This is to certify that the

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Role of Rhizobium cell surface carbohydrates in infection

presented by

Maria Gabriela Beconi-Barker

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date December 28, 1992

ROLE 01

ROLE OF *RHIZOBIUM* CELL-SURFACE CARBOHYDRATES IN INFECTION

By

Maria Gabriela Beconi-Barker

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1992

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ABSTRACT

ROLE OF RHIZOBIUM CELL-SURFACE CARBOHYDRATES IN INFECTION

By

Maria Gabriela Beconi-Barker

Surface carbohydrate epitopes of ***Rhizobium leguminosarum*** biovar ***viciae*** 300 cells grown in different environments were chemically characterized using ^{1}H- and ^{13}C-NMR spectroscopy and gas chromatography/mass spectrometry (GC/MS). Immunochemical studies were conducted on the same cell-surface carbohydrates using antibodies generated against immunogens synthesized from previously characterized tetra- and trisaccharide "core", and from the "O-antigen" components of the lipopolysaccharide (LPS). Total cell-surface charge was also determined for cell populations grown under different conditions. (Conditions included: alterations of pH, oxygen tension, and carbon source). The effects of the presence of flavone inducers was also investigated. The presence and distribution of these carbohydrate epitopes in free-living cells subjected to the environmental perturbations, and the influence of the perturbations on the cell-surface charge were correlated with changes which occur in the cell-surface chemistry of the bacteria during the infection process.

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Immunocytochemical studies indicated that, in the vegetative state, the capsular polysaccharide (CPS) was evenly exposed over the cell-surface. In contrast, the "O-antigen" and the tetra- and trisaccharide were either polarly exposed or localized, and might, therefore, have a role in mediating the well-characterized "polar attachment" of the bacteria to the host root.

Chemical and immunochemical studies indicated that, under low oxygen or low pH, the synthesis of new oligosaccharides was induced. These new oligosaccharides were devoid of negatively charged groups but contained amino sugars, which if not acetylated, would confer on them a net positive charge. One of these oligosaccharides, an unusual acid-induced trisaccharide, was found in ***R. leguminosarum*** and ***phaseoli*** strains, but in neither ***R. meliloti*** nor ***trifolii*** strains. The relative proportions synthesized of the usual tetra- and trisaccharide changed with growth conditions, indicating that these two oligomers were synthesized independently of each other. Cells grown under low oxygen or low pH were either devoid of capsule, or synthesized small amounts of a CPS that was devoid of the negatively charged pyruvyl non-carbohydrate substituents. The "O-antigen" epitopes, which were more positively charged than the capsule, were now exposed over the entire surface. Based on these observations, a model explaining bacterial release from the infection thread is proposed.

ACKNOWLEDGEMENTS

I sincerely appreciate the guidance given by Dr. Rawle I. Hollingsworth, my major professor throughout the course of this study. My extreme gratitude is expressed to Dr. Melvin. T. Yokoyama for his unselfish counseling, support, encouragement and timely advice. My appreciation is also expressed to the remaining members of my advisory committee: Dr. David McConnell, Dr. Charles Sweeley and Dr. Jack Watson for their valuable contributions to this study.

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I would like to acknowledge the Center for Microbial Ecology/Natural Science Foundation for awarding me a fellowship for the support of my graduate studies.

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Dr. Marjan

Lilli-Elgha

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possible.

Dr. Marjan Van der Woude, Dr. Silvia Rossbach and Deborah Lill-Elghanian for their friendship and helpful advice, and to fellow graduate students for their camaraderie and constant help.

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GC-MS

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KDO

LPS

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PBS

TEMED

LIST OF ABBREVIATIONS

BIII	Modified Bergensen's medium
BSA	Bovine serum albumin
CPS	Capsular polysaccharide
EDTA	Ethylene diamino tetraacetic acid
EI-MS	Electron impact-mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polysaccharide
FAB-MS	Fast atom bombardment-mass spectrometry
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HCl	Hydrochloric acid
KDO	2-keto-3-deoxy octulosonic acid
LPS	Lipopolysaccharide
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
TEMED	N,N,N',N'-tetramethyl ethylene diamine

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INTRODUCTION

The *Rhizobium*-legume system is an attractive, easy-to-work-with model of the infection of a eukaryote by a prokaryote. The processes that lead to the infection of eukaryotic cells by bacteria involve intriguing interactions. Studies of these interactions in suitable biological models provide insights that contribute to our understanding of the events that take place during other infection processes. In this dissertation, we will look, specifically, at the role of the bacterial cell-surface carbohydrates of *Rhizobium* in the infection of leguminous host plants.

One major reason which makes the *Rhizobium*-legume system an attractive one for study is its ability to fix atmospheric nitrogen. Molecular nitrogen, the major component of the atmospheric gases, is generally unavailable to higher organisms, including plants, unless it has been transformed into an assimilable organic form. Nitrogen is also a major limiting nutrient for more than 30 million hectares of grasslands in the humid areas of the United States. It is also the most costly of the major crop nutrients on a land-to-area basis. Nitrogen can be fixed into the soil directly from the

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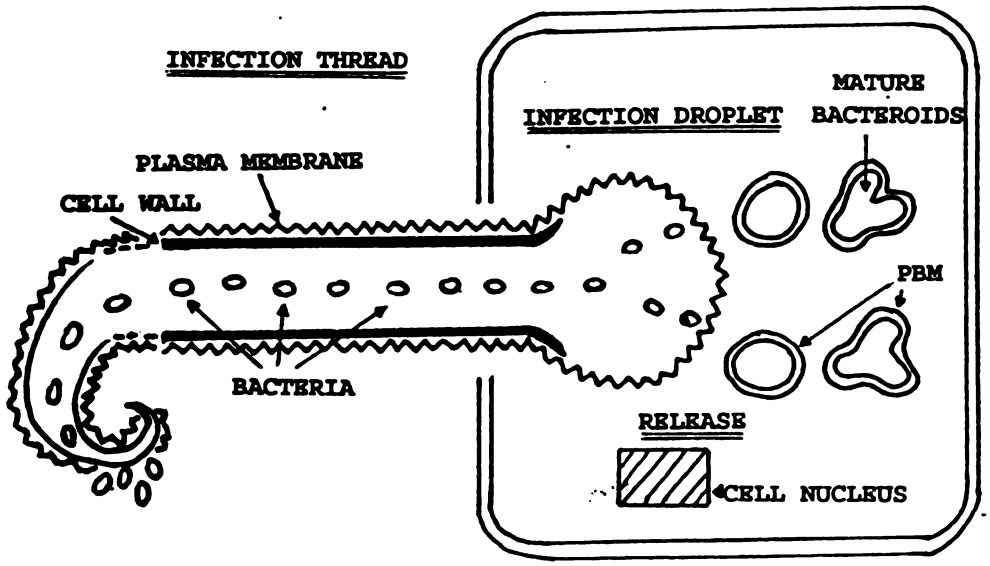
atmosphere or indirectly through combustion, fertilizers or by the action of microorganisms. Symbiotic nitrogen-fixing bacteria convert molecular nitrogen into ammonia, making it available for the host plant. They are responsible for fixing between 50 and 90% of the total soil nitrogen and are, therefore, the largest source of terrestrial organic nitrogen in the global nitrogen cycle.

Gram-negative soil bacteria of the genus ***Rhizobium*** are among the microorganisms which, when associated with specific species of plants of the family ***Leguminosae***, are capable of fixing nitrogen. This symbiotic association consists of a cooperative mechanism through which bacteria fix nitrogen and export it to the plant for assimilation. The host plant is rendered independent of soil nitrogen and provides the bacteria with photosynthates for fuel. This symbiotic association is a host-specific process that results from an infection process, leading eventually to the formation of nodules. In these nodules, bacteria differentiate into nitrogen-fixing forms called bacteroids.

During infection, Scheme 1, bacteria travel from the rhizosphere to the host root where they colonize the root hairs. From the tips of the root hairs they then travel to the base of the root in a structure of plant origin called the infection thread. This infection thread, which resembles an

SCHEME 1. Diagrammatical longitudinal section of root hair with an infection thread in pea nodule. Represents colonization of the root hair by ***Rhizobia***, tissue invasion, bacterial movement along the infection thread, and bacterial release from the infection thread into the host cell.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inside out root hair, exposes negative charges that interact with the invading bacteria. Finally, bacteria penetrate the root cortex to be subsequently released into newly-dividing plant cells in the nodules, where they differentiate into bacteroid forms.

During this dissertation we will test the following working hypothesis. Bacterial penetration to and release into the newly dividing host cells can only occur if the appropriate bacterium-plant interactions are favored. For this to happen, the net bacterial cell-surface charge has to be opposite in sign to that of the infection thread. In the vegetative state, bacteria are surrounded by a negatively charged capsule (Scheme 1), and the interaction with the negatively-charged infection thread is not favored. Changes in the bacterial cell-surface charge must, therefore, occur in order to facilitate the bacterial-plant interaction. These changes in bacterial cell-surface charge are brought about by changes in the chemistry of the major components of the bacterial cell-surface, the cell-surface carbohydrates. Changes in these cell-surface carbohydrates are triggered by changes in the environment of the bacteria. During their journey from the rhizosphere, through the root hair, to the root cortex, bacteria are exposed to constant variations in pH, oxygen and nutrient availability. Their membrane physiology must, therefore, change to allow them to adapt to

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The characteristics of the cell-surface of bacteria are important factors in determining the outcome of the bacterium-host interactions. In the *Rhizobium*/legume system, carbohydrates are the major surface components which are available to interact with the host plant. Since the early 1970s, *Rhizobium* cell-surface carbohydrates have been implicated as major participants in the initial steps of the nitrogen-fixing symbiotic relationship between these bacteria and their host legume plants. These initial steps involve recognition of a narrow range of host plants by the bacterium, and its subsequent attachment to the host root. More recently, research has addressed the importance of the role(s) of these cell-surface carbohydrates during later stages of the infection process.

Most of the studies addressing the role of bacterial cell-surface carbohydrates during the infection process have been done using antibodies against chemically undefined cell-surface carbohydrate epitopes. Structural information is only available for a few cell-surface carbohydrate epitopes obtained from free-living cells. No studies have ever been made correlating the presence of chemically defined cell-surface carbohydrate epitopes on free-living cells with their

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presence or absence on the cell-surface of bacteria during the infection process and stage of infection process. One of the major complications in obtaining structural information and in defining the role of specific carbohydrate structures in symbiosis is the extent of the dynamics of the bacterial cell-surface carbohydrate chemistry from one stage to the other of the symbiosis.

Direct chemical characterization of changes that occur in cell-surface carbohydrates of bacteria during root infection and nodule development is hampered by the amount of material needed, and complicated by the presence of carbohydrates of plant origin. Model systems where bacteria undergo changes that resemble those found under nodule conditions, therefore, need to be developed to facilitate these studies. Cell-surface chemistry observed in these artificial systems then needs to be correlated with the changes that cell-surface carbohydrates of bacteria actually undergo at different stages of the infection process.

Our studies have focused on the alterations of cell-surface carbohydrates of ***Rhizobium*** in response to changes in environmental parameters. Artificial systems were developed to facilitate structural studies of carbohydrate components of the rhizobial cell-surface. The changes in clonal (cell-to-cell) and spatial (within a cell) distribution of chemically-

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characterized cell-surface carbohydrate epitopes during the infection process were determined using a combination of chemical and immunochemical methods.

The review presented in Chapter 1 addresses topics of recent interest on the function of rhizobial cell-surface carbohydrates during the infection process. Chapter 2 describes the use of polyclonal antibodies generated against synthetic immunogens made from chemically characterized carbohydrate epitopes in defining the clonal and spatial distribution of the epitopes on the cell-surface of vegetative cells.

In order to assess the impact of the environment on the chemistry of cell-surface carbohydrates, ***Rhizobium leguminosarum*** was grown in liquid media under conditions representing the environment inside the nodule. Discussed in Chapter 3 is the chemical characterization of a new oligosaccharide induced in these free-living cells grown in acid conditions (pH 4.5). This unusual oligosaccharide appears to be species-specific and may have special significance in the bacterium-host recognition process. Chemical and immunochemical changes that occur in the cell-surface carbohydrates of free-living cells grown under different environmental conditions, and correlations of these changes with the ones that occur in bacteria within the nodule are described in Chapter 4.

