DATE DUE	DATE DUE	DATE DUE

CHARACTERIZATION OF BIOCHEMICAL ELEMENTS IMPORTANT FOR HOST-PATHOGEN INTERACTION IN THE *RHIZOBIUM*-LEGUME SYSTEM: A PARADIGM OF INFECTION

By

Jianjun Wang

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1997

ABSTRACT

CHARACTERIZATION OF BIOCHEMICAL ELEMENTS IMPORTANT FOR HOST-PATHOGEN INTERACTION IN THE *RHIZOBIUM*-LEGUME SYSTEM: A PARADIGM OF INFECTION

By

Jianjun Wang

In drastically different systems composed of a host-invasive bacterium and a plant or mammalian host, the infecting bacteria employ common strategies in the three phases of infection: cell surface attachment, cell invasion and intracellular survival. These strategies have been manifested in the infection process of host plants by *Rhizobium.*

It has been proposed that two tactics are used by *Rhizobium* in the attachment phase. One is a lectin-carbohydrate type and the other is a vitronectin-integrin type interaction. Both of these tactics are commonly employed by many mammalian pathogens such as *Haemophilus influenzae.* They are also used by *Agrobacterium*, a genus of plant pathogens and a close phylogenetic relative of *Rhizobium.*

Many bacterial virulence factors and toxins are actually enzymes which can degrade the cell surface components of the hosts. By this way, the invading bacteria can breach the cellular barrier and enter the host cells. These virulence factors include phospholipases, pectinases, cellulases, proteases and glycanases. The presence of phospholipases A and D activities in the membranes of *Rhizobium* is

demonstrated here. These phospholipases are proposed to be important for *Rhizobium* to enter plant root cells.

One of the strategies adopted by the infecting bacteria to evade host defense mechanisms is molecular mimicry. This is the process of altering certain structural features of the bacterial cells to mimic those of the invaded cells. It is possible because of the extraordinary ability of bacteria to adapt to new environmental challenges. *Rhizobium* and *Bradyrhizobium* are able to synthesize a variety of plant type glycolipids under conditions similar to those inside the nodule environment. Some of the glycolipids, α-sulfoquinovosyl diacylglycerol and diglucosyl diacylglycerol, are shown here to be located in the outer membrane of *Rhizobium*. This implies that they may function in mediating the interaction with the host cells at close range. The biosynthesis of α-sulfoquinovosyl diacylglycerol is investigated and discussed. The results show that *Rhizobium* shares metabolic features of α-sulfoquinovosyl diacylglycerol with plants, suggesting that *Rhizobium* and plants may have some common genes related to the metabolism of α-sulfoquinovosyl diacylglycerol. It is proposed that the use of molecular mimicry is one of the many conserved strategies used by diverse animal and plant-infecting bacteria including *Rhizobium*.

To Jesus Christ, my savior

ACKNOWLEDGMENTS

I am indebted to my mentor, Dr. Rawle I. Hollingsworth, who guided me through the course of this work. I thank him for giving me this very interesting and challenging project and being ready to discuss it with me at any moment. The excitement and joy from doing and appreciating this work would have been impossible without him.

Sincere thanks to my guidance committee, Drs. Loran Bieber, John Ohlrogge, Steven Trienzenberg, William Wells and Gregory Zeikus, who provided me invaluable comments and suggestions. I thank Dr. Laurie S. Kaguni for making me interested in mitochondrial transcription, which is still a favorite topic for me to read.

I thank all the members in Dr. Hollingsworth's laboratory for making such a friendly and international environment. I especially thank Jim Bradford for performing ES/MS and Ben Hummel for spending time and effort for others.

I thank the Getzs and the Yangs families, who showed me what a true Christian is and prayed for me during the past years. I thank Ruth Wallace for initiating the Bible study four years ago and being persistent when I was reluctant.

There are many other brothers and sisters such as Leslie, Anastatia, Hao, Bob, Shuxian, who have been in fellowship with me. My life would have been incomplete without these wonderful people.

My deep appreciation to my wife, Yin Tang, who was, is and will be an integral part of my life. Language is not enough to express my thanks. I particularly thank her for our wonderful and perfect boy, Lanson, who is such a joy and encouragement to me. He is an invisible but inseparable part of my work.

I thank God for giving me such a great family and an opportunity to be here in East Lansing for five and half years. The name of the city and the beautiful MSU campus will be in my memory for good, including the days in heaven.

TABLE OF CONTENTS

LIST OF TABLES

Introduction

Chapter 1

Chapter 2

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

CLOS	Chitolipooligosaccharides
DAG	Diacyl glycerol
DGDG	Diglactosyl diacylglycerols
ECM	Extracellular matrix
ED	Entner-Doudoroff (pathway)
EMP	Embden-Meyerhof-Parpas (pathway)
EPS	Exopolysaccharides
ES-MS	Electrospray mass spectrometry
FA	Fatty acids
GC-MS	Gas chromatography-Mass spectrometry
HDSF	Hexadecanesulfonyl fluoride
hrp	Hypersensitive response and pathogenicity genes
IM	Inner membrane
KDO	3-deoxy-D-manno-octulosonic acid
LLO	Listeriolysin O
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharides
LysoPLA	Lysophosphoslipase A

MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectrometry
OM	Outer membrane
Pais	Pathogenicity islands
PA	Phosphatidylacid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PI-PLC	Phosphatidylinosito-specific phospholipase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phosphomolybdic acid
PP	Pentose phosphate (pathway)
RGD	Arg-Gly-Asp tripeptide sequence
SDH	Succinate dehydrogenase
SQDG	α-D-sulfoquinovosyl diacylglycerols
SQG	Glyceryl-α-sulfoquinovose
TCA	Tricarboxylic acid (cycle)
TLC	Thin layer chromatography

INTRODUCTION

Rhizobium is a genus of Gram-negative soil bacteria that is able to infect plants and live inside the cytoplasm of plant root cells. This infection is beneficial to the host plant in the sense that *Rhizobium* reduces atmospheric nitrogen to much-needed ammonia for the host. In exchange, the host provides the infecting *Rhizobium* with nutrients in the form of carboxylic acids. This type of mutually beneficial association between two organisms is termed symbiosis. Symbiosis is a very common phenomenon in nature. Table 1 shows some of the known symbiotic systems. The infecting species are usually microorganisms, most commonly bacteria. The hosts include a wide range of organisms including fungi, ferns, higher plants and animals.

A similar type of interaction between a microorganism and a eukaryote is pathogenesis, defined as the infection of a host by a microorganism (as in symbiosis) but with a subsequent damaging effect on the host by the infecting microorganism rather than a beneficial one. In both symbiosis and pathogenesis, the infecting microorganisms need a way of entering their hosts and a strategy of surviving the host defense. The difference between symbiosis and pathogenesis is the final outcome of the infection, which depends on how the metabolisms of the invading microorganism and the host relate to each other.

Table 1 - Some examples of symbiosis in nature

Infecting organism	Host organism
Rhizobium	angiosperms(*Leguminosae*)
cyanobacterium	diatoms, ferns, mosses, liverworts, fungi, angiosperms
Bradyrhizobium	dicotyledons(*Leguminosae* and *Parasponia*)
Frankia (Gram-positive)	actinorhizal plants (e.g. Australian pine, alder, Californian lilac, bog myrtle, bitter brush, etc.)
mycorrhizal fungi	most angiosperms (74)
Vibrio fischeri	squids (118)
Photobacterium leiognathi	squids
Xenorhabdus	nematodes
protozoa	sheep

The parallel between symbiosis and pathogenesis lies in the ways by which they invade their hosts. Stanley Falkow, the president of American Society for Microbiology, defines a pathogen as being any microorganism whose survival is dependent upon its capacity to replicate and persist on or within another species by actively breaching or destroying a cellular or humoral host barrier that ordinarily restricts or inhibits other microorganisms (55).

The distinction between a symbiont and a pathogen becomes blurred when one realizes that, for a particular bacterial genus, one species can be a symbiont for a given host while another species can be a devastating pathogen for another. This is seen in *Vibrio* in which *V. fischeri* is symbiotically associated with squids (118) while *V. cholerae* is the one which causes cholera and has killed millions of people. In another case, *Agrobacterium*, which is a very close relative of *Rhizobium*, is famous for its ability to cause crown gall disease for a wide variety of plants. But *Bradyrhizobium*, which is far more distant from *Rhizobium* than *Agrobacterium* is by all kinds of criteria (Table 2), can establish a symbiotic relationship with leguminous plants, just like *Rhizobium*. Host specificity is often considered of primary importance. However, *Rhizobium* has been well documented to infect the roots of many non-legumes, including potato, rice and maize, etc, and to induce nodule structures (145, 3, 4, 35, 186). *Agrobacterium* shows no specificity at all. It can infect most dicotyledonous plants (133).

We propose that there must be some very important common biochemical features for infection, which are shared by host-benefitting symbionts and disease-

Table 2 - Phylogenetic and biochemical comparison of *Agrobacterium*, *Rhizobium* and *Bradyrhizobium*

	Agrobacterium	*Rhizobium*	*Bradyrhizobium*	Ref.
16S rRNA sequence	*Agrobacterium* and *Rhizobium* are in the same subgroup containing six genera while *Bradyrhizobium* in a different subgroup containing twelve genera			222, 111
Numerical taxonomy		The two genera are very distinct from each other		127
Genome organization	Pathogenic / symbiotic properties encoded by mega plasmids		symbiotic genes located in chromosome	9, 51
Genome size	5,500 kb	5,300 kb	8,700 kb	37, 46, 99
%GC content	59-63.5	59.5-63	63-66	9, 51
Growth rate	Fast	Fast	Slow	51
Growth on disaccharide	Yes	Yes	No	9, 187
Flagellae type	peritrichous	peritrichous	polar/ subpolar	51
Reaction on yeast extract manninol medium	Acid	Acid	base	51
Serum zone in litmus milk	Plus	Plus	Minus	51
Metabolism in free-living state		Pentose pathway	No pentose pathway	187

causing pathogens. It is these features, which confer to *Rhizobium* the abilities to infect and survive inside the host cells, that this thesis will be focused on. Although the establishment of intracellular symbiosis or pathogenesis is a continuity, it will be discussed as a three-phase process. The first phase involves the attachment of the microorganism to the host surface. This is a prerequisite for infection. Many cell surface components are responsible for the physical contact. The second phase involves the invasion of an individual host cell. The biochemical elements responsible for a pathogen to enter host cells are often called virulence factors, which are often used to include factors important for other phases. The final phase is the survival of a pathogen or endosymbiont inside the host cell, usually in the cytoplasm. Since the host cytoplasm is a completely different environment than that outside, it is expected that the invading microorganism in this phase has to go through a series of developmental changes to cope with the new demands. One of the tasks for an invading intracellular microorganism is to evade the host defense system. A second one is to multiply itself. For symbionts, a cooperation starts to be established somewhere in this phase, while for pathogens, it occurs at the expense of the well being of the host.

Chapter 2 describes the demonstration and characterization of phospholipases A and D in the membrane preparations of *Rhizobium*. These phospholipases may well serve as the virulence factors for invading plant cells. Consistent with this notion, cellulases and pectinases from *Rhizobium leguminosarum* have been reported to degrade plant cell wall polymers (113).

Chapter 3 describes the determination of membrane locations of some glycolipids of *Rhizobium* and *Bradyrhizobium*. Two of these glycolipids, α-sulfoquinovosyl diacylglycerols (SQDG) and diglucosyl diacylglycerols, are ubiquitous in plants but are very unusual in bacteria. The outer membrane location of these lipids of plant type suggests that they may function in interfacing the two organisms and avoiding the plant defense responses. This principle, termed molecular mimicry, has been commonly observed in host-pathogen interactions in general. Chapter 4 describes the metabolism of SQDG in *Rhizobium*, which is apparently very similar to that in photosynthetic organisms. Finally, our perspectives on *Rhizobium*-legume interaction will be summarized and extended in Chapter 5, along with the future direction of the research in this area.

CHAPTER 1

Review of *Rhizobium*-legume Interaction from a Pathobiochemical Perspective

During the infection of leguminous plants by *Rhizobium*, the bacteria first attach to the root hair of the host (Figure 1.1a), then infect the primordium cells by the formation of an infection thread (Figure 1.1b & 1.1c) and are released into the cytoplasm by endocytosis (Figure 1.1c), and finally survive inside the host cytoplasm as an organelle. This process closely resembles the infection of mammalian cells in terms of the entry of cells through endocytosis and the formation of organelles inside the host cytoplasm. The entire infection process stands out very similarly to a typical host-pathogen interaction. The question being asked is how far this similarity can go. If the infection processes of *Rhizobium* and pathogenic bacteria share essential features, the research in these two areas will greatly reinforce each other. On one hand, one can use the *Rhizobium*-legume system as an important model to study bacterium-eukaryote interactions in general. On the other hand, the principles obtained in the area of bacterial pathogenesis can be applied to *Rhizobium*-legume interactions and help to direct the future research to the essential aspects of bacterium-eukaryote interactions. In order to reveal the general principles of bacterial infection by studying the *Rhizobium*-legume system, a comparative dissection of the infection process from a pathobiochemical perspective will be necessary.

Figure 1.1 - Stages of host-cell infection by *Rhizobium*. (a) Attachment to root-hair cells; (b) root-hair deformation and initiation of the infection thread, which is coordinated by the plant cytoskeleton in association with the nucleus (N); (c) cell-to-cell spread of rhizobia through transcellular infection threads, followed by uptake of bacteria (endocytosis) from unwalled infection droplets; (d) growth and division of intracellular bacteria (bacteroids) enclosed by plant-derived peribacteroid membrane; (e) morphological and biochemical differentiation of bacteroids into nitrogen-fixing forms, individually enclosed within an organelle-like structure, termed the symbiosome (93)

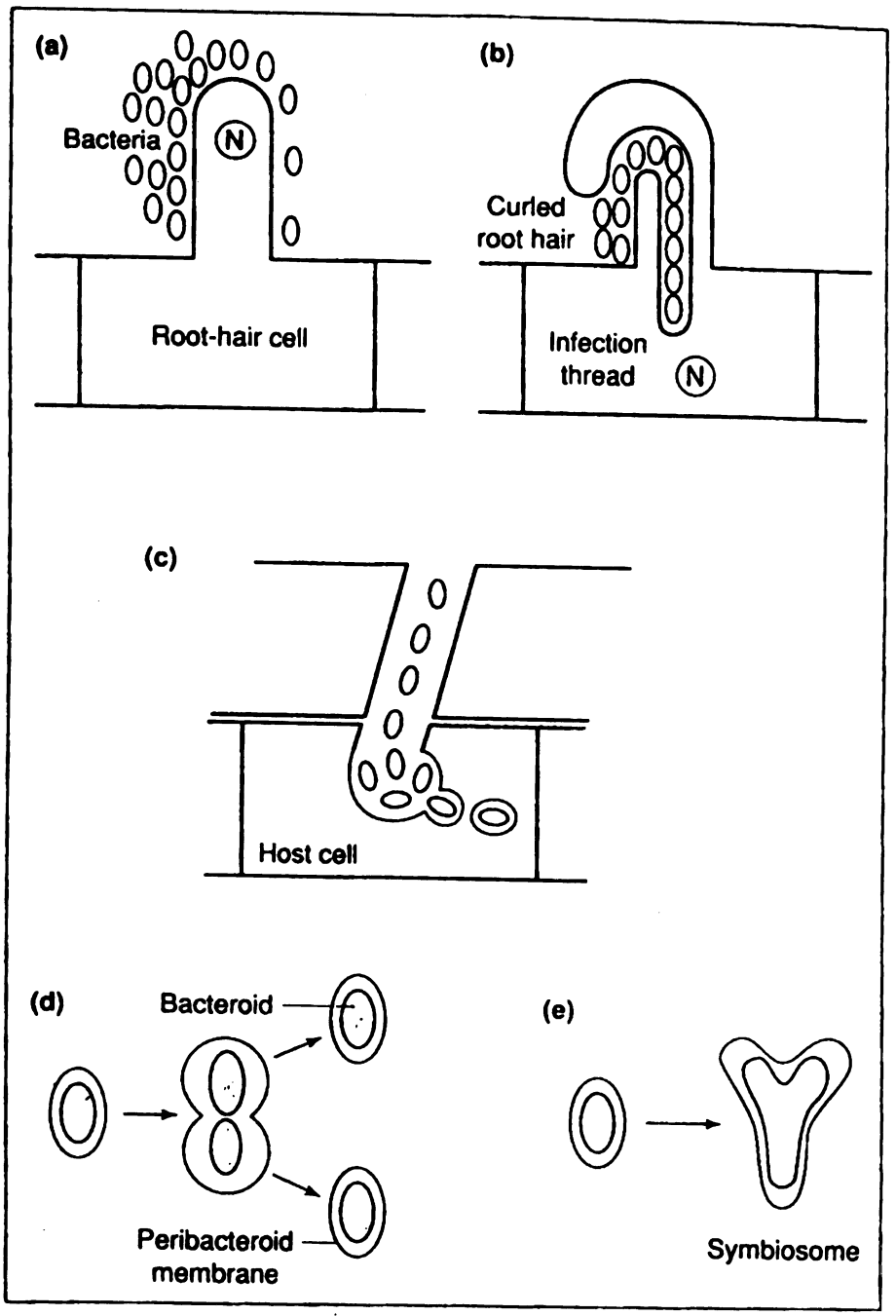
(a)
Bacteria
N
Root-hair cell
(b)
Curled
root hair
Infection
thread
N
(c)
Host cell
(d)
Bacteroid
Peribacteroid
membrane
(e)
Symbiosome

Figure 1.1

I. BACKGROUND

Before a comparative review of the interaction between bacteria and their eukaryote hosts, it would be helpful to have a brief review of bacterial and eukaryotic cell surfaces and the general defense mechanisms of eukaryotic organisms.

Bacterial Cell Surfaces

Bacteria are commonly classified as Gram-negative and Gram-positive, according to the structure of their cell envelopes. Gram-positive bacteria have a thick peptidoglycan layer outside the cytoplasmic membrane. This peptidoglycan layer is often referred to as the cell wall. Gram-positive bacteria commonly excrete hydrolytic enzymes into the surrounding medium. In Gram-negative bacteria, the peptidoglycan layer is thin and lies over the cytoplasmic membrane. Beyond the thin peptidoglycan layer is the lipopolysaccharide(LPS)-rich outer membrane. Between the two membranes and surrounding the peptidoglycan layer is the periplasm which contains amino acids, solute-binding proteins, hydrolytic enzymes and other substances. Worth-mentioning is some osmolarity regulators present in the periplasmic space. These regulators are usually in the form of oligosaccharides. The common examples are the membrane-derived oligosaccharides in *E. coli* and β-1,2 cyclic glucan in *Rhizobium* and *Agrobacterium*. Gram-negative bacteria often possess some other extracellular structures, including flagella, fimbriae (pili), capsular polysaccharides (CPS) and other fibrils of polysaccharide nature. They

also often release extracellularpolysacharides (EPS) into the environment. The structure of the Gram-negative cell surface is illustrated with the *Rhizobium* cell envelope as an example (Figure 1.2).

The bacterial membrane has its own unique lipid composition, quite different from that of animals and plants. It is rich of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and plasmalogens, but usually lack phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), sphingolipids, sterols and glycolipids.

Plant-invading bacteria are usually rod-shaped and most of them are the Gram-negative type (except coryneform bacteria). The flagella can be polar, as in *Pseudomonas* and *Bradyrhizobium*, or peritrichous, as in *Rhizobium*, *Agrobacteria* and *Erwinia*. Flagella are usually responsible for bacterial motility. Fimbriae, also called pili, are proteinaceous and are important for adhesion of bacteria to plant surfaces. This adhesion is not as specific as observed with mammalian pathogens. Mammalian pathogens comprise a broad range of bacteria, from Gram-negative to Gram-positive, from nonmotile to motile and from aerobic to anaerobic. Studies on them have been more or less focused on identifying virulence factors for preventive and therapeutic purposes.

Plant Cell Surface and Intracellular Organelles

Plants usually contain meristematic centers which are responsible for regenerating new cells. Meristematic cells are undifferentiated and do not show any

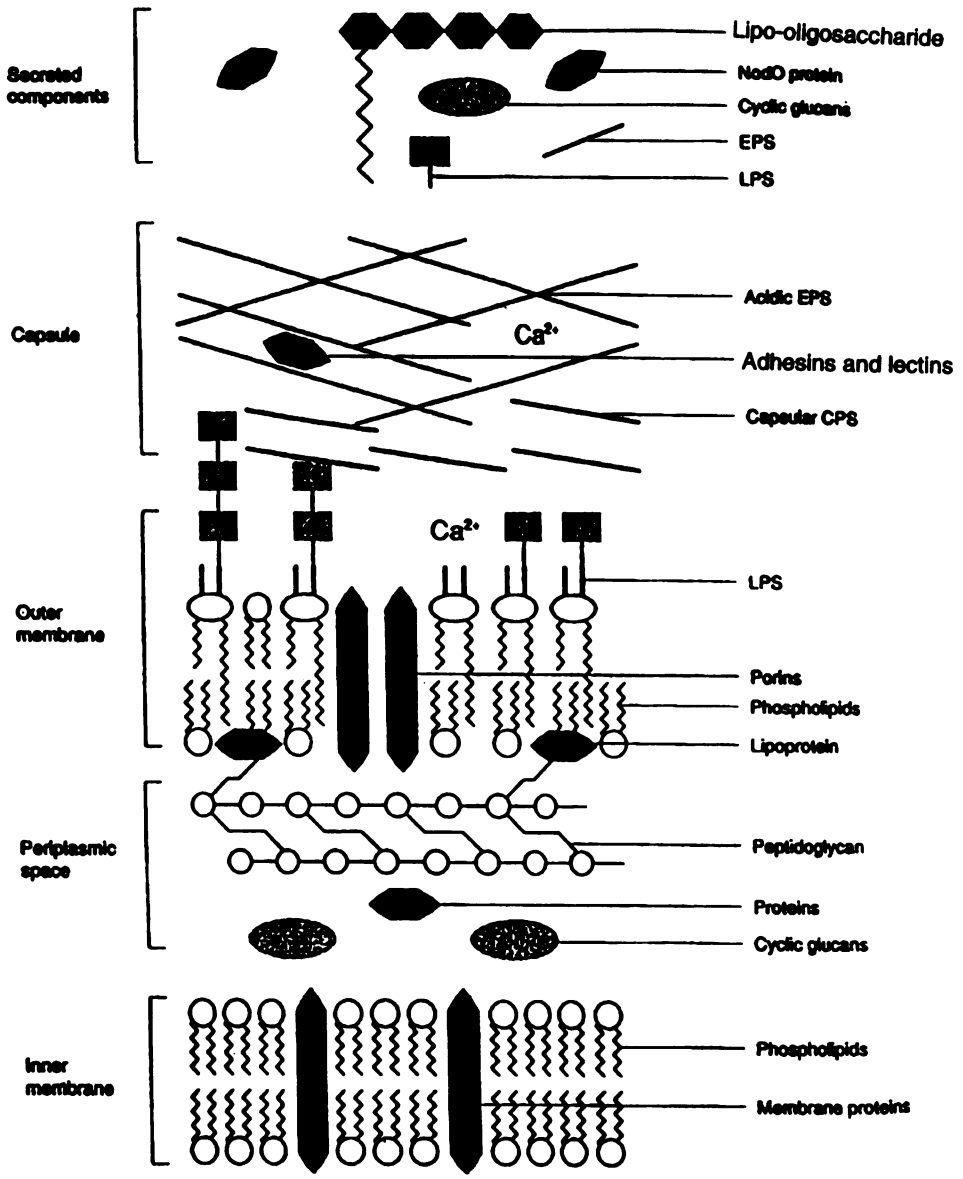

Figure 1.2 - Molecular components of the cell surface of Rhizobium (93)

drift in structure and behavior throughout the life of plants. The main merisematic regions are those in root tips and shoot tips. Unless specially noted, all the structural features discussed below will be of the meristem.

1. Cell Wall

The plant cells consists of a protoplast surrounded by a primary cell wall, without any type of thickening or lignification, which is found in many types of mature cells. Each cell is surrounded by others with which it shares its wall. During growth, material is contributed to the primary wall from both sides so that it retains a somewhat symmetrical structure across the middle lamella. In higher plants, the wall consists of fibrils of cellulose within a matrix of polysaccharides and proteins.

Cellulose constitutes about 20-30% of the dry weight of various primary walls that have been analyzed (146). It is an unbranched long-chained β-1,4-linked glucose polymer. The different chains associate laterally by extensive hydrogen bonding.

The matrix polysaccharides is mainly composed of hemicelluloses and pectic polysaccharides, taking up 20% and 35% of the dry weight of the primary wall in dicotyledons, respectively (28). The hemicelluloses, defined as the polysaccharides noncovalently associated with cellulose, are soluble in dilute alkali and, for the most part, neutral polysaccharides containing glucose, xylose and mannose. The major component of hemicelluloses is xyloglucan, which consists of a backbone of glucose units with side branches made up from xylose and some other sugars such

as galactose, fucose and arabinose. It is suggested that the hemicelluloses are capable of hydrogen bonding strongly to cellulose (38). The pectic polysaccharides contains two main components. The neutral pectin components include homopolymers of arabinose or galactose and heteropolymers built up from both of these sugars. The acidic pectin components include polygalacturonic acid and a mixed polymer of rhamnose and galacturonic acid which has neutral blocks of arabinan and galactan polymers attached to its backbone.

Proteins comprise 5-10% of the cell wall (dry weight) in dicotyledons (28, 38). They include both structural proteins and enzymes, for example, those responsible for cell wall growth. One feature of the proteins in cell walls is the high content of hydroxyproline as well as alanine, serine and threonine, as the structural protein collagen in animal cells. It has been found that these hydroxyproline-rich proteins are always glycosylated, but may or may not to cross-linled to any of the cell wall polymers (38). Studies suggested that a few hydroxyproline-rich glycoproteins extracted from plant tissues have carbohydrate-binding activities and that they may be present in the cell wall (38). Interestingly, a 32 kDa glycoprotein has recently been purified from the cell wall of pea (190). This protein has binding activity for rhicadhesin, which is a putative attachment protein of *Rhizobium* and will be discussed below. It is not known if it is hydroxyproline-rich. In other studies, cell wall glycoproteins have been implicated in plant-fungus interactions (135, 25).

2. Plasma Membrane

The plasma membrane is the second barrier that separates the living cell from the external environment. The cytoplasm of adjacent cells are actually connected through a membrane bridge called plasmadesmata. It was previously thought that only small molecules can diffuse through plasmadesmata. Recent data suggest that proteins, nucleic acids and viruses can all go through plasmadesmata channels (8). Plant plasma membranes are roughly composed of 40-50% lipids, 35-40% proteins and 20% carbohydrates (102). There are four major classes of lipids in plasma membrane: phospholipids, glycolipids, sterols and neutral lipids. The phospholipids are mainly PC, PE, PI, PG and DPG. A unique feature of plant membranes is the high content of glycolipids, representing 25-30% of the total amount of glycerolipids (92). Galactolipids and sulfolipids are the most characteristic plant lipids and comprise more than half of the total lipids in chloroplast membranes. The major galactolipid species are digalactosyl diacylglycerols (DGDG) and monogalactosyl diacylglycerols (MGDG). The sulfolipids, α-sulfoquinovosyl diacylglycerols (SQDG), is abundant in photosynthetic membranes but rarely found in other organisms. The structure of SQDG is shown in Figure 1.3.

The protein part of plasma membrane includes ATPases, glycosyl transferases, cellulases, enzymes involved in cell wall synthesis and some transporters. The carbohydrates are present mainly in the form of glycolipids and glycoproteins.

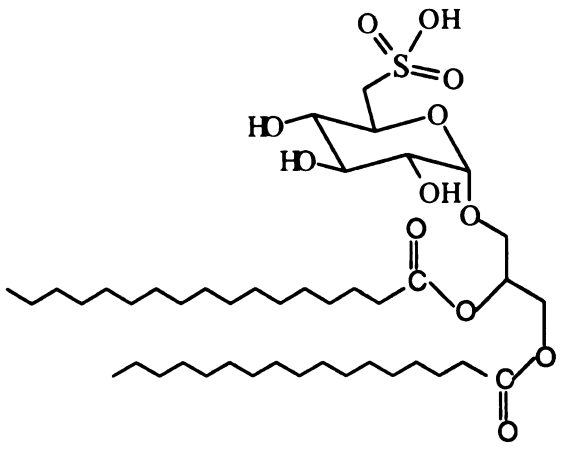

Figure 1.3 - The chemical structure of SQDG

The protein part of plasma membrane includes ATPases, glycosyl transferases, cellulases, enzymes involved in cell wall synthesis and some transporters. The carbohydrates are present mainly in the form of glycolipids and glycoproteins.

3. Plastids

Plants have various forms of plastids, including chloroplasts, amyloplasts and etioplasts. All kinds of plastids are derived from proplastids in meristematic cells. Plastids have a double membrane surface surrounding their own DNA, RNA and ribosomes. Three classes of plant lipids, MGDG, DGDG and SQDG are all very abundant in chloroplast membranes, especially in thylakoid membranes. Besides glycolipids, plastid membranes also contain PC and PG.

Mammalian Cell Surface

In the human body, there are over 200 different types of cell assembled into a variety of tissues such as epithelia, nervous tissue and muscle. The anatomy and physiology of animal tissues and organs are a subject too broad to cover. Given here is a brief description of the type of cells in epithelia, which is often the primary target of infection. Epithelia is a coherent cell sheets which line the inner and outer surfaces of the body. Three common types of cells are absorptive cells, ciliated cells and secretory cells. Absorptive cells have numerous hairlike microvilli projecting from their free surface to increase the area for absorption. Adjacent epithelial cells are bound together by junctions that give the sheet mechanical strength and also make it impermeable to small molecules. Ciliated cells have cilia on their free surface that beat in synchrony to move substances such as mucus over the epithelial sheet. Secretory cells are the specialized cells that secrete substances onto the surface of the cell sheet.

Mammalian and Plant Defense Mechanisms

Mammals have circulatory systems (blood and lymph), a sophisticated immune system involving phagocytes (cells which ingest foreign particles), lymphocytes (antibody-forming cells) and complement (a cascade system of enzymes). There are also bactericidal agents such as cationic proteins and iron-binding substances associated with phagocytes and free in the serum. In addition, there is a memory system whereby responses (antibody formation) on a

second encounter are rapid and at a high level and often prevent a second attack of a particular disease. In contrast to plants, the conditions in mammals are frequently anaerobic and the pH is more likely to be alkaline, especially in the intestinal tract.

Plants recognize and resist invading pathogenic bacteria by inducing a rapid response, termed the hypersensitive response. The hypersensitive response results in a localized cell and tissue death at the site of infection. This strategy constrains the further spread of the infection. It has been reported that infection threads containing wild type *Rhizobium* aborted within root cortical cells in legumes undergoing an hypersensitive response response (207). Sometimes, this local hypersensitive response triggers nonspecific resistance throughout the plant. This phenomenon is known as systemic acquired resistance. After being triggered, systemic acquired resistance can provide resistance to a wide range of pathogens for a few days. The hypersensitive response and systemic acquired resistance depend on the interactions between two proteins, the *R* (resistance) gene product in the plant and the *Avr* (avirulence) gene product in the plant-infecting bacteria. The hypersensitive response and pathogenicity (*hrp*) genes in bacteria are responsible for conferring on certain plant hosts the ability to elicit an hypersensitive response to a bacterium not compatible with the host. Some of these *hrp* genes encode a type III secretion system to export many factors important for infecting eukaryotic hosts (8). This type III system will be discussed later.

At the cellular level, a major difference between mammalian and plant cells

is that the former has no walls and cell-cell communication is via surface contact, not via plasmadesmata as in plants. At the enzyme level, lysozyme is present in secretions and phagocytes and thus equips mammals well to counter bacterial invaders. Lysozyme, also called muramidase, is an *N*-acetylhexosamidase, which acts upon the peptidoglycan layer of bacterial cell wall. In mammals, regulatory molecules are often high-molecular-weight glycopeptides with well-defined receptors at sites of action. Growth regulators in plants are of low molecular weight and varied composition with ill-defined receptors. Generally speaking, though there are profound differences between mammalian and plant systems at tissue level and above, similarities are more prominent at the cellular level.

II. REVIEW ON THE ATTACHMENT PHASE

Rhizoshere is a natural environment of all kinds of soil bacterial, also called rhizobacteria. A natural setting of rhizosphere is composed of the type of soil, the soil temperature, the degree of water saturation, the density and variety of microorganisms, the availability of nutrients and the ecology of plant roots.

Physical attachment is a prerequisite of infection by microorganisms. The mechanisms of attachment have been documented for many mammalian pathogens and more recently for *Agrobacterium* and *Rhizobium*, both of which are plant-infecting Gram-negative bacteria. What are the mechanisms of attachment for mammalian pathogens? What are the mechanisms of attachment for *Agrobacterium* and *Rhizobium*? What kind of selectivity is exerted by the attachment process toward the host? Are these mechanisms of attachment confined in a particular group of, or universal to all the infection systems? These are the questions this review will explore.

A necessary step in the successful infection of a host cell is the adhesion to cell surfaces. There could be multiple ways of attachment in any one particular system, but as far as we know, there are two major types of interaction implicated in the process of bacterial adherence to a eukaryotic cell surface. These two types of interaction will be discussed for mammalian- and plant-infecting bacteria, separately.

Mammalian Pathogens

Most bacteria produce surface lectins, in some cases as the tip subunit of fimbriae which are proteineous filaments on the outer surface. These fimbriae, like other lectins, are able to bind carbohydrate units in a specific manner. The sugar moiety are often glycoconjugates on the host cell surface. Bacterial lectin (adhesins) can also be of afimbrial type. This is seen in *Bordetella pertussis* which is a respiratory pathogen that causes whooping cough. There are at least four fimbrial genes and several afimbrial adhesins in *B. pertussis*, including pertussis toxin, which mimics eukaryotic adhesive molecules (165). By mimicking host molecules, bacterial adhesins can avoid triggering the host defense systems.

Examples of fimbriae adhesins are the family of P fimbriae (pyelonephritis-associated fimbriae), encoded by *pap* operon (87, 98). P fimbriae are very common throughout Gram-negative bacteria. Another group of fimbriae common in Gram-negative bacteria is the type IV family, which includes pili from *Pseudomonas aeruginosa*, *Neisseria* species, and *Vibrio cholerae* (188). There are actually many types of bacterial adhesins, each recognizing a specific carbohydrate structure (Table 3) (171).

Many lines of evidence suggests the importance of surface lectins of bacterial pathogens in the initiation of infection. The co-injection of methyl α-mannoide and *E. coli* with type 1 fimbriae into the urinary bladder of mice caused a three-fold decrease in the extent of bacteriuria (171). A PMF fimbriae mutant of

Table 3 - Sugar specificity of some bacterial surface lectins (171)

Saccharide	Bacteria	Fimbriae
Mannose	*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella spp.*, *Serratia marcescens*, *Shigella flexneri*	Type 1
Galactose	*E. coli*	
L-Fucose	*Vibrio cholerae*	
N-Acetylglucosamine	*E. coli*	Type G
N-Acetylgalactosamine	*E. coli*	
Galα4Galβ-	*E. coli*	Type P
Galβ4Glc	*Actinomyces naeslundii* *Actinomyces viscosus*	Type 2
Galβ4GlcNAc	*Staphylococcus saprophyticus*	
GlcNAcβ3Gal	*Staphylococcus pneumoniae*	
NeuAcα2-3Galβ3GalNAc	*E. coli* *Streptococcus mitis* *Mycoplasma pneumoniae*	Type S

Proteus mirabilis was found to colonize bladders of mice at an 83-fold-lower rate than that of the wild type strain (112).

