation into the final products, and seeing that if they are incorporated into more than one saccharide.

The most important question that can be answered in this dissertation is that by what mechanism rhizobia can survive the plant defense systems. Can rhizobia live inside of the plant as an organelle? The unusual occurrence of SQDG, PC and 27-hydroxyl-C28-fatty acid in the rhizobial species has strongly revealed similarities in the composition of membrane lipids among rhizobia, chloroplast, and cyanobacterium(a photosynthetic bacterium). Cyanobacterium is also a functional counterpart of rhizobium. According to endosymbiotic theory, chloroplast was originated through an endosymbiotic invasion of the plant cell by cyanobacterium (1, 2). Modern cyanobacteria are often found in symbiotic relationships with other organisms and live inside of these organisms as functional chloroplasts. In this sense, cyanobacterium is a CO_2- fixing symbiont and rhizobium is a N_2-fixing symbiont, both associated with plants. The similarities in both the function and the membrane composition suggest that rhizobium can live inside of the plant as an organelle. The work presented in this thesis further strengthened this hypothesis. The ability of these rhizobial

species to make the unusual plant-type lipids, such as SQDG, DGDG, PI, PC and di- and tri-saccharides-containing glycolipids, strongly suggests that the membrane surface of rhizobium mimics that of the plant (including that of plastids). This enables rhizobium to live inside of the plant successfully after the invasion and establish an effective symbiosis. The results obtained in Chapter 5 addressed this ability of rhizobia from another perspective. It is likely that the glycosyl transferases involved in the biosynthesis of these glycolipids have a relatively low substrate specificity. These enzymes can even incorporate monosaccharide analogue such as 3-O-methyl-glucose into membrane glycolipids. It is therefore not surprising that they can pick up different sugars in different growth conditions and make different glycolipids. In this way, rhizobium can quickly modify its membrane structure and adapt to a new environment.

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