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Conclusions from previously mentioned studies, coupled with direct measurements of changes that occur in the cell-surface charge of bacteria during the process of transformation into bacteroids, have led to the proposal of a mechanistic model of the infection process, which is described in Chapter 5. This model correlates the changes that occur in bacterial cell-surface carbohydrates during the infection process with cell-surface charge to explain how bacteria are released from the infection thread to populate plant cells. In the final chapter (Chapter 6), we summarize the future directions of this research and present preliminary data supporting them.

CHAPTER 1

LITERATURE REVIEW

PROPOSED ROLES OF RHIZOBIAL CELL-SURFACE CARBOHYDRATES IN THE NITROGEN-FIXING SYMBIOTIC RELATIONSHIP WITH LEGUMES.

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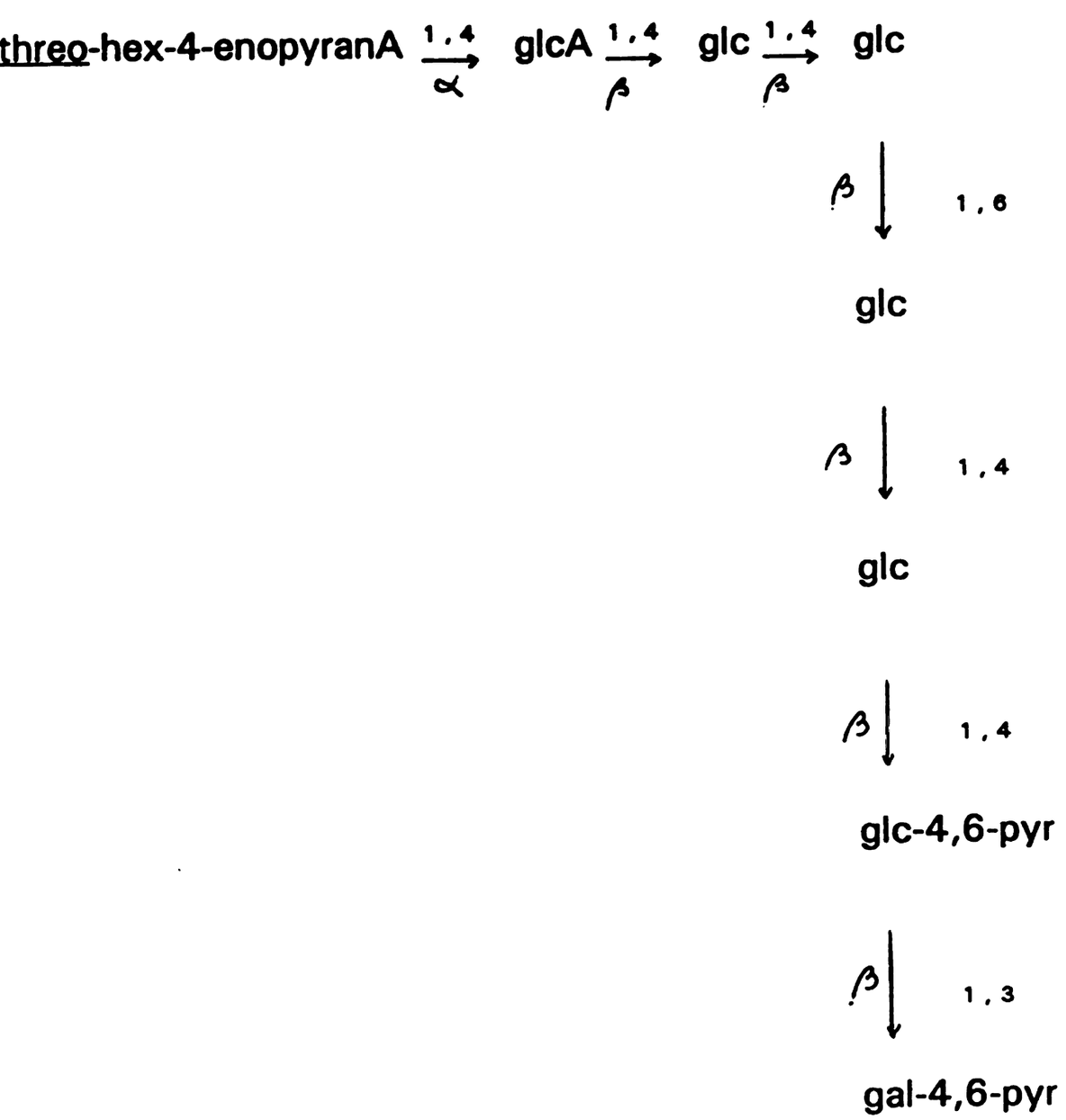
RHIZOBIAL Cell-surface carbohydrates. The known carbohydrate components of the ***Rhizobium*** cell are capsular polysaccharides (CPS) and lipopolysaccharides (LPS). Excreted extracellular polysaccharides (EPS) are glycoconjugates produced by bacteria which are not attached to their cell-surface but, rather, excreted to the medium. Typically, their common acidic components are uronic acids. Slow-growing ***Rhizobia*** such as ***Bradyrhizobium japonicum*** produce EPS that are different in structure from those produced by fast-growing ***Rhizobia*** like ***Rhizobium trifolii, leguminosarum*** and ***meliloti*** (6). Within the fast-growing ***Rhizobia***, the glycosyl sequences of the EPS from several strains of ***Rhizobium trifolii*** and ***Rhizobium leguminosarum*** are the same (39) (Figure 1), differing only in the type and location of their non-carbohydrate substituents (37). However, the EPS of ***Rhizobium meliloti*** are structurally unique, being succinylated and lacking the typical uronic acid components of all other EPS (6).

The structure of the CPS is usually very closely related, but not identical to that of the EPS. Within a given cell culture, the compositions of the CPS and EPS may change in a specific fashion with culture age. In the case of one strain

FIG. 1. Glycosyl sequence of the ***Rhizobium leguminosarum*** and ***Rhizobium trifolii*** repeating unit derived from enzymatic cleavage of the EPS. Adapted from Philip-Hollingsworth et al. (35). glcA = glucuronic acid, glc = glucose and gal = galactose.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of ***Bradyrhizobium japonicum*** this age effect results in the 4-O-methylation of the galactosyl residues of the CPS (31). Overall compositional analysis of these polysaccharides indicates that EPS originates from CPS which is lost or released from the capsule. The complete structure of the CPS of ***Rhizobium trifolii*** ANU 843 has been elucidated (22) (Figure 2), and the position of the non-carbohydrate substituents has also been determined for ***Rhizobium leguminosarum*** bv. ***viciae*** 300 and other related strains (27,37).

The LPS is the least characterized of the rhizobial cell-surface carbohydrates. A typical LPS of any gram negative bacterium is characterized by three structural domains: a lipid A, a core region and an O-antigen fragment (Figure 3). The lipid A is a hydrophobic region that anchors the LPS to the bacterial outer membrane. In most gram negative bacteria the lipid A contains a 1-6 β-linked glucosamine disaccharide substituted by 3-hydroxy fatty acids with chain lengths varying from 12 to 16 carbon atoms. The glucosamine disaccharide is usually phosphorylated at the C-1 position of the reducing end and at the C-4 position of the non-reducing glucosamine. The lipid A from ***Rhizobium trifolii*** ANU 843 is very different from the "typical" gram negative bacteria LPS. It is devoid of the phosphate, and bacteria have replaced this negatively charged moiety with a carboxyl group (2,23,24). A

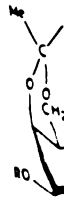

FIG. 2. Structure of the capsular polysaccharide synthesized by ***Rhizobium trifolii***. Adapted from Hollingsworth et al. (20).

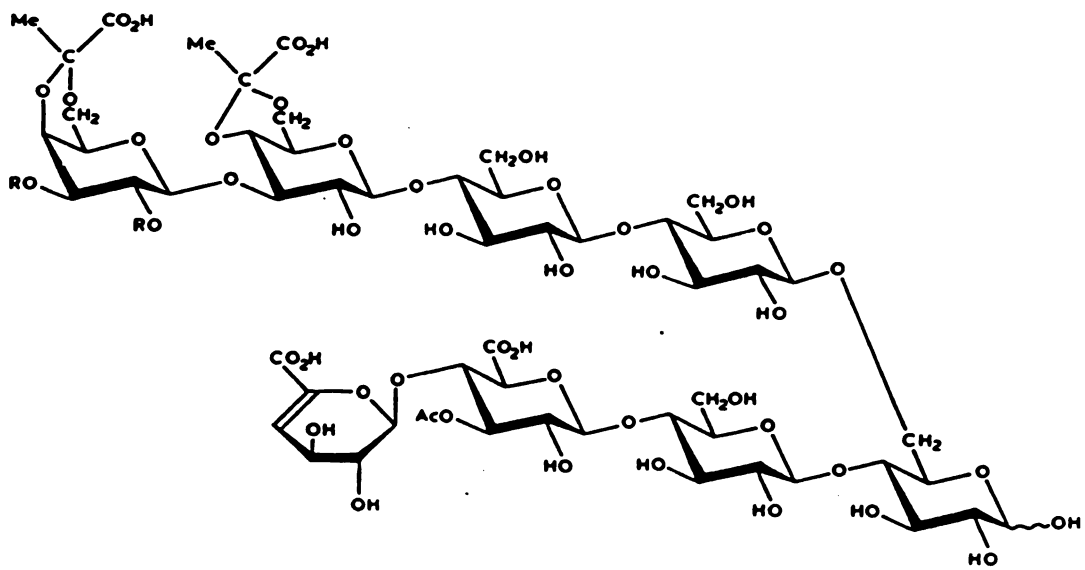
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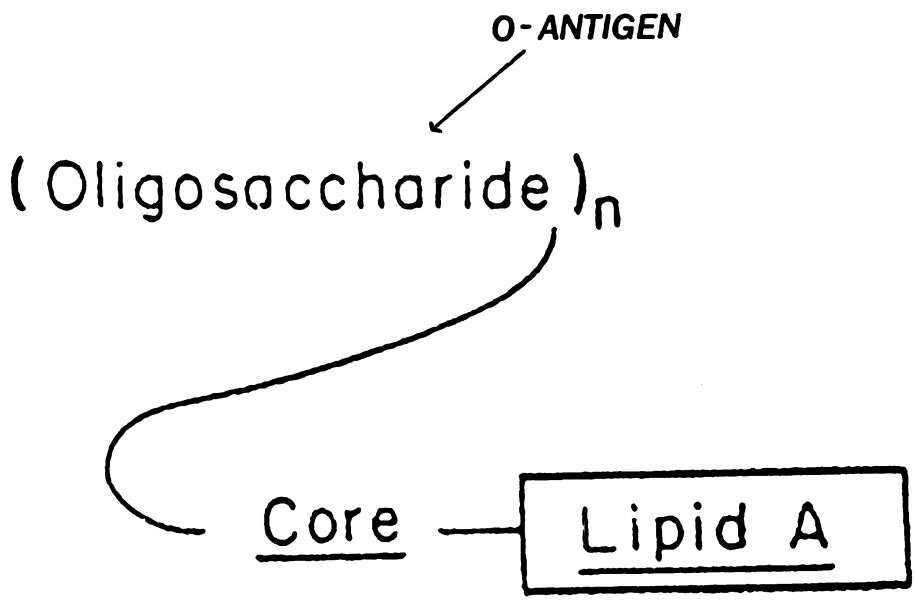
FIG. 3. Diagram representing the typical lipopolysaccharide architecture of a gram-negative bacterium. Three structural domains can be distinguished: the lipid A, a hydrophobic region which anchors this molecule to the bacterial outer membrane; a core region fairly conserved among strains of related species; and an O-antigenic side chain, which varies in a strain-specific manner.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2-amino-2-deoxy-gluco-hexuronic acid, glucosamine and galacturonic acid have been identified as carbohydrate components of the free lipid A, which is also substituted with a 27-hydroxyoctacosanoic acid not found in the enterobacteriaceae (23).