The second type of bacterial adherence is through the interaction between integrin and the adhesive fibrous glycoproteins such as fibronectin and vitronectin. In animals, there exists the extracellular matrix (ECM), which serves as the underlying foundation for the cells in various tissues like epithelial sheets and connective tissues. Some common ECM components are fibronectin, vitronectin and collagen, each of which has one or more RGD tripeptide sequence motif (161). Cells are usually attached to the ECM through the affinity of integrins for this RGD motif. The integrins are a family of transmembrane glycoproteins comprising noncovalent heterodimers (83). They interact with a wide variety of ligands including ECM glycoproteins, complement and other cells through their extracellular portion while their intracellular domains interact with the cytoskeleton.

There are two ways by which bacteria mimic mammalian integrin-ECM interaction. One is by binding integrins and the other is by binding ECM proteins. There are a number of examples for both cases. In the first case, the binding to integrin does not necessarily invoke the requirement for the RGD motif. As a matter of fact, the binding to some proteins without the tripeptide sequence is much stronger. The best known example is the invasin from *Yersinia pseudotuberculosis*, an enteric bacterium that enters host cells during the course of an oral infection of its animal hosts (85). Invasin has been shown to bind to a variety of integrins from epithelial cells, endothelial cells, lymphocytes and fibroblasts (86). In contrast, other

bacteria, like *Bordetella pertussis* and *Haemophilus influenzae*, use high-molecular-weight RGD-containing adhesins for the attachment to host cell surface integrins (58). In *B. pertussis*, one of the adhesins is FHA which has several domains that are homologous to those of other bacterial adherence molecules or to eukaryotic sequences that mediate cell-cell adhesion. The RGD motif mediates FHA binding to the leukocyte integrin CR3, which is responsible for the bacterial uptake into macrophages without triggering an oxidative burst (151).

Vitronectin is one of the adhesive glycoproteins in ECM and serum. It has been found that vitronectin mediates the internalization of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. Without the presence of vitronectin in the serum, the pathogen was adhered to but not internalized by Chinese hamster ovary cells (53). In *Staphylococcus aureus*, a cell surface protein of 60 kDa is responsible for binding vitronectin in a receptor-ligand type of interaction (105). Vitronectin-binding by *Hylicobacter pylori* is considered important for the pathogensis of helicobacter infection and type B gastritis (96, 154). The relationship of vitronectin to diseases has been studied from a pathological perspective (95). It was found that there was a negative correlation between the level of plasma vitronectin and the severity of pneumonia. The decrease of the level of vitronectin in serum was suggested due to the vitronectin consumption at the pneumonic site.

Plant-infecting Bacteria

Rhizobium and *Agrobacterium* can both infect plants. *Rhizobium* is, however, a symbiont which can benefit the host plants by providing ammonia as a precious nitrogen source, while *Agrobacterium* is a pathogen which can potentially infect a wide range of dicotyledonous plants. Despite this seemingly distinctive biological difference, they are essentially very similar to each other, both phylogenetically and biochemically. This has been addressed in Table 2. The similarity between *Rhizobium* and *Agrobacterium* can be extended to their interactions with plants. This high level of resemblance in their infection mechanism can be appreciated from a pathological perspective and extrapolated to mammalian pathogens as well.

Both *Rhizobium* and *Agrobacterium* are Gram-negative soil bacteria having peritrichous flagella. Flagella have been implicated in determining the ability of these bacteria to infect plants (34), even though numerous conflicting reports also exist (22). This is because most experimental conditions are different from the natural soil in terms of soil type, water saturation and many other factors. This is only one of many cases in which bacteria show their variability and changeability. The conclusion is that motility and chemotaxis are important for the infection by *Agrobacterium* and *Rhizobium* under some conditions but not others (173, 93, 22).

The attachment of *Rhizobium* to the root hair surface is generally divided into two steps (22, 42, 43). In step one, the bacteria loosely adhere to the root surface as single cells, possibly in a polar manner, i.e., with one particular pole in contact

with the host surface (43). The polarity of bacterial cells may be of great physiological significance, though no conclusion can be drawn yet (107).

One type of interaction involved in the first step of the attachment phase is between bacterial rhicadhesin and a glycoprotein from the plant host (183, 190). Rhicadhesin is a 14 kDa protein in the outer membrane of both *Rhizobium* and *Agrobacterium* (180, 182, 189) It mediates bacterial attachment to plant root hair tips (181, 189). This protein is ubiquitous in the family *Rhizobiaceae* but not present in *E. coli* or *Pseudomonas putida* (182). Furthermore, richadhesin-mediated adhesion of *Rhizobiaseae* or *Agrobacterium* cells is not restricted to legumes but occurs with a wide range of plants including dicotyledons and monocotyledons (182). A rhicadhesin receptor has been purified from the cell wall of pea root. The treatment with glycosidase reduced its size from 32 to 29 kDa. The binding between rhicadhesin and this glycoprotein was inhibited by both vitronectin and RGD-containing hexapeptides. It has been further shown that the RGD peptide inhibited the attachment of both *Rhizobium leguminosarum* biovar *viciae* and *Agrobacterium tumefaciens* to pea root hairs (190). This suggests that these plant-infecting soil bacteria may share the features of the ECM glycoprotein-integrin type interaction with mammalian pathogens.

It has long been suggested that plant root lectins are involved in the recognition between *Rhizobium* and host plants (18, 40, 49). In one study, it was suggested that the legume lectin present at the surface may interact specifically with a unique polysaccharide on the surface of *Rhizobium* (18). In another one, the

host specificity for *Rhizobium* infection was changed by the introduction of the lectin gene from another plant species (49). Some legume lectins, such as trifoliin A and a pea lectin PSL, have been found localized on the outer surface of epidermal cells around the tip area of root hair (42, 50). This correlates with the observation that the newly formed primary cell wall is usually the target of enzyme degradation and the fact that infection threads always start from the tip area. Fluorescence-labeled CPS isolated from *Rhizobium trifolii* was demonstrated to bind to clover root hair tips. The specificity of this binding was shown by the ability of unlabeled CPS from *R. trifolii* but not from *R. meliloti*, to block the binding by the labeled CPS (42). Many carbohydrate-containing molecules on the *Rhizobium* cell surface are important for infection. These include LPS, EPS, CPS, cyclic glucans and glycolipids (93, 137). There are about 100 lectins already isolated from various leguminous plants (172). Taking into account those not yet isolated and unknown, this number could go up tremendously. It is very likely that there are multiple lectin-carbohydrate interactions involved for a single *Rhizobium*-legume pair (42).

It is proposed that both *Rhizobium* and mammalian pathogens are able to attach to their host cell surface through lectin-carbohydrate interaction, even though the origin of lectin or adhesin in the two cases is different. In one case the lectin is on the host cell and in the other it is on the surface of the bacterium. However, this does not necessarily mean that *Rhizobium* does not have fimbriae or afimbrial lectin on its cell surface. In *Agrobacterium tumefaciens*, a lectin was isolated from the cell envelope (48). It has a strong haemagglutination activity. In *Bradyrhizobium*

japonicum, a galactose-binding pili has been proposed to mediate the firm attachment to soybean roots (211). A 38 kDa galactose-specific lectin has been isolated from *Bradyrhizobium japonicum* cell surface (77). It has further been shown to be located on one pole of the bacteria and to mediate the polar attachment of the bacteria to soybean roots (106).

The second step in the attachment phase of either *Rhizobium* or *Agrobacterium* to plant surfaces involves the adhesion of additional bacteria to the bound bacterial cells and the formation of aggregates at the attachment site. This is accomplished by its extracellular cellulose fibrils (180, 43, 114, 115), which have been characterized to be *N*-glucosaminyl polymers (79). This step is believed to be irreversible and important for infection especially during rain, flooding, or heavy dew. Similar condition is also seen in mammalian bladder and intestine. This firm attachment allows the infecting bacterium enough time to invade the host. Presumably this type of aggregation will also help to exclude other microorganisms in the surrounding area.

Two types of interaction are proposed to be employed by *Rhizobium* when adhering to plant root hair tips at the initial attachment phase. One is a lectin-carbohydrate type and the other is related to vitronectin. Both types of the interaction are commonly used by mammalian pathogens to infect their hosts. The interaction between leguminous lectins and *Rhizobium* surface carbohydrates is analogous to the interaction between the bacterial adhesins and mammalian cell surface carbohydrates. The interaction between rhicadhesin and the root surface

glycoprotein is similar to that between mammalian vitronectin and bacterial integrin. Both the mammalian ECM and plant cell wall are glycoprotein-containing extracellular polysaccharide matrices. Both rhicadhesin and integrin are cell-membrane-located proteins. Furthermore, vitronectin inhibits the binding between rhicadhesin and the plant cell-wall-located glycoprotein. This argues that rhicadhesin may have some structural similarity with integrin. Since integrin is involved in the endocytosis of infecting bacteria by linking the response to the cytoskeleton, it does not appear likely that rhicadhesin can be functionally related to integrin in terms of the polymerization of actin. However, it can not be excluded that rhicadhesin may be an analog of the binding domain of integrin. This point has not been addressed in the literature. No experimental data is available to help to answer this question. As will be discussed, plant- and animal-invading bacteria share many more features in their ways to infect eukaryotic host organisms.

By no means can we say that a successful attachment can be established by a certain type of interaction. We propose that the attachment between a bacterium and a eukaryotic cell has to depend on multiple factors or interactions, one or some of which may become prominent under certain conditions. The lectin-carbohydrate and vitronectin-integrin types of interaction may be merely two that have been manifested in favorable circumstances.

III. REVIEW ON THE INVASION PHASE

The strategies bacteria use to penetrate eukaryotic cell envelopes can be divided into two classes. One class is via endocytosis and the other involves breaching the cell surface continuity by enzymatic activities. These two mechanisms are not mutually exclusive.

The invasion of host cells is not an isolated event. On one hand, it depends on the result of previous attachment phase and it decides, by integrating the contributions from the relevant bacterial and host factors, the intracellular destination of the infecting bacteria. On the other hand, the attachment and invasion phases may actually be a single event so that it is not appropriate to divide them into two phases.

Endocytosis

1. Mammalian Pathogens

Endocytosis is a general term to describe the process of taking up foreign objects into a cell by enclosing it in the plasma membrane. Phagocytosis refers to the endocytosis of relatively large particles such as microorganisms. Normally, the invading bacteria in mammalian cells are engulfed by phagocytes to form a subcellular organelle called a phagosome. The fusion of phagosomes with lysosomes generates phagolysosome which contains many kinds of hydrolytic enzymes such as lysozyme and toxins such as oxygen radicals. Mammalian

pathogens have two ways of avoiding the phagocytic killing process. One is to survive the life inside phagolysosomes. For example, some pathogens make enzymes to neutralize oxygen radicals and others secrete proteolytic enzymes that degrade host lysosomal proteins. *Coxiella burnetii* and *Salmonella typhimurium* even depend on the acidic pH inside phagolysosome to initiate their intracellular replication and to synthesize factors to allow them to persist in the intracellular environment (72, 150). The other way is to enter structural cells such as epithelial and endothelial cells to avoid being engulfed by phagocytes.

The mechanism of the phagocytosis of mammalian pathogens into nonphagocytes are usually studied in cultured cell lines. Even though the data from this kind of *in vitro* studies may be very off from the *in vivo* situations, it does provide much insight into the bacteria-host cell interaction. Out of the few bacterial species (mainly enteropathogenic bacteria) studied, *Yersinia* can probably be used as a prototype.

A single chromosomal gene encoding an outer membrane protein, named invasin, has been found essential for invading the host cells (85). Invasin was found to mediate the attachment and entry of *Yersinia pseudotuberculosis* and *Y. enterocolitica* into nonphagocytic cells. It was shown that phospholipid vesicles containing isolated integrin proteins were able to attach to invasin. Inert beads coupled with purified invasin were taken up by the cultured human cells (85). Invasin was found to mediate the attachment and entry of *Yersinia pseudotuberculosis* and *Y. enterocolitica* into nonphagocytic cells. It has been found

that host actin polymerization is needed for bacterial uptake since cytochalasins inhibit particle uptake (56). In addition, tyrosine kinase activation was also reported to be part of the host cell response because its inhibitor blocked the endocytosis but not the bacterial adhesion to the cell surface (158). A subset of the integrin superfamily has been shown to be the host cell surface component to bind invasin prior to bacterial penetration into mammalian cells. The structure of integrin is illustrated in Figure 1.4 (83). It was shown that the β1 subunit is critical for invasin binding (85). The β subunit of integrin is a transmembrane glycoprotein of about 100 kDa. Whether the glycoconjugate is responsible for the binding is not known. Two other *Yersinia* proteins, Ail and YadA have also been known to mediates high levels of adherence to epithelial cells and low levels of bacterial invasion (125). Ail belongs to a family of bacterial outer membrane proteins that mediate several different virulence functions (148). YadA also binds to host β1 integrins and is encoded by the virulence plasmid in *Yersinia* species (169). Proteins with functions similar to *Yersinia* invasin have been found in other bacterial

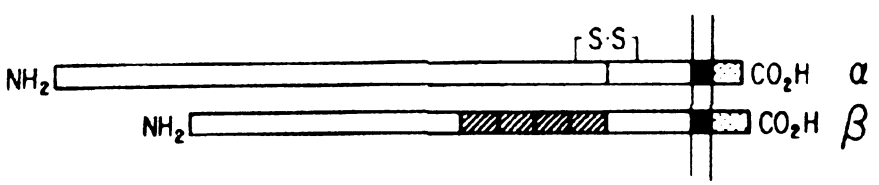

Figure 1.4 - The structure of integrin (83)

species. These are: internalin in *Listeria monocytogenes* (62), IpaB,C and D in *Shigella flexneri* (123), invasin in enteropathogenic *E. coli* (58), and SipB-D in *Salmonella typhimurium* (58). All of these virulence proteins have roles in bacterial adhesion by binding to integrins and in bacteria endocytosis by provoking the cytoskeletal rearragement in host cells.

2. Plant-infecting Bacteria

Endocytosis in the plant kingdom is not as ubiquitous as in the animal world. A better studied case is the release of *Rhizobium* from infection threads into the plant cytoplasm.

After the firm attachment to root hair tips, *Rhizobium* enters plant by breaking the local cell wall. Meanwhile, the cytoplasm of the root epiderms is reorganized. A process of synthesizing inward new wall and plasma membrane starts (Figure 1.1). When the infection thread reaches a nodule primordium cell, synthesis of the infection thread wall stops and only the plasma membrane remains. *Rhizobium* cells at the tip of the infection thread are then released into the host cytoplasm, enclosed in peribacteroid membrane, which is originally from the plasma membrane and which eventually develops to a unique mosaic membrane (210).

Although the nature of this endocytic process is not known. it is clear that contributions from both *Rhizobium* and the plant are needed for it. A Tn5 mutant of *Bradyrhizobium japonicum* was not released from its infection thread and the bacterial entry was stopped at the stage of endocytosis. Infection thread and nodule

differentiation proceeded normally (128). On the other hand, the plant cytoskeleton is required for endocytosis (209). Some additional insights were provided by the attachment of the peribacteroid membrane to components of the bacterial surface (20). It has been shown that an LPS O antigen epitope was abruptly lost right before or during endocytosis of *Rhizobium leguminosarum* bv. *viciae* (67). This suggests that the cell surface of *Rhizobium* is involved in endocytosis. Consistent with this, there are a number of rhizobial mutants which do not enter into the plant cytoplasm, although nodule morphogenesis proceeds through the different stages (209). Whether there is a plant counterpart of the invasin of mammalian pathogens remains elusive, although analogies with bacterial parasites suggest that receptor-mediated endocytosis is likely (128). Such a protein, if any, would be expected to be located in the outer membrane of the invading *Rhizobium*. One clue is that a *Erwinia* virulence gene has sequence similarity to the Spa proteins of *Shigella flexneri* and a pathogenicity protein from the plant pathogen *Xanthomonas campestris* pv. *glycines* (130).

Entry by Breaching Host Cell Envelopes

One important group of virulence factors are hydrolytic enzymes which target a variety of host molecules. By destroying host structural and functional molecules, the infecting bacteria can achieve many specific goals. This strategy can be used at every stage during the infection process and found in diverse host-invading organisms.

Soil Gram-negative bacteria *Bdellovibrio bacteriovorus* can destroy the cell envelops of the *E. coli* by two enzymatic activities, one glycanase and a peptidase (197). Together they were capable of solubilizing 10 to 15% of the *E. coli* peptidoglycan. This enzymatic degradation process appears to be under certain regulatory control because the glycanase activity came to a sharp halt with the completion of the penetration process while the peptidase activity continued, but at a diminishing rate.

The genus *Rickettsia* is a group of small, Gram-negative and obligately intracellular pathogenic bacteria. The interaction of *Rickettsia prowazekii* with mouse fibroblasts (L-cells) results in the expressin of a phospholipase A (PLA) activity with the concomitant release of free fatty acids and lysophospholipids from the phospholipids of the L-cells (219). *Rickettsia* can also lyse erythrocytes of a number of mammalian species. This hemolysis is dependent on the contact of the pathogen with erythrocyte membrane (149). The effect of inhibitors on hemolysis closely parallels their effect on entry into L cells and the release of free fatty acids and lysophosphatides (129). More recent studies shows that both PLA and phospholipase C (PLC) activities were increased during the infection by *R. prowazekii* (219).

There are many other hydrolytic enzymes associated with mammalian pathogens. For example, a protease is anchored by glycosyl phosphatidylinositol in the membrane of *Plasmodium falciparum* (21). PLC and phospholipase D (PLD) can modify the charged head of phospholipids. Because the charged head group

stabilizes the lipid bilayer structure of the host cell plasma membrane, removal of this group destabilizes the membrane. Some PLCs, such as those from *Clostridium perfringens* (196, 164) and from *Vibrio cholerae* (60), are well known for their cytotoxicity. *Listeria monocytogenes* is able to produce at least three phopholipases, all of which are involved in the pathogenicity and encoded in the virulence cluster on the chromosome (164).

On the plant side, the involvement of hydrolytic enzymes are also well-documented. Bacterial soft rot of vegetables and fruits is induced by many bacteria belonging to *Erwinia*, *Pseduomonas*, *Xanthomonas*, *Bacillus* and *Clostridium*. *Erwinia carotocora* and *Erwinia chrysanthemi* attack a wide range of dicotyledons and some monocotyledons by digesting the plant cell wall with a number of pectinases such as pectate lyase and polygalacturonase (68). *Botrytis cinerea* can infect undamaged plant tissue directly by penetration of the cuticle and cutinase A is expressed during the early stages of infection (205). Endoglucanase activity was found associated with a 28 kDa protein in *Clavibacter michiganensis* subsp. *sepedonicus* (7).

Electron microscopy studies of legume infection by *Rhizobium* indicates that hydrolytic enzymes are involved in various steps in the infection process. These include entry of *Rhizobium* into root hairs (153, 200), crossing of root cortical cell walls by *Rhizobium* in the advancing infection thread (33), and release of *Rhizobium* from infection threads into the nodule cell cytoplasm (33, 208). The cell wall degradation is a *Rhizobium*-dependent process, suggesting that *Rhizobium*

enzymes participate in the local cell wall degradation process (206).

IV. REVIEW ON THE SURVIVAL PHASE

Intracellular life is completely different from that in the free-living state. All the bacteria capable of living intracellularly have to adapt to the new environmental challenges. The strategies of this adaptation can be divided into two classes according to whether a particular strategy is constitutive or not. Some infecting bacteria adapt by employing the features that are pre-existing and compatible with their extracellular lives. Some other features needed for an intracellular life may not be present when the bacteria are living in the free-living state, thus have to be induced upon or after invading the host cell. The number of ways for various kinds of bacterial adaptation can be very many. The following review will cover only two very important and common types of strategy for a bacteria to live inside of the cytoplasm of an eukaryotic cell. These strategies are molecular mimicry and metabolism integration.

Bacteroid Formation

After *Rhizobium* is released from infection thread, the peribacteroid unit formed by endocytosis starts to differentiate to become the symbiosome, able to fix nitrogen for the host. The series of transformation also requires the contribution from both *Rhizobium* and the plant. Mutations leading to defects of many rhizobial cell surface molecules can cause problems at this stage. While the endoplasmic reticulum and Golgi bodies are responsible for the tremendous increase of PBM, many nodule-specific proteins, called nodulins, are synthesized by the host cell in

a time-dependent fashion. Obviously, a set of highly coordinated molecular and biochemical events from both organisms are necessary. For example, the nodules formed in alfalfa after inoculation with an EPS mutant of *Rhizobium meliloti* are devoid of bacteria because the infection threads abort within the peripheral cells of the developing nodule (221).

As the interface between the bacteroid and the host cytoplasm, the peribacteroid membrane deserves special attention. During the development of the peribacteroid unit to a symbiosome, *Rhizobium* differentiates from a more free-living state to a bacteroid form while the peribacteroid membrane changes to a more mosaic type, possessing features of both plasma and vacuolar membranes (210). Many new materials are deposited into the peribacteroid membrane to make it function in mediating the interaction between the two partners. A glucosamine-containing PI has been found on the peribacteroid membrane surrounding actively dividing bacteria in root nodules induced by *Rhizobium leguminosarum* (143). Concomitant with the accumulation of starch granule, which is a marker for bacteroid differentiation, this lipid was abruptly lost as the differentiation progressed. Glycosyl phosphatidylinositols are the well-known anchors for many macromolecules on the cell surface of parasitic protozoa and are essential for their infectivity in insect and mammalian host (116). Human pathogen *Leishmania*, like many other protozoan parasites, is also able to synthesize a group of heterogeneous protein-free glycoinositol phospholipids, which may be very important for parasite survival in hosts (117).

Within the peribacteroid unit, there is usually a peribacteroid space surrounding the bacteroid. However, a direct contact between LPS of bacteroid outer membranes and peribacteroid membranes has been observed (22). It suggests that *Rhizobium* play an active role during the differentiation process. This notion has been supported by another observation that the expression of a rhizobial outer membrane protein RopA is developmentally controlled with its disappearance coinciding with the increase of *nifH* mRN A levels (47).

Molecular Mimicry

Eukaryotic hosts have many ways to protect themselves from bacterial infection. As discussed earlier, mammalian hosts possess very sophisticated defense systems both at the cellular level and at the whole body level.

A growing number of examples are suggesting a strategy, called molecular mimicry, used by many bacteria to manage living inside host cells. Gram-negative bacteria *Neisseria* are capable of very quickly changing and modifying the surface carbohydrate structures in such a way that it always mimics the surface antigens of the host (109). Sialylation of LPS is commonly used in *Neisseria* species to evade macrophages by avoiding the opsonization by complement. This is because sialic acid is a ubiquitous host molecule (164). Thus the modification by sialylation will camouflage the invading *Neisseria*. This phenomenon is also observed in *Haemophilus* species (108). By constantly changing the surface antigens, *Neisseria* can also make any antibody response mounted by the host ineffective.

Another example of molecular mimicry is *Streptococcal pyogenes*, which causes sore throats to humans. The pathogen colonizing throat mucosa has a thick hyaluronic acid capsule. Hyaluronic acid is a common constituent of human tissues and is therefore normally not immunogenic. Such a capsule is antiphagocytic and protect *Streptococcal pyogenes* from being killed by phagocytes (199, 164).

As Gram-negative bacteria, *Rhizobium* and *Bradyrhizobium* are very unusual in their ability to synthesize a broad spectrum of glycolipids, some of which are rarely found outside of photosynthetic organisms. They are able to synthesize SQDG (Figure 1.3) in the free-living state (31), DGDG in bacteroids (193) and PI and glycosyl diacylglycerols in conditions similar to that in the peribacteroid unit (192, 194). One of these glycolipids has been shown to be important for symbiosis (137). Some other phospholipids such as PC, which are uncommon for Gram-negative bacteria but common for plants, are also made by *Rhizobium.* This whole spectrum of plant type lipids strongly suggest that they serve to camouflage the intracellular bacteria and to mediate the interaction between *Rhizobium* and host cells. This is a good example of molecular mimicry.

Metabolism Integration

Intracellular bacteria differ in their duration of living inside the host cytoplasm. Normally, the more pathgenic the bacteria, the shorter they live intracellularly. Less devastating bacteria, like parasites or even symbionts, are expected to live much longer intracellularly. It is very reasonable to think that the longer the bacteria live

intracellularly, the more they depend on the nutrients from the host and the more the requirement for the metabolisms of the host and infecting bacteria to fit each other.

One example of metabolism integration is observed with *Plasmodium falciparum*, which is the protozoa causing malarial infection in humans. Plasmodia are incapable of *de novo* fatty acid or cholesterol biosynthesis so that they must obtain these lipid components from their host (212, 213). They achieve this by digesting host phospholipids with PLA and lysoPLA activities. The activity of lysoPLA of human erythrocytes increased by three orders of magnitude upon infection with *Plasmodium falciparum* (223). This would bring about the increased phospholipid biosynthesis by the host. By this way, the phospholipid metabolism of the parasite and human cells are linked together. This particular integration is in favor of the parasite but not as devastating to the host as in the cases of pathogens.

The inter-dependency of virulence and metabolic activities of pathogenic bacteria has been illustrated by the effect of the pathogenicity islands (Pais) on enteropathogenic *E. coli* (155). The insertion of the Pais not only contributes to the recipient strain many trans-acting virulence factors and regulators but also abolishes many metabolic functions due to the insertional inactivation of the tRNA genes where the Pais are inserted.

The best example of metabolism integration is probably the *Rhizobium*-legume symbiosis. Each one of the symbionts depends on the other one for

nutrients and each fulfills its own role in the integrated metabolism (216). This metabolism integration depends on the genetics of either organism to "fit" each other. On one hand, they have to be compatible to each other to avoid any conflicts during the course of the metabolite exchange. On the other hand, they have to complement and take advantage of each other. Since *Rhizobium* is able to synthesize the host glycolipid, SQDG (31), it is expected that it shares features of SQDG metabolism with plants. If this were true, it would mean the compatibility of the host-infecting bacteria and the plant hosts at the genome level, strongly arguing the importance of SQDG in the establishment of the symbiotic relationship between the two organisms.

V. Common Themes in Bacterium-Eukaryote Interaction

Two major classes of bacterium-eukaryote interaction are the bacterium-mammal and bacterium-plant types. The word "major" refers to the importance or interest of human being and thus the availability of experimental data. Since most eukaryote-infecting bacteria are of Gram-negative type, the discussion will be focused on the interaction between eukaryotes and Gram-negative bacteria for the sake of simplicity. However, this does not necessarily mean that Gram-positive pathogens are not important. As a matter of fact, many Gram-positive bacteria pose serious thread to human health by causing various diseases.

First of all, the commonality is seen through the infection process of mammals and plants by bacteria. Both types of interaction can be divided into a three-phase process. In the attachment phase, lectin-carbohydrate and protein-protein recognition are the possible mechanisms for both bacteria-plant and bacteria-mammalian interaction. In the invasion phase, enzymatic degradation and endocytosis are the two common approaches for bacteria to invade host body and host cell. In the intracellular survival phase, the analogy between the pathogen-containing vacuole in mammalian cytoplasm and the bacteroid-containing peribacteroid unit is obvious. Both vacuole membrane and the peribacteroid membrane are biosynthesized by the host cell and are unique in their composition. This uniqueness is apparently related to their biological function, which is to interface the intracellular bacteria and the eukaryotic cytoplasm. For mammalian pathogens, the primary goal is to avoid the fusion of lysosomes and vacuoles. For

Rhizobium, the primary function of the peribacteroid membrane is to mediate material exchange and to protect bacteroids from causing the hypersensitive response in host cells. The coexistence of two organisms will require a series of strategies including molecular mimicry.

It has been found that both mammal- and plant-infecting bacteria use type III secretion systems to export the virulence factors (203, 10). Because nearly all bacterial virulence factors are located on the bacterial surface or are secreted, the cross-membrane transportation of these factors is very important for bacterial pathogenicity. There are three general secretion pathways in Gram-negative bacteria that export bacterial virulence factors (162). The type III export system is triggered specifically by contact with host cells and delivers its content directly into those cells. This system was originally discovered in *Yersinia* to be responsible for the secretion of Yops in early 1991. Soon after, these proteins were found in *Salmonella*, *Shigella*, enteropathogenic *E. coli* and a number of plant pathogens including *Erwinia*, *Xanthomonas* and *Pseudomonas* (58). The recent sequencing of the 536 kb megaplasmid in *Rhizobium* revealed a cluster of six ORFs showing strong homology to the components of other type III machineries (61). Homologues of these genes are also sequenced in another *Rhizobium* species and are responsible for the secretion of various proteins. In both mammalian and plant pathogens, most proteins secreted via this route are virulence factors. These include the Yops in *Yersinia*, the invasion factors in *Salmonella* and *Shigella*, AvrB in *Pseudomonas*. Furthermore, these type III secretion genes are often located in

the Pais on the chromosome (for example, in *Salmonella*) or on a large virulence plasmid (for example, in *Shigella*) (57). The strong sequence homology and the megaplasmid-location of the genes suggest the presence of the type III secretion pathway in *Rhizobium*.

The regulation of gene expression of virulence factors is a key in the process of adaptation of pathogens to their host environment. This can be accomplished in many different ways and at many different levels. One proposed strategy employed by the bacteria living in high density (for example, inside eukaryotic cell, which is equivalent to rich medium *in vitro*) is termed quorum sensing. It is thought that bacteria are able to measure their population density and only produce virulence factors at a certain threshold. The molecules important for quorum sensing are a class of diffusible communication signals, *N*-acyl homoserine lactones, which are widespread in Gram-negative bacteria. This phenomenon has been observed in quite a few eukaryote-inhabiting bacterial species including *Pseudomonas aeruginaosa* (141), *Vibrio fisheri* (121), *Erwinia carotovora*, *Yersinia enterocolitica*, *Agrobacterium tumefaciens* (36) and *Rhizobium leguminosarum* (70, 168). The protein responsible for the synthesis of the *Pseudomonas* autoinducer is a virulence factor LasI, which is a homologue of LuxI, the enzyme responsible for synthesizing the autoinducer in *Vibrio fisheri* (141). Clearly, this quorum sensing mechanism is highly conserved among many eukaryote-infecting Gram-negative bacteria including *Rhizobium*.

It has been mentioned earlier that flagella is important for virulence under

certain conditions and that type III secretion systems are responsible for exporting virulence factors and are conserved among many eukaryote-infecting Gram-negative bacteria. At first glance, there seems no connection between these two phenomena, except that they are both related to bacterial infectivity. Interestingly, a *Rhizobium meliloti* gene, which is essential for motility and similar to the FliP proteins of *E. coli* and *Bacillus subtilis*, has been found to share significant sequence similarity with HrpT from *Pseudomonas solanacearum* and *Xanthomonas campestris*, LsaB from *Shigella flexneri* and Spa24 from *Yersinia pestis* (59). All of these pathogen proteins are involved in the export or presentation of proteins on the cell surface. In *Pseudomonas solanacearum* and *Pseudomonas syringae*, the *hrp* gene cluster controls both the production of type III secretion system and the biogenesis of flagella (82, 204). In both cases, one of the *hrp* genes is related to the *nolT* gene in *Rhizobium fredii*. *nolT* is megaplasmid-encoded and hybridizable to the DNA from *Rhizobium* sp. NGR234 but not other *Rhizobium* species (122). The function of *nolT* is unknown. It is reasonable to ask whether *nolT* has something to do with exporting factors for interacting with plants. Once again, the connection between protein export and the proteins responsible for infectivity seen in host-infecting Gram-negative bacteria is also observed in *Rhizobium*.

CHAPTER 2

The Membrane Phospholipases of *Rhizobium*

(Potential Virulence Factors for Invading Plant Cells)

ABSTRACT

Hydrolytic enzymes have been implicated in the infection of eukaryotic organisms by bacteria, either to change the membrane compositions and thus to modify its properties or to disrupt the membranes and allow the bacterial entry or escape. It has been demonstrated that *Rhizobium* hydrolyzes cell wall locally to enter the plant roots and that phospholipases are virulence factors in a number of mammalian pathogens. We propose that phospholipases are critical for the infection of plant cells by *Rhizobium*. The activities of phospholipases D and A have been demonstrated with *Rhizobium* membranes. Phospholipase A has further been localized to the outer membrane, suggesting a direct role in the interaction with the host.

INTRODUCTION

Phospholipases are ubiquitous enzymes which degrade phospholipids. Major types of phospholipases are phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD). These are classified according to the chemical bond they attack in phospholipids. PLA attackes the acyl bond and releases fatty acids from phospholipids. Both PLC and PLD work on phosphodiesters. The hydrolysis by PLC generates diacylglycerol (DAG) and the phosphorylated headgroup while the hydrolysis by PLD results in the accumulation of phosphatidic acid (PA) and the appropriate headgroup. These phospholipases have been found in a wide range of organisms including animals, plants, fungi and bacteria. They can be cytosolic, membrane-bound or secreted extracellularly. Quite a few of these enzymes have been purified, cloned, sequenced, and crystalized. Since the major location of phospholipids is biological membranes, the functions of phospholipases are usually related to membranes.

Biological membranes are the boundaries between the intracellular spaces and the extracellular world and are responsible for maintaining the integrity of living cells. All biological membranes, including the plasma membrane and the internal membranes of eukaryotic cells, have a common general structural assembly of lipid and protein molecules held together mainly by noncovalent interactions. The membrane lipid composition varies with the type of organisms. In general, all membranes contain large amounts of phospholipids. Mammalian membranes tend to have sphigolipids, sterols and some glycolipids while plant membranes usually

contain large amount of glycolipids and sterols. A unique feature for the membrane of Gram-negative bacteria is the presence of LPS, which is the predominant lipid component in the outer leaflet of the outer membrane.

As the most basic building block of biological membranes, phospholipids stands out in their contribution and importance to normal cell function. This explains the lethal effects of various phospholipases in all kinds of situations, either *in vitro* or *in vivo*. All the three types of phospholipases have been implicated in toxicity, hemolysis and virulence. PLAs are the lethal factors in snake and insect venoms. PLCs are the toxins secreted by many disease-causing bacteria. PLDs are the essential virulence determinants in a group of human and animal pathogens (120).

Phospholipases are also important for many normal biochemical and physiological processes. These include the turnover of phospholipids and the supply of metabolic intermediates. The focus here, however, is the roles of phospholipases during the bacterial infection of plants and animals. As discussed in Chapter 1, one strategy for bacteria to penetrate the host membrane is to breach it with hydrolytic enzymes. One important group of such enzymes is the phospholipases. Phospholipases have proven to be the virulence factors of many bacterial pathogens such as *Listeria*, *Pseudomonas*, *Campylobacter*, *mycobacteria*, *Clostridium*, *Legionella*, *Helicobacter*, *Vibrio*, etc. In some fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus*, phospholipases have also been demonstrated to be important for pathogenicity (16, 84).