The core region of the LPS is usually conserved among gram-negative bacteria of related strains. A tetrasaccharide and trisaccharide isolated from the LPS of ***Rhizobium trifolii*** (8,20,21) and found to be common to the ***Rhizobium leguminosarum*** biovars (7) are thought to be components of the core of rhizobial LPS. The tetrasaccharide contains galactose, mannose, 2-keto-3-deoxyoctusolonic acid (KDO) and galacturonic acid (20,21). The trisaccharide contains two galacturonic acid residues and KDO (8).

No structural information is available on the O-antigenic side chain of the ***Rhizobium*** LPS. Early theories proposed that this chain consisted of an oligosaccharide repeating unit which varied in a strain-specific manner and that it was responsible for bacterial antigenicity (6). Recent studies have addressed the magnitude of the variations that occur in this LPS epitope in response to genetic mutations and have correlated changes in LPS structure with changes in symbiotic phenotype (7,14,26,30,34,37,38).

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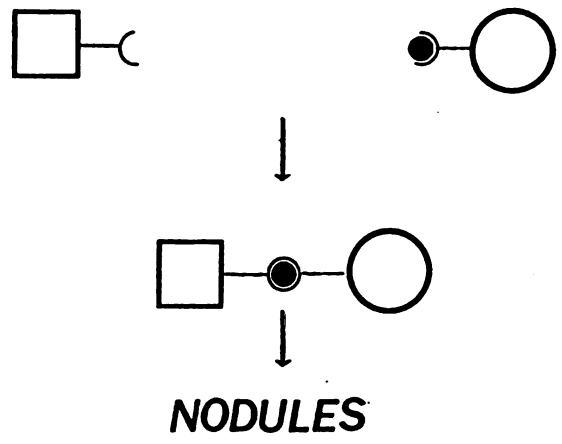
ROLE OF THE RHIZOBIAL CELL-SURFACE CARBOHYDRATES IN RECOGNITION OF THE HOST PLANT. Recognition is a biological phenomenon where one cell or organism elicits a selective response from another cell or organism. In 1974, Bohlool and Schmidt (3) reported the first experimental evidence of involvement of bacterial cell-surface carbohydrates in the legume recognition process. Their theory stated that carbohydrate receptors on the bacterial cell-surface interact with lectins released from the plant; in turn, these lectins also bind to receptors on the host root. An early model proposed by Dazzo and Hubbel (11) attempted to explain how this interaction between the plant lectin and the rhizobial cell-surface carbohydrates could be responsible for determining host specificity (Scheme 1). This model, in which the lectin released by the plant is proposed to recognize common antigens on both the root and bacterial surface, does not explain rhizobial/legume recognition processes that occur in the absence of plant lectins. The involvement of bacterial capsular polysaccharides (10) and the O-antigen containing LPS (45) have both been proposed as the bacterial lectin receptors. In the clover/*Rhizobium trifolii* system, it has been proposed (14) that a specific receptor binding site for a plant protein, trifoliin A, sits on the cell-surface of encapsulated *Rhizobium trifolii* cells. Later (13), a model was proposed where an alteration of the capsule of unattached cells results in polarly attached capsular material which

SHEME 1. Early model that was proposed to explain the host specific recognition by the bacterium in the *Rhizobium*/legume symbiosis (14).



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binds the root lectin in a species-specific manner. The time necessary for this capsule modification to occur, however, would not allow this model to explain findings (4) where host specificity was expressed during the initial stages of adsorption of ***Rhizobium meliloti*** to alfalfa roots. New evidence exists supporting the rhizobial cell-surface carbohydrate receptors/plant lectin recognition-mediated process (17,20). Halverson and Stacey (20), found a new soybean lectin involved in the nodulation process. Recently, Dias et al. (17) found that the normal symbiont of peas bound specifically to transgenic alfalfa plants containing genes encoding for the pea lectin. Since then, numerous studies have been conducted addressing the involvement of specific cell-surface carbohydrates in recognition and attachment. Specifically, researchers have tried to determine whether small changes in side groups and non-carbohydrate substituents on conserved structures can be determinants of host specificity. The relevance of these changes to the outcome of the infection process is still in question (6,36,37). Research has also focused on the involvement of specific extracellular signals in eliciting a host-specific response. In the ***Rhizobium meliloti*** system, a sulfated lipo-oligosaccharide was found which is thought to be the critical determinant of host specificity (29,30). However, this idea has been questioned and it is now well accepted that this simplistic model is not very realistic. In any event, the nature of the plant lectins and

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their purported bacterial cell-surface receptors and extracellular signals still needs to be defined.

ROLE OF RHIZOBIAL CELL-SURFACE CARBOHYDRATES IN HOST ROOT INFECTION, NODULE DEVELOPMENT AND MAINTENANCE. The necessity for an intact LPS O-antigen structure as a critical requirement for host root infection, nodule development or nodule maintenance was recognized several years ago (31). In this work, it was demonstrated that mutants of ***Bradyrhizobium japonicum*** which appeared to be defective in LPS biosynthesis were incapable of forming nitrogen fixing nodules in plants. Later, this was also demonstrated in ***Rhizobium phaseoli*** mutants (35) and in ***Rhizobium leguminosarum*** mutants (1). While these studies lacked direct chemical analysis of the LPS structure, this component was provided in recent structural studies of LPS components of symbiotically defective ***Rhizobium phaseoli*** (8) and ***Rhizobium leguminosarum*** (49) mutants.

A role in bacterial release from the infection thread was proposed for the O-antigen fragment of the LPS of ***Rhizobium leguminosarum*** (19). In this study, nodule development with mutants that lacked the O-antigen containing LPS species or mutants with only small amounts of an antigenically altered O-antigen-containing LPS in place of the wild type LPS, was blocked at the stage of bacterial release from the infection

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The structural work conducted on mutants defective in a specific symbiotic function has been a major contribution towards understanding the role of rhizobial cell-surface carbohydrates in symbiosis. Still the exact nature of this role needs to be defined.

DYNAMICS OF THE RHIZOBIAL CELL-SURFACE CARBOHYDRATES. Defining a role for cell-surface carbohydrates in symbiosis is complicated by the constant changes that bacterial cell-surface components undergo while trying to adapt to new environmental conditions. Several studies have found that, as a function of growth stage, glycosyl components of the O-antigen chain and EPS are methylated (13,26,33,34). An increase in methylation of the glycosyl components of the O-antigen was also observed *for Rhizobium leguminosarum* free-living cells grown in acidic media when compared to free-living cells grown in neutral media (39). Oxygen and pH are environmental

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parameters which influence the expression of cell-surface antigens (27) and could partially explain developmental differences in the antigenicity of bacteria observed during the initiation and growth of root nodules (43). Another complication arises from the fact that bacteria are able to synthesize more than one EPS. In two instances, ***Rhizobium*** mutants which were incapable of synthesizing the usual EPS, and were impaired in symbiosis, recovered the wild-type phenotype after mutations which led to the synthesis of a new alternate EPS (18,47). It has also been demonstrated that ***Rhizobium meliloti*** strains are able to synthesize more than one LPS type (44,45). In these two studies, a gene that regulates the synthesis of an alternate LPS type was characterized. Recently, it was found that a ***Rhizobium leguminosarum*** defective mutant, which is incapable of synthesizing the usual tetrasaccharide component of the LPS, synthesized instead an LPS type which contained an unusual deoxy-glycosyl component (49). The complications of the genetic and environmental control of synthesis and post-synthetic modifications of bacterial cell-surface carbohydrates present a challenge in defining their role in symbiosis.

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CHAPTER 2

CHEMISTRY AND IMMUNOCYTOCHEMISTRY OF RHIZOBIUM LEGUMINOSARUM BIOVAR VICIAE CELL-SURFACE CARBOHYDRATE ANTIGENS.

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ABSTRACT

Chemical and immunochemical studies on the bacterial cell-surface carbohydrate antigens of ***Rhizobium leguminosarum*** biovar ***viciae*** 300 were conducted. Antibodies were generated against immunogens synthesized from the purified tetra- and trisaccharide components of the lipopolysaccharide (LPS) and from fragments of the so-called "O-antigen". Fluorescence and immunogold labels were then used to probe the distribution of these pre-determined carbohydrate structures on the cell-surface of cells grown in neutral media. Results indicated that the capsule was exposed around the entire cell-surface, but that the "O-antigen", the tetrasaccharide, and the trisaccharide were polarly exposed having, therefore, a potential role in the cell's "polar attachment" to the host root. Antibodies against the trisaccharide fragment showed greater reactivity than the ones against the tetrasaccharide fragment. This indicated that the trisaccharide was more abundantly synthesized by the cells, and/or that it was better exposed to react with antibodies.

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INTRODUCTION

The cell-surface carbohydrates of *Rhizobium* species have been implicated in the nitrogen-fixing symbiotic relationship between these bacteria and their host legume plants. One of the first theories on the role of cell-surface carbohydrates states that carbohydrate receptors on the bacterial cell-surface interact with lectins released from the plant. These lectins also bind to receptors in the host root (1). This cross bridging was suggested to be a symbiont-specific interaction between the bacterium and its host. Potential lectin receptors on the bacterial cell-surface were thought to be the lipopolysaccharides (LPS) (42) and the capsular polysaccharides (CPS) (6,8).

More recently, efforts to understand the role(s) of bacterial cell-surface carbohydrates in symbiosis have focused on the LPS. The necessity for an intact LPS O-antigen structure as a critical requirement for host root infection, nodule development or nodule maintenance was recognized several years ago (27), and has been confirmed in several systems since then. Bacterial phenotypes with defective LPS O-antigens are associated with defective

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infection thread development, failure of bacteria to be released from infection threads and the formation of empty, ineffective nodules (3,10,14,28,30,31,36).

One complication in defining the roles of the extracellular polysaccharides (EPS) and LPS in symbiosis, arises from the ability of any given bacterial strain to synthesize more than one CPS or LPS type, and from the fact that the synthesis of these types is regulated in a very complex fashion. Symbiotically impaired mutants of ***Rhizobium meliloti***, incapable of synthesizing the usual succinylated EPS, recovered the wild type phenotype after mutations which led to the synthesis of a surrogate EPS (13). This new EPS had a completely different structure from the wild type EPS. Similar phenomena were observed in other studies (43). These findings lead one to question the relevance of a single exact CPS or EPS structure in determining the outcome of symbiosis. More recently, duplication of genes responsible for polysaccharide structure was demonstrated for the LPS of ***Rhizobium meliloti*** (40,41). In these studies, a lesion in LPS synthesis in a defective mutant was repaired by mutations at other sites that led to synthesis of different LPS structures.

Another complication in defining the role of bacterial cell-surface carbohydrates in symbiosis is the dynamics of

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bacterial cell-surface antigenicity as a function of pH (24, 37). This raises several questions about the clonality of cell-surface carbohydrate expression, and the spatial relationship between CPS and LPS types on any given bacterial cell. The fate of the bacterial capsule during early events in the symbiosis has been addressed in one study (34) where it was demonstrated that, at one stage of the attachment process, the capsule has a non-uniform distribution on the bacterial cell-surface.

The elucidation of partial and complete structures of the CPS and some aspects of LPS (4,17,18,19,20,26,29,32), was a very important contribution towards understanding the role of rhizobial cell-surface carbohydrates in symbiosis. In earlier work we defined the exact structures of the CPS and of two components of the LPS of *Rhizobium trifolii* ANU 843 (4,17, 18,19). One of the LPS components is a tetrasaccharide containing galactose, mannose, 2-keto-3-deoxyoctusolonic acid (KDO) and galacturonic acid (17,18). The other one is a trisaccharide containing two galacturonic acid residues and KDO (4,18). These two structures have been found in *Rhizobium phaseoli* strains (3) and appear to be quite common to *Rhizobium trifolii* biovars.

In this study on *Rhizobium leguminosarum* bv. *viciae* 300 we

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used these exact, purified tetra- and trisaccharides from the LPS to make synthetic immunogens which were then used to generate specific antibodies to the pre-determined cell-surface carbohydrate structures. Antibodies were also generated against purified fragments of the O-antigen chain and purified CPS oligomers. This allowed us to define the cell—surface immunochemistry of ***Rhizobium leguminosarum*** bv. ***viciae*** 300 in a very specific fashion and answer questions about the clonal and spatial distribution of these chemically defined species using light and electron microscopy. This is the first study in the ***Rhizobium***/legume system to combine structural chemistry and immunochemistry in a specific fashion.

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MATERIALS AND METHODS

General methods: Compositional analysis of the O-antigenic fragments was determined as their alditol acetate derivatives (33). These derivatives were analyzed on a Hewlett-Packard gas chromatograph (GC) equiped with a Supelco DB225 capillary column and a flame ionization detector. The temperature program used was the following: initial temperature of 200°C, rate of 2 deg/min, final temperature of 230°C, final hold time 55.00 min, and a run length of 70.00 min. Glycosyl composition was confirmed by combined gas chromatography/mass spectrometry (GC-MS) on a JEOL 505 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR300 spectrometer operating at 300 MHz for protons and at 75.43 MHz for ^{13}C. Spectra were obtained in deuterium oxide. Carbohydrate content in fractions from gel filtration columns was monitored by the phenol/sulfuric acid method (12).

LPS isolation and purification: *Rhizobium leguminosarum* bv. *viciae* 300 was grown aerobically in modified Bergensen's liquid medium (pH 6.9) at 30°C as previously described (5). LPS were extracted from cells using a hot phenol/water method (39). The aqueous phase was dialyzed against four

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changes of water using 12,000-14,000 molecular weight cut-off dialysis tubing, concentrated and treated with an aqueous solution of DNAse I, RNAse A and $MgCl_2.6H_2O$ to remove contaminating nucleic acid material. The LPS was further purified using size exclusion chromatography (Sepharose 4B, with 0.05 M formic acid adjusted to pH 5.5 with ammonia, as the mobile phase). The carbohydrate-containing peak that voided the column, the LPS, was hydrolyzed with 1% acetic acid at 100°C for 2 h and partitioned between water and chloroform. The water-soluble portion was fractionated by gel filtration chromatography (Bio Gel P-2 with 0.1% formic acid as the mobile phase). The fractions which eluted as a peak were pooled, lyophilized, and analyzed by ^{1}H-NMR and ^{13}C-NMR spectroscopy. Compositional analysis of the glycosyl components was performed on the alditol acetates after hydrolysis,reduction and acetylation.

Synthesis of the carbohydrate antigens

(a) Preparation of allyl glycosides: Allyl glycosides were prepared from the tetrasaccharide and trisaccharide and from the "O-antigen" fragments of the LPS of ***Rhizobium leguminosarum*** bv. ***viciae*** 300 vegetative cells by Fisher glycosidation with allyl alcohol (21). Briefly, 2 mg of the carbohydrate were dissolved in N,N-dimethylformamide (200 µl), allyl alcohol (40 µl) and trifluoroacetic acid (10 µl) and stirred at 50°C. After 4 h, the mixture was dried under

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nitrogen, and 1M sodium hydroxide (100 µl) was added to the resulting product while the mixture was kept at 5°C. Formic acid was added to bring the pH to 7.5. The allylated oligomers were isolated from the reaction mixture by gel permeation chromatography over Bio Gel P-2 with water as the mobile phase and characterized by ^{1}H-NMR spectroscopy. During the formation of allyl glycosides, the O-antigen fragment was solvolyzed into what appeared to be two fragments. Both were recovered by size exclusion chromatography over Bio Gel P-6 with water as the mobile phase and characterized by ^{1}H-NMR spectroscopy. A small fraction of each was analyzed by GC/MS for composition after converting the glycosyl components to alditol acetate derivatives.

(b) Polymerization with acrylamide: The purified allylated glycosides (2 mg) were dissolved in water (1 ml) and copolymerized with acrylamide by modification of previously described procedures (22). Acrylamide (0.6 mg), TEMED (5 µl) (N,N,N′,N′-Tetramethylethylenediamine, Bio-Rad) and 10% ammonium persulfate (20 µl) were subsequently added. The mixture was vortexed immediately and left at room temperature for 4 h. The resulting gel was mechanically broken, homogenized in water and desalted by passing through a Bio Gel P-2 column with water as the mobile phase. Synthesis of the CPS antigen was done by directly polymerizing an enzymatically derived octasaccharide of this

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molecule (29) with acrylamide, via the α-L-threo-hex-4-eno-pyranosyl residue.

Production of polyclonal antibodies: An amount of polymer corresponding to 0.05 mg of carbohydrate was redissolved in 0.5 ml of sterile saline, mixed with 0.5 ml of Freund complete adjuvant and injected subcutaneously in adult New Zealand rabbits. Injection was repeated 3 and 6 weeks later with Freund incomplete adjuvant. The rabbits were bled 2 weeks after the last injection.

Indirect enzyme linked immunosorbent assay (ELISA): Microtiter plates (96-well clear polystyrene Nunc-Immuno Plate Maxisorp) were coated with LPS, 4 µg in 100 µl of phosphate buffered saline (PBS)/well (two wells were coated with 100 µl PBS each and used as blanks). Coated plates were incubated overnight at 4°C, washed 15 times with 0.2% Tween 20 (SIGMA) in PBS (PBS-Tween) and then blocked by incubating for 1 h at 37°C with 300 µl/well of 3% Bovine Serum Albumin (SIGMA) in PBS-Tween (BSA-PBS-Tween). After washing plates 15 times with PBS-Tween, dilutions of rabbit sera (post-immune sera, 1:50 and 1:100 in BSA-PBS-Tween; pre-immune sera, 1:50 in BSA-PBS-Tween and control, BSA-PBS-Tween) were added to the plates (100 µl/well) followed by 1-h incubation at 37°C. The microtiter plates were washed 15 times with PBS—Tween and then incubated with goat-antirabbit Ig immunoglobulin alkaline phosphatase conjugate (SIGMA) diluted in 1:300 BSA-PBS-Tween (100 µl/well) for 0.5 h at

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37°C. Plates were washed 15 times with PBS-Tween and were developed by adding 100 µl/well of 1 mg/ml p-nitrophenylphosphate (SIGMA) in 10% diethanolamine buffer (pH 9.8) containing 0.2% sodium azide. Following incubation at room temperature for 1 h in the dark, the reaction was stopped by adding 100 µl/well of 0.05 M ethylene diamino tetra-acetic acid (EDTA) and read at λ 405 nm (Bio-Tek EL-308 EIA Reader). To characterize the reactivity of the antibodies, some of the wells in each plate were used for a competitive indirect ELISA: after the BSA-PBS-Tween blocking and washing steps, sera were diluted (1:100) in BSA-PBS-Tween containing 2 mg/ml of the corresponding antigen and 100 µl were added to each well of the plates. The remaining procedure was carried out as described above.

Immunofluorescence microscopy: Procedures were adapted from (34). Briefly *Rhizobium leguminosarum* bv. *viciae* 300 cells in late exponential growth were centrifuged at 3,000 X g for 20 min at 4°C. The pellet was suspended and washed in PBS twice. The pellet was then resuspended in PBS, heat-fixed to microscope slides, rinsed with distilled water and air-dried. Non-specific binding sites were blocked by incubating the fixed cells with 3% BSA in PBS (BSA-PBS) for 1 h at room temperature. Slides were washed with distilled water, air-dried and incubated with 1:10 dilution of post-immune sera in PBS for 1 h at room temperature. In parallel, two slides (controls) were incubated with PBS. After washing with

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distilled water, 100 µl of a 1:3 dilution of goat-antirabbit FITC conjugate (SIGMA) in PBS was added to each slide and incubated in the dark for 1 h at room temperature. Slides were washed with distilled water, air-dried and observed under the microscope using phase-contrast and fluorescence optics.

Immuno-gold labelling for transmission electron microscopy (TEM): *Rhizobium leguminosarum* bv. *viciae* 300 cells (amount contained in 1 ml of media) in late exponential growth were centrifuged at 3,000 X g for 20 min at 4°C. The pellet was suspended and washed in PBS twice. Cells were suspended in BSA-PBS and incubated at 37°C for 1 h with shaking. After spinning the cells and resuspending the pellet in PBS 3 times, the pellet was suspended in a 0.3 ml of 1:10 dilution of post-immune sera in PBS, and incubated at 37°C with shaking. In parallel, two vials were incubated with PBS as a control. After 1 h, cells were pelleted by centrifugation and washed in PBS 3 times. The pellet was then suspended in 0.3 ml of a 1:3 dilution of goat-antirabbit gold conjugate, 10 nm (SIGMA) in PBS and incubated at 37°C for 1 h with shaking. After washing with PBS 3 times the pellet was suspended in 0.3 ml PBS, cells were sprayed on polyvinyl formal (Formvar) coated grids (200-300 mesh) (25) and air-dried. After carbon coating, cells were observed with a Phillips 201 transmission electron microscope.

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RESULTS

Isolation, purification and characterization of oligosaccharides. LPS isolated from *Rhizobium leguminosarum* bv. *viciae* 300 vegetative cells was hydrolyzed with mild acid and partitioned between water and chloroform as described in the materials and methods section. The gel filtration chromatography profile (Bio Gel P-2) of the aqueous portion of the LPS indicated the presence of three major carbohydrate peaks.

The ^{1}H-NMR spectrum of the oligosaccharides eluting in the void volume, and normally referred to as "O-antigen" in literature, is shown in Figure 1A. Resonances between δ 0.9 and 1.3 ppm (relative to external Me_4Si) corresponded to methyl protons of 6-deoxy hexoses. The signals corresponding to methyls of O- and N-attached acetyl groups appeared between δ 1.8 and 2.2 ppm. The N-methyl signals appeared at 2.6 ppm. The O-methyl signals appeared as sharp singlets between δ 3.2 and 3.4 ppm. A multiplet at δ 5.1 was assigned to signals of the anomeric protons. The solvent line (HOD) appeared as a broad singlet at δ 4.65. The rest of the signals corresponded to carbohydrate ring protons.

FIG. 1. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of the oligosaccharide eluting as peak A after gel permeation chromatography (Bio Gel P-2). (A) Resonances between δ 0.9 and 1.3 ppm (relative to Me_4Si) correspond to the methyl protons of 6-deoxy hexoses. The signals corresponding to methyls of O- and N-attached acetyl groups appear between δ 1.8 and 2.2 ppm. The signals at around δ 5.1 are due to the anomeric protons. The solvent line (HOD) appears as a broad singlet at δ 4.65. The rest of the signals correspond to ring protons. (B) The five resonances between δ 15 and 24 ppm correspond to the methyl carbons of 6-deoxy hexoses and of acetate groups. Resonances for carbonyl carbons belonging to O-acetyl groups appear between δ 173 and 176 ppm, ring carbons attached to nitrogen appear at δ 56 ppm and the anomeric carbon resonances appear between δ 100 and 105 ppm. The rest of the signals correspond to carbons of the sugar rings. (The broad peak centered at about 112 ppm is due to the vortex plug in the NMR tube. This was necessary because of the small volume).

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Figure 1B shows the ^{13}C-NMR spectrum corresponding to the oligosaccharide eluting in the void volume. In this figure, the five resonances between δ 15 and 24 ppm corresponded to the methyl carbons of 6-deoxy hexoses and methyl carbons of acetate groups. Resonances for carbonyl carbons belonging to O-acetyl groups appeared between δ 173 and 176 ppm. Ring carbons attached to nitrogen appeared at δ 56 ppm and the anomeric carbons between δ 100 and 105 ppm. The rest of the signals corresponded to other carbons in the sugar ring.

The glycosyl composition of this oligosaccharide was determined by GC and GC/MS analyses. Under our hydrolysis conditions the proportions of glucose, 6-deoxyhexoses (rhamnose and fucose) and 2-amino,2,6-dideoxyhexose were approximately 2:2:1 according to the detector response. There were also small amounts of O-methyl, 6-deoxy hexoses. This oligosaccharide, which corresponded to the so-called "O-antigen fragment" of *Rhizobium leguminosarum* bv. *viciae* 300, contained neutral sugars and an amino sugar, the latter when not acetylated would confer a net positive charge to the O-antigen.