The genus *Listeria* can cause severe diseases like meningitis and

meningoencephalities in human. The infection of human cells by *Listeria* can be divided into four major events: internalization, escape from the intracellular vacuole, nucleation of actin filaments and cell-to-cell spread. The intracellular growth begins after the lysis of the primary vacuole formed upon bacterial entry into a host cell. *Listeria monocytogenes* produces three hemolysins, listeriolysin O (LLO), PI-specific PLC (PI-PLC) and a broad range PLC. In human epithelial cells, a LLO-mutant is capable of growth, suggesting that some gene products other than LLO are also critical for mediating the escape of *L. monocytogenes* from vacuoles. It has been shown that in the absence of LLO, both the broad range PLC and a metalloprotease are needed for the lysis of vacuoles in human cells. PI-PLC is also needed for the high efficiency of escape (110).

In most cases, the virulence-associated phospholipases are secreted by bacteria. This type of cytotoxicity is manifested without a need for the bacteria to be in contact with the host cells. However, evidence is accumulating for the presence of membrane-associated phospholipases which are involved in bacterial infection. A PLA from the outer membrane of *E. coli* has been purified (166). Its structural gene, *pldA*, has been cloned (45) and sequenced (80). The overexpression of this PLA enabled it to be crystallized and structurally characterized to be a member of serine protease family (24). The further cloning and sequencing of the *pldA* genes from *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Proteus vulgaris* imply that it is well conserved among *Enterobacteriaceae*, which includes many species capable of infecting animals, suggesting a possible role for the outer membrane phospholipase A during the

infection of host cells (23). Consistently, PLA activity has been found in a high degree of correlation to other virulence parameters like enterotoxin production in *Salmonella typhimurium* (174). More direct evidence comes from a recent study which shows that a homologue of *E. coli* outer membrane PLA is present in *Campylobacter coli*. The inactivation of this gene resulted in reduced cell-associated hemolytic activity of *C. coli* (69).

It has long been known that phospholipase activities, including PLA, lysoPLA and PLC, are needed for the internalization process of rickettsiae into eukaryotic cells (220). To distinguish from the common type of phagocytosis, this internalization mechanism is often referred to as "induced phagocytosis" to emphasize the active role of *Rickettsia*. It has been demonstrated that the phospholipase activities are from *Rickettsia* rather than the host cells (178), suggesting that a reorganization of the host plasma membrane is necessary and that the modification is initiated by the infecting bacteria.

Rhizobium share many pathogenic features with other plant- and animal-infecting bacteria. During the infection of a target nodule primordium cell, *Rhizobium* cells need to be enclosed in a membrane and endocytosed into the host cytoplasm. This peribacteroid membrane is mainly of the plant origin. But this does not necessarily mean that the participation from *Rhizobium* is excluded. As a matter of fact, *Rhizobium* plays an active role during this process. It has been suggested that the fusion of plasma membrane and *Rhizobium* outer membrane is very possible. This has been discussed earlier in Chapter 1. The phospholipases have to be

involved in membrane reorganization, which is one of the basic events during the infection of plant root cells by *Rhizobium*. The formation of infection thread involves the change of the membrane from a more lamellar type to a more tubular one. This requires a reduction of the size of the phospholipid headgroups and the adjustment of membrane topology and fluidity. The former change would need phospholipase C and D activities and the later one would need phospholipase A and lysophospholipase activities. We propose that phospholipases are important for the infection of legume root cells by *Rhizobium* and that these phospholipase activities are from *Rhizobium*. The first step for research along this line is to detect and characterize phospholipase activities in *Rhizobium*.

MATERIALS AND METHODS

Bacterial Strains and Cultures

Rhizobium meliloti 2011 was obtained from Dr. Ethan Signer in Massachusetts Institute of Technology. Cells were grown at 30° C to mid-log phase in BIII medium (41) with constant shaking at 175 rpm, in the presence or absence of 2 mg/L luteolin.

Membrane Preparation

Cells were harvested with centrifugation at 8 k rpm in a Sorvall GSA rotor for 15 minutes. The cell pellet was washed in 15 ml of 25 mM Tris/HCl buffer at pH 7.9 and re-suspended in 15 mL of 10 mM Tris/HCl buffer at pH 7.9 containing 20% sucrose (w/w) and 2 mM EDTA. Dithiothreitol (0.2 mM) and RNase A (Boehringer Mannheim, 0.2 mg/mL of lysate) were added after quick freezing-thawing. The cells were passed twice through a French-press at 15,000 psi. $MgCl_2$ (3 mM), DNase I (Boehringer Mannheim, 0.2 mg/mL) and lysozyme (Boehringer Mannheim, 0 .2 mg /mL) were then added and the lysate further incubated at room temperature for 30 minutes with stirring. For assaying phospholipase A activities, SDS was added to 0.05% at this point. The lysate then was centrifuged at 4,000 rpm in a Sorvall SS34 rotor for 15 minutes to spin down the unlysed cells. The supernatant was then centrifuged at 165,000 x g in a Beckman 70Ti rotor for two hours. The resulting pellet was the total membrane preparation used for assaying the phospholipase activities.

Detection of Phospholipase D

The buffer for assaying the PLD activities contained 25 mM Tris/HCl at pH 7.5, 10 mM $CaCl_2$, 0.1% SDS and 10 μL of membrane preparation in 60 mM MES buffer at pH 6.0 in a total volume of 100 μL. The substrate used was TLC-purified ^{14}C-1,2-dipalmyltoyl phosphatidylcholine (specific activity of 0.16 Ci/mole) in emulsion at 20 mM. 25 mM NaF was used to inhibit phosphatase activity. The assay was done at 30 °C with moderate shaking for two hours. The assay mixture was extracted with 0.4 mL 200:100:0.5 chloroform/ methanol/ HCl. The organic layers were condensed and subject to TLC analysis. The solvent system for TLC was 65:35:5 chloroform/ methanol/ concentrated ammonia.

Characterization and Localization of Membrane Phospholipase A

Membrane suspensions were prepared the same as described above. The assay buffer contained 40 mM MES buffer at pH 6.0, 0.01% α-octyl glucoside, 10 mM $MgCl_2$ and 1% MeOH, unless otherwise specified, in a total of 200 μL. The substrate and the method used to extract the reaction product was the same as in the phospholipase D assay. PhosphoImager system was used for quantitating the TLC plates. The intensity of a radioactive spot was normalized by substracting with the background and by the subsequent division of the total count of each sample. The potential PLA inhibitors were added to the reaction buffer before the reaction. Hexadecanesulfonyl fluoride (HDSF) was incubated with the membrane preparation at various concentrations for one hour before the start of reactions.

For membrane separation, the outer membrane was released from the intact cells by incubating in 50 mM TrisHCl at pH7.9, 0.5M sucrose and 0.2 mM EDTA at 30°C. After lysozyme digestion, the spheroplast formed was broken by sonicating on ice for 10 min with a 1.5 sec-4.0 sec program. Inner membrane fraction was separated from cytosol by centrifugation at 150,000 xg for 20 minutes. The buffer containing outer membrane and periplasm was lyophilized. The solid was then resuspended in a buffer containing 60 mM MES and 10 mM $MgCl_2$. The protein contents were measured with Bradford method.

Spectroscopic Analysis

NMR spectroscopy was carried out on a Varian VXR 500 instrument operating at 500 MHz for protons. All spectra obtained in deuterium oxide and the residual water line was suppressed by selective inversion - recovery. Spectra were recorded at 25 °C. Mass spectra were obtained in the positive ion mode on a Fisons Platform electrospray instrument. The sample was infused in methanol.

RESULTS

Phospholipase D

The phospholipase D activity was first observed during the separation of *Rhizobium* membranes (Materials and Methods in Chapter 3). After the density gradient centrifugation of membrane pellets in the presence of sucrose of high concentration, it was observed that bulk of the phospholipids disappeared and that a new lipid species of large quantity was formed (Figure 3.3A). This newly formed slow-moving lipid was purified with preparative TLC and subject to spectroscopic NMR and ES-MS analysis. Figure 2.1 shows the NMR spectrum of this slow-moving lipid. The resonances between 1.0 and 1.5 ppm indicates the presence of hydrocarbon chains. The complex signals between 3.0 and 3.8 ppm indicates the presence of a sugar moiety and the signal further downfield at 5.4 ppm the presence of an anomeric proton. Resonances at 4.2, 4.3, and 5.1 ppm are characteristic signals for glycerol backbone, indicating that this is a glycerolipid. The lipid sample was then analyzed in deuterium oxide in a different NMR experiment. The spectrum obtained was compared with that of sucrose recorded under the same condition. This is shown in Figure 2.2. It is clear that the headgroup of this glycerolipid is sucrose. This phosphatidylsucrose apparently resulted from the headgroup exchange during the membrane separation process due to a transphosphatidyl activity. The identity of phosphatidylsucrose was further confirmed by ES-MS (Figure 2.3). The molecular ion m/z 1049 is a sodium adduct of phosphatidylsucrose containing two fatty acyl chains with a total of 36 carbon

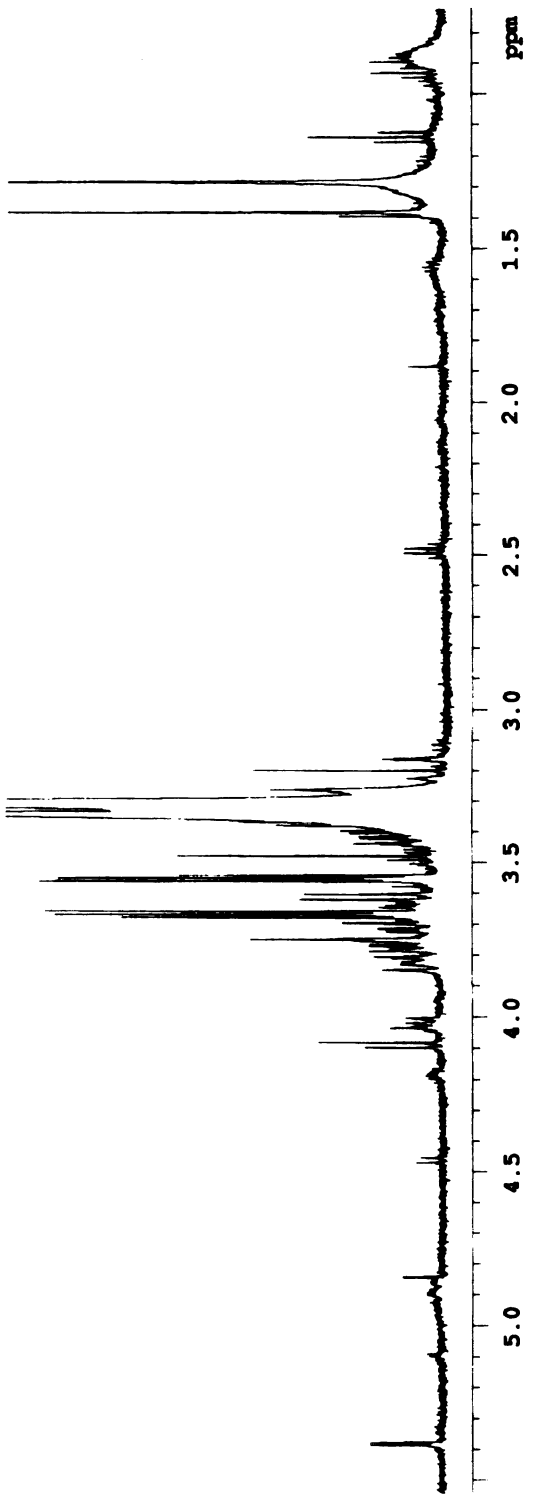

Figure 2.1 - ^{1}H-NMR spectrum of phosphatidylsucrose

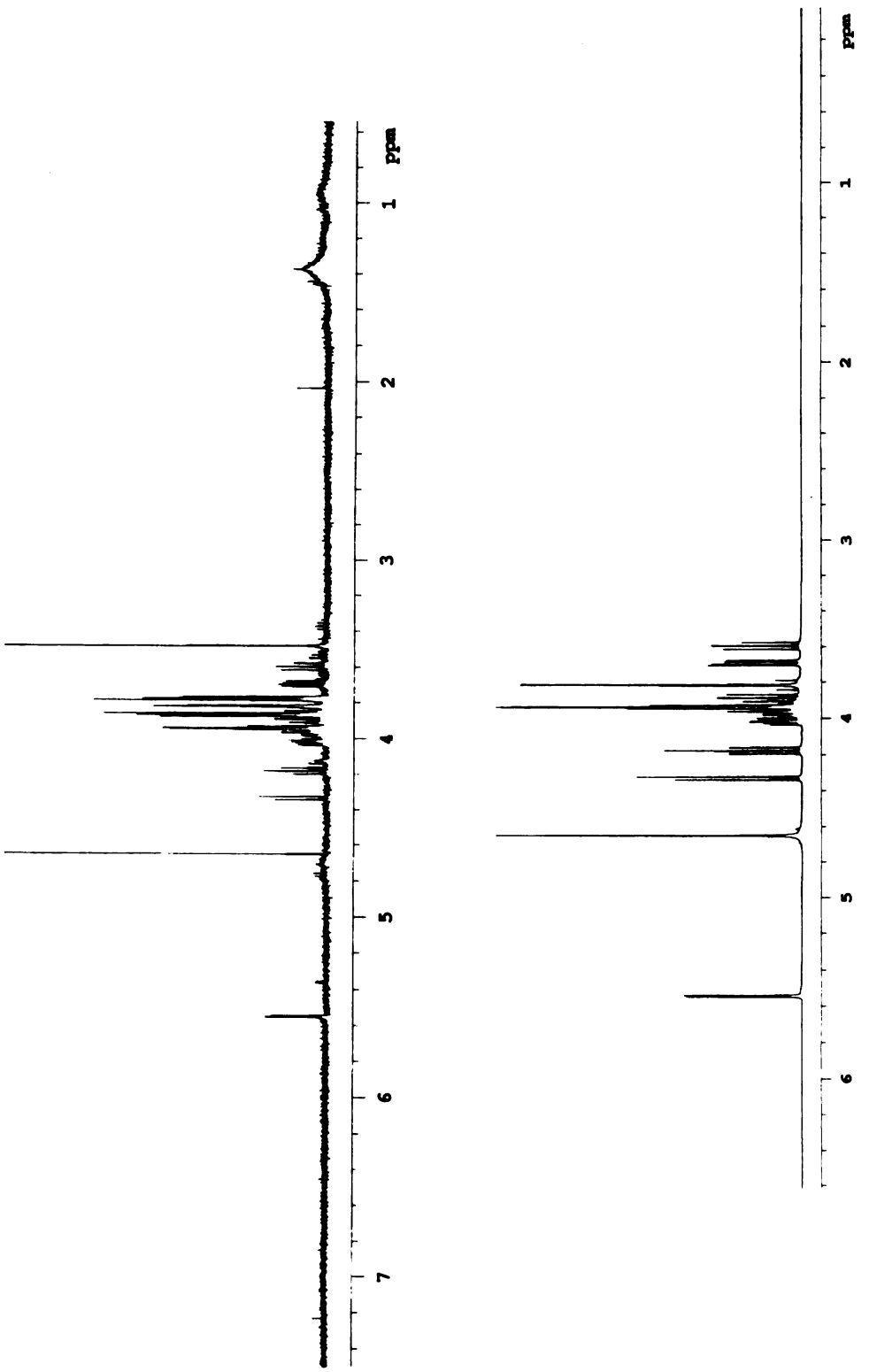

Figure 2.2 - ^{1}H-NMR spectra of phosphatidylsucrose (Top) and sucrose in D_2O

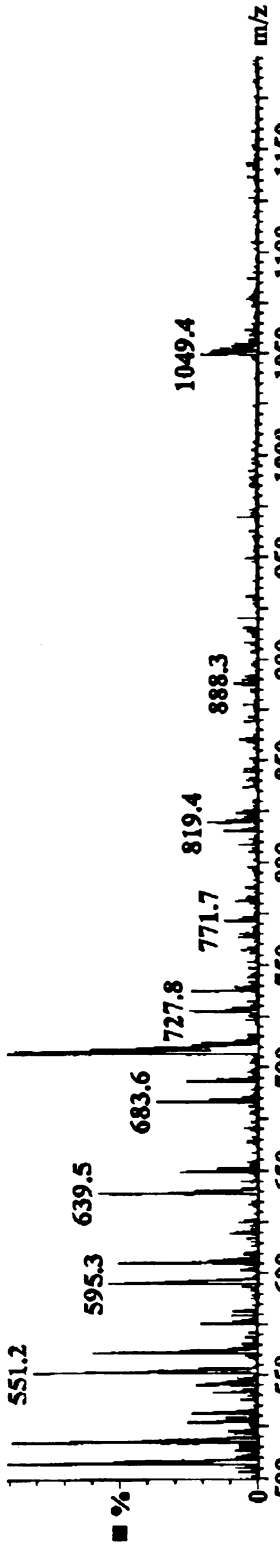

Figure 2.3 - The ES-MS spectrum of phosphatidylsucrose

atoms.

Phospholipase D is well documented to possess this transphosphatidyl activity (214). The formation of the phospholipids with some alcohols, such as ethanol, as headgroups is often used as a way to measure the PLD activity (215). It is very likely that the formation of phosphatidylsucrose was due to the transphosphatidyl activity of PLD in *Rhizobium* membranes.

The assay of PLD shows the formation of PA in a calcium-dependent manner (Figure 2.4). Luteolin, a plant flavonoid often used to induce *Rhizobium* genes associated with symbiosis, did not seem to have any effect on PA formation. This result further suggests the presence of membrane-associated phospholipase D in *Rhizobium*.

Phospholipase A

Phospholipase A (PLA) assay conditions were optimized by changing the different factors one at a time. As shown from Figure 2.5 through Figure 2.12 , the release of fatty acids by the membrane preparation of *Rhizobium meliloti* 2011 was divalent cation-dependent, time-dependent, temperature-dependent, pH-dependent, and heat-inactivated. The fact that fatty acids can be released from LPC indicates the presence of lysoPLA activity. The presence of alcohol did not seem to matter very much for the release of fatty acid, though the membranes showed higher PLA and, especially, lysoPLA activities in methanol. Neither the potential membrane perturbant *cis*- or *trans*-decalenes nor the inhibitors to other PLAs, *p*-bromophenacyl

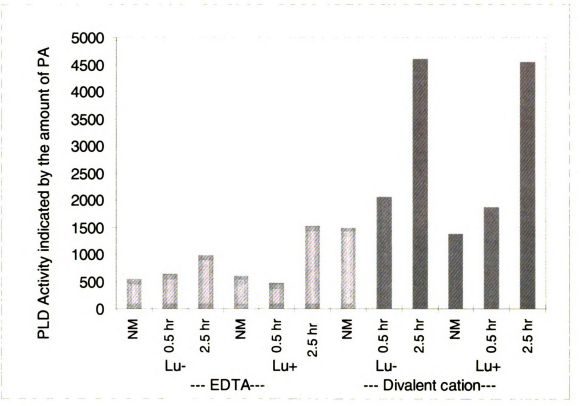

Figure 2.4 - Formation of PA from PC by *Rhizobium* membranes
NM, no membrane; Lu, luteolin

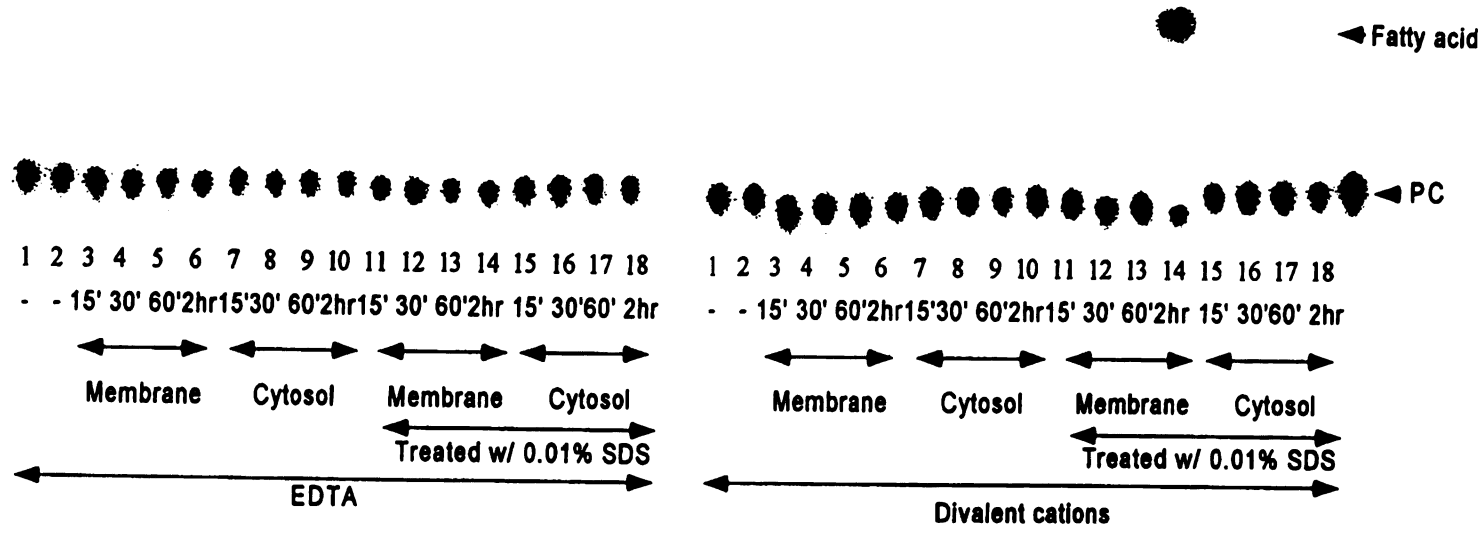

Figure 2.5 - Divalent cation-dependent release of fatty acid from PC by *Rhizobium* membranes

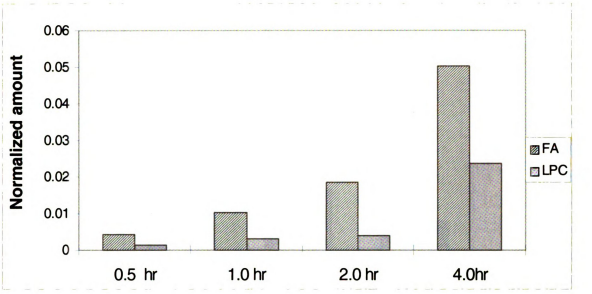

Figure 2.6 - Time course of FA and LPC formation from PC

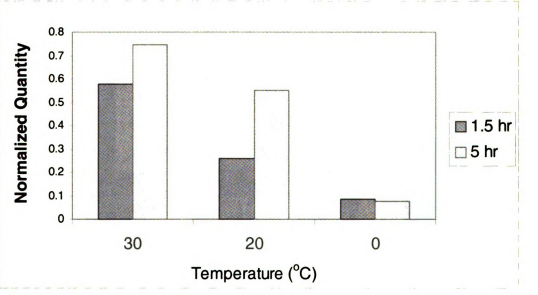

Figure 2.7 - The temperature-dependence of FA release from PC

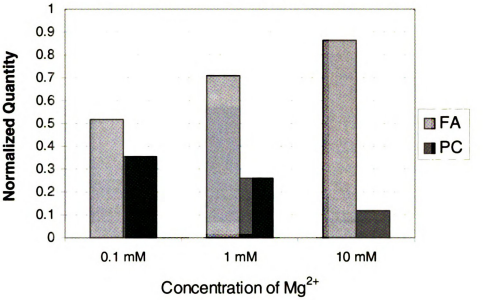

Figure 2.8 - The effect of Mg^{2+} concentration on FA release from PC

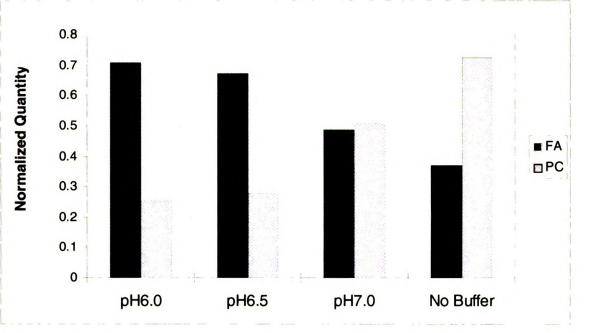

Figure 2.9 - The effect of pH on the release of FA from PC

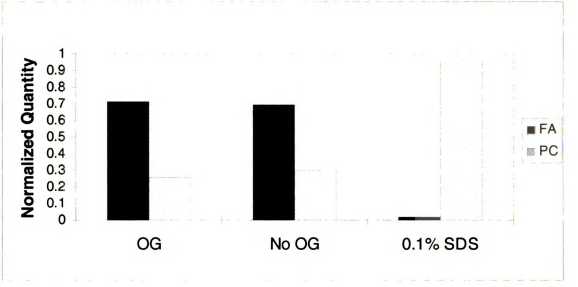

Figure 2.10 - The effect of detergent on the release of FA from PC

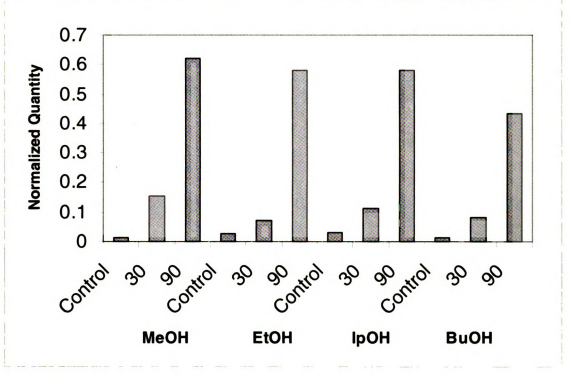

Figure 2.11A - The effect of alcohol on FA release from PC

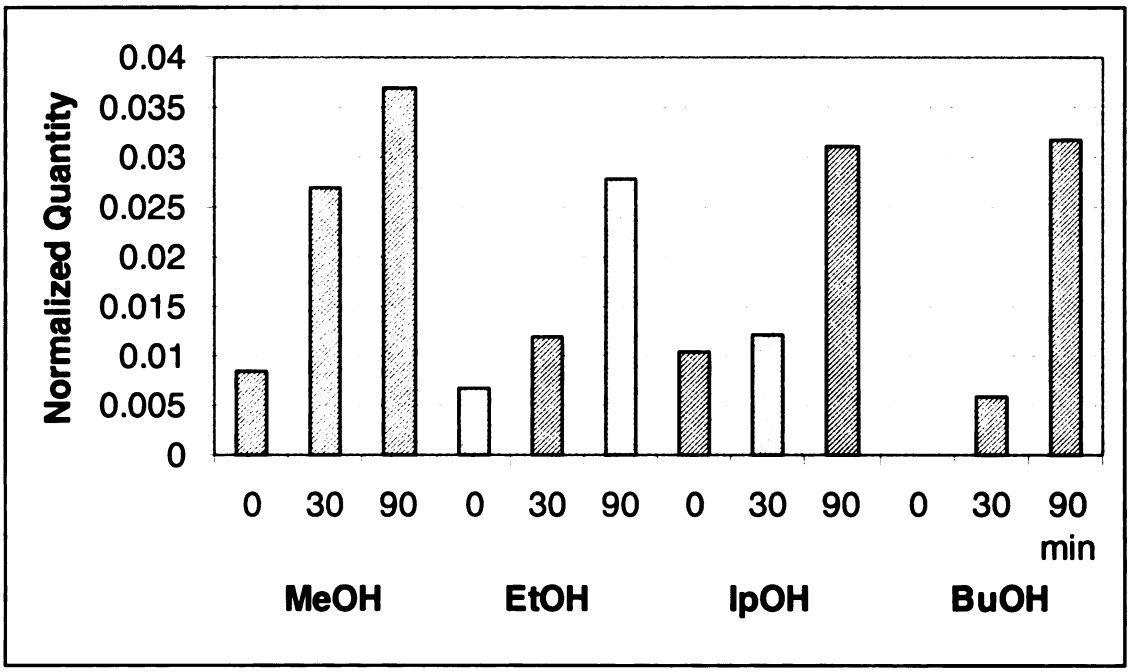

Figure 2.11B - The effect of alcohol on LPC formation from PC

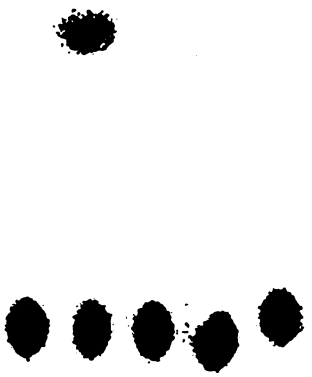

Figure 2.12 - Heat inactivation of PLA from *Rhizobium* membranes
H, PLA heated at 100 °C for 5 minutes. N, not heated.
1 and 2, two different membrane preparations

bromide and 2-acetyl salicylic acid, showed inhibitory effects on this membrane PLA activity (Figures 2.13, 2.14 and 2.15).

The purified *E. coli* outer membrane PLA has been reported to be inhibited by hexadecanesulfonyl fluride (HDSF) but not by the well-known serine-protease inhibitor phenylmethanesulfonyl fluoride (PMSF) (81). An experiment was carried out to synthesize HDSF from hexadecanesulfonyl chloride by refluxing in anhydrous acetone for three hours in the presence of tenfold molar excess of anhydrous ammonium. NMR analysis showed the successful synthesis of HDSF (Data not shown). However, PLA activities did not seem to be inhibited by HDSF under standard assay conditions (Figure 2.16).

Since the DR(detergent resistant)-PLA of *E. coli* is located in the outer membrane, an experiment was designed to determine the PLA membrane localization in *Rhizobium*. The fractions of outer membrane and periplasm, inner membrane and cytosol were obtained as described in Materials and Methods. The protein distribution in these fractions are shown in Table 2.1, in comparison with that from *E. coli*. Figure 2.17 shows that the PLA and lysoPLA activities were associated with the outer membranes rather than the inner membranes and the cytosol.

Table 4 - Comparison of protein cellular distribution between *Rhizobium* and *E. coli*

	IM	OM + periplasm	cytosol
Rhizobium meliloti 2011	13%	7%	80%
E. coli	6-9%	4-6%	85-90%

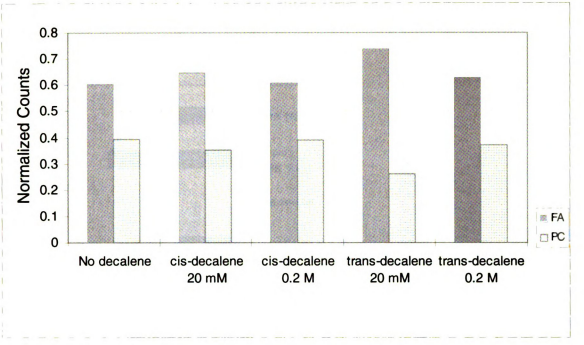

Figure 2.13 - The effect of decalenes on FA release from PC

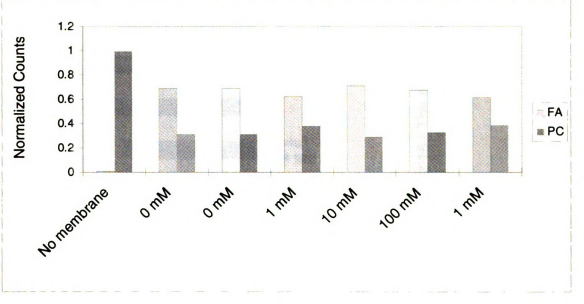

Figure 2.14 - The effect of 2-acetyl salicylic acid on FA release from PC

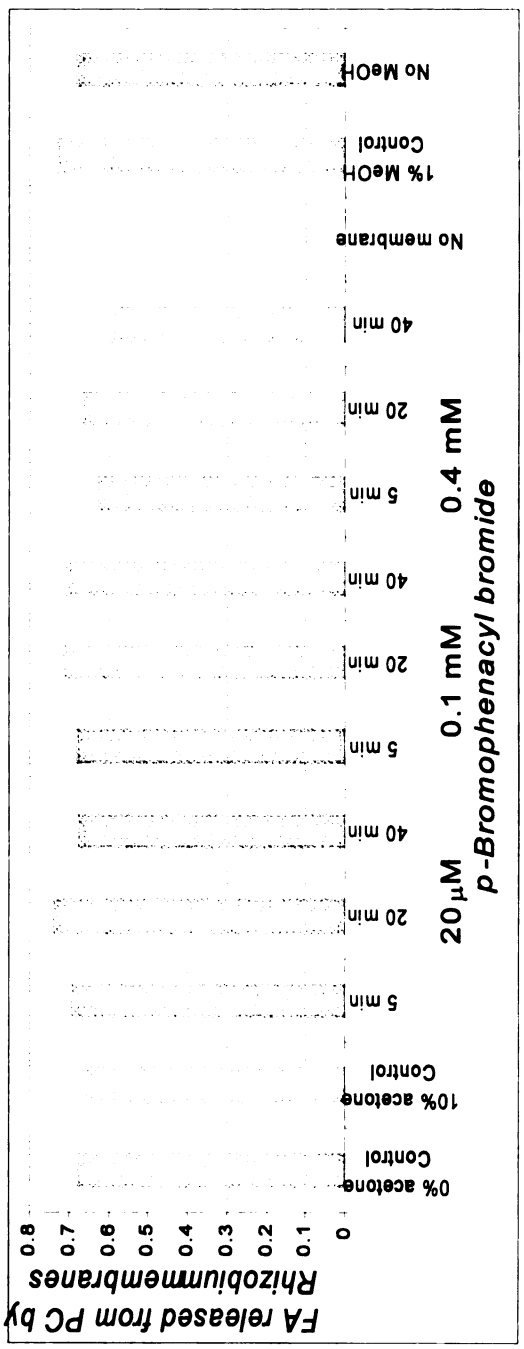

Figure 2.15 - The effect of *p*-bromophenacyl bromide on the release of FA from PC by *Rhizobium* membranes

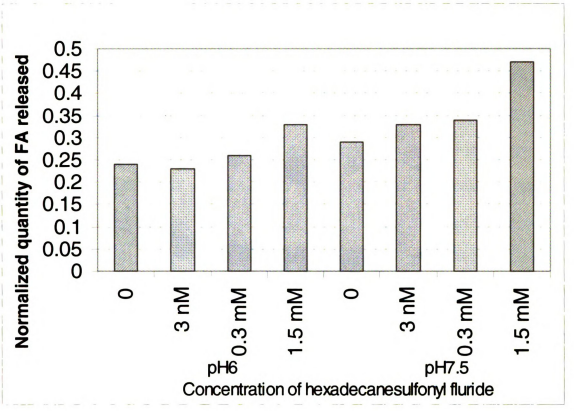

Figure 2.16 -The effect of hexadecanesulfonyl fluride on FA release from PC

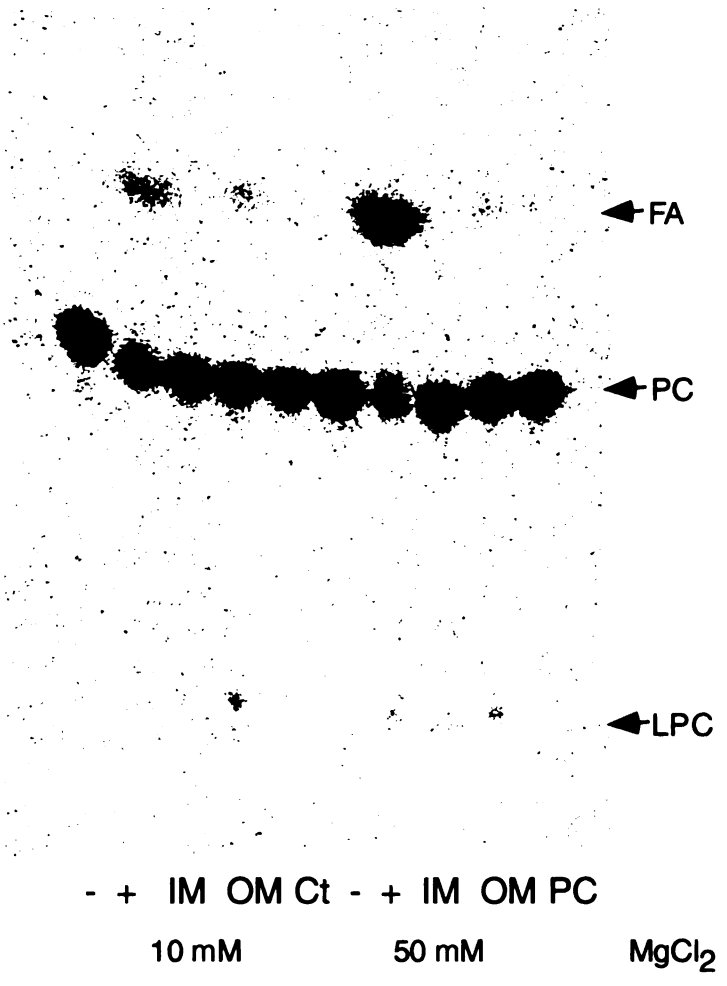

Figure 2.17 - The PLA activities are found in the outer membrane rather than the inner membrane of *Rhizobium*

DISCUSSION

If membranes are essential to all organisms, phospholipases have to be important to many basic biochemical processes. As a matter of fact, PLA, PLC and PLD have all been implicated in the bacterial pathogenesis of both animal and plant hosts. We propose that phospholipases are important during the infection of the host plants by *Rhizobium*. In addition to be secreted, phospholipases are also present in membranes, although few of them has been purified and characterized. Some known examples of membrane phospholipases include the PLD in yeast, the PLC in one mycoplasma species and the DR-PLA from two *E. coli* strains. Here we report the discovery of PLA and PLD associated with *Rhizobium* membranes. This is the first time that phospholipases are detected in *Rhizobium*.