Further chromatography on the Bio Gel P-2 void volume using ion-exchange chromatography (DEAE with a linear gradient of 0.01 M formic acid adjusted to pH 5.5 with

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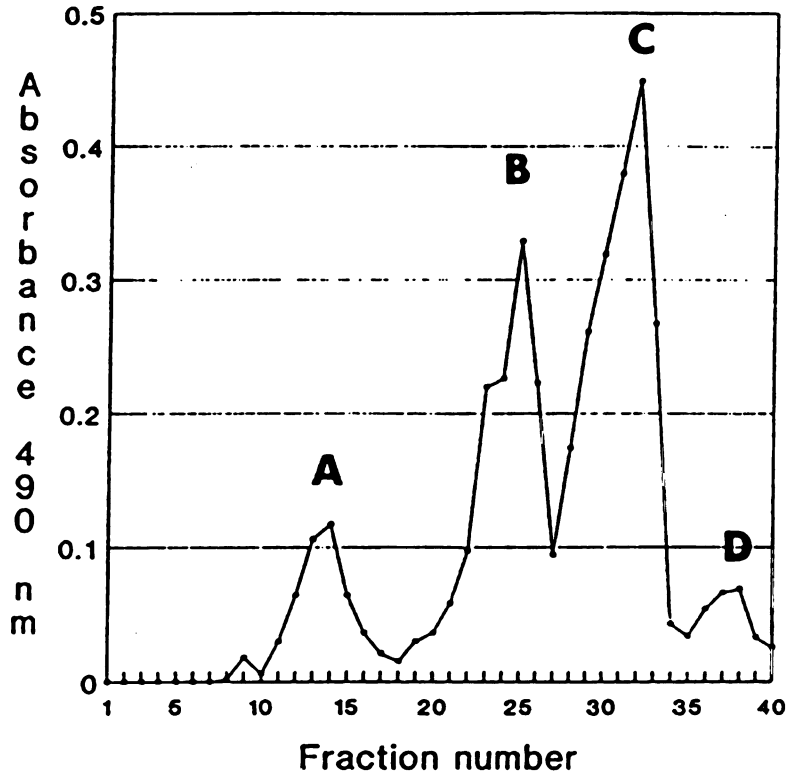
ammonium and 0.2 M ammonium chloride as the mobile phase) indicated that it was a mixture of four oligomers (Figure 2). Because the carbohydrate elution profile was monitored by the phenol/sulfuric method, the intensity of the peaks does not correlate with the amount of material recovered from each peak. These four oligomers were further characterized by ^{1}H-NMR spectroscopy and their glycosyl composition determined. The smaller oligomer (Figure 3A) contained an approximate ratio of 1:1 rhamnose:glucose and smaller proportions of 3-O-methyl,6-deoxyhexose, fucose, mannose, galactose, glucose and 2-amino,2,6-dideoxyhexose, suggesting the possibility of a repeating unit of rhamnose and glucose with several side chain substituents. The second eluting peak (Figure 3B) consisted primarily of 3-O-methyl,6-deoxyhexose, fucose, glucose and 2-amino,2,6-dideoxyhexose in an approximate ratio of 1:1:2:1. In this second peak, trace amounts of rhamnose, mannose and galactose were found. The peak eluting third (Figure 3C) appeared to have the most complicated glycosyl composition containing an approximate 1:1:1 ratio of 3-O-methyl,6-deoxyhexose, fucose and 2-amino,2,6-dideoxyhexose, which could be part of a trisaccharide repeating unit. 3,4-O-methyl,6-deoxyhexose, mannose, galactose and an unidentified amino sugar were also present. The last peak (Figure 3D) contained the following four sugars in similar ratios: 3-O-methyl,6-deoxyhexose, fucose, the same unidentified amino

FIG. 2. Carbohydrate profile of the ion-exchange chromatography column (DEAE Sephadex) of the "O-antigen" oligosaccharides of ***Rhizobium leguminosarum*** bv. ***viciae*** 300. Components were eluted with a linear gradient of ammonium chloride.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D
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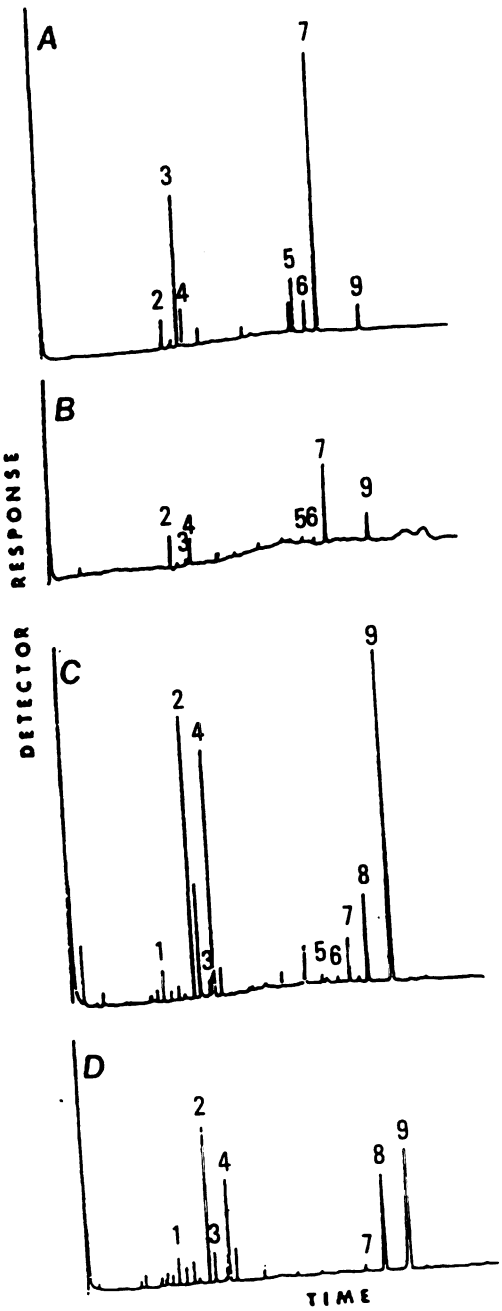
30
35
40
Fraction number

FIG. 3. Gas chromatography profiles of the alditol acetate derivatives of the glycosyl components of the oligosaccharides eluting from the DEAE column (Figure 2). Profiles labeled A through D correspond to peaks in Figure 2 with the same letter. Glycosyl peaks labeled 1 through 9 correspond, respectively, to 3,4-O-methyl hexose, 3-O-methyl hexose, rhamnose, fucose, mannose, galactose, glucose, an unidentified amino sugar and 2-amino,2,6-dideoxyhexose.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The large disparity in both the relative proportions the compositions of the four components isolated in the E Sepharose column is difficult to reconcile with the epted model of LPS structure in which one O-antigen chain identical repeat units is proposed. It is clear that this not the case for this strain of Rhizobium leguminosarum var viciae. It is obvious that several different "O-igen" structures are being synthesized at once. The plete lack of overlap between the compositions of some of four components supports this notion. The question of ther these differences are spatial or developmental then ains to be answered.

The ^{1}H-NMR spectra of the oligosaccharide peaks eluting ond and third from the P-2 column corresponded to the uctures reported (4,17,18) for the tetra- and saccharide components of the LPS of ***Rhizobium trifolii*** ANU with the difference that in ***Rhizobium leguminosarum*** bv. e 300 the tetrasaccharide was devoid of acetyl groups. s has also been observed for strains of ***Rhizobium phaseoli***

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Synthesis and characterization of carbohydrate immunogens. Carbohydrate immunogens were synthesized from the so-called "O-antigen fragment" (a mixture of oligosaccharides) by linking them through glycosyl allylation into a polymer matrix as described in the materials and methods section. Figure 4 shows a scheme of this two-step copolymerization. During the glycosyl allylation (Step 1, Figure 4) the O-antigen component was solvolyzed into oligosaccharides with two distinct size distributions that were separable by gel filtration chromatography on Bio Gel P-6. These are referred to as O-antigen 1 and O-antigen 2. Each distribution was characterized by ^{1}H-NMR spectroscopy (Figure 5A and 5B). Signals for a ^{1}H multiplet at approximately δ 5.8 ppm (relative to external Me_4Si), were ascribed to the methine proton of the allyl group. The ^{1}H multiplets at approximately δ 5.3 and 5.1 ppm were due to the vinylic methylene protons which are cis and trans, respectively, to the allyl oxygen. The signal at δ 1.8 ppm corresponded to free acetate. The O-antigen 1 fraction eluted first from the P-6 column indicating that it contained oligosaccharides of larger size than those in the O-antigen 2 fraction. It contained eight of the sugars found in the original Bio Gel

FIG. 4. Scheme indicating the glycosyl allylation step (1) and the copolymerization with acrylamide (2) in the synthesis of the carbohydrate antigens.