Experiments indicated that *Rhizobium meliloti* did not produce extracellular phospholipases because the incubation of PC with neither live cells nor culture media resulted in any PC degradation, even after prolonged time. PLD and PLA activities were later detected and manifested in membrane preparations of the bacteria. The production of diacylglycerol (DAG) was observed, indicating a PLC activity. However, because of the variability associated with DAG production, the membrane PLC activity was not further pursued.

The presence of PLD in the membranes of *Rhizobium meliloti* was demonstrated by the formation of phosphatidylsucrose during sucrose density gradient ultracentrifugation and by the release of PA from PC after the incubation

of the membrane preparation with PC. The production of PA was calcium-dependent, suggesting the involvement of an enzymatic activity. However, a transphosphatidylation activity was not observed with unnatural headgroups such as enthanol and butanol. It could be due to the reaction condition or to the nature of *Rhizobium* enzyme. The transphosphatidylation activity was manifested only with extremely high concentration of sucrose.

We also showed that PLA activities were associated with *Rhizobium* membranes. The release of fatty acid was more significant at slightly acid pH than at more alkaline pH, at 30 °C than at lower temperatures, and at higher concentration of divalent cations than at lower one. Furthermore, the activity was abolished by boiling for five minutes. These results strongly suggest that the release of fatty acid from PC was because of enzymatic activities rather than nonspecific chemical degradation. These activities were demonstrated to be associated with the outer membrane rather than the inner membrane or the cytosol fraction. This outer membrane PLA (OM-PLA) resembles that found in *E. coli* in this aspect. The *E. coli* OM-PLA possess both PLA and lysoPLA activities (134). It is currently not known whether the *Rhizobium* OM-PLA has both of PLA and lysoPLA activities since the enzyme has not been purified. However, it is likely that the *Rhizobium* OM-PLA does possess both of these activities because they tend to be optimized by the same reaction conditions. The lysoPLA activity was very prominent since the accumulation of lysoPC was not very significant and even not observed in some cases.

The inhibition of some potential PLA or serine protease inhibitors was not observed. It is very difficult to evaluate these results, however. First, the effect of these inhibitors are highly varied to different kinds of PLAs. Secondly, there are many variables contributing to a crude membrane preparation. The solubilization of these PLA and lysoPLA activities proved to be difficult. This is shown to certain degree by their stability in crude membrane preparations. Diminishing activities can last for over a month. They can also survive some harsh treatments such as freezing-thawing, lyophilization and running through columns at room temperature. Solubilization and purification would be necessary to further characterize the outer membrane PLA and lysoPLA activities in *Rhizobium*.

Phospholipases A and D have been demonstrated in *Rhizobium* membranes. The PLA is located in the outer membrane. It is likely a homologue of the OM-PLAs in enterobacteria and in *Campylobacter coli*. The roles of phospholipases in the bacterial infection of eukaryotes have been well demonstrated. Considering the similar infection process and biochemical strategies employed by *Rhizobium* and other eukaryote-infecting bacteria, we propose that phospholipases are important for the *Rhizobium* invasion of the host cells.

It is likely that some of these phospholipases are encoded in the symplasmids. A very preliminary search of the available protein sequences related to nodulation showed that the OM-PLA of *E. coli* had a 21% identity match with RosR over a sequence of 91 amino acid sequences. The active site sequence of the *E. coli* OM-PLA also matched a different region of RosR with a 38% identity in

a 16 amino acid overlap. RosR has been reported to be related to the nodulation competitiveness in *Rhizobium etli* (15). It is a homologue of the Ros protein from *Agrobacterium tumefaciens* and the MucR protein from *Rhizobium meliloti.* Both Ros and MucR have been reported to be transcriptional repressors and to contain a DNA-binding domain. It is difficult to evaluate if this match is significant without more information.

Interestingly, the secreted NodO in *Rhizobium* is an ion channel-forming protein and similar to *E. coli* hymolysin. Hymolysins possess PLA activities. How NodO functionally relates to the outer membrane PLA in *Rhizobium* deserves further attention.

CHAPTER 3

The Presence of α-Sulfoquinovosyl Diacylglycerol and Other Glycolipids in The Outer Membrane of *Rhizobium* Is An Example of Molecular Mimicry for Intracelluar Pathogens to Survive

ABSTRACT

Recently, several new glycolipids have been identified in the cell membranes of the gram negative bacteria *Rhizobium* and *Bradyrhizobium.* Four such classes of glycolipids are sulfoquinovosyl diacylglycerol, diglycosyl diacylglycerols, triglycosyl diacylglycerols and the chitolipooligosaccharide *nod* factors. There are several theories that have been proposed to explain the functions these molecules may play in the infection and subsequent symbiotic process between *Rhizobium* and legume plants. One role that has been suggested for the chitolipooligosaccharides is that they come in contact with the plants at an early stage and trigger the early, primary events of the process. It has been proposed that a similar role might be played by the diglycosyl diacyl glycerols, although a bacterium - plant compatibility function is also likely given the known preponderence of glycolipids in plant cells. A compatibility function has been proposed for sulfoquinovosyl diacyl glycerol since its occurance is very restricted to plants and photosynthetic bacteria. Here we show that sulfoquinovosyl diacylglycerol and the diglycosyl diacylglycerols are localized in the outer membrane of the bacteria and that the chitolipooligosaccharides are localized in the inner membrane. These findings are consistent with the roles proposed for sulfoquinovosyl diacylglycerols and diglycosyl diacylglycerols but suggest that some modification should be made to the models on how chitolipooligosaccharides are delivered to plants and how they might function. They also provide several important clues to the biosynthesis of chitolipooligosaccharides.

INTRODUCTION

SQDG and CLOS are glycolipids that are found in the Gram-negative bacterium *Rhizobium*, a plant symbiont responsible for nitrogen fixation in legume plants. SQDG has been proposed to be important for *Rhodobacter* under phosphate-limiting conditions (13a). The accepted role for CLOS is that they are the recognition or specificity factors that trigger the plant cell transformations that eventually lead to the development of nodules and the establishment of the symbiosis. Since SQDG is found only in photosynthetic organisms, a role in establishing compatibility through similar surface chemistry between host and symbiont is a reasonable guess of its function. Here we show that SQDG is localized in the outer membrane of the bacteria and that the CLOS are localized in the inner membrane. These findings are consistent with the roles proposed for SQDGs. Although they do not conflict with the proposed modes of action of CLOS, they do suggest that some modification should be made to the models on how these molecules are delivered to the plants and how they might function. They also provide several important clues to the biosynthesis of CLOS.

Despite their importance in determining the outcome of the biological interaction between *Rhizobium* and plants, little is known about the localization of membrane-associated glycolipids. One prominent member of this class of molecules is CLOS, also called *nod* factors. This is a group of molecules which are believed to determine the host range of *Rhizobium* and the early events of the nodulation process (103). It was at first believed that these molecules were

synthesized and excreted, but it has subsequently been shown that they are present in the membrane in levels that are orders higher than the trace amounts found in the culture medium (32, 138). One possible mechanism is that CLOS reside on the bacterial cell surface and exert their effects at a close range through contact with the host plant. Although it is not an absolute requirement, this mode of action would be facilitated if the CLOS were located in the outer membrane preferentially in the outer leaflet. Determining in which membrane CLOS is actually located would be a significant step forward in constructing models explaining its function in the early events of infection.

We have previously shown that SQDG, a typical plant lipid that is highly restricted in the plant kingdom except for photosynthetic bacteria (76), is a component of the membrane lipids of *Rhizobium* (31). We proposed that it might be localized in the outer membrane (on the outer surface) of the cell where it serves some recognition or compatibility function so that the invading bacteria do not trigger host defenses. Determining in which membrane this glycolipid is located is therefore an important test of this idea.

An important gain from determining where these glycolipids are localized is that such information sheds much light on where and when these molecules are synthesized especially in relation to other carbohydrate molecules. For instance, it has been demonstrated that sulfation of CLOS is, somehow, tied to sulfation of LPS (32). If CLOS are found exclusively in the bacterial inner membrane, then the enzymes that are responsible for sulfating them, along with LPS, must also be in the

inner membrane. This would mean that LPS is sulfated in the inner membrane before it becomes translocated to the outer membrane. It would also suggest that the *nod* genes are inner membrane proteins. There has been much debate about this latter issue. In this study, we address where SQDG and CLOS are localized in *Rhizobium meliloti*.

MATERIALS AND METHODS

Bacterial Strains and Cultures

Rhizobium meliloti 2011 was obtained from Dr. Ethan Signer, Massachusetts Institute of Technology. Cells were grown at 30° C to mid-log phase in BIII medium (Dazzo82) with constant shaking at 175 rpm. The cultures contained 150 mCi/L ^{35}S-labeled free sulfuric acid (New England Nuclear) and 2 mg/L luteolin.

Cell Harvesting and Membrane Preparation

For cell harvesting and the preparation of total membrane fraction, refer to Materials and Methods in Chapter 2.

A discontinuous sucrose density gradient was used to separate the bacterial membranes. The sucrose solutions of different density were made up in 50 mM Tris/HCl buffer at pH7.9 containing dithiothreitol (0.2 mM) and EDTA (5 mM). The membrane pellet was resuspended in 20% sucrose solution, homogenized by passing through a $22^1/_2$G needle and loaded on a step gradient prepared with 4 mL 55%, 4 mL 52.5%, 6 mL 50%, 4 mL 47.5%, 4 mL 45%, 4 mL 40%, 4 mL 37.5%, 4 mL 35% and 2 mL 30% sucrose (w/w). The tubes were then centrifuged at 26,000 rpm in a Beckman SW27 rotor for 18 hours. Fractions of 0.5 mL volume were collected by inserting a capillary tube attached to narrow bore Tygon tubing close to the bottom of tubes and removing samples in a continuous stream with the aid of a peristaltic pump.

Assays of Membrane Fractions

Protein content was determined using the Bradford method (19). A small aliquot (60 µL) was counted for ^{35}S-radioactivity in a RackBeta Primo liquid scintillation counter (LKB Wallace). Succinate dehydrogenase (SDH) and NADH oxidase activities were used as inner membrane markers (39). The assay mixture for SDH was composed of 2 mL of 150 mM sodium phosphate buffer at pH 7.4 containing NaCN (5 mM), DCPIP (25 mg/mL) sodium succinate (50 mM) and 25 µl of membrane fraction. The activity was observed as the decreased optical density at 600 nm over a period of ten minutes. The assay for NADH oxidase was performed as described (39). The outer membrane fraction was identified by analyzing each fraction for the content of 3-hydroxy fatty acids (78). Total fatty acids from each fraction were released by 2 M TFA hydrolysis at 120 °C of 100 µl sample for 30 minutes. The solutions were brought to dryness and then methanolysed in 0.2% HCl in methanol at 70 °C for 30 hours with frequent sonication. The mixture was then dried under a nitrogen stream and partitioned between water and chloroform. The layers were separated and the aqueous layer was extracted twice more with chloroform. The chloroform layers were pooled, washed with water and concentrated to 10 µL for GC-MS analysis. The samples were analyzed on a Hewlett-Packard 5995C GC/MS instrument using a J & W Scientific DB5 column. The program used was: 100-180 °C at 40 °C/min with a two-minute hold at 180 °C and 180-320 at 10 °C/min with a two-minute hold at 320 °C.

Extraction and Analysis of Glycolipids from Membrane Fractions of *Rhizobium meliloti* 2011

Total lipids containing SQDG were extracted from membrane fractions and analyzed by thin layer chromatography using silica layers as described earlier (31). SQDG was identified by its R_f, characteristic carbohydrate response with the orcinol detection reagent and by ^{35}S autoradiography using a PhosphoImager instrument (Molecular Dynamics, Inc.).

To isolate CLOS, the membrane fractions were lyophilized and then sonicated in methanol for 30 minutes. After brief centrifugation, the supernatant was recovered and dried under a stream of nitrogen. The residue was redissolved in ethanol/isopropanol (1:1, v/v) and again centrifuged. The supernatants were moved to fresh glass vials and dried under a stream of nitrogen and the residue after evaporation dissolved in ethanol/isopropanol (1:1, v/v) and subjected to TLC analysis on silica gel (LHP-KDF plates, Whatman). The solvent system used was isopropanol / concentrated ammonia / water (6:4:1). Authentic CLOS standards were used as reference (138). The TLC plates for lipids were visualized with orcinol spray and those for CLOS by spraying with 10% PMA. CLOS were also detected by ^{35}S autoradiography using a PhosphoImager instrument (Molecular Dynamics).

RESULTS

Preparation of *Rhizobium* Membranes

The protein profile for the membrane fractions of *R. meliloti* 2011 is shown in Figure 1, along with the profiles for SDH and NADH oxidase assays, both used as inner membrane markers (139, 167). The ^{35}S radioactivity profile essentially revealed the protein content. This was expected because sulfuric acid was the only source of sulfur under the growth condition used and, therefore, the only source for sulfur in methionine and cystine. A total of five protein peaks were observed. Two of them, namely II and III, had SDH and NADH oxidase activities, indicating that they contained inner membrane material. Little enzyme activity was detected in the other three peaks, indicating that peaks I, IV and V did not contain significant amount of inner membrane material.

Figure 2 shows the profile of 3-hydroxy fatty acids, characteristic components of lipopolysaccharides which are located in the outer membrane of Gram negative bacteria. The use of m/z 103 from mass spectra as an indication of the presence of lipid A in LPS has been published before (78). The outer membrane was with peak I as evidenced by the high relative abundance of the characteristic ion fragment at m/z 103 in the total ion current from GC/MS analysis (Figure 2). This was further supported by the lack of significant level of inner membrane marker enzymes and the fact that the outer membrane is the most dense band 139, 167). Minor levels of m/z 103 fragments were detected in peaks II and III. This was

Figure 3.1 - Protein content and SDH activity of the membrane fractions. All assays were done as described in Materials and Methods. Open circles connected by broken lines represent data points of ^{35}S radioactivity. Protein concentrations are indicated by x with solid line and the Y axis on the left side. The relative SDH activity is indicated by filled circles with shaded lines. NADH dehydrogenase (NADHD) activity is indicated by the trigangles with solid lines. The protein peaks are named I through V in the direction of high density to low density.

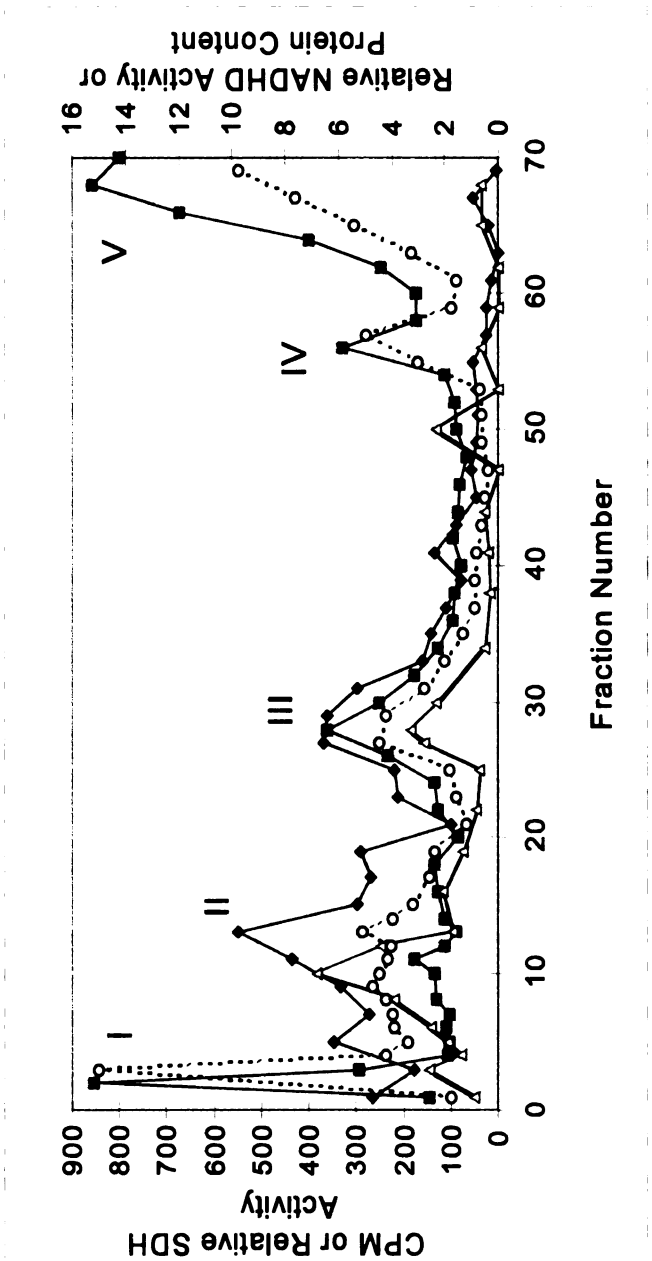
CPM or Relative SDH Activity
Relative NADHD Activity or Protein Content
Fraction Number
I
II
III
IV
V
0
100
200
300
400
500
600
700
800
900
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70

Figure 3.1

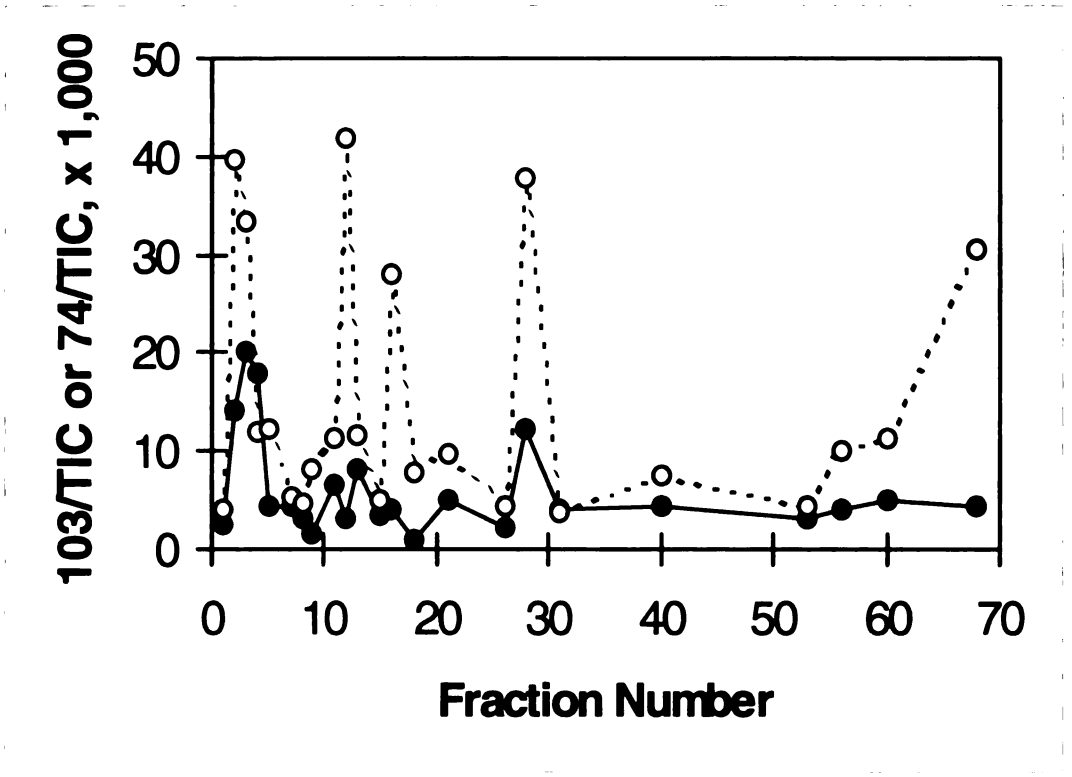

Figure 3.2 - GC/MS analysis of fatty acids isolated from membrane fractions of *R. meliloti* 2011. The open circle represents the ratio of ion current for m/z 74 to total ion current (TIC) in each fraction The closed circle represents that for m/z 103. m/z 74 was due to the fragmentation of common fatty acid methyl esters. m/z 103 was due to the fragmentation of methyl ester of 3-hydroxy fatty acid, which is a characteristiccomponent of LPS in the outer membrane of Gram-negative bacteria.

because CLOS, shown by this study to be in the inner membrane, also contain some 3-hydroxy fatty acids (138). Peak IV did not contain any membrane lipids as evidenced by the absence of common fatty acid methyl ester fragment at m/z 74. Peak V contained solublized proteins.

The results shown in Figures 1 and 2 suggest that peaks III and I represent inner and outer membranes, respectively. The inner membrane peak was uniform in its containing protein, enzyme and fatty acid profiles, indicating a relatively pure preparation. The outer membrane peak, peak I in Figure 1, was the most dense one among all and contained proteins as well as a high content of 3-hydroxy fatty acid. Moreover, it lacked SDH and NADH oxidase activities, which are common markers for the bacterial inner membrane. Between the inner and outer membranes was a heterogeneous group of peaks called peak II. Peak II was quite broad and had an intermediate buoyant density. It had a 3-hydroxy fatty acid content and both enzyme activities associated with the inner membrane. All these observations strongly suggest that it is an inner membrane/ outer membrane fusion peak. Fatty acid analysis rather than the KDO assay was used because *Rhizobium* synthesizes extracellular or capsular polysachharides with a high KDO content (152) and these contribute significantly to the background since they appear in all fractions by virtue of their solubility.

Identification of SQDG in Membrane Fractions

SQDG is a membrane glycolipid found in organisms capable of photosynthesis. The presence of SQDG in rhizobia is very unusual and interesting

(31). To elucidate the function and biosynthesis of SQDG, it would be necessary to know its cellular location. Thin layer chromatography analysis of membrane fractions (Figure 3A) showed that SQDG was localized almost exclusively in the outer membrane fractions (peak I). It was readily revealed by its characteristic pink color after orcinol spray, its mobility which is slightly lower than PC (31) and the confirmation of ^{35}S in the autoradiograph (Figure 3B). Another orcinol-positive species (in minor amounts) which moved faster than PE was also observed, as indicated by the arrows in Figure 3. PC is another lipid species, in addition to SQDG, common to both plants and *Rhizobium*, but uncommon to other Gram-negative bacteria (27, 124, 64). The fast-moving orcinol-positive spot was also labeled by ^{35}S. It has not been identified because of its low abundance. Small amounts of radioactivity were associated with peak II. This was consistent with its being a fusion peak. Little radioactivity were found in the inner membrane fractions (peak III). This is reasonable since SQDG, like other glycolipids, should be synthesized there and then transported to the outer membrane.

Localization of CLOS in *Rhizobium* Membranes

To determine their exact membrane location, inner and outer membrane pools were extracted with methanol and subsequently a mixture of ethanol and isopropanol. The extracts were analyzed by TLC using PMA detection in addition to autoradiography (Figure 4A & 4B). Authentic CLOS were used as standards for comparison (138). The PMA spray revealed a major spot, which was due to sucrose, in both outer and inner membrane and a component in the inner

A

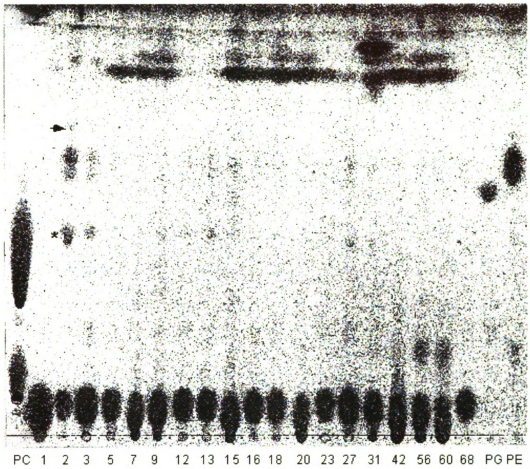

Figure 3.3 - A. TLC of total lipids from *Rhizobium* membrane fractions. The fraction numbers are indicated for each lane. The star indicates SQDG. The arrow indicates the fast-moving and sulfur-containing glycolipid. The TLC plate was air-dried and sprayed with orcinol. Lipids were visualized by heating at 120 °C for two hours. There are two major components in PC. The slower moving one is lysophosphatidylcholine, from the degradation of PC.

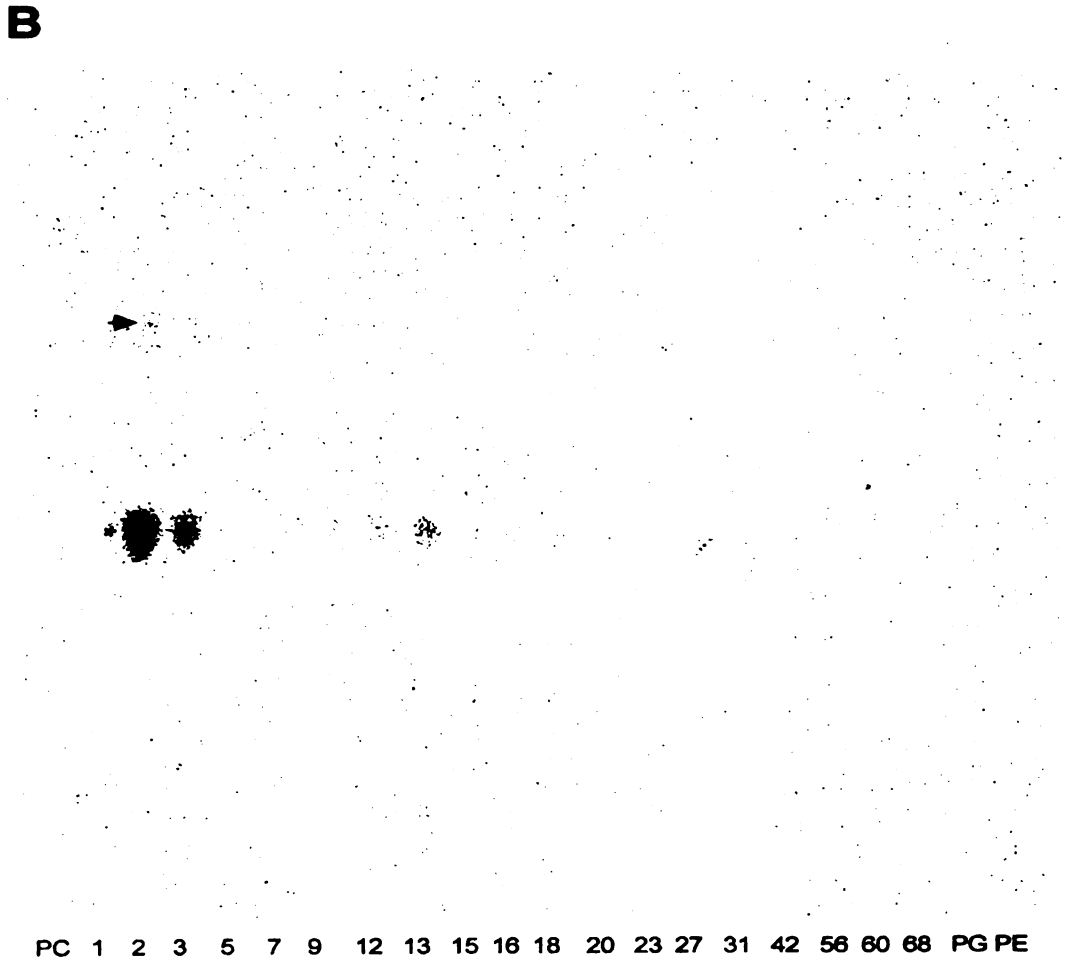

Figure 3.3 - B. Autoradiograph of the TLC plate in Figure 3.3A. The star indicates SQDG and the arrow indicates the fast-moving sulfur-labeled lipid species.

Figure 3.4 - TLC analysis of methanol extracts of membrane pools from *R. meliloti* 2011. NF, authentic CLOS (Nod factors). O,outer membrane pool. I, inner membrane pool. (A) The channel TLC was visualized with PMA spray. (B) The corresponding autoradiograph.

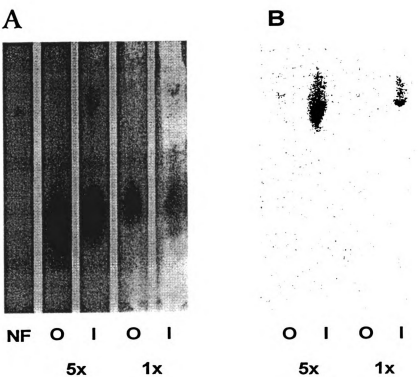

Figure 3.4

membrane that was identical in retention time to that of the purified CLOS samples. This component was radiolabeled with ^{35}S since the *R. meliloti* CLOS are sulfated (156, 198). The nonhomogeneity of the spot was consistent with the fact that CLOS are usually very heterogeneous in fatty acid and carbohydrate contents. A radioactive spot at the same R_f was barely detected in the corresponding extract of the outer membrane pool.

Localization of glycolipids in *B. japonicum* USDA 110 membranes

The inner and outer membranes of bacteroid forms of *B. japonicum* USDA 110 were separated using a similar method as for *R. meliloti* 2011. The outer membrane was readily recognizable by the presence of 3-hydroxy fatty acids (from LPS) in the membrane lipids (Figure 5). Two major fatty acid containing peaks were observed. The first one (centered at fraction 8) corresponded to the outer membrane. The mass spectra of the fatty acid methyl ester derivatives from the second peak were abundant in m/z 74 but lacked fragments at m/z 103. This latter fragment is diagnostic for 3-hydroxy fatty acids. The second peak was centered at fraction 41 and was the major SDH positive peak indicating that it was the inner membrane fraction. The total protein profile was consistent with the location of these two peaks but a third less intense one centered at fraction 19 was observed (data not shown). The behavior of the membrane lipids of the bacteroid forms of *B. japonicum* was fairly different from that of the *R. meliloti* membrane. The two membrane peaks emerged significantly later. This is not surprising because of the known fragility of bacteroid membranes and the lack of structure of bacteroids in

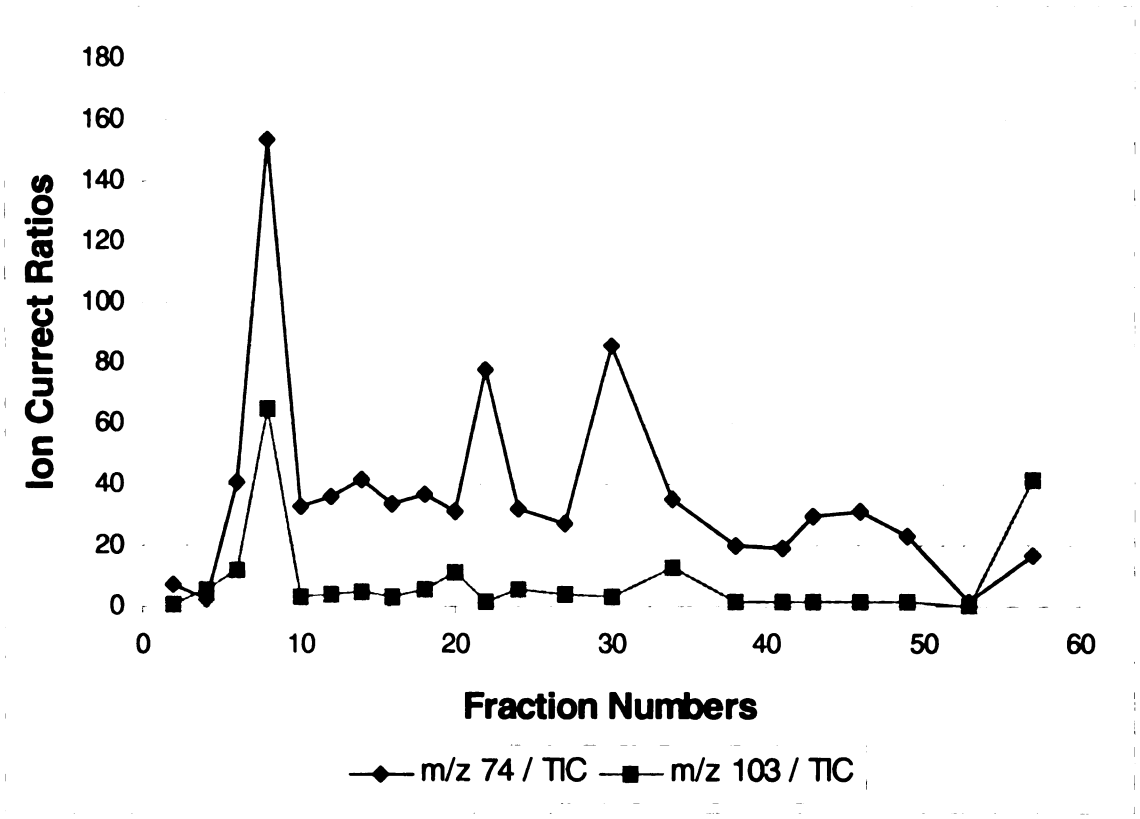

Figure 3.5 - GC/MS analysis of FA from *Bradyrhizobium* membrane fractions

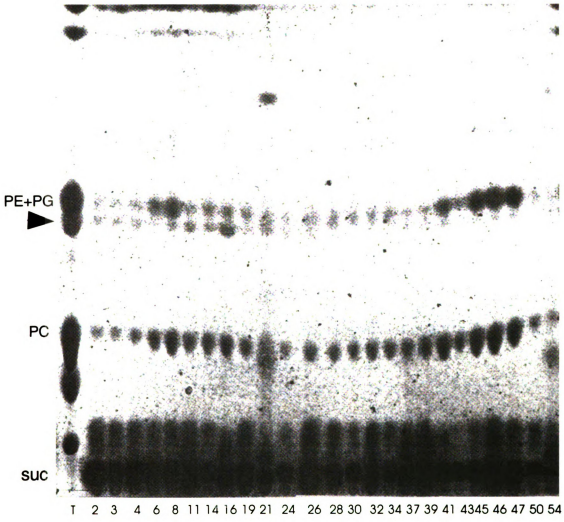

Figure 3.6 - TLC analysis of total lipids from *Bradyrhizobium* membrane fractions:T, total lipids; suc, phosphatidylsucrose; the arrowhead indicates diglucosyl diacylglycerols.

general.