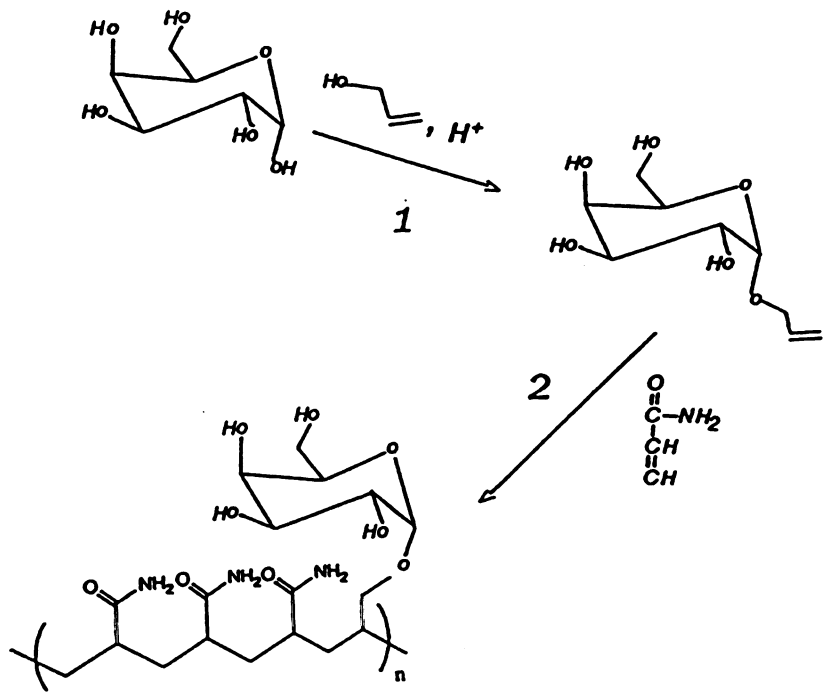
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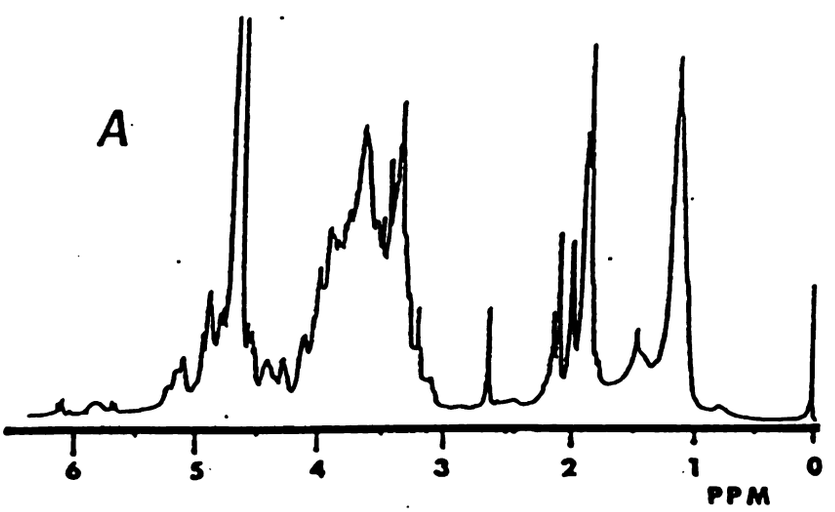
FIG. 5. ^{1}H-NMR of the O-antigen 1 (A) and O-antigen 2 (B) fractions, respectively, generated during the glycosyl allylation step. The ^{1}H multiplet at approximately δ 5.8 ppm (relative to external Me_4Si), reveals the presence of the methine proton of the allyl group, and the ^{1}H multiplets at approximately δ 5.3 and 5.1 ppm due to the vinylic methylene protons cis and trans respectively to the allyl oxygen. The signal at δ 1.8 ppm corresponds to free acetate.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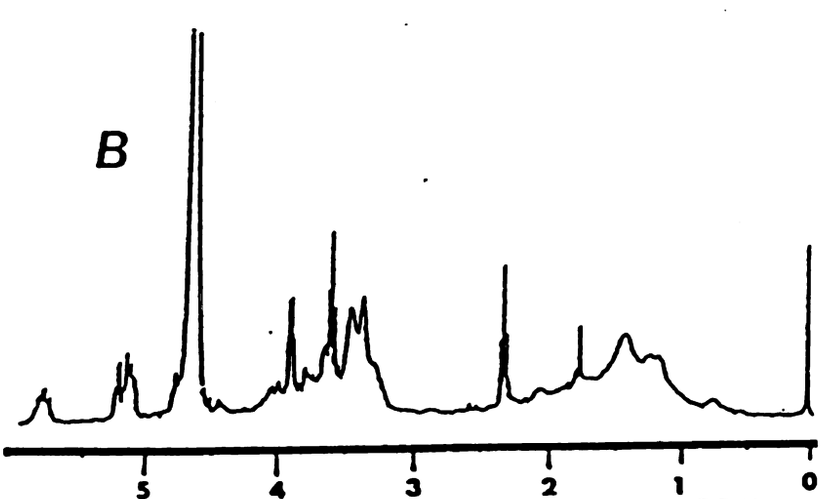
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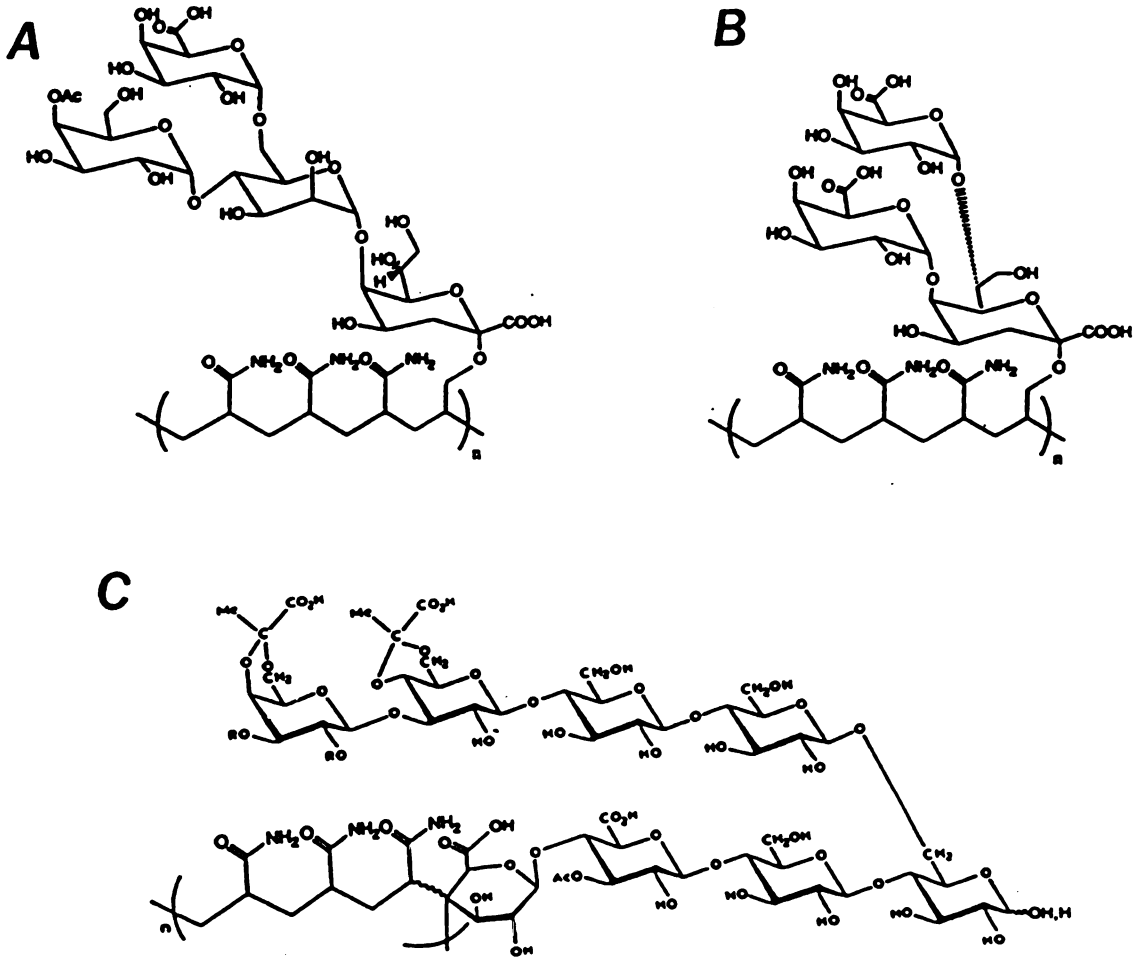
P-2 fraction. Six(6)-deoxyhexoses (primarily rhamnose), glucose and 2-amino,2,6-dideoxyhexose were the predominant components and were found in an approximate proportion of 2:2:1 judged by the detector response. All glycosyl components were neutral except for the amino sugar which when unacetylated, would confer to these fragments an overall positive character. The O-antigen 2 fraction contained smaller size oligomers (eluted second on the P-6 column) and its main glycosyl component was glucose (approximately 85% of the total detector response). It also contained all of the other sugars in smaller amounts. The amino sugar, the only non-neutral sugar if unacetylated, would confer to the O-antigen 2 a positive character.

Carbohydrate antigens were also synthesized by incorporating the tetra- and trisaccharide through glycosyl allylation into a polymer carrier to give the structures shown in Figures 6A and 6B. The glycosyl allylated species were characterized by ^{1}H-NMR spectroscopy. Signals for a ^{1}H multiplet at approximately δ 5.8 ppm (relative to external Me_4Si), were observed for the methine proton of the allyl group. ^{1}H multiplets at approximately δ 5.3 and 5.1 were assigned to the vinylic methylene protons which are cis and trans, respectively, to the allyl oxygen. A signal at δ 1.8 ppm in the trisaccharide spectrum corresponded to free acetate. The remaining signals corresponded to those

FIG. 6. Structure of the synthetic immunogens from the tetrasaccharide (A) and trisaccharide (B) of the LPS and of the capsular polysaccharide (C). In the case of A and B immunogens were synthesized by incorporating the oligosaccharides into a polymer matrix by polymerization of the allyl glycosides with acrylamide, in the case of C the hapten was directly polymerized.


A
B
C

previously reported (4,18,20) for the tetra- and trisaccharide of ***Rhizobium trifolii*** ANU 843.

Antibodies to the capsule were raised against an octasaccharide fragment isolated and purified by enzymatic degradation by a phage-induced enzyme (3,16). This fragment contained the unsaturated glycosyl residue α-L-threo-hex-4-enopyranosyl uronic acid, which was utilized in the direct copolymerization reaction with acrylamide (Figure 6C).

Antibodies raised in rabbits against the synthetic carbohydrate antigens were characterized for their reactivity with crude LPS and with inhibition assays with the corresponding carbohydrate of interest in ELISA tests (Figure 7). In all cases, binding of the antibody to the LPS was effectively blocked by addition of the respective carbohydrate to the corresponding post-immune serum. Based on the inhibition assay, the tetrasaccharide and trisaccharide exhibited approximately the same blocking effect on their respective antibodies, followed by the O-antigen 1 and the O-antigen 2, respectively.

Spatial distribution of Cell-surface Carbohydrates. The antibodies raised specifically against the tetrasaccharide, the trisaccharide, the O-antigen 1, the O-antigen 2 and the

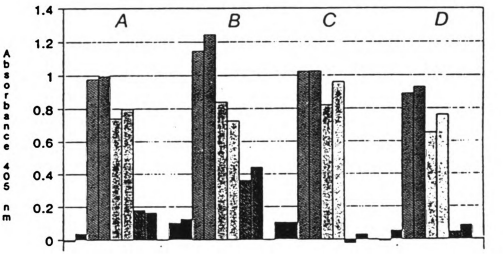
FIG. 7. Indirect ELISA results utilizing the polyclonal antibodies raised against the O-antigen 1 fraction (A), the O-antigen 2 fraction (B), the tetrasaccharide (C) and the trisaccharide (D).

 Pre-immune serum (1:50 dilution in BSA-PBS),

 post-immune serum (1:50 dilution in BSA-PBS),

 post-immune serum (1:100 dilution in BSA-PBA),

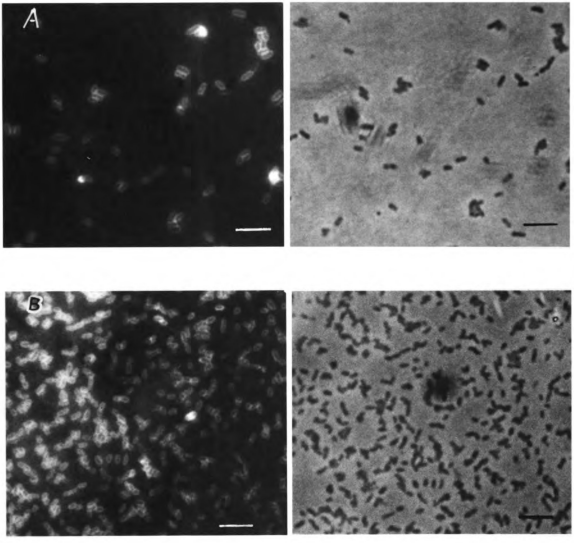
 inhibition assay: post-immune serum (1:100 dilution in BSA-PBS containing 1 mg/ml of the corresponding antigen).


A
B
C
D
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance 405 nm

CPS were used to determine the spatial distribution of these LPS domains on vegetative cells using indirect immuno-fluorescence. When vegetative cells were incubated with antibodies against the tetrasaccharide, the trisaccharide, the O-antigen 1 and O-antigen 2, the secondary antibodies were not found evenly distributed on the surface of the cells, but were located at certain poles. As an example, Figure 8A shows vegetative cells incubated against the trisaccharide where it can be noted that fluorescence was concentrated only at the "tip" of most cells (see arrows). These observations suggested that the different LPS epitopes are available to react with the respective antibodies only in a few localized areas of the cell-surface. To find out if there was another cell-surface carbohydrate that could be blocking the LPS and causing it to be exposed only in a "polar" fashion, we incubated the vegetative cells with antibodies raised against the CPS. Figure 8B shows that in this case almost all cells fluoresced and that the secondary antibody was distributed over the entire cell-surface. The CPS therefore was available to react with its respective antibody all around the cell-surface in almost all vegetative cells. From the immunofluorescence microscopy results it was not clear if both poles of the vegetative cells fluoresced when incubated with the anti-CPS antibody.

These observations were further investigated by TEM

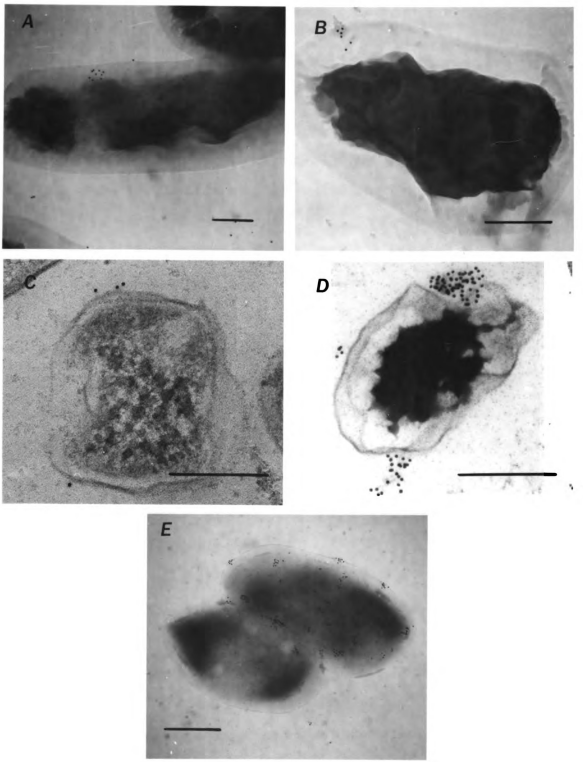
FIG. 8. Spatial distribution of the trisaccharide (A) and CPS (B) on the cell-surface of vegetative ***Rhizobium leguminosarum*** bv. ***viciae*** 300 cells visualized using immunofluorescence microscopy. Arrows indicate the polar exposure of the trisaccharide (A); the same phenomenon was observed in cells incubated with antibodies against the O-antigen 1, O-antigen 2 and tetrasaccharide. The CPS was available to react with its respective antibodies all around the cell (B). These observations were complemented with transmission electron microscopy as shown in Figure 9. (Bar = 5 μm). Panels on the right are the corresponding phase contrast images.