In a recent study under the condition of nutrient enrichment, which is known to induce bacteroid formation in *B. japonicum*, diglucosyldiacylglycerides were identified as major new lipid components formed under these conditions (193). They were readily identifiable on the TLC plate as bright purple spots (shortly after orcinol spray) moving slightly slower than PE, as indicated by the arrowhead in Figure 6. These lipids were scraped from TLC plates, with their glycosyl components converted to alditol acetate derivatives and subjected to GC/MS analysis. The GC-MS analysis confirmed the findings in the previous study (193) that glucose was the only cabohydrate components of these lipids. Here diglucosyl diacylglycerides were found exclusively in outer membrane lipid fractions (Figure 6), while the membrane location of triglycosyl diacylglycerides were not determined due to the presence of sucrose which co-migrated with them on the TLC plate.

DISCUSSION

This study shows that SQDG is localized in the outer membrane and that CLOS are localized in the inner membrane. The outer membrane localization of SQDG supports the conjecture that it has a role as a compatibility or recognition factor to reduce the impact of the foreign invading bacteria on host defense responses. Determining the site of localization of CLOS can help in defining the site of their assembly and, therefore, the localization of the various proteins encoded by the *nod* genes. Three genes, namely *nod*A, B and C, are responsible for the biosynthesis of the oligosaccharide backbone. *nod*C is the most critical gene involved in oligosaccharide assembly and is believed to code for an N-acetylglucosaminyltransferase (63). In the past, there has been some controversy about the location and function of the *nod*C gene product. It was first thought to be an outer membrane protein with some receptor function (89, 90 and 91). Later reports indicated a chitin synthase function (26, 6 and 44). Subsequent to these findings, it was demonstrated that the *nod*C gene product is an inner membrane protein (11). This is consistent with the inner membrane location of CLOS, indicating that it is synthesized in the inner membrane and only small amounts make it to the outer membrane. This would mean that the other *nod* gene products that are involved in its assembly and modification are also located in the inner membrane, periplasm or cytoplasm where they can act on the inner membrane surface. It is likely that the synthesis takes place on the cytoplasmic face of the inner membrane since the access to the UDP-sugars would then be assured. An inner membrane site of the synthesis of CLOS is also consistent with the fact that there is

considerable overlap between LPS and CLOS modification with LPS sulfation being tied to CLOS sulfation (32). LPS are synthesized on the inner membrane.

The results of the CLOS localization may require a more convoluted model for CLOS synthesis, and transport. One may have to invoke various transport and other specific excretion functions to explain their extracellular appearance. On the other hand, the route by which it is excreted may be a non-specific one that simply involves the shedding of membrane vesicles. The former transport mechanism would not explain the excretion of both charged (sulfated) and neutral CLOS by the same organism.

The outer-membrane location of SQDG in *Rhizobium* hints its role to avoid the hydrolytic functions in the host cell. In addition, SQDG may be of significance to *Rhizobium* under the phosphate-limiting condition inside plant cells. It has recently been found that SQDG enhances the growth of *Rhodobacter speharroides* under phosphate limitation and that it may function as a surrogate for phospholipids (13a).

CHAPTER 4

The Metabolism of α-Sulfoquinovosyl Diacylglycerols, A Host Glycolipid, in *Rhizobium*

ABSTRACT

An intracellular life demands a bacterial species to avoid the hostile host defenses and take advantage of its cellular provisions. This has been manifested by the ability of *Rhizobium* to synthesize SQDG and a number of other "plant type" lipids. The presence of SQDG in *Rhizobium* is unusual because of its highly restricted distribution in living organisms and the intimate relationship between *Rhizobium* and plants. We propose that the presence of SQDG and the capability of *Rhizobium* to synthesize it are important for bacteroid's intracellular living. The SQDG metabolism in *Rhizobium* has been investigated. Evidence suggests that SQDG can be formed by an aldol condensation of cysteic acid and another three-carbon intermediate, consistent with one of the mechanisms proposed for plants. In addition, the presence of glyceryl-α-sulfoquinovose in *Rhizobium* indicates that it also shares features with plants in SQDG catabolism.

INTRODUCTION

The unusual presence of SQDG in *Rhizobium* prompted us to study its biosynthesis in this organism. This will provide us information on the molecular overlap between *Rhizobium* and plants on another level and should help to solve the mystery of SQDG biosynthesis in photosynthetic organisms. The hypothesis of horizontal gene transfer has been under intensive discussion (185). It is particularly relevant to the *Rhizobium*-legume symbiosis because of the close developmental and biochemical ties between the two organisms. A strong piece of evidence for this hypothesis is that one of the two glutamine synthetases in *Rhizobium* has a striking homology to that from plant while the other one is of prokaryotic type (30).

SQDG Metabolism in Photosynthetic Organisms

SQDG is associated with all photosynthetic membranes. However, neither its function nor its biosynthesis is completely known (92). Research on SQDG has so far been limited in biochemical studies, except in the cyanobacterium *Synechococcus sp.* PCC7942 (71) and, especially, the purple bacterium *Rhodobacter sphaeroides*, four mutants of which have been obtained (12, 13, 159). With the identification of UDP-sulfoquinovose in algae (175) and the recent demonstration of its accumulation in a *Rhodobacter sphaeroides* mutant (159), plus the characterization of a UDP-sulfoquinovose: diacylglycerol sulfoquinovosyl transferase from spinach (170), the last step of SQDG biosynthesis has been strongly suggested: transfer of the sulfoquinovosyl group from UDP-sulfoquinovose

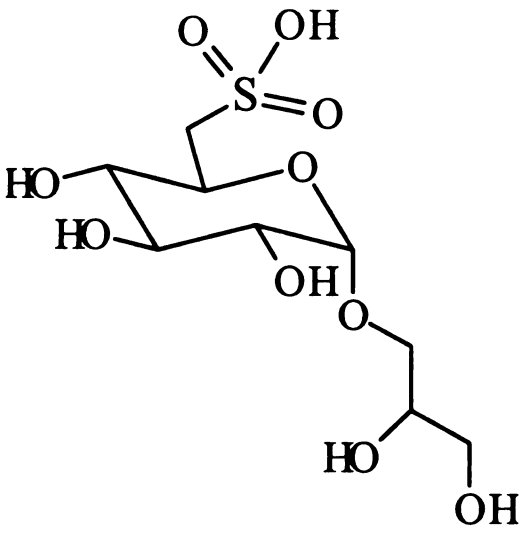

Figure 4.1 - The chemical structure of glyceryl-α-sulfoquinovose

to the 3-position of diacylglycerol. Glyceryl-α-sulfoquinovose (SQG, Figure 4.1) was found to be a metabolite in algae (14, 176) and considered a product of SQDG breakdown rather than a precursor (177) because the level of SQG labeled in etiolated cells remained unchanged during the process of greening. This conclusion was later confirmed by a study which showed that the transfer of ^{35}S-labeled *Chlorella elipsoidea* to a system devoid of CO_2 caused a decrease in sulpholipid and an increase in SQG (126).

The biosynthesis of the head group of SQDG is unclear. In green algae *Euglena gracilis*, cysteic acid was found to decrease the uptake of sulfate into SQDG significantly (39). In alfalfa, radioactive cysteic acid was incorporated with higher efficiency than sulfate into SQDG and decreased the incorporation of sulfate into SQDG (75). These data are in agreement with the sulfoglycolytic pathway for

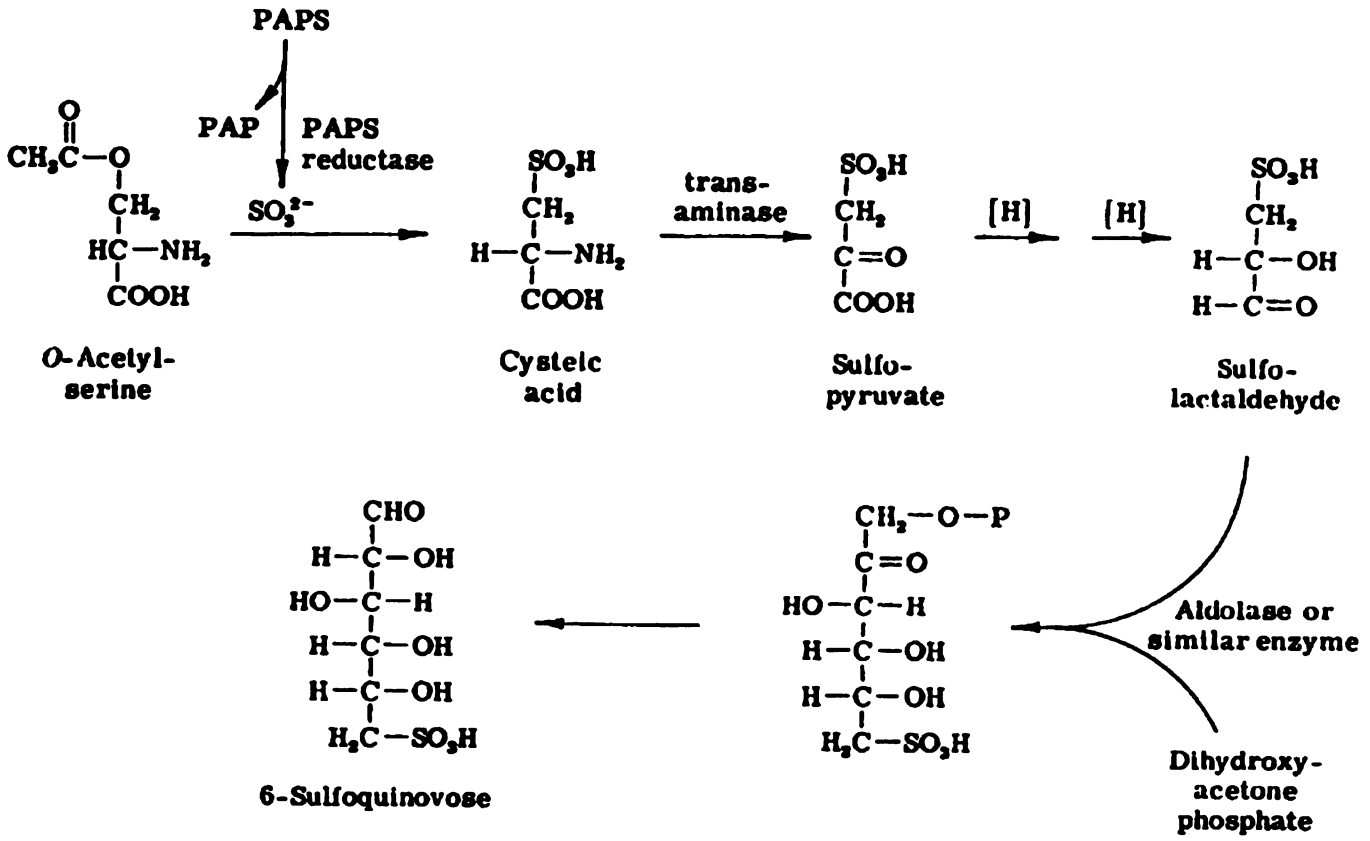

Figure 4.2 - The sulfoglycolytic pathway proposed for the biosynthesis of 6-sulfoquinovose (73)

SQDG biosynthesis (39, 73) (Figure 4.2).

The other pathway under debate involves the formation of C-S bond at the six-carbon level (224, 147). A UDP-4-keto-6-deoxyglucose intermediate could be generated by the oxidation at C-4 position (Figure 4.3). Subsequently the attack of this keto-deoxy intermediate by sulfite through a nucleophilic replacement or free radical mechanism would form the critical C-S bond. UDP-sulfoquinovose, which is ready to be transferred to a diacyglycerol moiety to form the final product SQDG, can be generated with an appropriate reduction reaction. This hypothesis is supported by the results obtained with *in vitro* labeling using isolated chloroplast (147), the chemical reaction of sulfite with glucose-enide (101) and the slight sequence homology between the *sqdB* gene involved in SQDG biosynthesis in *Rhodobacter sphaeroides* and the UDP-glucose epimerase from various organisms (12). Overall, both pathways have so far been supported by some data obtained in both higher plants and microorganisms. It seems that evidence from different species is conflicting, even under investigation by the same research group (75, 147).

The common rule for the biosynthesis of glycerolipids is that the head group is transferred to the diacylglycerol intermediate at a membrane location. The evidence obtained in photosynthetic organisms is consistent with this. According to this rule, SQG is possibly a degradation intermediate of SQDG. A sulfoglycolytic pathway for SQDG degradation has been suggested by Benson (Figure 4.4) (100).

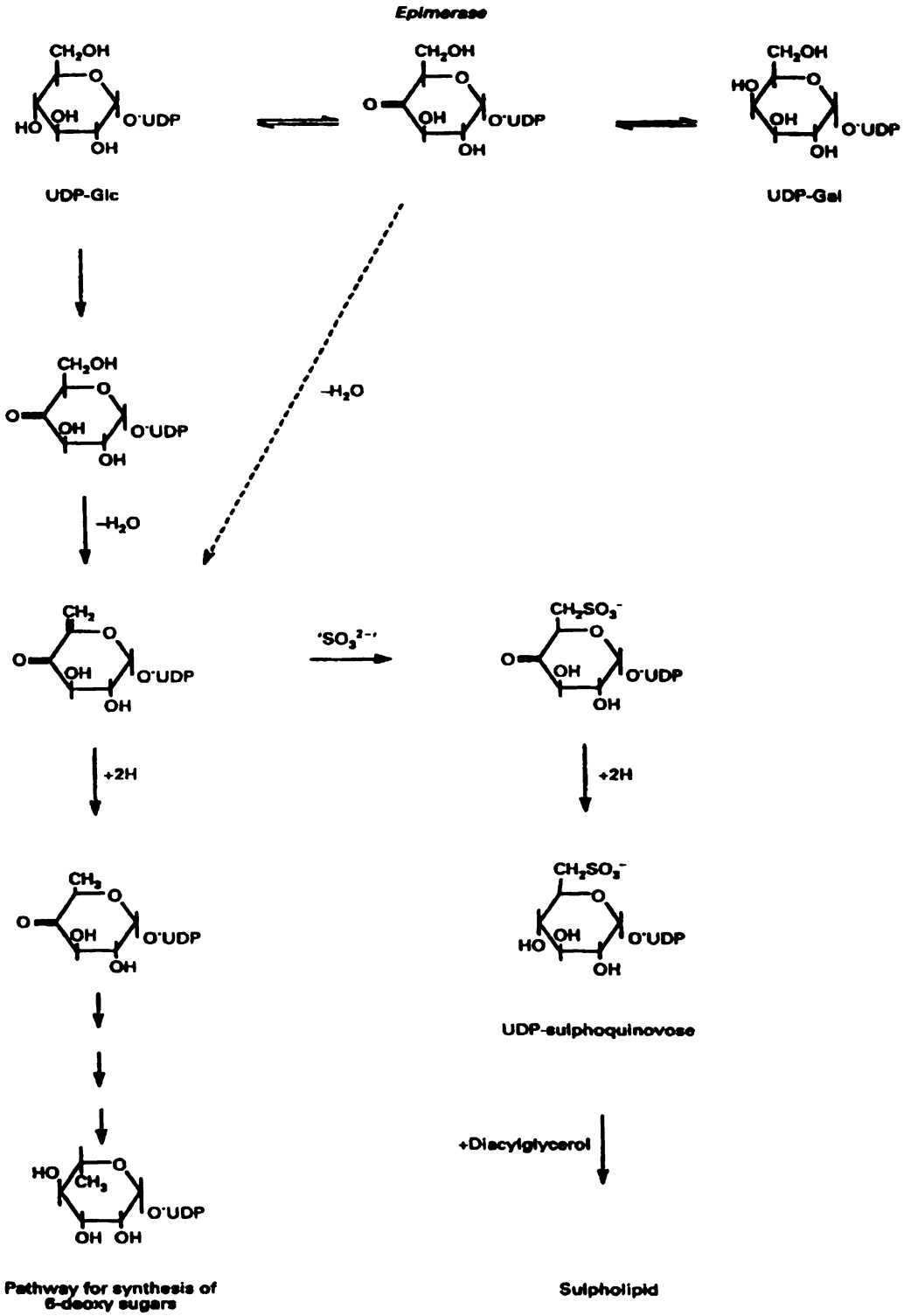

Figure 4.3 - The UDP-glucose pathway proposed for the biosynthesis of SQDG (147)

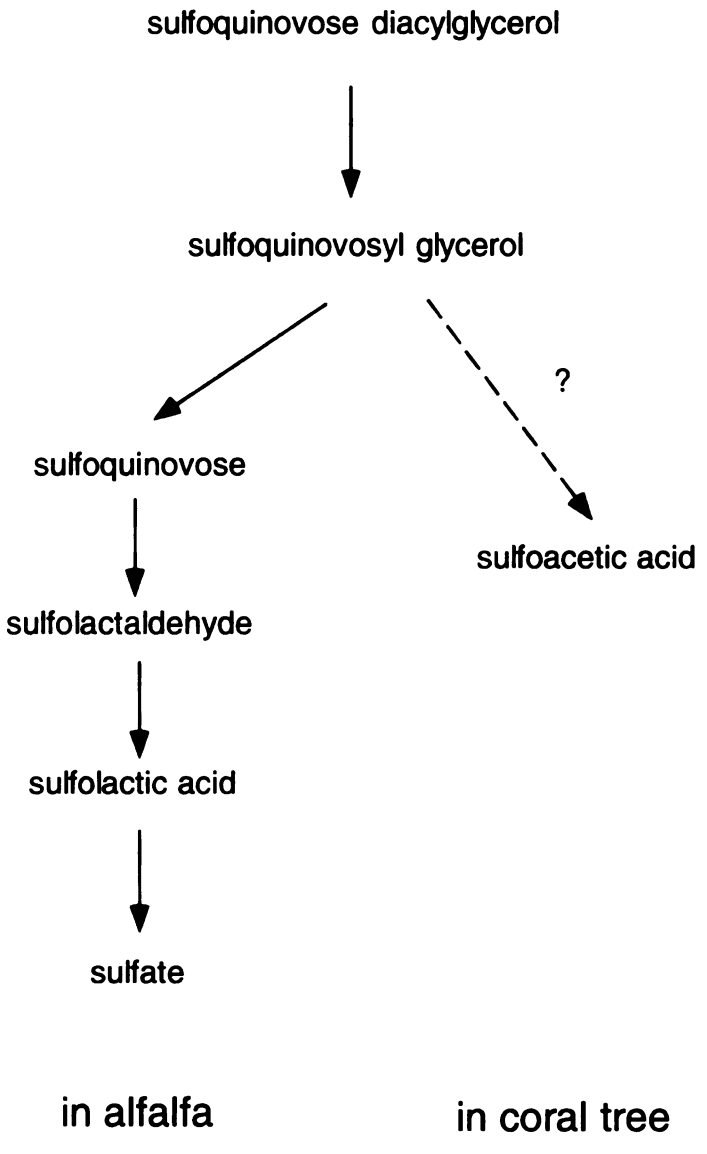

Figure 4.4 - The proposed pathway for SQDG degradation (100)

Sulfur Metabolism in Gram-negative Bacteria

Sulfate is the most common sulfur source for living organisms. There are two sulfate reduction mechanisms, assimilatory and dissimilatory pathways. The dissimilatory sulfate reduction is carried out only by certain anaerobic microorganisms as part of a respiratory pathway that utilizes sulfate as a terminal electron acceptor and produces large quantities of sulfide as an end product. In contrast, the mechanism of sulfate reduction by most microorganisms and plants is the assimilatory type, in which only sufficient sulfur for biosynthetic purposes is reduced. In enteric bacteria, the synthesis of *L*-cysteine from inorganic sulfur is the predominant mechanism by which reduced sulfur is incorporated into organic compounds. This process has been well studied and shown in Figure 4.5A and 4.5B (97). Two other mechanisms of sulfur reduction have also been suggested. But neither of them is significant under normal conditions (132, 179).

One group of Gram-negative gliding bacteria, including the genera *Cytophaga, Capnocytophaga*, *Flexibacter* and *Sporocytophaga*, and the nonphotosynthetic diatom *Nitzschia alba* are able to synthesize an unusual sulfonolipoid called capnine (66, 5). This sulfonolipid has been suggested to be critical for the gliding motility of the *Cytophaga-Flexibactor* group of bacteria (2) The structure of capnine is shown in Figure 4.6, along with that of sphingosine. Since sphingosine is formed by the condensation of serine with palmitoyl coenzyme A, it was suggested that capnine would be formed by condensation of an appropriate amino acid with isopentadecanoyl CoA (65). It was later shown that L-cysteate is

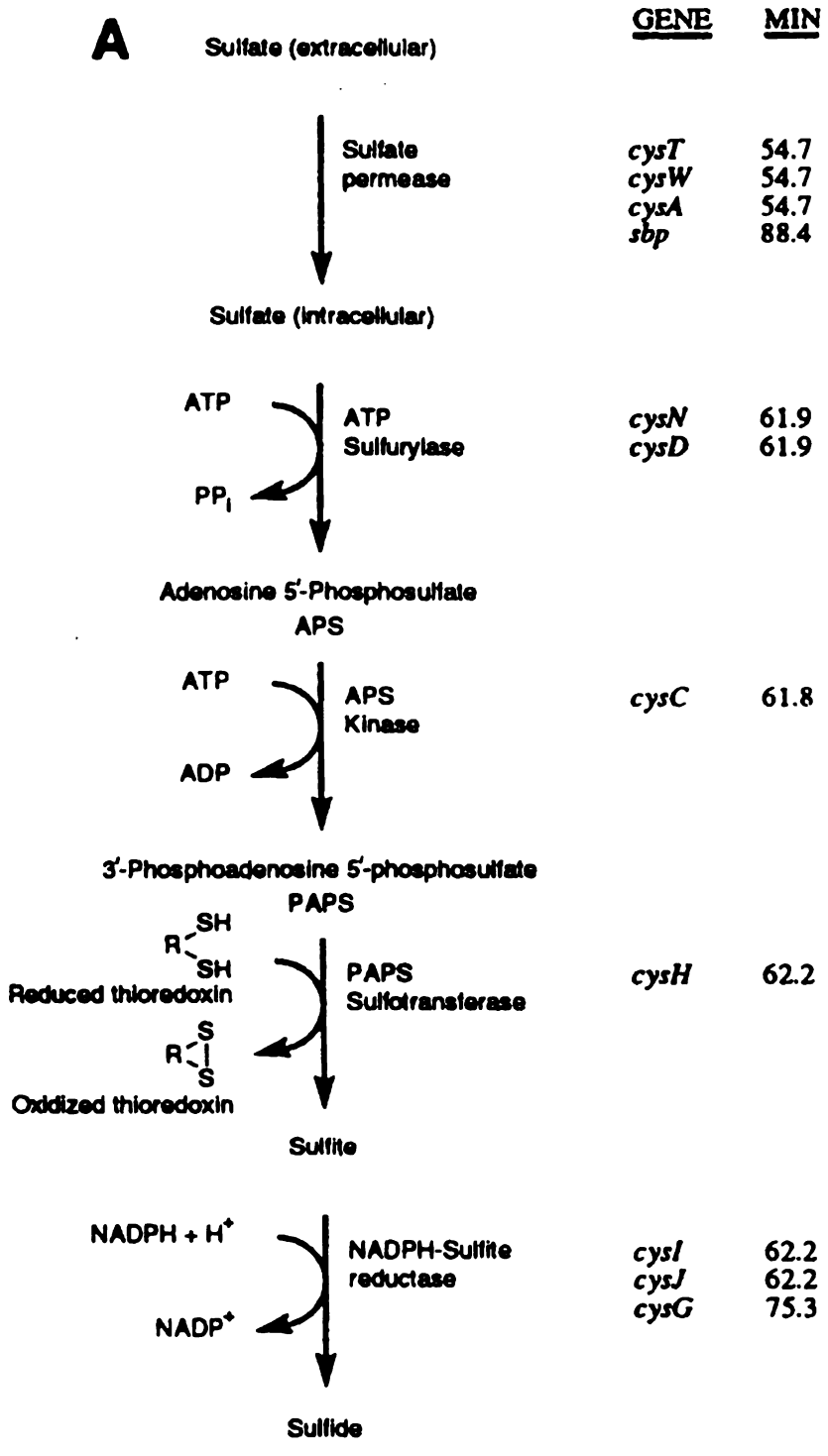

Figure 4.5A - Sulfate reduction in *Salmonella* and *E. coli* (97)

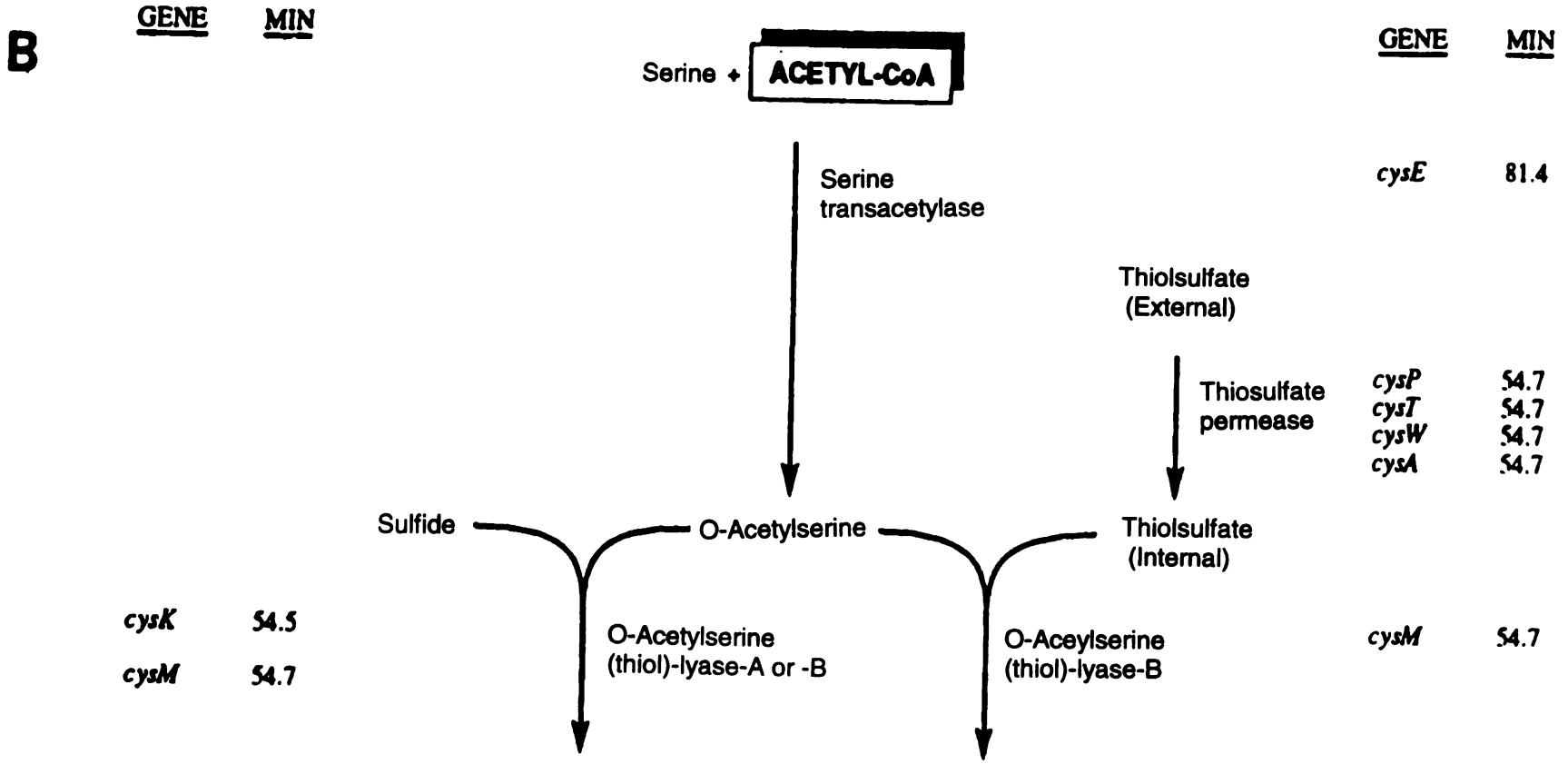

Figure 4.5B - Biosynthesis of L-cystein in *Salmonella* and *E. coli* (97)

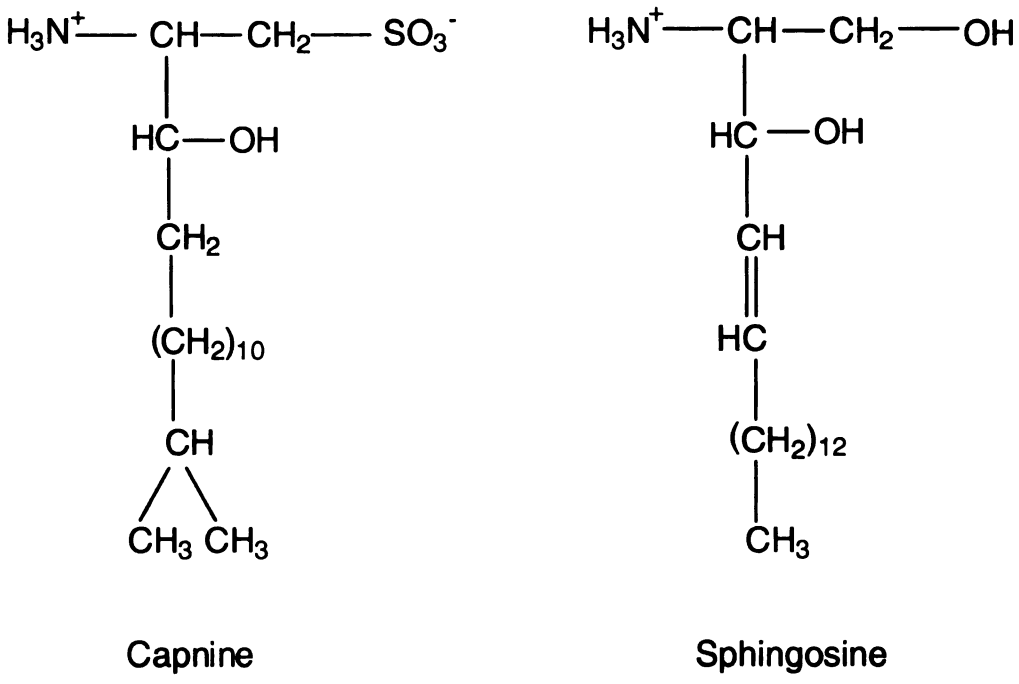

Figure 4.6 - The chemical structures of capnine and sphingosine

utilized preferentially to both cystine and sulfate as a precursor of capnine sulfur and to both cystine and serine as a precursor of carbons 1 and 2 of capnine (1, 218). It has also been observed that the cells can not grow with cysteate as sole sulfur source (1). Therefore, L-cysteate is the immediate precursor of capnine, consistent with the hypothesis that capnine is formed by an amino acid with a fatty acyl CoA.

Carbon Metabolism in *Rhizobium*

Rhizobium is capable of living as an independent organism in the rhizosphere or as an endosymbiont in the cytoplasm of a plant host. In the free-living state, its metabolism conforms to the rules governing a typical aerobic Gram-negative

bacteria. In the symbiotic state, it has to depend on the supply of carboxylic acids, and possibly some other compounds like proline (88), from the host cytoplasm as the carbon and energy source. This two-state life style would demand very complex regulatory mechanisms for *Rhizobium*.

In free-living state, *Rhizobium* is able to use a broad range of compounds as carbon source. These include hexoses, pentoses, oligosaccharides, organic acids and aromatic compounds (187). The ability of *Rhizobium* to utilize such a variety of compounds indicates the presence of a complex web of pathways, as illustrated in Figure 4.7. The central pathways operating in *Rhizobium* include Entner-Doudoroff (ED), pentose phosphate (PP), Embden-Meyerhof-Parpas (EMP) and tricarboxylic acid (TCA) cycle. In addition, there exist a number of anapleurotic pathways such as ketogluconate pathway, glyoxylate cycle (187), a special malic enzyme (52), pyruvate carboxylase (54) and so on. It is very reasonable to propose that such a complex of events has to be highly coordinated and regulated according to the growth conditions and for the benefit of *Rhizobium*. This has so far proved true. In *R. trifolii*, glucose has been demonstrated to mediate catabolite repression and catabolite inhibition of enzymes of polyol metabolism (157). On the other hand, polyol induces not only mannitol dehydrogenase but aslo NAD-dependent 6-phosphogluconate dehydrogenase (131). It is worthy to mention that there exists in *Agrobacterium* a De Ley-Doudoroff oxidative pathway for galactose catabolism, leading to glyceraldehyde 3-phosphate (201). Whether there is a similar pathway in *Rhizobium* is not known.