A
B

using immunogold labelling. All cells showed few gold particles on their surface when incubated with antibodies against either of the O-antigen fractions (see arrows on Figures 9A and 9B). There was also some labeling of background material that was probably sloughed-off LPS. General labeling of cells was not appreciably above this background level except at a localized area on only a few cells. The number of particles attached at a single point on such a cell was never greater than ten. These observations confirmed that the O-antigen fragments were only available to react with antibodies in few specific regions of the cell-surface.

When cells were incubated with antibodies against the trisaccharide (Figure 9D) gold particles were also localized on two very defined areas of the cell-surface (see arrows). Antibodies raised against the trisaccharide were more reactive than antibodies raised against the tetrasaccharide (Figure 9C). In the former case, the number of particles found together in the area where the antibodies bound was larger than twenty and up to sixty in some cells, as opposed to no more than three particles per similar area per cell for the tetrasaccharide. Whether this reflects differences in binding efficiency between the two antibodies or differences in availability of the different epitopes on the bacterial cell-surface is an obvious question. The more

FIG. 9. Spatial distribution of the O-antigen 1 fraction (A), O-antigen 2 fraction (B), tetrasaccharide (C), trisaccharide (D) and CPS (E) on the cell-surface of vegetative ***Rhizobium leguminosarum*** bv. ***viciae*** 300 cells visualized using immunogold labeling transmission electron microscopy. O-antigen fractions (A,B) were only available to react with antibodies in few specific regions of the cell-surface (see arrows). Antibodies raised against the trisaccharide (D) were more reactive than antibodies raised against the tetrasaccharide (C). The number of particles found together in areas with antibodies against the trisaccharide was larger than twenty and up to sixty in some cells, as opposed to no more than three particles per similar area per cell for the tetrasaccharide. The more efficient labeling showed by antibodies raised against the trisaccharide indicated that the tetrasaccharide and trisaccharide antigens were synthesized and/or exposed to very different extents. The large number of gold particles reacting with the trisaccharide in a specific area could be indicative of the existence of a longer polymer. This polymer, could be cleaved into trisaccharide units during the hydrolysis step. The CPS was exposed on the cell-surface in a fairly homogeneous fashion; only few areas on the cell-surface did not react with antibodies (E). These non-reactive areas could be due to capsule modification that did not allow for antibody recognition, or to synthesis of a different CPS structure by the cell. (Bar = .4 μm).


A
B
C
D
E

efficient labeling obtained with antibodies raised against the trisaccharide when incubated with ***Rhizobium leguminosarum*** bv. ***viciae*** 300 vegetative cells indicated that the tetrasaccharide and trisaccharide antigens were exposed to very different extent and/or that they are synthesized independently of each other. The large number of gold particles reacting with the trisaccharide in a specific area, could be indicative of the existence of a longer polymer. This polymer could then be cleaved into trisaccharide units during the hydrolysis step.

When incubated with anti-CPS antibody, almost all cells were covered with gold particles in a fairly homogeneous fashion (Figure 9E). Few localized surface areas did not react with the antibodies. Only a small percentage of the cells were covered by few gold particles indicating that, for a few cases, cells were either devoid of capsule or that the capsule had been modified and was no longer recognized by the antibody (Figure not shown). For these few cases, if cells were devoid of capsule, then the different domains of the LPS would have been available to react with the antibodies all over the surface. This was not observed in our study, leading us to believe that the capsule in some instances undergoes modifications that do not allow for antibody recognition or that they might be synthesizing a different CPS structure. This is also indicative of cell

heterogeneity within the same population.

DISCUSSION

Extensive work has been conducted to define the role of the CPS and the LPS in the symbiont-host specificity of the ***Rhizobium***/legume symbiosis. The idea that ***Rhizobium*** cell-surface receptors are recognized by plant root lectins was introduced several years ago (1,7). There is new evidence supporting this hypothesis (11,15), however, the nature of the lectins' and cell-surface receptors' role still needs to be defined.

Special attention has been given to these potential cell-surface receptors that could be responsible for host specificity and cell attachment to the host root. An early report (9) established that a receptor binding site for a plant protein, trifoliin A, that specifically recognizes ***Rhizobium*** is on the cell-surface of encapsulated cells of ***Rhizobium trifolii***. Later, a model was proposed (8) where an alteration of the capsule of unattached cells results in a polar capsule which binds the root lectin in a species-specific manner. In later findings (29) several groups of ***Rhizobium trifolii*** were defined based upon their acidic EPS

(CPS) structure; and their similarities to and distinct differences from the acidic EPS (CPS) of ***Rhizobium leguminosarum***. The main differences between CPS were found in the proportions of non-carbohydrate substituents. Though these differences might, at most, be contributing factors in determining the host range of the ***Rhizobium trifolii-Rhizobium leguminosarum*** complex, they are not enough to completely explain host specificity.

Our findings suggest that the negatively charged CPS surrounds the cell and that the LPS is exposed in very small localized regions on the surface of ***Rhizobium leguminosarum*** bv. ***viciae*** 300 vegetative cells. This might contribute to the "polar attachment" of bacteria to root hairs that is normally observed during the adsorption stage (8), but does not rule out the possibility of a complementary action with local CPS structures and the role of pili, fimbriae or even glycoproteins in attachment. The outermost domain of the LPS, the O-antigenic side chain, which varies in a strain-dependent manner could then be responsible for specific attachment. Differences in O-antigenic side chains between microsymbiont strains could be enough to explain the high selectivity observed in the ***Rhizobium meliloti***-alfalfa symbiosis in the presence of other heterologous bacteria (38). Also, it could explain earlier findings (2) where host

specificity has been shown to be expressed during the initial stages of adsorption of ***Rhizobium meliloti*** to alfalfa roots, and where the time in which attachment occurred was not enough for capsule modification to occur as proposed earlier (8).

Electrostatically, the O-antigenic side chain in ***Rhizobium leguminosarum*** biovar ***viciae*** 300, which is more positively charged than the capsule, will also favor attachment to the negatively charged host root. It is important to note that host specificity is determined at the level of bacterium/root hair interactions and not at the level of nodule formation.

In this study, we showed that the so-called "O-antigen fragment" of ***Rhizobium leguminosarum*** bv. ***viciae*** is a mixture of oligosaccharides of completely different composition and not fragments of uniform composition and different length cleaved from the same repeating polymer unit. This allows for the possibility of several different antigenic LPS side chains to be independently synthesized. If several O-antigenic side chains independently synthesized are held together by non-covalent interactions, then they will elute as only one polymer after gel permeation chromatography. Separation methods that destroy these non-covalent interactions should be used when studying the different O-

antigen side chains. We now need to address which are the variable and which are the conserved features of these "O-antigens" when cells are grown under different conditions. These findings stress the importance of the dynamics of the total cell-surface carbohydrate chemistry.

It has been suggested that bacterial nutrition and growth stage influence the polysaccharide chemistry of the cell-surface in relation to lectin binding (1). Growth stage effects on the lectin-binding activity of the ***Rhizobium trifolii*** LPS to the clover lectin trifoliin A have been demonstrated in vivo (23). These observations could be explained by our theory where environmental conditions and/or growth stage could regulate ***Rhizobium***-root hair attachment by causing changes in the O-antigenic side chain. Cells devoid of capsule will have more O-antigen exposed and could explain the random attachment of ***Rhizobium*** cells to host roots previously observed (8). Together with the involvement of cellulose fibrils observed in the attachment of ***Rhizobium leguminosarum*** to pea root hair tips (35), the CPS could have a potential role stabilizing the attachment through non-covalent forces. Though CPS is negatively charged and cellulose is nominally neutral, non-carbohydrate substituents can cause changes in their net charge. Environmental conditions and/or bacterial growth stage could

affect the kinds and amounts of non-carbohydrate substituents found, resulting in changes in the cell-surface overall net charge, thus affecting non-covalent forces involved in the attachment process.

The bacterial cell-surface is a dynamic entity, constantly changing to adapt to different environmental conditions. We have shown by the use of antibodies raised against defined chemical domains of the LPS that during the vegetative state the CPS surrounds the cells; and that in the LPS different domains are only exposed in very small regions notably at one pole, and may be responsible for "polar attachment". Our data suggest that the tetra- and trisaccharide components of the LPS could be synthesized independently of each other and that both do not necessarily form part of the same LPS molecule. Also, there could be more than one O-antigen side chain synthesized at a given time and the resulting LPS molecules may be associated by strong non-covalent interactions. The localization of the tetra- and trisaccharide components of the LPS within each cell and their population distribution in cells grown under different environmental conditions can now be directly observed on the cells. Issues about the relevance of these molecules in cells exposed to different field growth conditions and during the nodulation steps can now be answered by directly probing these molecules on the

bacterial cell-surface. Finally, we will now be able to study the environmental influences on the cell-surface immunochemistry and the fate of these carbohydrate antigens in real bacteroids during nodule development.

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CHAPTER 3

THE SYNTHESIS OF A COMPLETELY NEW ALTERNATE LIPOPOLYSACCHARIDE IS INDUCED IN RHIZOBIUM AT LOW pH, IN ADDITION TO METHYLATION OF THE EXISTING LIPOPOLYSACCHARIDE.

ABSTRACT

A new lipopolysaccharide is synthesized by wild-type strains of *Rhizobium leguminosarum* biovar *viciae* when the bacteria are grown at pH 4.5. There is no evidence for the production of this molecule when cells are grown at pH 7.0. The lipopolysaccharide contains a trisaccharide component that contains mannose, galactose and an unidentified deoxy sugar. The synthesis of a previously described 3-deoxy-2-octulosonic acid-containing tetrasaccharide component, which is one of the dominant structures in cells grown under neutral conditions, is severely repressed at pH 4.5. The same acid-induced trisaccharide has been isolated from the lipopolysaccharide of cells of a *Rhizobium leguminosarum* biovar *viciae* mutant strain which is impaired in the synthesis of a normal tetrasaccharide component. In addition to synthesis of the new LPS type, an increase in methylation of the "O-antigen" of the usual LPS components was observed for *Rhizobium leguminosarum* bv. *viciae* 300 cells. A similar increase in methylation of the "O-antigen" of the usual LPS type, is observed in *Rhizobium* cells as they enter the stationary phase, when the pH is known to be depressed. Analyses of lipopolysaccharides from two strains of

the trifolii biovar indicate that the unusual trisaccharide is not synthesized in these strains. However, it was observed in the one strain of the phaseoli biovar which was analyzed. This makes the unusual lipopolysaccharide a possible candidate for a species-dependent surface carbohydrate, with special significance for the infection process.

INTRODUCTION

Environmental factors are known to modulate the expression of cell-surface carbohydrates of *Rhizobium*. These conditions include oxygen and pH and have been demonstrated both in planta and ex planta (1,19,30,33). Understanding the magnitude of the impact of environmental factors on bacterial cell-surface chemistry is very important, since bacterial cell-surface carbohydrates such as the capsular polysaccharide (CPS) and lipopolysaccharide (LPS), have been suggested to be involved in aspects of the *Rhizobium*/legume symbiosis. This involvement is exerted throughout recognition and attachment, nodule development and maintenance, and release of bacteria from infection threads (2,3,7,9,10,12,20,23,27,29). There has been much debate about the roles of certain extracellular polysaccharides in symbiosis, as well as the exact structural details of very small magnitudes relative to the overall structures (4,24,26). However, the question of the relevance both of the structures and of the growth conditions of the bacteria from which the molecules were isolated, to the conditions that might more accurately reflect the rhizosphere, has been addressed only once (1). In this case, an increase in methylation of 2-O-methyl rhamnose and a decrease in

methylation of 2,3,4, tri-O-methyl fucose were observed. It is possible that antigens which are dominant under the conditions normally used for the growth and maintenance of laboratory cultures (pH 7 and adequate aeration) might not even exist in the rhizosphere, infection thread or nodule. The potential for environmental conditions to alter bacterial surface chemistry should be rigorously examined. The earlier work of Kannenberg and Brewin, and Vanden Bosch et al., (19,33), coupled with that of others (11,13,30,31,32,37), clearly demonstrates a need to understand the effects of environmental factors on surface chemistry. The work described here focuses on the effect of pH on the surface chemistry of biovars of ***Rhizobium leguminosarum***.