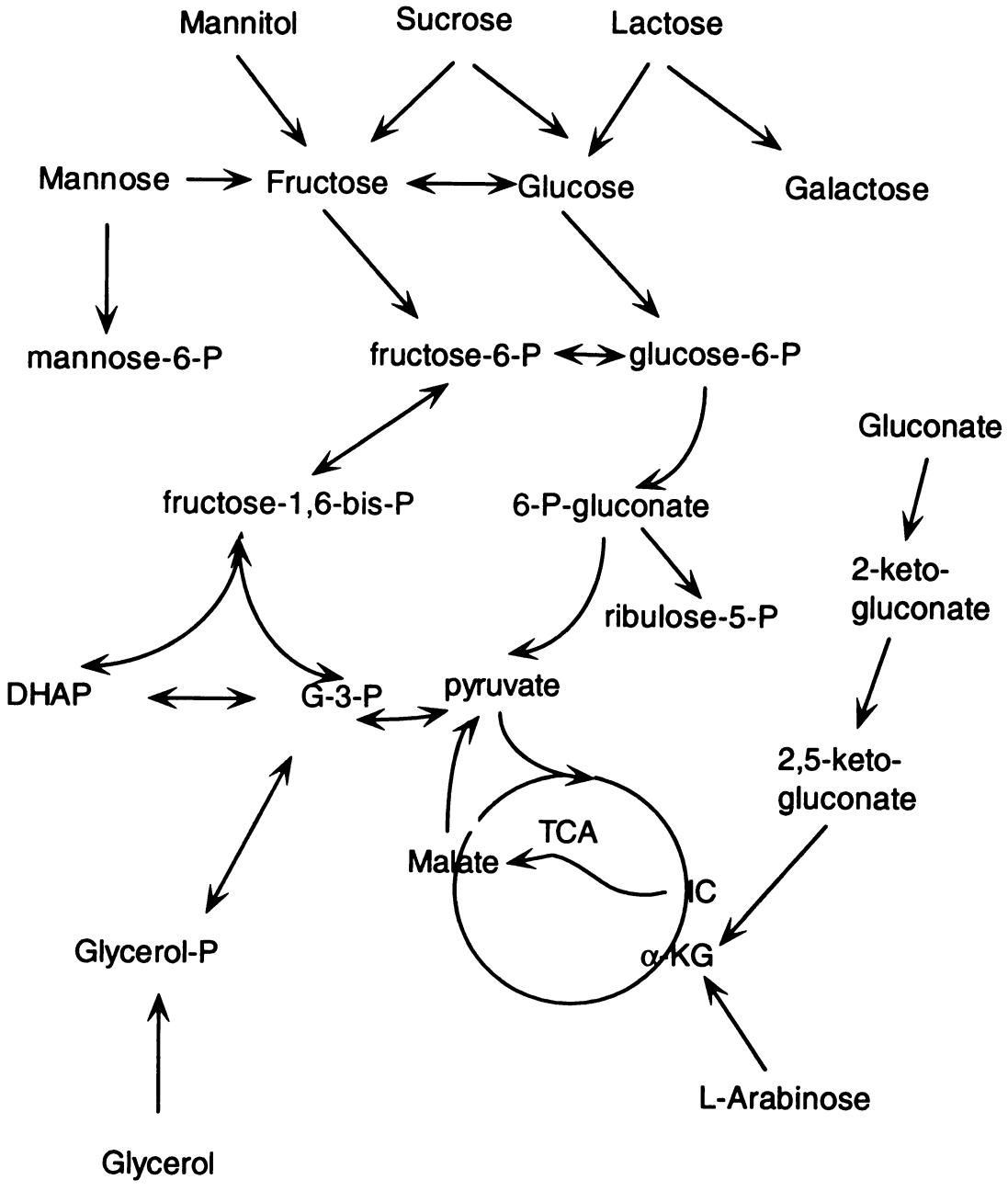

Figure 4.7 - The metabolic pathways in *Rhizbium*

It has been generally believed that nodule cytosol contains pathways of anaerobic metabolism, in addition to the EMP and PP pathways and the TCA cycle (187). This may explain the sufficient supply of carboxylic acids to *Rhizobium* bacteroids. In the bacteroid form, ED and PP pathways do exist, but at a much lower level than in the free-living state. Instead, gluconeogenesis becomes important, though not essential (119). What is interesting is the presence of an alternative gluconeogenic pathway in *Rhizobium* (140), suggesting that the metabolic versatility of this organism well equips it to deal with a variety of environmental conditions. Overall, it seems that *Rhizobium* can bypass many metabolic pathways.

The ability of *Rhizobium* to make SQDG adds to the challenges to solving the mysteries of metabolism and regulatory mechanism in *Rhizobium*. As discussed previously, SQDG may be a very critical part of the *Rhizobium*-legume interactions. The regulation of its biosynthesis and degradation would be important to the well-being of *Rhizobium* during the infection process. As a matter of fact, the presence of SQDG in *Rhizobium* gives us an advantage to study its biosynthesis and catabolism compared to such studies in higher plants. More importantly, the elucidation of SQDG metabolism will detail one of the mechanisms for establishing molecular mimicry by eukaryote-infecting bacteria.

MATERIALS AND METHODS

Bacterial Strains and Cell Cultures

Rhizobium wild type and mutant stains were obtained from Drs. Ethan Signer and Gram Walker at MIT. The culture medium used was liquid modified BIII medium (41), unless specified. For radio-labeling the sulfur-containing intermediates, $^{35}SO_4^{2-}$ (0.3 mCi) was added at a specific activity of 380 μCi/mmole. In the ^{13}C-labeling experiment, half of the mannitol was replaced with glucose containing 5% ^{13}C-1-glucose. In the labeling experiments with ^{14}C-glucose, the amount of mannitol was reduced by three quarters and an equal amount of glucose was added. *Rhizobium meliloti* 2011 cells were grown without the label at the initial stage of growth. 25 μCi ^{14}C-1-glucose was added at the mid-log phase to a specific activity of 7 μCi/mmole. In the uptake kinetic experiment with cysteine label, the amount of mannitol was reduced by three-quarters. Glucose was added at a level of one-fifth the amount of mannitol. Half of the magnesium sulfate was replaced with five times the molar proportion of cysteine. 100 μCi ^{35}S-cysteine was added at the mid-log phase to give a specific activity of 90 μCi/mmole.

Isolation and Identification of Sulfur-containing Intermediates

Rhizobium meliloti 2011 cells were harvested by centrifugation at 8,000 xg for 20 minutes. The total metabolites were extracted by stirring the cell pellet with a mixture of ethanol and water (50 / 50, v/v) for one hour. The aqueous ethanol

extract was subjected to chromatography on a silica gel column (1.5 cm X 2 cm) using 1-propanol : concentrated ammonia : water (60:40:10). Fractions of 0.5 mL were collected and analyzed for radioactivity and those corresponding to peaks containing radioactive material were pooled and concentrated. The early-eluting pool was passed through a C-18 reverse phase column in pure water and the fore-run which contained most of the radioactivity was collected. The fore-run was then subjected to gel-filtration chromatography on a Biogel P2 column (1.5 cm X 90 cm) using pure water as the eluant. The major peak containing radioactivity was subjected to anion exchange column chromatography (0.5 cm x 2 cm, DEAE-cellulose). The column was washed with pure water and then eluted sequentially with 0.5 M and 1.0 M formic acid (5 ml each). The compounds of interest are analyzed by NMR spectroscopy at various stages. When applicable, approximately one-third of the samples containing the signals of interest was hydrolyzed by heating in 2 M trifluoroacetic acid at 120 °C for 1 hour. The hydrolysate was concentrated to dryness and passed through a short column of anion exchange resin (0.2 cm X 0.4 cm) in pure water and the flow-through collected. It was concentrated to dryness and analyzed by NMR spectroscopy.

NMR spectroscopy was carried out on a Varian VXR 500 instrument operating at 500 MHz for protons. All spectra were obtained in deuterium oxide and the residual water line was suppressed by selective inversion-recovery. Spectra were recorded at 50 °C. Mass spectra were obtained in the negative ion mode on a Fisons Platform electrospray instrument. The sample was infused in pure water.

SQDG Labeling from Carbon-labeled Glucose

Rhizobium meliloti 2011 cells were grown without label at the initial stage of growth. The label was added at mid-log phase to a specific activity of 0.4 μCi/ mmole. The cells were then harvested at 0.5, 1, 2, 4, 6, 8 and 24 hours after the addition of the label. Samples were then processed as described in Chapter 3. The lipid samples were loaded onto TLC and the TLC was performed in a mixture of chloroform/ acetone/ methanol/ acetic acid/ water in a ratio of 10:4:2:2:1 (v/v). The TLC plate was exposed to a PhosphoImager. The image was obtained with a scanner, STORM (Molecular Dynamics, Inc.), and processed with an ImageQuant software (Molecular Dynamics, Inc.).

In the ^{13}C-labeling experiment, *Rhizobium meliloti* 2011 cells were labeled and lipids extracted. SQDG was isolated by preparative TLC. The identity of SQDG was confirmed by ES-MS and by autoradiography on a TLC plate using standards. Both ^{1}H and ^{13}C spectra were obtained for ^{13}C-labeled SQDG in deuterium-labeled methanol on a Varian VXR 500 instrument. The proton spectrum was obtained with 100 scans and the ^{13}C spectrum with 50,000 scans.

^{35}S-labeling of SQDG

Rhizobium meliloti 2011 cells were grown as described above. Cell samples were taken at 0.5, 1, 2, 3 and 4 hours after ^{35}S-cysteine had been added. Lipids were extracted and analyzed as described above and in Chapter 3.

To investigate the ability of *Rhizobium* to utilize cysteic acid, the cell lysate were prepared from *Rhizobium meliloti* 2011 by French-press. For details about the use of French-press, refer to Materials and Methods in Chapters 2 and 3. Cysteic acid was obtained in a reaction containing 2:3:1 acetic acid/ hydrogen peroxide/ cysteine in molar ratio in methanol. 0.5 mL of cell lysate was incubated with 3 μCi ^{35}S-labeled cysteic acid with a specific activity of 1 mCi/mmole at 37 °C for 5, 10, 30 and 60 minutes. SQDG was analyzed as described previously.

RESULTS

Identification of Key Intermediates of SQDG Metabolism

One strategy for elucidating the metabolism of SQDG in *Rhizobium* is to identify the key intermediates involved. This is aided by the fact that sulfur is present only in a limited variety of biomolecules and one can therefore target molecules that are radio-labeled.

Figure 4.8 shows the ^{1}H-NMR spectrum of partially purified cysteic acid. Resonances at 2.85 and 3.18 ppm are due to the methylene protons adjacent to a sulfonic acid group. Both signals are doublet of doublet because of the splitting by one germinal proton and one vicinal proton. This is also observed in the spectrum of standard cysteic acid and the purified SQDG (Figure 4.9). The signals at 3.93 and 3.13 ppm are the signature resonances of sulfoquinovose headgroup. The multiplet at 3.8 ppm is due to the methine proton. The chemical shift is critical here, indicating an amino group rather than a hydroxyl group. Signals at 2.05 and 2.42 ppm are due to impurities from glutamic acid as manifested by the greatly reduced intensity during the purification process. The presence of cysteic acid hints at the sulfoglycolytic biosynthetic pathway for SQDG.

Figure 4.10 shows another isolated sulfur-containing compound. The complex signals between 3.2 and 4.6 ppm indicates that this is a carbohydrate molecule. The number of proton resonances indicated a hexose. The absence of an anomeric proton suggests that this is a keto sugar. The presence of two

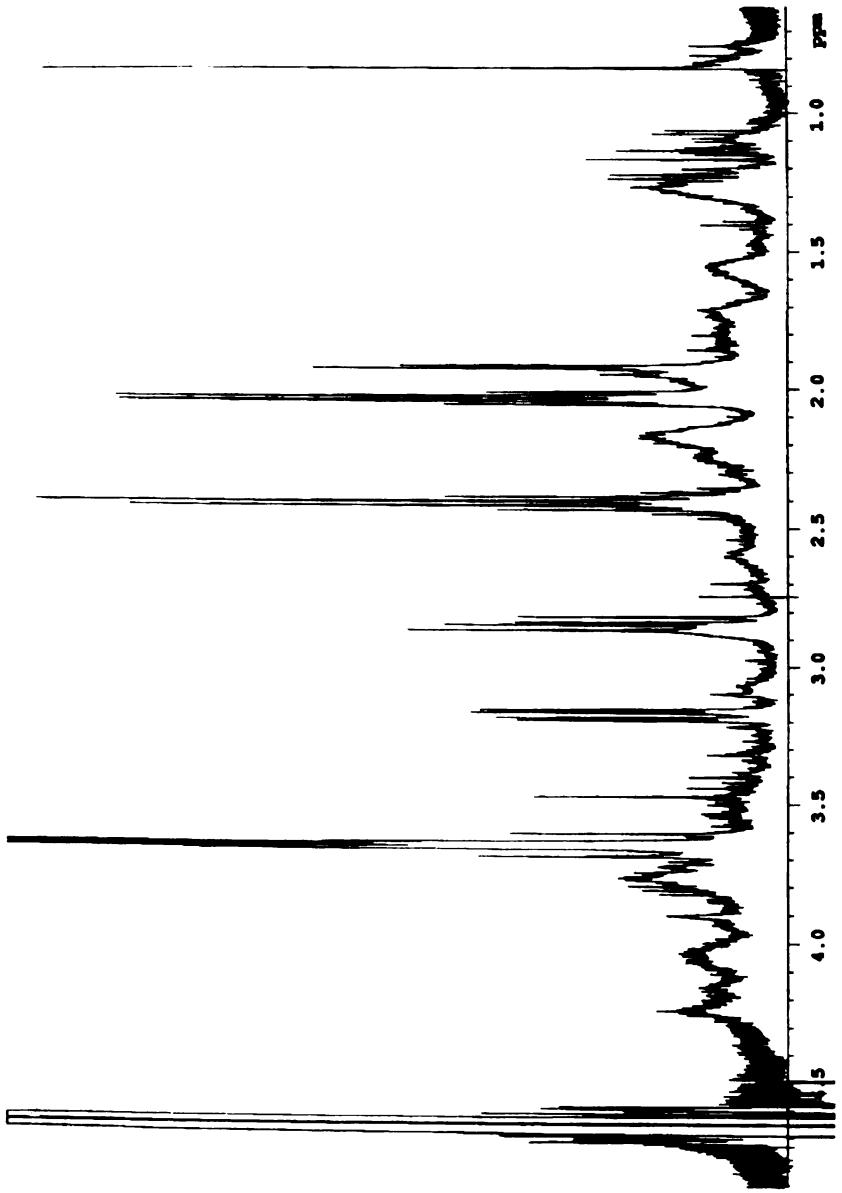

Figure 4.8 - ^{1}H-NMR spectrum of partially purified cysteic acid

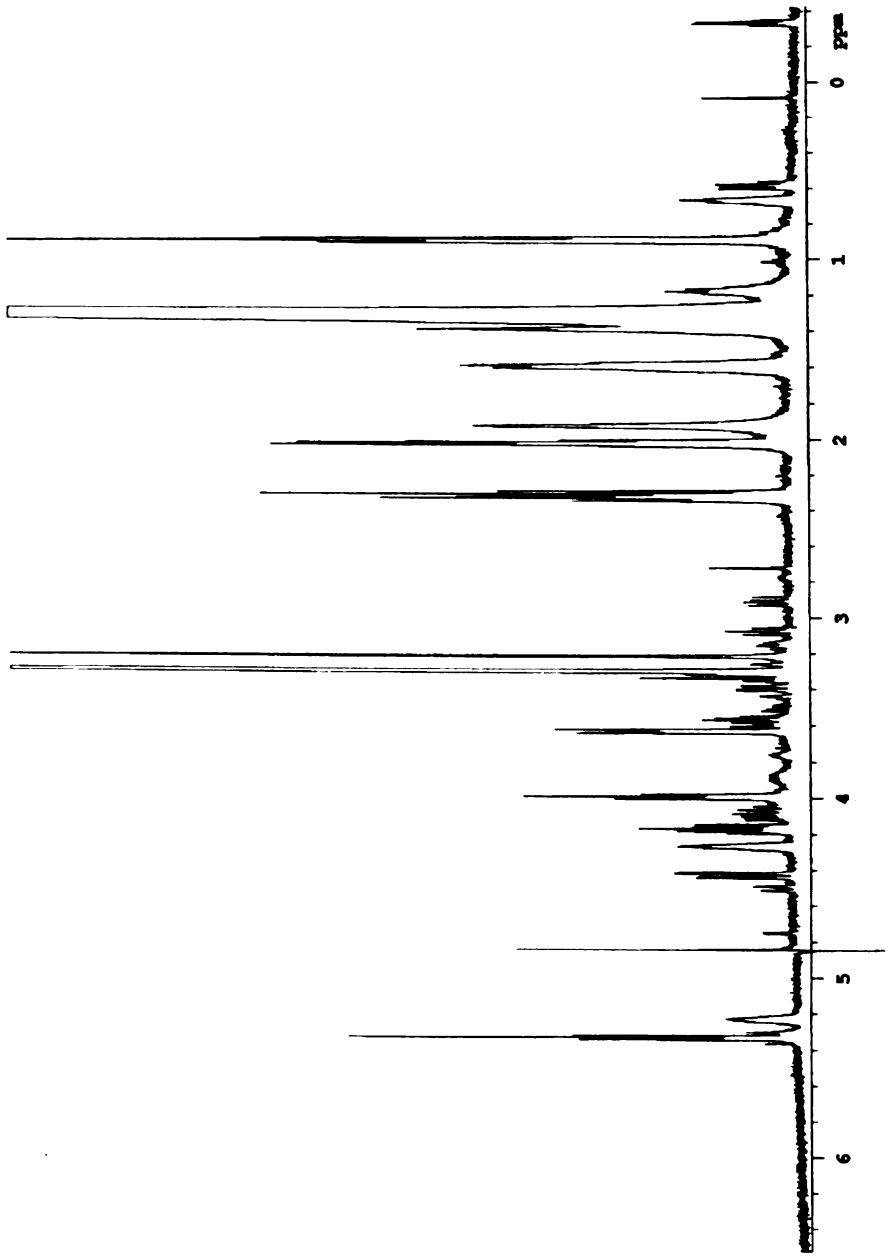

Figure 4.9 - The ^{1}H-NMR spectrum of isolated SQDG

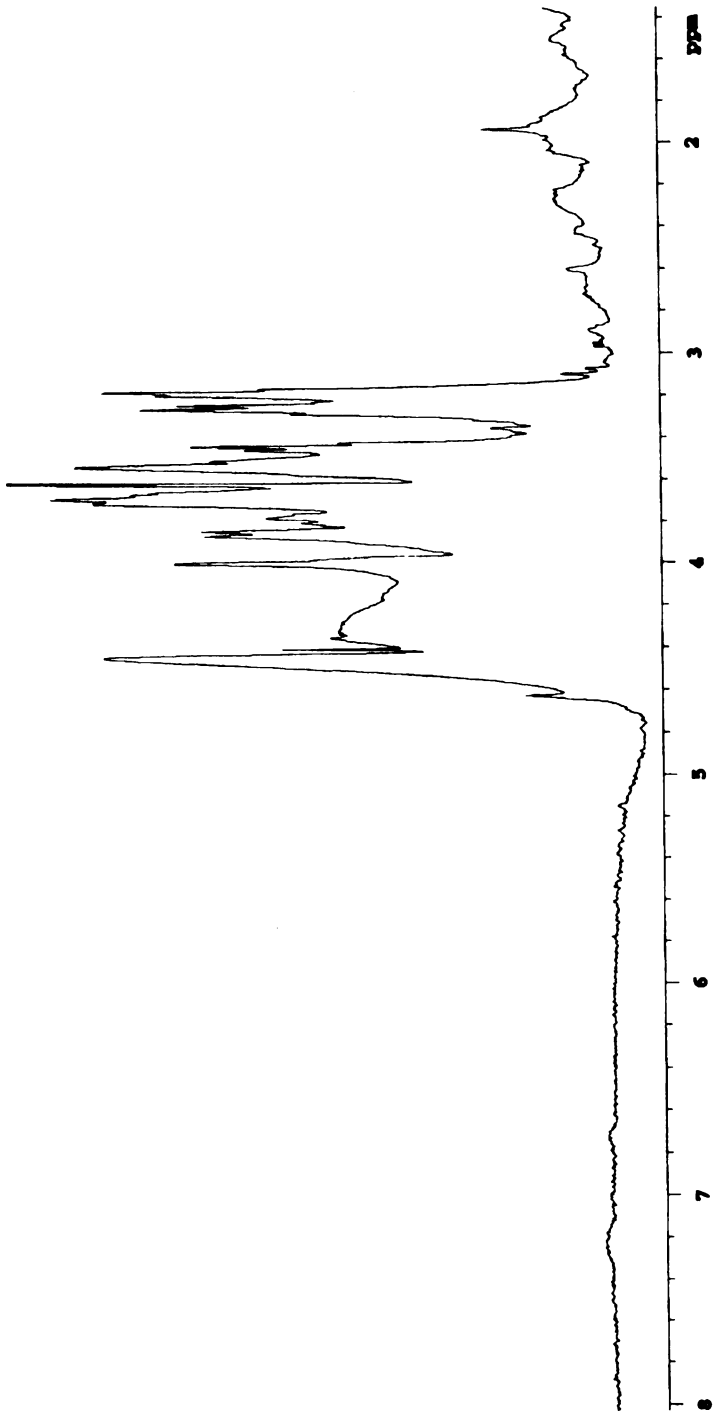

Figure 4.10 - ^{1}H-NMR spectrum of 6-sulfono-fructose-1-phosphate

Rhizobium leguminosarum sulfur metabolites labeled by ^{14}C-glucose and $^{32}PO_4^{2-}$ and with a similar mobility on TLC agrees with this molecule (data not shown). All these are consistent with 6-sulfono-fructose-1-phosphate, the product of aldol condensation from sulfolactaldehyde and DHAP, in the sulfoglycolytic pathway for SQDG biosynthesis proposed by Davies (39) (Figure 4.2). The negative ion electrospray mass spectrum of this compound is consistent with the structural assignment. It contains ions at m/z 345 and m/z 381, assignable to the M-H ion of the mono-sodiated and mono- sodiated and di-ammoniated species, respectively (Figure 4.11)

Another sulfur-containing component was isolated and characterized. Its ^{1}H-NMR spectrum is shown in Figure 4.12, which contained signals that were immediately attributable to α-linked sulfoquinovose (31). There is a pair of doublet of doublets between 2.8 and 3.3 ppm attributable to the protons at the 6-position of sulfoquinovose. The first doublet of doublets (J = 10 + 15 Hz) appeared at 2.93 ppm and second one (J = 0.1 + 15 Hz) appeared at 3.24 ppm. The larger splitting was due to geminal coupling. The anomeric proton signal appeared at 4.76 ppm as a narrow doublet (J = 3.7 Hz) indicating that the ring was in the pyranosyl configuration. The signals for H3 (a triplet (J = 9 Hz) at 3.34 ppm) and H4 (a triplet with 9.4 Hz coupling at 3.13 ppm) were also readily assignable. Signals with large geminal couplings between 3.4 and 3.9 ppm indicative of two different sets of methylene groups meant that glycerol linked through a primary hydroxyl group was the aglycon. The glycosidic linkage was confirmed by hydrolyzing the metabolite and passing the hydrolysis products through an anion-exchange column to trap the

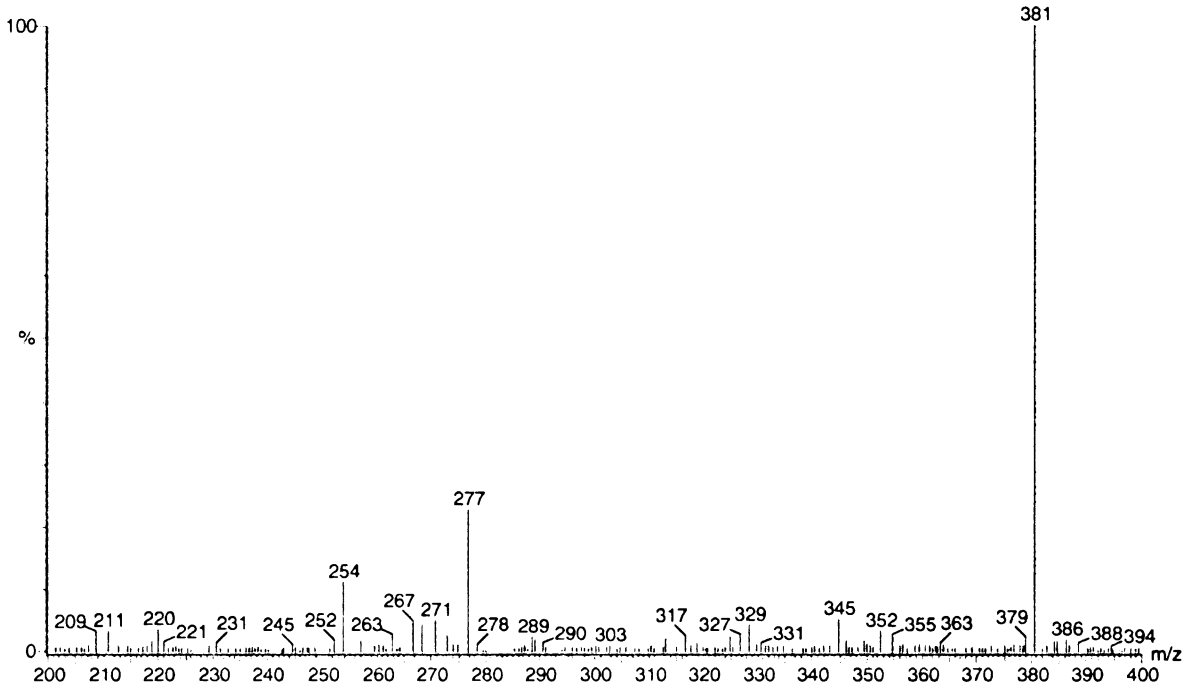

Figure 4.11 - The ES/MS spectrum of 6-sulfono-fructose -1-phosphate

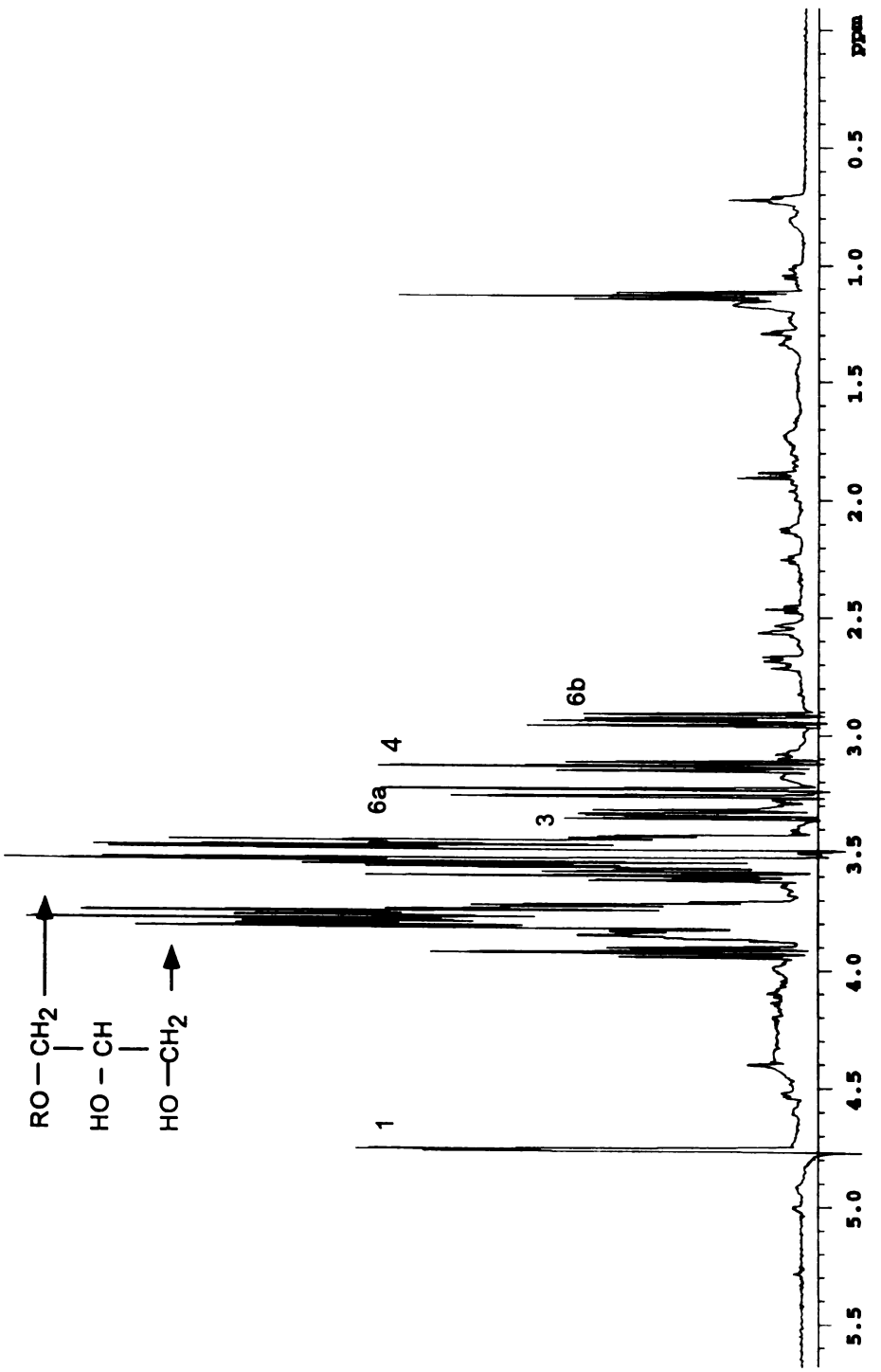

Figure 4.12 - The ^{1}H-NMR spectrum of isolated SQG

free sulfonic acid. Glycerol was identified as the sole component of the column flow-through by NMR spectroscopy (Figure 4.13). The molecular weight of the intact glycoside was confirmed by negative ion electrospray mass spectrometry which yielded a peak at m/z 317 for the M-H ion (Figure 4.14). As discussed earlier, SQG has been implicated as an intermediate of SQDG degradation in photosynthetic organisms. The significance of the discovery of SQG in *Rhizobium* will be discussed later.

SQDG Labeling by Carbon-labeled Glucose

Two possible biosynthetic pathways have been proposed for photosynthetic organisms. They are referred to as sulfoglycolytic pathway (Figure 4.2) and UDP-glucose pathway (Figure 4.3), respectively. To investigate the SQDG biosynthesis in *Rhizobium*, a series of SQDG labeling experiments have been performed.

After ^{13}C-1-glucose was fed to the bacteria, SQDG was isolated by preparative TLC and its identity confirmed by proton NMR and ES-MS (data not shown). To reveal the metabolic destiny of the ^{13}C label, a ^{13}C spectrum of the isolated SQDG sample was obtained by using a triple probe (Figure 4.15). The spectrum does not show any signal around 100 ppm, indicating the loss of the ^{13}C label at the carbon-1 position in sulfoquinovose. In stead, the ^{13}C label was found at other positions in sulfoquinovose, as indicated by the presence of ^{13}C signals between 70 and 80 ppm. The ^{13}C also ended up in the fatty acyl chains in SQDG, as shown by the signals around 130 ppm and 15 ppm (Figure 4.15).

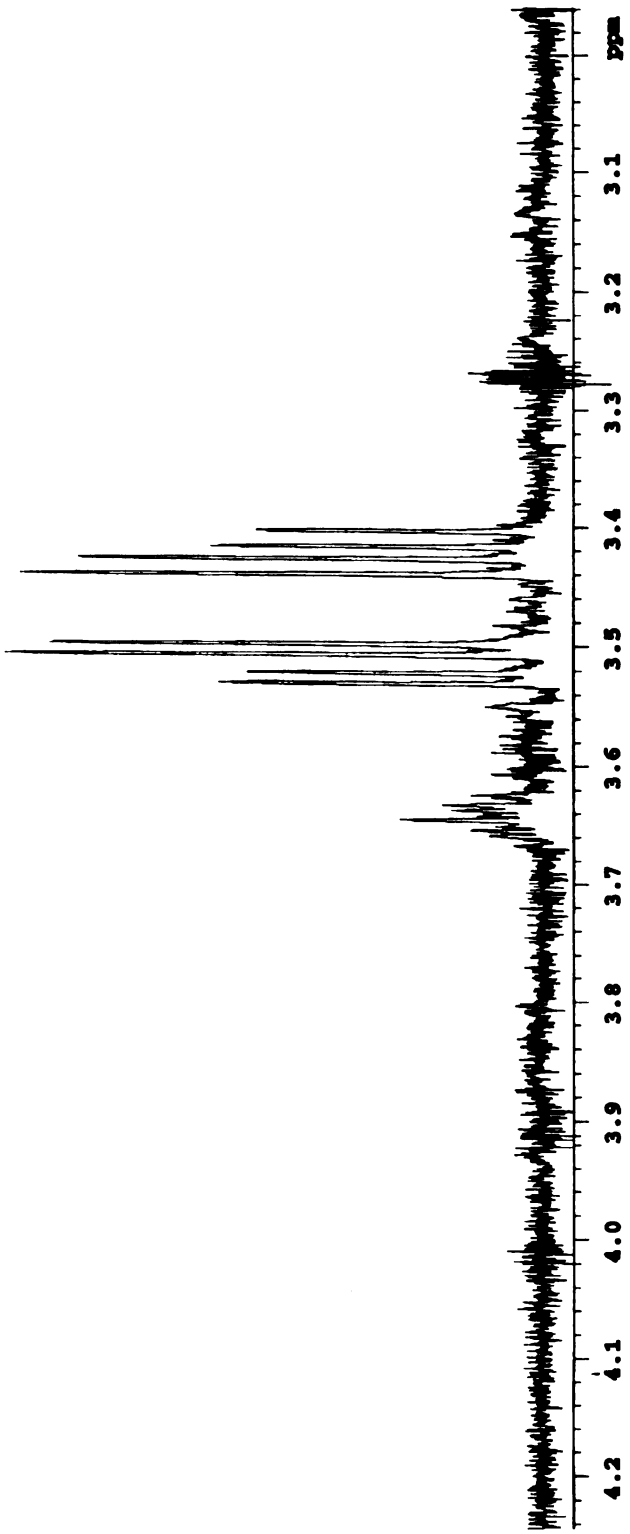

Figure 4.13 - The ^{1}H-NMR spectrum of glycerol from SQG

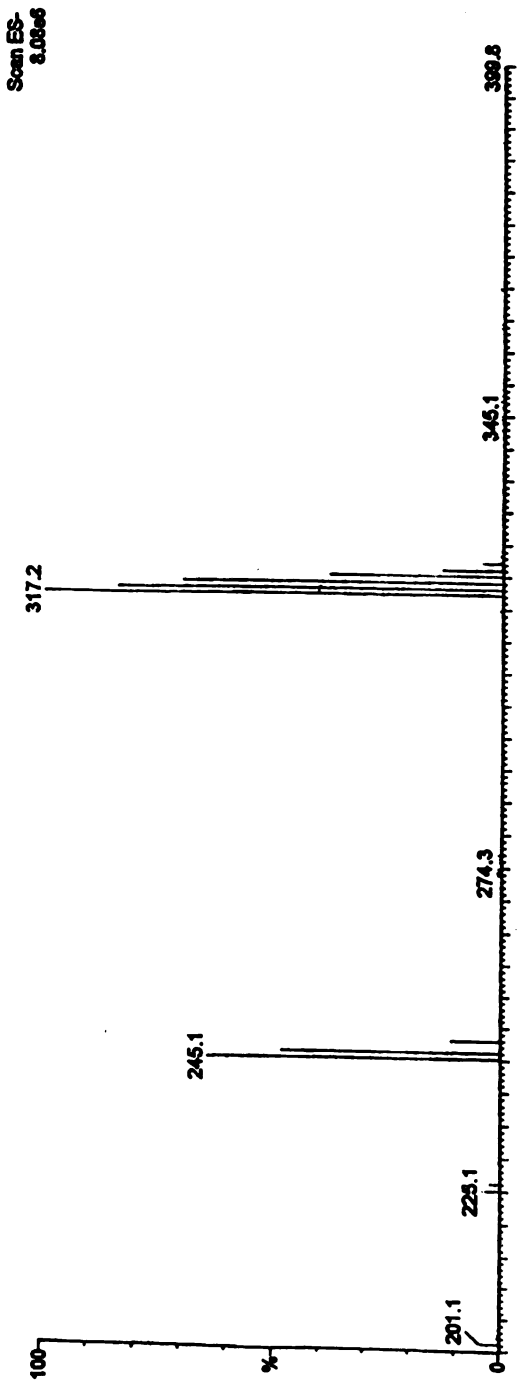

Figure 4.14 - The ES/MS spectrum of isolated SQG

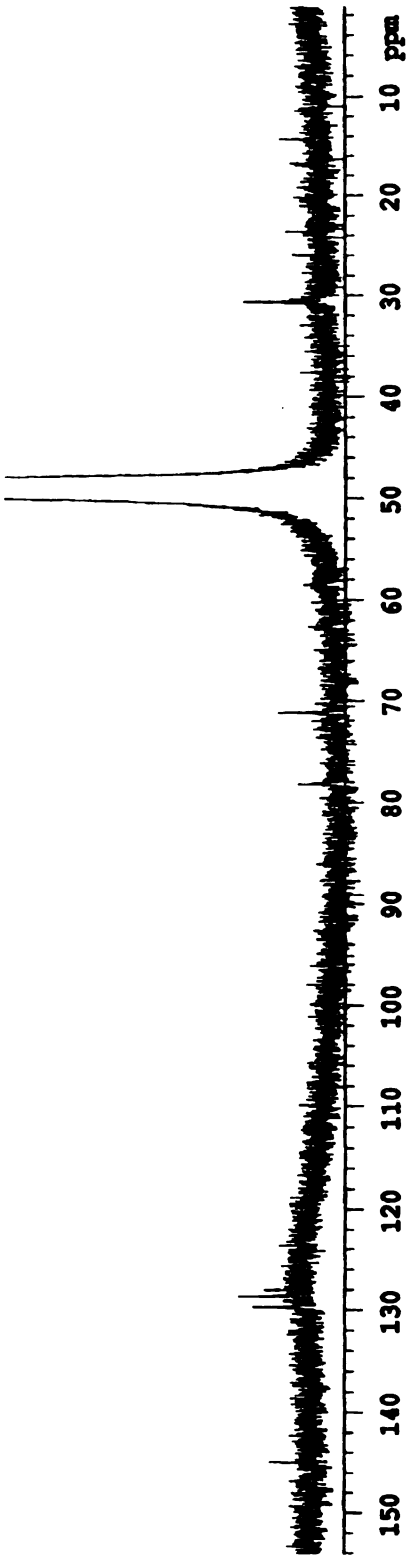

Figure 4.15 - ^{13}C-NMR spectrum of SQDG isolated from *Rhizobium meliloti*

The resonance at 30 ppm was due to the carbon atom adjacent to the sulfonate group in sulfoquinovose . The intensity of this signal is more than three times of others, suggesting a biased labeling effect. This phenomenon is very meaningful for both SQDG biosynthesis and carbon metabolism in *Rhizobium* in general. The high intensity of the signal at 30 ppm is well explained by the sulfoglycolytic pathway (Figure 4.2). According to this pathway, the carbon at 6-position of sulfoquinovose is derived from the 3-carbon of serine, which is biosynthesized from 3-phospho-glycerate. In the EMP pathway, this carbon comes from the carbon at 1-position of glucose. Since ^{13}C-1-glucose was fed to the bacteria, the label was enriched at the carbon at 6-position, adjacent to the sulfonate group of sulfoquinovose.

These results suggest that glucose was not directly incorporated into SQDG. In another word, the sugar backbone of the sulfoquinovose moiety did not come from the intact glucose taken up by the bacteria. This means that the formation of the carbon-sulfur bond in sulfoquinovose may not be at the six-carbon level.

The possibility of a UDP-glucose pathway has further been investigated with an uptake kinetics experiment using ^{14}C-1-glucose. Lipids were extracted at seven time points after the addition of the ^{14}C-label in the mid-log phase. The lipid samples were loaded onto TLC and developed as described in Materials and Methods. Figure 4.16 shows the image of the TLC plate after spraying with orcinol after heating at 120 °C for ten minutes. This revealed the phospholipids and glycolipids present on the TLC plate. PE, PG, SQDG and PC were the major lipid species

observed. Figure 4.17 shows the same TLC plate after spraying with orcinol for approximately two minutes. By this way, only glycolipids were stained in a very characteristic pink color. Showed up in the figure is SQDG only. The same TLC plate was also subjected to PhospholImager analysis. The autoradiograph is shown in Figure 4.18. No radioactive spots were observed half an hour after the addition of the label. PE was labeled at one-hour time point and the labeling reached a plateau quickly at the four-hour point. PG started to be labeled between one and two hours after the chase. The labeling went all the way up until the last sample taken at 24-hour time point. The spots of SQDG and PC moved very closely to each other, with SQDG sitting right on top of PC, as judged with SQDG and PC standards, the pink color for SQDG. The total amount of labeling for SQDG and PC was apparently less than that for PE or PG. The rate of label incorporation into SQDG and PC was also slower. This result supports that glucose was incorporated into fatty acyl chains or glyceryl backbones of phospholipids earlier than into SQDG.

SQDG Labeling by Sulfur-labeled Cysteine and Cysteate

Since the evidence obtained strongly suggests the sulfoglycolytic pathway rather than the UTP-glucose one, another time course experiment was carried out with ^{35}S-cysteine as sulfur source. Figure 4.19 is the autoradiograph of the TLC plate with lipid samples taken at different time points. It is clear that SQDG was labeled with ^{35}S within the first half hour of the addition of the label. This is sooner than the labeling of SQDG by ^{14}C-glucose, consistent with a sufoglycolytic pathway.

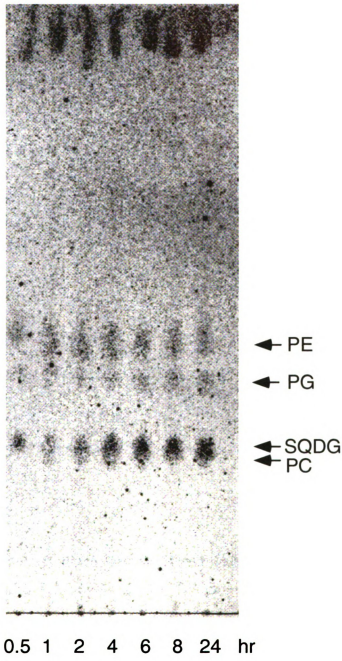

Figure 4.16 - Lipid TLC of time course labeling with ^{14}C-1-glucose: showing all lipids

Figure 4.17 - Lipid TLC of time course labeling with ^{14}C-1-glucose: showing glycolipids

Furthermore, SQDG was labeled with ^{35}S-cysteic acid as early as 10 minutes after the incubation of the cell lysate and the label (Figure 4.20A). The synthesis of SQDG is time-dependent as shown by the increase of SQDG level with time (Figure 4.20B). The quantitation was performed with ImageQuant software. This supports a sulfoglycolytic pathway for SQDG biosynthesis in *Rhizobium* in a more direct way. It is highly likely that cysteic acid is directly incorporated into SQDG in stead of being metabolized first.

exo-mutant Screening

Rhizobium meliloti exo mutants, including *exoB*, which has an impaired UDP-glucose 4-epimerase, were used to screen for SQDG. Lipids were extracted and the lipid samples were subjected to TLC and PhosphoImager analysis (Figure 4.21). The result shows that *exoB* mutant was able to synthesize SQDG. The loss of the ability to make UDP-galactose from UDP-glucose does not affect the ability of *Rhizobium* to make SQDG.

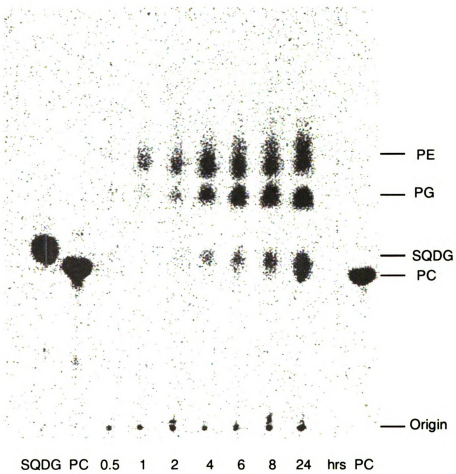

Figure 4.18 - Lipid TLC of time course labeling with ^{14}C-1-glucose: showing radio-labeled lipid species

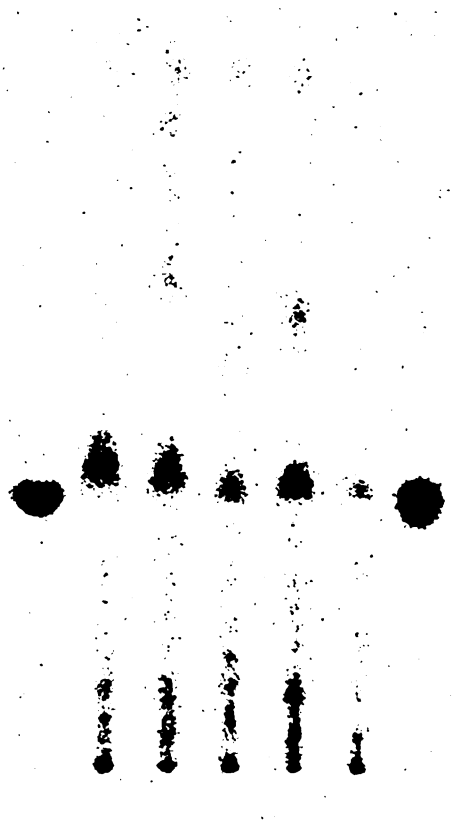

Figure 4.19 - Lipid TLC of time course labeling with ^{35}S-cysteine: The numbers indicate the time points (hour) when the cells were taken.

Figure 4.20A - *in vitro* labeling of SQDG with ^{35}S-cysteic acid

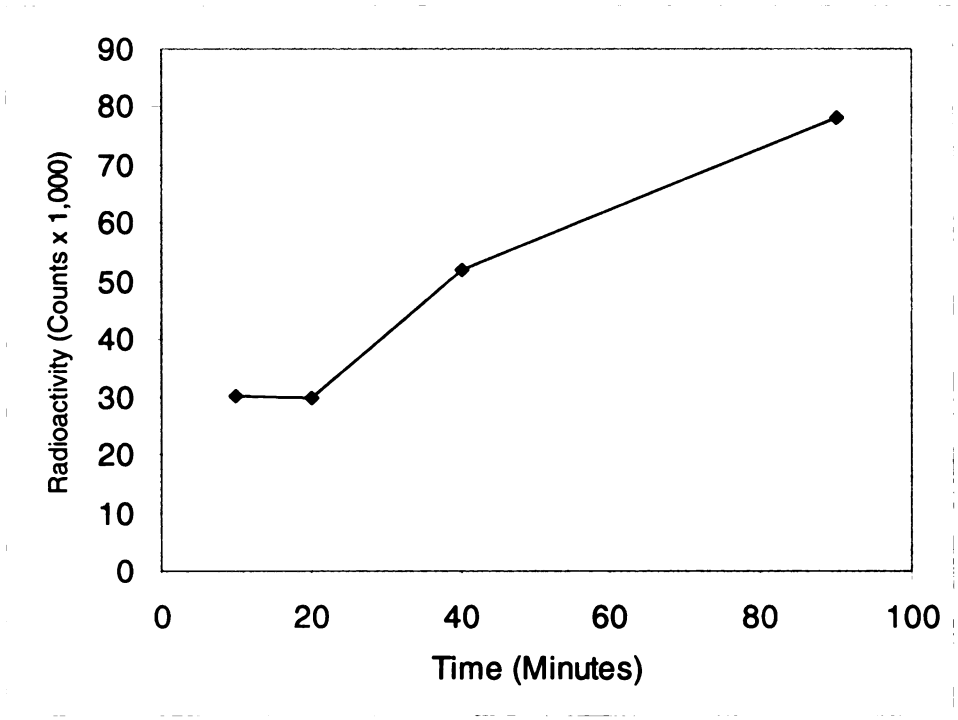

Figure 4.20B - Quantitation of *in vitro* labeling of SQDG with ^{35}S-cysteic acid

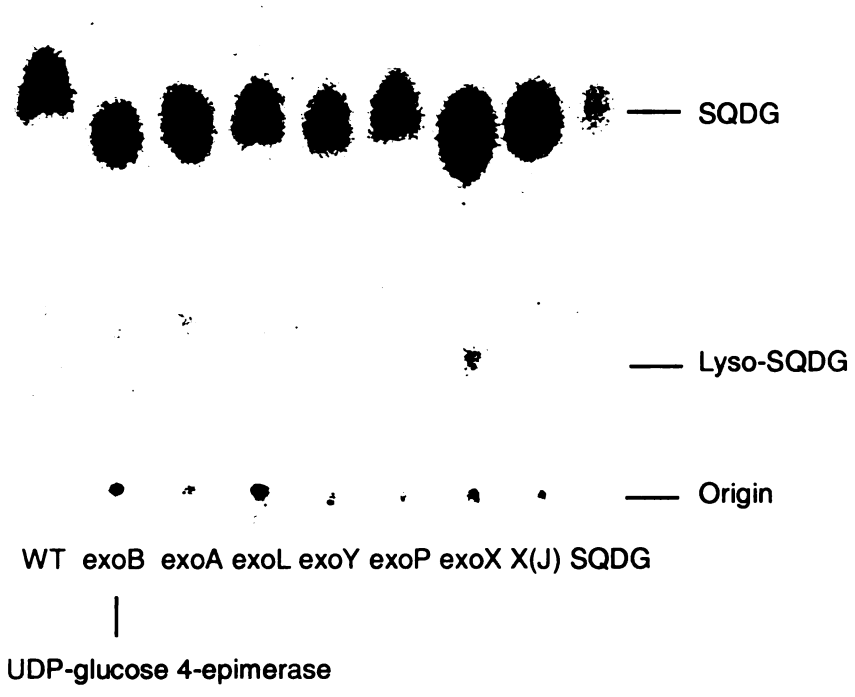

Figure 4.21 - TLC autoradiograph of lipids from *exo* mutants of *Rhizobium*

DISCUSSION

It is very unusual for *Rhizobium* to contain SQDG in their outer membranes. In view of their close ties to plants, we propose that the ability for *Rhizobium* to share the SQDG commonality with plants is meaningful and important for the interactions between *Rhizobium* and the host plant, very likely between the bacteroids and the host cytoplasm.

To better understand the SQDG biosynthesis in *Rhizobium* and thus to further the understanding of *Rhizobium*-plant interactions, we initiated the investigation of SQDG metabolism. The key issue to SQDG biosynthesis is when the carbon-sulfur bond in the sulfoquinovose headgroup is formed, at the three-carbon or six-carbon level. Two pathways have been proposed for photosynthetic organisms regarding to this. The sulfoglycolytic pathway explains the formation of carbon-sulfur bond at the three-carbon level and the UDP-glucose pathway at the six-carbon level. We took two approaches toward this goal. One is to trace the metabolic pathways with radio-labeled glucose. The other is to identify key intermediates of sulfur metabolism.

Various labeling experiments have been performed to investigate the biosynthesis of SQDG in *Rhizobium*. The carbon label in glucose has been found to be incorporated into PE earlier than into SQDG. The anomeric carbon in glucose was not retained in SQDG, suggesting that sulfoquinovose is not formed by the modification of glucose backbone directly. Importantly, the ^{13}C label at 1-position of glucose was found enriched at the 6-position of sulfolquinovose. The sulfur label in

cysteine was found incorporated into sulfoquinovose within the first half an hour of the addition of the label. This is sooner than the incorporation of the carbon label from glucose into SQDG.

How the sulfur from cysteine is incorporated, however, is not known. In *Cytophaga*, cysteine is not directly oxidized to cysteic acid (1). *Rhizobium meliloti* was not able to grow with cysteic acid as the sole sulfur source. This has also been observed in *Cytophaga* and strongly suggest that cysteic acid is incorporated into capnine without the cleavage of carbon-sulfur bond (1). These are consistent with the fact that *Rhizobium* cell lysate was able to incorporate the sulfur in cysteic acid into SQDG in a short time..

The biosynthesis of cysteic acid is an interesting question. The answer will reveal how the sulfur is oxidized. It has been suggested that the sulfur is in an oxidation state higher than the sulfohydryl level before cysteic acid is made (1). It seems true that cysteic acid is not an oxidation product of cysteine when it serves as a precursor for sulfonolipids, in contrast to when it is mere a degradation intermediate. This has been observed in *Cytophaga* (218, 1). The data obtained with *Rhizobium* also agrees with it. The result from ^{13}C-labeling experiment strongly suggests that cysteic acid is formed from serine by incorporating a sulfur moiety, very possibly sulfite. This notion is in agreement with the sulfoglycolytic pathway for SQDG synthesis in plants.

We conclude that cysteic acid is condensed with another three-carbon compound to yield sulfoquinovose based on the following considerations: i. the

UDP-glucose pathway is not likely as seen from the failure for SQDG to contain the anomeric carbon in glucose. In stead, a strong signal at 30 ppm indicates that the carbon atom attached to the sulfonate group comes from the carbon at 3-position of glycerate-3-phosphate, which derives from the carbon at 1-position of glucose; ii. the ^{14}C-label in glucose showed up earlier in PE than in SQDG, suggesting that the label was quickly incorporated into the headgroup of PE after going through the EMP pathway. If sulfoquinovose were made from glucose directly, it would be expected that SQDG had been labeled immediately; iii. cells were able to incorporate the sulfur from cysteine into SQDG earlier than to incorporate the anomeric carbon from glucose into SQDG; iv. the sulfur in cysteic acid was taken into SQDG by the cell lysate very quickly and this incorporation is time-dependent. v. a mutant impaired in UDP-glucose 4-epimerase could synthesize SQDG normally, suggesting that UDP-glucose 4-epimerase is not involved in SQDG biosynthesis; vi. cysteic acid and 6-sulfono-fructose-1-phosphate have been demonstrated to be present metabolites of *Rhizobium meliloti*. Both of these compounds are the key intermediates in the sulfoglycolytic pathway; vii. cysteic acid has been demonstrated to be an immediate precursor for capnine, another sulfonoglycolipid species in Gram-negative bacteria and suggested not to be made from cysteine oxidization; viii, the sulfoglycolytic pathway was originally proposed for photosynthetic algea and higher plants and bulk of evidence has been present.

Some potential pitfalls are: i. There is a possibility that *Rhizobium* is capable of both pathways, depending on the conditions. ii. Regarding to the point ii above, it may not be true that UDP-glucose pathway is quicker for the cells to make

SQDG than the sulfoglycolytic one, even though it is easier and more straightforward. In the same line, we haven't been able to demonstrate that the cells are able to synthesize galactose within the first half an hour of the chase, though data suggests that galactose has been incorporated into EPS at the two hour point (not shown). iii. Regarding to the point v above, it is possible that there exists in *Rhizobium* another way for making the 4-keto-UDP-glucose, which is necessary for synthesizing SQDG along this pathway. However, the ^{13}C-glucose experiment provided definitive evidence that the direct incorporation pathway is not the case.

Overall, the results strongly suggest that the formation of carbon-sulfur bond in sulfoquinovose is not at the six carbon level and that a sulfoglycolytic pathway for SQDG biosynthesis is in operation in the free-living state of *Rhizobium*.

Whether SQG is an intermediate of biosynthesis or degradation in *Rhizobium* is not clear. The common rule for the biosynthesis of glycerolipids is that the head group is transferred to the diacylglycerol intermediate on an membrane location. The evidence obtained in photosynthetic organisms is consistent with this. If this rule also applies to *Rhizobium*, the discovery of SQG means that *Rhizobium* may share a common catabolic pathway with plants, which is possibly the sulfoglycolytic one as suggested by Benson (100). If SQG is a biosynthetic intermediate, it would suggest that *Rhizobium* have a distinct mechanism for SQDG biosynthesis from that of plants. However, no valid argument is available to lead to this conclusion.

Our experiments reveal that the mechanism of SQDG biosynthesis in *Rhizobium* is very similar to that in plants, although there are some discrepancies

about the biosynthesis of SQDG in plants. We believe that the existence of SQDG in *Rhizobium* and its ability to make it is not a coincidence. It is difficult to surmise whether the presence of SQDG or the ability to synthesize it is more critical for the well-beings of the two organisms, if any. We propose that SQDG is something *Rhizobium* uses to evade the host defense. This type of feature-sharing has been commonly observed in bacteria for both LPS and proteins in some host-pathogen systems. The phenomenon is called molecular mimicry. We generalize the above proposal and hypothesize that it is applied to all the host-pathogen systems.

CHAPTER 5

Perspectives on *Rhizobium*-legume Interactions

SUMMARY AND PERSPECTIVES

We have demonstrated that the phospholipases A and D activities are associated with *Rhizobium* membranes. We have found that both phospholipase A and α-sulfoquinovosyl diacylglycerol are located in the outer membrane of *Rhizobium* and that chitolipooligosaccharides (Nod factors) are in the inner membrane. We have identified some key intermediates of sulfur metabolism in *Rhizobium* such as cysteic acid, 6-sulfono-fructose-1-phosphate and glyceryl-α-sulfoquinovose. The results from a series of labeling experiments suggest that α-sulfoquinovosyl diacylglycerol is synthesized by an aldol condensation reaction of cysteic acid and another three-carbon intermediate. The presence of cysteic acid and 6-sulfono-fructose-1-phosphate strongly support this. In this model, it is proposed that Nod factors function as secreted signal molecules in nano-molar to pico-molar level to mediate the species-specific interactions between *Rhizobium* and legumes. The inner membrane location of chitolipooligosaccharide Nod factors in relatively large quantity argues against this role.

These results are in line with our current knowledge about eukaryote-infecting bacteria, the metabolism of α-sulfoquinovosyl diacylglyerol in photosynthetic organisms and the structurally similar sulfonolipids in other Gram-negative bacteria. However, they are against the model of Nod factor about the so-called host specificity in *Rhizobium*-legume interactions.

The biosynthesis of SQDG has been investigated with two approaches. One

is to trace SQDG synthesis by radioactive labels, including the carbon labeled at 1-position of glucose and the sulfur labeled in free sulfate, cysteine and cysteic acid. The other one is to isolate the sulfur-containing intermediates from *Rhizobium*. Theoretically, either of these two approaches should be able to lead us to the complete understanding of SQDG biosynthesis. We have clearly and definitively demonstrated from the first approach (labeling experiments) that SQDG is synthesized in *Rhizobium* through the aldo condensation of cysteic acid and another three-carbon species, very possibly DHAP rather than the direct incorporation of glucose followed by the addition of sulfur-containing group. We have also obtained strong evidence from the second approach, suggesting that the conclusion above is indeed true. However, the data along this line is not perfect yet. Two more experiments should be performed in the near future to further complete the work of SQDG biosynthesis. One is to test the incorporation of cysteic acid labeled by both carbon and sulfur. This will tell us in a direct way that cysteic acid is indeed a biosynthetic precursor of SQDG. The other is to test the ability of DHAP to affect the incorporation of cysteic acid during the *in vitro* labeling experiments. This will confirm the notion that DHAP is the three-carbon species condensed with cysteic acid during the formation of six-carbon and sulfur-containing precursor of SQDG.

We propose that there is a set of biochemical principles governing the mechanisms and strategies for the infection of higher organisms by prokaryotes in general. These principles are conserved in bacterium-animal and bacterium-plant interactions, which include a variety of biological phenomena such as pathogenesis, parasitism and symbiosis. Some specific phases of the bacterial infection process

of higher organisms are attachment, invasion and survival. The biochemical features of each of these phases are not necessarily restricted to a certain pair of organisms involved. The ability of bacteria to infect and the outcome of the infection are not determined by one or a few factors but by the infecting bacteria as a whole. After the invasion, bacteria strive to integrate the host metabolism. The outcome of this integration determines whether the bacteria will be a harm or a benefit to the host organism.

The implication of the above principles in the field of *Rhizobium*-legume symbiosis is that the research should not be focused so much on one particular type of "magic" molecule to solve everything. It is naive to think that bacteria rely on merely one way to cope with the environment. As a matter of fact, many *Rhizobium* biomolecules have been implicated in its infection of plants. These include EPS, LPS, CPS, β-1,2-glucan, glycolipids, Nod factors and various enzymes involved in the metabolisms. Even these may well be just the tip of an iceberg. A wealth of evidence suggests that many of the *nod* gene products are general enzymes working on all kinds of substrates rather than being "specific" to chitolipooligosaccharides. On the other hand, it has turned out that *Rhizobium* is not that specific, neither is its phylogenetic brother *Agrobacterium*. Therefore, "host specificity" is not one of the most fundamental biochemical features associated with *Rhizobium*-legume interactions.

The bacterial genes related to the infection of higher organisms are usually carried by plasmids or clustered on the chromosomes. There is good evidence

suggesting that gene transfer is common within the bacterial world. It has been hypothesized that gene transfer also occurs between plants and bacteria horizontally. This would explain the unusual ability of *Rhizobium* to synthesize α-sulfoquinovosyl diacylglycerol in the same way as plants.

A practical implication of this notion of the universal principles is that infections can be controlled by limiting the ability of the bacteria to attach to prevent further damage to the host. Therefore, better understanding of the attachment phase will help to develop antibacterial strategies for the benefit of human being.

Furthermore, since the *Rhizobium*-legume system is one of a set of bacterial infections, which is governed by some common principles, it can be used as a model system to study many aspects of host-pathogen interactions without any human or even animal risk.

BIBLIOGRAPHY

1. Abbanat, D. R., Godchaux III, W., Polychroniou, G., and Leadbetter, E. R. 1985. Biosynthesis of a sulfonolipid in gliding bacteria. Biochem. Biophys. Res. Commun. 130:873-878.

2. Abbanat, D. R., Leadbetter, E. R., Godchaux III, W., and Escher, A. 1986. Sulphonolipids are molecular determinants of gliding motility. Nature. 324:367-369.

3. Al-Mallah, M. K., Davey, M. R., and Cocking, E. C. 1989. Formation of nodular structures on rice seedlings by rhizobia. J. Exp. Botany 40:473-478.

4. Al-Mallah, M. K., Davey, M. r., and Cocking, E. C. 1990. Nodulation of oilseed rape (Brassica napus) by rhizobia. J. Exp. Botany 41:1567-1572.

5. Anderson, R., Kates, K., and Volcani, B. E. 1979. Identification of the sulfolipids in the non-photosynthetic diatom *Nitzschia alba*. Biochem. Biophys. Acta 528:89-106.

6. Atkinson, E. M. and Long, S. R. 1992. Homology of *Rhizobium melitloti* NodC to polysaccharide polymerizing enzymes. Mol. Plant-Microbe Interact. 5:439-442.

7. Baer, D. and Gudmestad, N. C. 1995. In vitro cellulolytic activity of the plant pathogen *Clavibacter michiganensis* subsp. *sepedonicus*. Can. J. Microbiol. 41:877-888.

8. Baker, B., Zamgryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. 1997. Signaling in plant-microbe interactions. Science 276:726-733.

9. Barbour, W. M., Wang, S., and Stacey, G. 1992. Molecular genetics of *Bradyrhizobium* symbiosis. *In*: Biological Nitrogen Fixation, Stacey, G., Burris, R. H., and Evans, H. J. (eds.), pp 648-656, Chapman and Hall, Inc., New York, London.

10. Barinaga, M. 1996. A shared strategy for virulence. Science 272:1261-1263.

11. Barny, M. A. and J. A. Downie. 1993. Identification of the NodC protein in the inner but not the outer membrane of *Rhizobium leguminosarum*. Mol. Plant-Microbe Interact. 6:669-672.

12. Benning, C. and Somerville, C. R. 1992. Identification of an operon involved in sulfolipid biosynthesis in *Rhodobacter sphaeroides*. J. Bacteriol. 174:6479-6487.

13. Benning, C. and Somerville, C. R. 1992. Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. J. Bacteriol. 174:2352-2360.

13a. Benning, C., Beatty, J. T., Prince, R. C., and Somerville C. R. 1993. The sulfolipid sulfoquinovosyl diacylglycerol is not required for photosynthetic electron transport in *Rhodobacter sphaeroids* but enhances growth under phosphate limitation. Proc. Natl. Acad. Sci. 90:1561-1565.

14. Benson, A. A. and Shibuya, I. 1961. Sulfocarbohydrate metabolism. Fed. Proc. 20:79.

15. Bittinger, M. A., Milner, J. L., Saville, B. J. and Handelsman, J. 1997. *rosR*, a determinant of nodulation competitiveness in *Rhizobium etli*. Mol. Plant-Microbe Interact. 10:180-186.

16. Birch, M., Robson, G., Law, D., and Denning, D. W. 1996. Evidence of multiple extracellular phospholipase activities of *Aspergillus fumigatus*. Infect. Immun. 64:751-755.

17. Boettcher, K. J. and Ruby, E. G. 1995. Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid light organs. J. Bacteriol. 177:1053-1058.

18. Bohlool, B. B. and Schmidt, E. L. 1974. Lectins: a possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185:269-271.

19. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem.72:248-254.

20. Bradley, D. J., Butcher, G. W., and Galfre, G. 1986. Physical association between the peribacteroid membrane and lipopolysaccharide from the bacteroid outer membrane in *Rhizobium*-infected pea root nodule cells. J. Cell Sci. 85:47-61.

21. Braun-Breton, C., Rosenberry, T. L., and da Silva, L. P. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidylinositol-specific phospholipase C. Nature 332:457-459.

22. Broek, A. V., and Vanderleyden, J. 1995. The role of bacterial motility, chemotaxis, and attachment in bacteria-plant interactions. Mol. Plant-Microbe Interact. 8:800-810.

23. Brok, R. G. P. M., Brinkman, E., van Boxtel, R., Bekkers, A. C. A. P. A., Verheij, H. M., and Tommassen, J. 1994. Molecular characterization of enterobacterial *pldA* genes encoding outer membrane phospholipase A. J. Bacteriol. 176:861-870.

24. Brok, R. G. P. M., Belandia, I. U., Dekker, N., Tommassen, J., and Verheij, H. M. 1996. *Escherichia coli* outer membrane phospholipase A: a role of two serines in enzymatic activity. Biochemistry 35:7787-7793.

25. Brownleader, M. D. and Dey, P. M. 1993. Purification of extensin from cell walls of tomato cells in suspension culture. Planta 191:457-469.

26. Bulawa, C. E. 1992. *CSD2*, *CSD3*, and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: The *CSD2* gene product is relatated to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. 12:1764-1776.

27. Bunn, C. R. and Elkan, G. H. 1971. The phospholipid composition of *Rhizobium japonicum*. Can. J. Microbiol. 17:291-295.

28. Burgess, J. 1985. An Introduction to Plant Cell Development, pp 71, Cambridge University Press, Cambridge.

29. Canter Cremers, H. C. J., Batley, M., Redmond, J. W., Eydems, L., Breedveld, M. W., Zevenhuizen, L. P. T. M., Pees, E., Wijffelman, C. A., and Lugtemberg, B. J. J. 1990. *Rhizobium leguminosarum exoB* mutants are deficient in the synthesis of UdP-glucose 4'-epimerase. J. Biol. Chem. 265:21122-21127.

30. Carlson, T. A., and Chelm, B. K. 1986. Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature 322:568-570.

31. Cedergren, R. A., and Hollingsworth, R. I. 1994. Occurrence of sulfoquinovosyl diacylglycerol in some members of the family *Rhizobiaceae*. J. Lipid Res. 35:1452-1461.

32. Cedergren, R. A., Lee, J., Ross, K. L., and Hollingsworth, R. I. 1995. Common links in the struture and cellular localization of *Rhizobium* CLOS and general *Rhizobium* membrane phospholipid and glycolipid components. Biochemistry 34:4467-4477.

33. Chalifour, F. and Benhamou, A. 1989. Indirect evidence for cellulase production by *Rhizobium* in pea root nodules during bacteroid differentiation: cytochemical aspects of cellulose breakdown in rhizobial droplets. Can. J. Microbiol. 35:821-829.

34. Chesnokova, O., Coutinho, J. B., Khan, I. H., Mikhail, M. S., and Kado, C. I. 1997. Characterization of flagella genes of *Agrobacterium tumefaciens* and the effect of a bald strain on virulence. Mol. Microbiol. 23:579-590.

35. Cocking, E. C., Al-Mallah, M. K., Benson, E., Davey, M. R. 1990. Nodulation of non-legumes by rhizobia. *In*: Nitrogen Fixation: achievements and Objectives, Gressoff, P. M., Roth, L., E., Stacey, G., Newton, W. E.(eds.), pp 813-823, Chapman and Hall Inc., New York.

36. Cook, D. M., Li, P. L., Ruchaud, F., Padden, S., and Farrand, S. K. 1997. Ti plasmid conjugation is independent of *vir*: reconstitution of the *tra* functions from ptic58 as a binary system. J. Bacteriol. 179:1291-1297.

37. Crow, V. L., Jarvis, B. D. W., and Greenwood, R. M. 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium*. Int. J. Syst. Bacteriol. 31:152.

38. Darvill, A., McNeil, M, Albersheim, P., and Delmer, D. P. 1980. The primary cell walls of flowering plants. *In*: The Plant Cell, Tolbert, N. E. (ed.), Vol 1 of The Biochemistry of Plants, A Comprehensive Treatise, Stumpf, P. K. and Conn, E. E. (eds), Academic Press, New York, etc.

39. Davies, W. H., Mercer, E. I., and Goodwin, T. W. 1966. Some observations on the biosynthesis of the plant sulpholipid by *Euglena gracilis*. Biochem. J. 98:369-373.

40. Dazzo, F. B. and Hubell, D. H. 1975. Cross-reactive antigens and lectins as determinants of symbiotic specificity in the *Rhizobium*-clover assiciation. Appl. Microbiol. 30:1018-1033.

41. Dazzo, F. B. 1982. In: Experimental Microbial Ecology (R.G. Burns and J.H. Slater, eds.), pp 431-446, Blackwell Scientific Publications, Oxford.

42. Dazzo, F. B. and Truchet, G. L. 1983. Interactions of lectins and their saccharide receptors in the *Rhizobium*-legume symbiosis. J. Membrane Biol. 73:1-16.

43. Dazzo, F. B., Truchet, G. L., Sherwood, J. E., Hrabak, E. M., Abe, M., and Pankratz, S. H. 1984. Specific phases of root hair attachment in the *Rhizobium* trifolii-clover symbiosis. Appl. Environ. Microbiol. 48:1140-1150.

44. Debellé, F., Rosenberg, C., and J. Dénarié. 1992. The *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* NodC proteins are homologous to yeast chitin synthases. Mol. Plant-Microbe Interact. 5:443-446.

45. de Geus, P., van Die, I., Bergmans, H., Tommassen, J., and de Haas, G. 1983. Molecular cloning of *pldA*, the structural gene for outer membrane phospholipase of *E. coli* K12. Mol. Gen. Genet. 190:150-155.

46. de Ley, J. 1974. Phylogeny of prokaryotes. Taxon. 23:291-8.

47. de Maagd, R. A., Yang, W., Roo, L. G., Mulders, I. H. M., Roest, H. P., Spaink, H. P., Bisseling, T., and Lugtenberg, B. J. J. 1994. Down-regulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *ropA* occurs abruptly in interzone II-III of pea nodules and can be uncoupled from *nif* gene activation. Mol. Plant-Microbe Interact. 7:276-281

48. Depierreux, C., Kang, H. C., Guerin, B., Monsigny, M., Delmotte, F. 1991. Characterization of an *Agrobacterium tumefaciens* lectin. Glycobiology. 1:643-649.

49. Díaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. Nature 338:579-581.

50. Díaz, C. L., Trudy, J. J., Stam, H. C., and Kijne, J. W. 1995. Sugar-bingind activity of pea lectin expressed in white clover hairy roots. Plant Physiol. 109:1167-1177.

51. Dixon, R. O. D. and Wheeler, C. T. 1986. Nitrogen fixation in plants, pp 9-11, Chapman and Hall, Inc., New York.

52. Driscoll, B. T. and Finan, T. M. 1996. $NADP^+$-dependent malic enzyme of *Rhizobium meliloti*. J. Bacteriol. 178:2224-2231.

53. Duensing, T., D. and van Putten, J. P. 1997. Vitronectin mediates internalization of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. Infect. Immun. 65:964-970.

54. Dunn, M. F., Encarnación, S., Araíza, G., Vargas, M. C., Dávalos, A., Peralta, H., Mora, Y., and Mora, J. 1996. Pyruvate carboxylase from *Rhizobium elti*: mutant characterization, nucleotide sequence, and physiological role. J. Bacteriol. 178:5960-5970.

55. Falkow, S. 1997. What is a pathogen. ASM News 63:359-365.

56. Finlay, B. B. and Falkow, S. 1990. Comparisonn of the invasion strtegies used by *Salmonella cholerae-suis*, *Shigella flexneri* and *Yersinia enterocolitica* to enter cultured animal cells: endosome acidificationnn is not required for bacterial invasion or intracellular replication. Biochemie 70:1089-1099.

57. Finlay, B. B. and Cossart, P. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science 276:718-725.

58. Finlay, B. B. and Falkow, S. 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev.61:136-169.

59. Finnan, T. M., Gough, C., and Truchet, G. 1995. Similarity between the *Rhizobium meliloti fliP* gene and pathogenicity-associated genes from animal and plant pathogens. Gene 152:65-67.

60. Fiore, A. E., Michalski, J. M., Russell, R. G., Sears, C. L., and Kaper, J. B. 1997. Cloning, characterization, and chromosomal mapping of a phospholipase (lecithinase) produced b *Vibrio cholerae*. Infect. Immun. 65:3112-3117.

61. Freiberg, C., Fellay, R., Bairoch, A., Broughton, W. J., Rosenthal, A. and Perret, X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Natrue 387:39-401.

62. Gaillard, J. L., Berche, P., Frehel, C., Gouin, Cosart, P. 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell 65:1127-1141.

63. Geremia, R. A., Mergaert, P., Geelen, D., Van Montagu, M., and Holsters, M. 1994. The NodC protein of *Azorhizobium caulinodans* is an *N*-acetylglucosaminyltransferase. Proc. Natl. Acad. Sci. USA 91:2669-2673.

64. Gerson, T. and Patel, J. J. 1975. Neutral lipids and phospholipids of free-living and bacteroid forms of two strains of *Rhizobium* infective on *Lotus pedunculatus*. Applied Microbiol. 30:193-198.

65. Godchaux III, W. and Leadbetter, E. R. 1980. *Capnocytophaga spp.* contain sulfonolipids that are novel in procaryotes. J. Bacteriol. 144:592-602.

66. Godchaux III, W. and Leadbetter, E. R. 1983. Unusual sulfonolipids are characteristic of the *Cytophaga-Flexibacter* group. J. Bacteriol. 153:1238-1246.

67. Goosen, D. R. L, de Maagd, R. A., and Lugtenberg, B. J. 1991. Antigeic changes in lipopolysaccharide I of *Rhizobium leguminosarum* bv. *viciae* in root nodules of *Vicia sativa subsp. nigra* occur during release from infection threads. J. Bacteriol. 173:3177-83.

68. Goto, M. 1992. Fundamentals of bacterial plant pathology. pp142-167. Academic Press, Inc., San Diego.

69. Grant, K. A., Belandia, I. U. , Dekker, N., Richardson, P. T., and Park, S. F. 1997. Molecular characterization of *pldA*, the structural gene for a phospholipase A from *Campylobacter coli*, and its contribution to cell-associated hemolysis. Infect. Immun. 65:1172-1180.

70. Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. E., Greenberg, E. P. 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. J. Bacteriol. 178:372-6.

71. Güler, S., Seeliger, A., Härtel, H., Renger, G., and Benning C. 1996. A null mutant of *Synechococcus sp.* PCC7942 deficient in the sulfolipid sulfoquinovosyl diacylglycerol. J. Biol. Chem. 271:7501-7507.

72. Hackstadt, T. and Williams, J. C. 1981. Biochemical strategy for obligate parasitism of eukaryotic cells *Coxiella burnetii*. Proc. Natl. Acad, Sci. USA 78:3240-3244.

73. Haines, T. H. 1973. The plant sulfonolipid. *In*: Lipids and Biomembranes of Eukaryotic Microorganisms, Erwin, J. A.(ed.), pp 200-212, Academic Press, London.

74. Harrison, M. J. and van Buuren, M. L. 1995. A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:626-729.

75. Harwood, J. L. 1975. Synthesis of sulphoquinovosyl diacylglycerol by higher plants. Biochem. Biophys. Acta 398:224-230.

76. Harwood, J. L. 1980. Sulfolipids, *In* The Biochemistry of Plants, Vol 4, Academic Press, Inc.

77. Ho, S. C., Schindler, M., and Wang, J. L. 1990. Carbohydrate binding activities of *Bradyrhizobium japonicum*. II. Isolation and characterization of a galactose-specific lectin. J. Cell. Biol. 111:1639-1643.

78. Hollingsworth, R. I. and Dazzo, F. B. 1988. Use of phenyl carbanate derivatives for the GC/MS characterization of 3-hydroxyalkanoic acids in bacterial lipopolysaccharides. Anal. Microbiol. 7:295-302.

79. Hollingsworth, R. I. 1997. Biochemistry, submitted.

80. Homma, H., Kobayashi, T., Chiba, N., Karasawa, K., Mizushima, H., Kudo, I., Inoue, K., Ikeda, H., Sekiguchi, M., and Nojima, S. 1984. The DNA sequence encoding *pldA* gene, the structural gene for detergent-resistant phospholiase A of *E. coli.* J. Biochem. 96:1655-1664.

81. Horrevoets, A. J. G., Verheij. H. M. , and de Haas, G. H. 1991. Inactivation of *Escherichia coli* outer-membrane phopholipase A by the affinity label hexadecanesulfonyl fluoride. Eur. J. Biochem. 198:247-253.

82. Huang, H. C., Lin, R. H., Chang, C. J., Collmer, A., and Deng, W. L. 1995. The complete *hrp* gene cluster of *Pseudomonas syringae* pv. *syringae* 61 includes two blocks of genes required for harpinPss secretion that are arranged colinearly with *Yersinia ysc* homologs. Mol. Plant-Microb. Interact. 8:733-746.

83. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. Cell 48:549-554.

84. Ibrahim, A. S., Mirbod, F., Filler, S. G., Banno, Y., Cole, G. T., Kitajima, Y., Edwards, J. E., Nozawa, Y., and Ghannom, M. A. 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans.* Infect. Immun. 63:1993-1998.

85. Isberg, R. R. and Leong, J. M. 1990. Multiple β1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell 60:861-871.

86. Isberg, R. R. 1991. Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. Science 252:934-938.

87. Jacab-Dubuisson, T., Kuehn, M., and Hulgren, S. J. 1993. A novel secretion apparatus for the assembly of adhesive bacterial pili. Trends Microbiol. 1:50-55.

88. Jiménez-Zurdo, J. I., Garcia-Rodríguez, F. M. and Toro, N. 1997. The *Rhizobium meliloti putA* gene: its role in the establishment of the symbiotic interaction with alfalfa. Mol. Microbiol. 23:85-93.

89. John, M., Schmidt, J., Wieneke, U., Kondorosi, E., Kondorosi, A., and Schell, J. 1985. Expression of the nodulation gene *nod C* of *Rhizobium meliloti* in *Escherichia coli*: role of the *nod C* gene product in nodulation. EMBO J. 4:2425-2430.

90. John, M., Schmidt, J., Wieneke, U., Krussmann, H., and Schell, J. 1988. Transmembrane orientation and receptor-like structure of the *Rhizobium meliloti* common nodulation protein NodC. EMBO J. 7:583-588.

91. Johnson, D., Roth, L. E., and Stacey, G. 1989. Immunogold localization of the NodC and NodA proteins of *Rhizobium meliloti*. J. Bacteriol. 171:4583-4588.

92. Joyard, J., Block, M. A., Malherbe, A., Maréchal, E., and Douce, R. 1993. Origin and synthesis of galactolipid and sulfolipid head groups. *In*: Lipid Metabolism in Plants, Moore, T. S. Jr. (ed.), CRC Press Inc., Florida.

93. Kannenberg, E. L., and Brewin, N. J. 1994. Host-plant invasion by *Rhizobium*: the role of cell -surface ocmponents. Trens in Microbiol. 2:277-283.

94. Kao, C. C. and Sequeira, L. 1994. The function and regulation of genes required for extracellular polysaccharide synthesis and virulence in *Pseudomonas solanacearum*. In: Molecular Mechanisms of Bacterial Virulence, Kado, C. I. and Crosa, J. H. (eds.), pp 93-108, Kluwer Academic Publishers, Netherlands.

95. Kawahira, M. 1990. Clinical and experimental studies on vitronectin in bacterial pneumonia. Kansenshogaku Zasshi 64:741-751.

96. Khin, M. M., Ringner, M., Aleljung, P., Wadstrom, T., and Ho, B. 1996. Binding of human plasminogen and lactoferrin by *Helicobacter pylori* coccoid forms. J. Med. Microbiol. 45:433-439.

97. Kredich, N. M. 1996. Biosynthesis of cysteine. *In*: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, vol.1 (2nd ed.), F. C. Neidhardt (ed. chief), Curtiss III, R., Ingrahsm, J. L., Lin, E. C. C., Low, K. B., Magasnik, B., Reznifoff, W. s., Riley, M., schaechter, M., and Umbarger, H. E., (eds.), ASM Press, Washington, D.C.

98. Kuehn, M. J, Jacob-Dubuisson, F., Daeson, K, Slonim, L., Striker, R., and Hultgren, S. J. 1994. Genetic, biochemical and structural studies of biogenesis of adhesive pili in bacteria. Methods Enzymol. 236:282-306.

99. Kündig, C., Hennecke, H., and Göttfert, M. 1993. Correlated physical and genetic map of the Bradyrhizobium japonicum 110 genome. J. Bacteriol. 175:613-622.

100. Lee, R. F. and Benson, A. A. 1972. The metabolism of glyceryl sulfoquinovoside by the coral tree, *Erythrina crista-galli*, and alfalfa, *Medicago sativa*. Biochem. Biophy. Acta. 261:35-37.

101. Lehmann, J. and Benson, A. A. 1964. The plant sulfolipid. IX. Sulfosugar synthesis from methyl hexoseenides. J. Am. Chem. Soc. 86,4469-4472.

102. Leonard, R. T. and Hodges, T. K. 1980. The plasma membrane. *In*: The Comprehensive Treatise, Stumpf, P. K. and Conn, E. E. (eds.), Academic Press, New York.

103. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by sulphated and acylated glucosamine oligosaccharide. Nature 344:781-784.

104. Liang, O. D., Maccarana, M., Flock, J. I., Paulsson, M., and Wadstrom, T. 1993. Multiple interactions between human vitroneçtin and *Staphylococcus aureus*. Biochim. Biophys. Acta. 1225:57-63.

105. Liang, O. D., Flock, J. I., and Wadstrom, T. 1995. Isolation and characterization of a vitronectin-binding surface protein from *Staphylococcus aureus*. Biochim. Biophys. Acta. 1250:110-116.

106. Loh, J. T., Ho, S. C., de Feijter, A. W., Wang, J. L., and Schindler, M. 1993. Carbohydrate binding activities of *Bradyrhizobium japonicum*: Unipolar localization of the lectin BJ38 on the bacterial cell surface. Proc. Natl. Acad. Sci. USA 90:3033-3037.

107. Maddock, J. R., Akkey, M. R. K., and Shapiro, L. 1993. Polarized cells, polar actions. J. Bacteriol. 175:7125-7129.

108. Mandrell, R. E., McLaughlin, R., Kwaik, Y. A., Lesse, A., Yamasaki, R., Gibson, B., Spinola, S. M., and Apicella, M. A. 1992. Lipooligosaccharedes (LOS) of some *Haemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. Infect. Immun, 60:1322-1328.

109. Mandrell, R. E. and Apicella, M. A. 1993. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. Immunobiol. 187:382-402.

110. Marquis, H., Doshi, V., and Portnoy, D. A. 1995. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. Infect. Immun. 63:4531-4534.

111. Martínez-Romero, E. and Caballero-Mellado J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. 15:113-140.

112. Massad, G., Lockatell, C. V., Johnson, D. E., and Mobley, H. L. T. 1994. *Proteus mirabilis* fimbriae: construction of an isogenic pmfA mutant and analysis of virulence in a CBA mouse model of ascending urinary tract infection. Infect. Immun. 62:536-542.

113. Mateos, P. F., Jimenez-Zurdo, J. I., Chen, J., Squartini, A. S., Haack, S. K., Martines-Molina, E., Hubbell, D. H., and Dazzo, F. B. 1992. Cell-associated pectinolytic and cellulolytic enzymes in *Rhizobium leguminosarum* biovar trifolii. Appl. Environ. Microbiol. 56:1816-1822.

114. Matthysse, A. G., Holmes, K. V., and Gurlitz, R. H., G. 1981. Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. J. Bacteriol. 145:583-595.

115. Matthysse, A. G. 1983. Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. J. Bacteriol. 154:906-915.

116. McConville, M. J. and Ferguson, M. A. J. 1993. The structure, biosynthesis and function of glucosylated phosphatidylinositol in the parasitic protozoa and higher enkaryotes. Biochem. J. 294:305-324.

117. McConville, M. J., Collidge, T. A. C., Ferguson, M. A. J., and Schneider P. 1993. The glycoinositol phospholipids of *Leishmania mexicana* promastigotes. J. Biol. Chem. 268:15595-15604.

118. McFall-Ngai, M. J. and Ruby, E. G. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254:1491-1494.

119. McKay, I. A., Glenn, A. R. and Dilworth, M. J. 1985. Gluconeogenesis in *Rhizobium leguminosarum* MNF3841. J. Gen. Mirobiol. 131:2067-2073.

120. McNamara, P. J., Cuevas, W. A., and Songer, J. G. 1995. Toxic phospholipase D of *Corynebacterium pseudotuberculosis*, *C. ulcerans* and *Arcanobacterium haemolyticum*: cloning and sequence homology. Gene 156:113-118.

121. Meighen, E. A. 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55:123-142.

122. Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.

123. Menard, R., Prevost, M. C., Gounon, P., Sansonetti, P., and Dehio, C. 1996. The secreted Ipa complex of *Shigella flexneri* promotes entry into mammalian cells. Proc. Natl. Acad, Sci. USA 93:12541258.

124. Miller, K. J., Shon, B. C., Gore, R. S., and Hunt, W. P. 1990. The phospholipid composition of *Bradyrhizobium spp.* Curr. Microbiol. 21:205-210.

125. Miller, V. L. and Falkow, S. 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. 56:1242-1284.

126. Miyachi, S. and Miyachi, S. 1966. Sulfolipid metabolism in *Chlorella.* Plant Physiol. 41:479-486.

127. Moffet, M. L. and Colwell, R. R. 1968. Adansonian analysis of the *Rhizobiaceae.* J. Gen. Microbiol. 51:245-266.

128. Morrison, N. and Verma, D. P. S. 1987. A block in the endocytosis of *Rhizobium* allows cellular differentiation in nodules but affects the expression of some peribacteroid membrane nodulins. Plant Mol. Biol. 9:185-196.

129. Moulder, J. W. 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. 49:298-337.

130. Mulholland, V., Hinton, J. C., Sidebotham, J., Toth, I. K., Hyman, L. J., and Perombelon, M. C. 1993. A pleiotropic reduced virulence mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. 9:343-356.

131. Mulongoy, K. and Elkan, G. H. 1978. Some effects of mannitol on the glucose metabolism of two derivatives of a strain of *Rhizobium japonicum* differing in mannitol dehydrogenase. Curr. Microbiol. 1:335-340.

132. Nakamura, T., Iwahashi, J., and Eguchi, Y. 1984. Enzymatic proof for the identity of the S-sulfocysteine synthase and cysteine synthase B of *Salmonella typhimurium.* J. Bacteriol 158:1122-1127.

133. Nester, E. W. and Gordon, M. P. 1991. Molecular strategies in the interaction between *Agrobacterium* and its hosts. *In*: Molecular Strategies of Pathogens and Host Plants, Patil, S. S., Ouchi, S., Mills, D., and Vance, C., (eds.), Springer-Verlag, New York, etc.

134. Nishijima, M,, Nakaike, S., Tamori, Y., and Nojima, S. 1977. Detergent-resistant phopholipase A of *Escherichia coli* K-12: purification and properties. Eur. J. Biochem. 73:115-124.

135. Nurnberger, T., Wirtzz, W., Nennstiel, D., Hahlbrock, K., Jabs, T., Zimmermann, S., and Scheel, D. 1997. Signal perception and intracellular signal transduction in plant pathogen defense. J. Recept. Signal Transduct. Res. 17:127-136.

136. Olsén, A., Jonsson, A., and Normark, S. 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli.* Nature 338:652-655.

137. Orgambide, G., Philip-Hollingsworth, S., Hollingsworth, R., I., and Dazzo, F. B. 1994. Flavone-enhanced accumulation and symbiosis-related biologicalactivity of a diglycosyl diacylglycerol membrane glycolipids from *Rhizobium leguminosarum* biovar *trifolii.* J. Bacteriol. 176:4338-4347.

138. Orgambide, G. G., Lee, J., Hollingsworth, R. I., and Dazzo, F. B.1995. Structurally diverse CLOS Nod factors accumulate primarily in membranes of wild type *Rhizobium leguminosarum* Biovar *trifolii. B*iochemistry 34:3832-3840.

139. Osborn., M. J., Gander., J. E., Parisi, E., and Carson, J. 1972. Mechanism of assembly of the outer membrane in *Salmonella typhimurium.* J.Biol. Chem. 247:3962-3972.

140. Østerås,M., Driscoll, B. T., and Finan, T. M. 1997. Increased pyruvate orthophosphate dikinase activity results in an alternative gluconeogenic pathway in *Rhizobium* (*Sinorhizobium*) *meliloti.* Microbiol. 143:1639-1648.

141. Passador, L., Cook, J. M., Gambello, M. J., Rust, L., and Iglewski, B. H. 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science 260:1127-1130.

142. Pepe, J. C., Wachtel, M. R., and Wagar, E., and Miller, V. L. 1995. Pathogensis of defined invasion mutants of *Yersinia enterocolitica* in a BALB/c mouse model of infection. Infect. Immun. 63:4928-4948.

143. Perotto, S., Donovan, N., Drøbak, B. K., and Brewin N. J. 1995. Differential expression of a glycosylinositol phospholipid antigen on the peribacteroid membrane during pea nodule development. Mol. Plant-Microbe Interact. 8:560-568.

144. Philip-Hollingsworth, S. Hollingsworth, R. I., Dazzo, F. B., Djordjevic, M. A., Rolfe, B. G. 1989. The effect of interspecies transfer of *Rhizobium* host-specific nodulation genes on acidic polysaccharide structure and in situ binding by host lectin. J. Biol. Chem. 264:5710-5714.

145. Plazinski, J., Innes, R. W., and Rolfe, B. G. 1985. Expression of *Rhizobium trifolii* early nodulation genes on maize and rice plants. J. Bacteriol. 163:812-815.

146. Preston, R. D. 1974. The Physical Biology of Plant Cell Walls. Chapman & Hall, Inc., London.

147. Pugh, C. E., Roy, A. B., Hawkes, T., and Harwood, J. L. 1995. A new pathway for the synthesis of the plant sulpholipid sulphoquinovosyl diacylglycerol. Biochem. J. 309:513-519.

148. Pulkkinen, W. S. and Miller, S. I. 1991. A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. J. Bacteriol. 173:86-93.

149. Ramm, L. E. and Winkler, H. H. 1973. Rickettsial hemolysis: adsorption of richettsiae to erythrocytes. Infect. Immun. 7:93-99.

150. Rathman, M., Sjaastad, M. D., and Falkow, S. 1996. Acidification of phagosomes containing *Salmonella typhimurium* in murine macrophages. Infect. Immun. 64:2765-2773.

151. Relman, D. Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K., and Wright, S. D. 1990. Recognition of a bacterial adhesion by an integrin macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis.* Cell 61:1375-1382.

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

153. Ridge, R. W. and Rolfe, B. G. 1985. *Rhizobium sp.* degradation of legume root hair cell wall at the site of infection thread origin. Appl. Environ. Microbiol. 50:717-720.

154. Ringer, M, Paulsson, M., and Wadstrom, T. 1992. Vitronectin binding by *Helicobacter pylori.* FEMS Mibrobiol. Immunol. 5:219-224.

155. Ritter, A., Blum, G., Emödy, L., Kerenyi, M., Böck, A., Neuhierl, B., Rabsch, W., Scheutz, F., and Hacker, J. 1995. tRNA genes and pathogenicity islands: influence on virulence and metabolic properties of uropathogenic *Escherichia coli*. Mol. Microbiol. 17:109-121.

156. Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher., C., Truchet., G., Denarie, J., and Prome, J. 1991. Molecular basis of symbiotic host specificity in *Rhizobium meliloti*: *nodH* and *nodPQ* genes encode the sulfation of lipooligosaccharide signals. Cell 67:1131-1143.

157. Ronson, C. W. and Primrose, S. B. 1979. Effect of glucose on polyol metabolism by *Rhizobium trifolii*. J. Bacteriol. 139:1075-1078.

158. Rosenshine, I., Duronio, V., and Finlay, B. B. 1992. Tyrosine protein kinase inhibitors block invain-promoted bacterial uptake by epithelial cells. Infect. Immun. 60:22112217.

159. Rossak, M., Tietje, C., Heinz, E., and Benning, C. 1995. Accumulation of UDP-sulfoquinovose in a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. J. Biol. Chem. 270:25792-25797.

160. Ruby, E. g. and McFall-Ngai, M. J. 1992. A squid that glows in the night: development of an animal-bacterial mutualism. J. Bacteriol. 174:4865-4870.

161. Ruoslahti, E. and Pierschbacher, M. D. 1987. New perspectives in cell adhesion: RGD and integrins. Science 238:491-497.

162. Salmond, G. P. and Reeves, P. J. 1993. Membrane traffic wardens and protein secretion in gram-negative bacteria. Trends Biochem. Sci. 18:7-12.

163. Salmond, G. P., Gycroft, B. W., Stewart, G. S., and Williams, P. 1995. The bacterial 'enigma': cracking the code of cell-cell communication. Mol. Microbiol. 16:615-624.

164. Salyers, A. A. and Whitt, D. D. 1994. Bacterial Pathogenesis, a Molecular Approach, American Society for Microbiology Press, Washington, D.C.

165. Sandros, J. and Tuomanen, E. 1993. Attachment factors of *Bordetella pertussis*: mimicry of eukaryotic cell recognition molecules. Trens Microbiol. 1:192-196.

166. Scandella, C. J. and Kornberg, A. 1971. A membrane-bound phospholipase A1 purified from *Escherichia coli*. Biochemistry 10:4447-4456.

167. Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli.* J. Bacteriol. 104:890-901.

168. Schripsema, J., de Rudder K. E., van Vliet T. B., Lankhorst P. P.; de Vroom, E., Kijne J. W., van Brussel, A. A. 1996. Bacteriocin small of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. J. Bacteriol. 178:366-71.

169. Schulze-Koops, H., Burkhardt, H., Heesemann, J., Kirsch, T., Swoboda, B., Bull, C., Goodman, S., and Emmrich, F. 1993. Outer membrane protein YadA of enteropathogenic yeriniae mediates specific binding to cellular but not plasma fibronectin. Infect. Immun. 61:2513-2519.

170. Seifert, U. and Heinz, E. 1992. Enzymatic characteristics of UDP-sulfoquinovose: diacylglycerol sulfoquinovosyltransferase from chloroplast envelopes. Bot. Acta. 105:197-205.

171. Sharon, N. 1987. Bacterial lectins, cell-cell recognition and infectious disease. FEBS Lett. 217:145-157.

172. Sharon, N. 1993. Lectin-carbohydrate complexes of plants and animals: an atomic view. Trends Biochem Sci. 18:221-226.

173. Shaw, C. H., Loake, G. J., Brown, A. P., Garrett, C. S. 1991. The early events in *Agrobacterium* infection. In: Biochemistry and Molecular Biology of Plant-Pathogen Interactions, C. J. Smith (ed.), Procedures of the Phytochemical Society of Europe, Oxford Science Publications, London.

174. Shetty, M., Bairy, K. L., Krishnananda, S., and Shivananda, P. G. 1994. Correlation of phospholipase A and enterotoxin production by *Salmonella typhimurium* with reference to virulence parameters. Indian J. Med. Res. 99:162-166.

175. Shibuya, I. and Benson, A. A. 1961. Hydrolysis of a-sulphoquinovosides by b-galactosidase. Nature 192:1186-1187.

176. Shibuya, I., Yagi, T., and Benson, A. A. 1963. Sulfonic acids in algae. In: Studies on microalgae and photosynthetic bacteria, Jpn. Soc. Plant Physiol., Ed. Pp. 627-636, Univ. Tokyo Press, Tokyo.

177. Shibuya, I. and Hase, E. 1965. Degradation and formation of sulfolipid occurring concurrently with de- and re-generation of chloroplasts in the cells of *Chlorella protothecoides.* Plant & Cell Physiol. 6:267-283.

178. Silverman, D. J., santucci, L. A., Meyers, N., and Sekeyova, Z. 1992. Penetration of host cells by *Rickettsia rickettsii* appears to be mediated by a phospholipase of rickettsial origin. Infect. Immun. 60:2733-2740.

179. Simon, M. and Hong, J. S. 1983. Direct homocysteine biocynthesis from O-succinylhomoserine in *Escherichia coli*: an alternative pathway that by-pass cystathionie. J. Bacteriol. 153:558-561.

180. Smit, G., Kijne, J. W., and Lugtenberg, B. J. J. 1987. Involvement of both cellulose fibrils and a Ca^{2+} -dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. 169:4294-4301

181. Smit, G., Kijne, J. W., and Lugtenberg, B. J. J. 1989. Roles of flagella lipopolysaccharide and a Ca^{+2}-dependent adhesin are involved in the attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. 169:4294-4301.

182. Smit, G., Logman, T. J. J., Boerrigter, M. E. T. I., Kijne, J. W., and Lugtenberg, B. J. J. 1989. Purification and partial characterization of the Ca^{+2}-dependent adhesin from *Rhizobium leguminosarum* biovar *viciae*, which mediates the first step in attachment of *Rhizobiaceae* cells to plant root hair tips. J. Bacteriol. 171:4054-4062.

183. Smit, G., Tubbing, D. M. J., Kijne, J. W., and Lugtenberg, B., J. J. 1991. Role of Ca^{2+} in the activity of rhicadhesin from *Rhizobium leguminosarum* biovar *viciae*, which mediates the first step in attachment of *Rhizobiaceae* cells to plant root hair tips. Arch. Microbiol. 155:278-283.

184. Smit, G., Swart, S., Lugtenberg, B., J., and Kijne, J. W. 1992. Molecular mechanisms of attachment of *Rhizobium* bacteria to plant roots. Mol. Microbil. 6:2897-2903.

185. Smith, M. W., Feng, D., and Doolittle, R. F. 1992. Evolution by acquisition: the case for horizontal gene transfers. Trends Biochem. Sci. 17:489-493.

186. Spencer, D., James, E. K., Ellis, G. J., Shaw, J. E., and Sprent, J. I. 1994. Interaction between rhizobia and potato tissues. J. Exp. Botany. 45:1475-1482.

187. Stowers, M. D. 1985. Carbon metabolism in *Rhizobium* species. Ann. Rev. Microbiol. 39:89-108.

188. Strom, M. S. and Lory, S. 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. 47:569-596.

189. Swart, S., Smit, G., Lugtenberg, B. J. J., and Kijne, J. W. 1993. Restoration of attachment, virulence and nodulation of *Agrobacterium tumefaciens chvB* mutant by rhicadhesin. Mol. Microbiol. 10:597-605.

190. Swart, S., Logman, T. J. J., Smit, G., Lugtenberg, B. J. J., Kijne, J. W. 1994. Purification and partial characterization of a glycoprotein from pea (*Pisum sativum*) with receptor activity for rhicadhesin, an attachment protein of *Rhizobiaceae*. Plant Mol. Biol. 24. 171-183.

191. Swart, S., Lugtenberg, B. J. J., Smit, G., and Kijne, J. W. 1994. Rhicadhesin-mediated attachment and virulence of an *Agrobacterium tumefaciens chvB* mutant can be restored by growth in a highly osmotic medium. J. Bacteriol. 176:3816-3819.

192. Tang, Y. and Hollingsworth, R. I. 1997. Regulation of lipid Synthesis in *Bradyrhizobium*: Low Oxygen Triggers Phosphatidylinositol Biosynthesis. Appl. Environ. Microbiol. In press.

193. Tang, Y. and Hollingsworth, R. I. 1997. Diglactosyl diacylglycerols, plant glycolipids rarely found in bacteria, are major membrane components of bacteroid forms of *Bradyrhizobium japonicum*. Glycobiol. 7:935-942.

194. Tang, Y. and Hollingsworth, R. I. 1997. Synthesis of di- and tri-glycosyl diacylglycerols in bacteroid forms of *Bradyrhizobium japonicum*. Carbohydr. Res. Submitted.

195. Tertti, R., Skurnik, M., Vartio, T., and Kuusela, P. 1992. Adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectcin. Infect. Immun. 64:2584-2594.

196. Thelestam, M.and Mollby, R. 1975. Sensitive assay for the detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts. Infect. Immun. 12:225-232.

197. Thomashow, M. F. and Rittenberg, S. C. 1978. Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: solubilization of *Escherichia coli* peptidoglycan. J. Bacteriol. 135:998-1007.

198. Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Prome, J., and Denarie, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium melitoti* elicit root nodule organogenesis in alfalfa. Nature 351: 670-673.

199. Tsuchiya, N., and Williams, R. C. J. 1992. Molecular mimicry-hypothesis or reality. West. J. Med. 157:133-138.

200. Turgeon, B. and Bauer, W. 1985. Ultrastructure of infection thread development during the infection of soybean by *Rhizobium japonicum*. Planta 163:328-349.

201. Uttaro, A. D., Ielpi, L., and Ugalde, R. A. 1993. Galactose metabolism in *Rhizobiaceae*: characterizationnn of *Agrobacterium tumefaciens exoB* mutants. J. Gen. Microbiol. 139:1055-1062.

202. van Brussel, A. A. N., Bakhuizen, R., van Spronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J. J., and Kijne, J. W. 1992. Induction of pre-infection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium*. Science 257:70-72.

203. van Gijsegem, F., Genin, S., and Boucher, C. 1993. Conservationn of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol. 1:175-180.

204. van Gijsegem, F., Gough, C., Zischek, C., Niqueuz, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095-1114.

205. van Kan, J. A., van't Klooster, J. W., Wagemakers, C. A., and Dees, D. C. 1997. Cutinase A of Botrytis cinerea is expressed, but not essential, during penetration of gerbera and tomato. Mol. Plant-Microb. Interact. 10:30-38.

206. van Spronsen, P. C., Bakjuizen, R., van Brussel, A. A. N., and Kijne, J. W. 1994. Cell wall degradation during infection thread formation by the root nodule bacterium *Rhizobium leguminosarum* is a two-step process. Eur. J. Cell. Biol. 64:88-94.

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

208. Verma, D. P. S., Zogbi, V., and Bal, A. 1978. A cooperative action of plant and *Rhizobium* to dissolve the host cell wall during development of root nodule symbiosis. Plant Sci. Lett. 13:137-142.

209. Verma, D. P. S. 1992. Signals in root nodule organogenesis and endocytosis of *Rhizbogium*. Plant Cell 4:373-382.

210. Verma, D. P. S., and Hong, Z. 1996. Biogenesis of the peribacteroid membrane in root nodules. Trends in Microbiol. 4:364-368.

211. Vesper, S. J. and Bhuvaneswari, T. V. 1988. Nodulation of soybean roots by an isolate of *Bradyrhizobium japonicum* with reduced firm attachment capability. Arch. Microbiol. 150:15-19.

212. Vial , H. J., Thuet, M. J., Broussal, J. L., and Philippot, J. R. 1982. Phospholipid biosynthesis: the incorporation of phopholipid precursors and the identification of previously undetected metabolic pathways. J. Parasitol. 68:379-391.

213. Vial , H. J., Philippot, J. R., and Wallach, D. F. H. 1984. A reevaluation of the status of cholesterol in erythrocytes infected by *Plasmodium knowlesi* and *P. falciparum*. Mol. Biochem. Parasitol. 13:53-65.

214. Wang, P., Schuster, M., Wang, Y., and Wong, C. 1993. Synthesis of phospholipid-inhibitor conjugates by enzymatic transphosphatidylation with phospholipase D. J. Am. Chem. Soc. 115, 10487-10491.

215. Wang, X., Xu, L., and Zheng, L. 1994. Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from *Ricinus communis* L. J. Biol. Chem. 269:20312-20317.

216. Werner, D. 1992. Physiology of nitrogen-fixing legume nodules: compartments and functions. *In*: Biological Nitrogen Fixation, Stacey, G., Burris, R. H., and Evans, H. J. (eds.), pp 399-431, Chapman and Hall, Inc., New York, London.

217. Westerlund, B. and Korhonen, T. K. 1993. Bacterial proteins binding to the mammalian extracellular matrix. Mol. Microbiol. 9:687-694.

218. White, R. H. 1984. Biosynthesis of the sulfonolipid 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid in the gliding bacterium *Cytophaga johnsonae*. J. Bacteriol. 159:42-46.

219. Winkler, H. H. and Miller, E. T. 1984. Activated complex of L-cells and *Rickettsia prowazekii* with *N*-ethylmaleimide-insensitive phospholipase A. Infect. Immun. 45:577-588.

220. Winkler, H. H., Day, L., and Daugherty, R. 1994. Analysis of hydrolytic products from choline-labeled host cell phospholipids during growth *Richettsia prowaxekii*. Infect. Immun. 62:1457-1459.

221. Yang, C., Signer, E. R., and Hirsch, A. M. 1992. Nodules initiated by *Rhizobium meliloti* exopolysaccharide mutants lack a discrete, persistent nodule meristem. Plant Physiol. 98:143-151.

222. Young, J. P. W. 1992. Phytogenetic classification of nitrogen-fixing organisms. *In*: Biological Nitrogen Fixation, Stacey, G., Burris, R. H., and Evans, H. J. (eds.), pp 58-64, Chapman and Hall, Inc., New York, London.

223. Zidovetzki, R., Sherman, I. W., Prudhomme, J., and Crawford, J. 1994. Inhibition of *Plasmodium falciparum* lysophospholipase by anti-malarial drugs and sulphydryl reagents. Parasitol. 108:249-255.

224. Zill, L. P. and Cheniae, G. M. 1962. Lipid metabolism. Annu. Rev. Plant Physiol. 13:225-264.