MATERIALS AND METHODS

General methods. Compositional analysis of the LPS fragments was determined for the glycosyl components by converting them to their alditol acetate derivatives (28). Analysis was performed by gas chromatography (GC), with a Hewlett Packard gas chromatograph, using a capillary DB225 column and flame ionization detector. Glycosyl composition was confirmed by gas chromatography/mass spectrometry (GC/MS) analysis on a JEOL 505 mass spectrometer, in the electron impact mode. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR500 spectrometer, operating at 500 MHz for protons. Spectra were obtained in D_2O solutions, and chemical shifts were referenced relative to external TMS. Carbohydrate content in fractions from gel filtration columns was monitored by the phenol-sulfuric acid method (14).

LPS and LPS fragment isolation and purification. ***Rhizobium leguminosarum*** cells of the following biovars: ***viciae,*** strains 300 and 128C53; ***phaseoli,*** strain UMR1632; and ***trifolii,*** strains ANU843 and 0403, were grown aerobically in modified Bergensen's media (B-III) (pH 6.9) at 30°C, as previously described (8). Acidic conditions were obtained by adjusting the culture medium to pH 4.5 as follows. Bacteria were inoculated on B-III agar plates,

and transferred after 3 days to 500 ml Erlenmeyer flasks containing 250 ml of B-III liquid medium (pH 6.9). At late log phase, each 250 ml inoculum was transferred to a separate 4-liter Erlenmeyer flask containing 2 liters of B-III medium (pH 6.9). Once the cells reached mid to late exponential growth, 2 liters of B-III medium at a pH of 3.2 (obtained using free glutamic acid in place of sodium glutamate and adjusting the pH with 1 HCl) were added into each flask, and the pH of each adjusted to give a final value of 4.5. The contents of each flask were then split into two 4-liter Erlenmeyer flasks, each containing about two liters of culture. Cells were grown until the beginning of stationary phase. Cultures were constantly shaken at 265 rpm. LPSs were extracted by the hot phenol-water method (34), treated with DNAse-RNAse, and purified by gel permeation chromatography over Sepharose 4B, with aqueous formic acid (0.05M, adjusted to pH 5.5 with ammonia) as the mobile phase. Carbohydrate fragments were released by hydrolyzing the LPS with 1% acetic acid at 100°C for 2h, and partitioned between water and chloroform. The aqueous layer was lyophilized and fractionated by size exclusion chromatography over Biogel P2, with aqueous formic acid (0.1%) as the mobile phase. Fractions eluting as a peak were pooled, lyophilized and analyzed by ^{1}H-NMR spectroscopy and by GC and GC/MS, after derivatization into alditol acetates as described in the general methods section. Fractions which appeared impure by these two analyses were subjected to further

purification by ion-exchange chromatography over DEAE sephadex, in aqueous formic acid (0.01%, adjusted to pH 5.5 with ammonia), and eluting with a linear gradient of 0-0.2M ammonium chloride.

RESULTS

The gel filtration chromatography profile (Biogel P2) of the aqueous portion of the LPS from ***Rhizobium leguminosarum*** bv. ***viciae*** strain 300 cells, grown in neutral media and in acidic (pH 4.5) media, are shown in Figures 1A and 1B respectively. ^{1}H-NMR spectra and glycosyl composition of the carbohydrate components of peak 1, normally referred to as "O-antigen fragment", for cells grown under normal and acidic conditions, indicated that there were differences in the methylation levels of the glycosyl constituents. There was an increase in the amount of 3-O-methyl,6-deoxy hexose as well as the appearance of a new peak with a mass spectrum which was assignable to a 3,4-di-O-methyl,6-deoxy glycosyl component (Figure 2, Scheme 1).

Glycosyl composition of the "O-antigen" fragment of the one strain of the ***Rhizobium leguminosarum*** bv. ***phaseoli*** analyzed indicated the appearance of a peak with a mass spectrum which was assignable to a 2,3-di-O-methyl,6-deoxy glycosyl component (Figure 3, Scheme 2).

FIG. 1. Gel permeation chromatography (Biogel P2) profile of the water soluble components of the LPS after hydrolysis with 1% acetic acid, for ***Rhizobium leguminosarum*** bv. ***viciae*** 300 cells grown under normal conditions (A), and under acidic conditions (B). Carbohydrate concentration was monitored with the phenol-sulfuric acid assay. Note the relative decrease in production of carbohydrate fragments eluting as peak 1, over the relatively increased production of carbohydrate fragments eluting as peak 2 for cells grown under acidic conditions (pH 4.5), when compared to cells grown in neutral media.

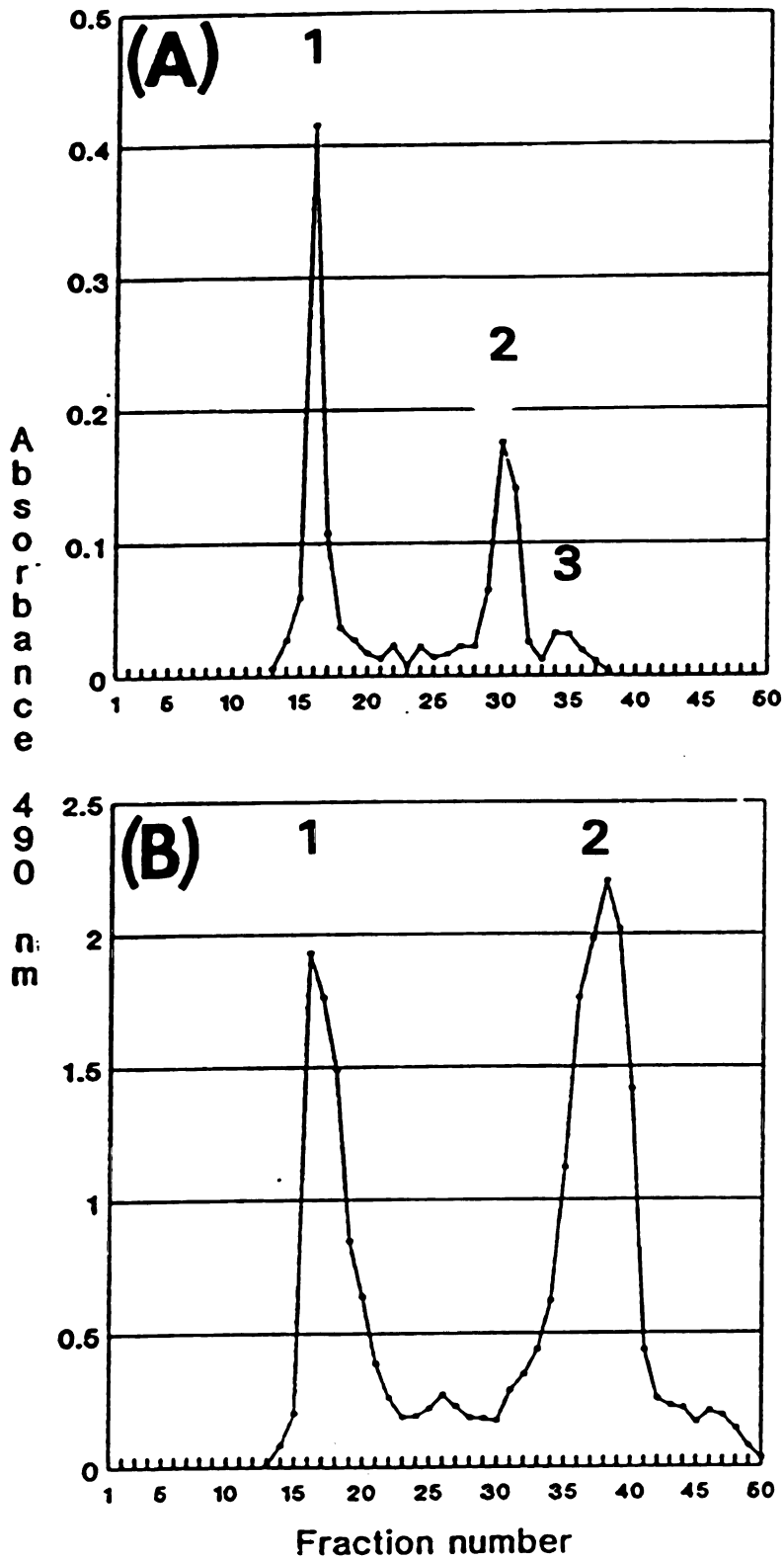

(A)
1
2
3
Absorbance 490 nm
0.5
0.4
0.3
0.2
0.1
0
1 5 10 15 20 25 30 35 40 45 50
(B)
1
2
2.5
2
1.5
1
0.5
0
1 5 10 15 20 25 30 35 40 45 50
Fraction number

FIG. 2. Electron impact mass spectrum of the di-O-methyl 6-deoxy hexose alditol acetate derivative, which is synthesized by ***R. leguminosarum*** bv. ***viciae*** in response to depression of the pH of the growth medium.

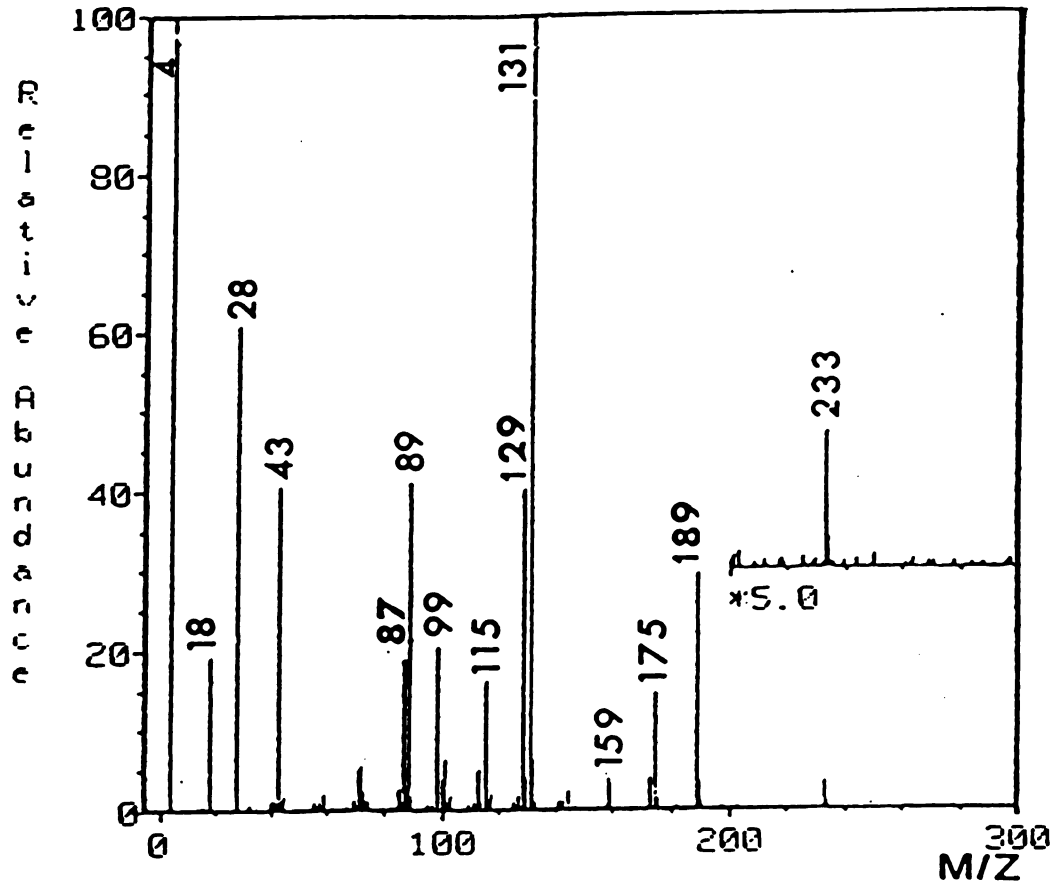
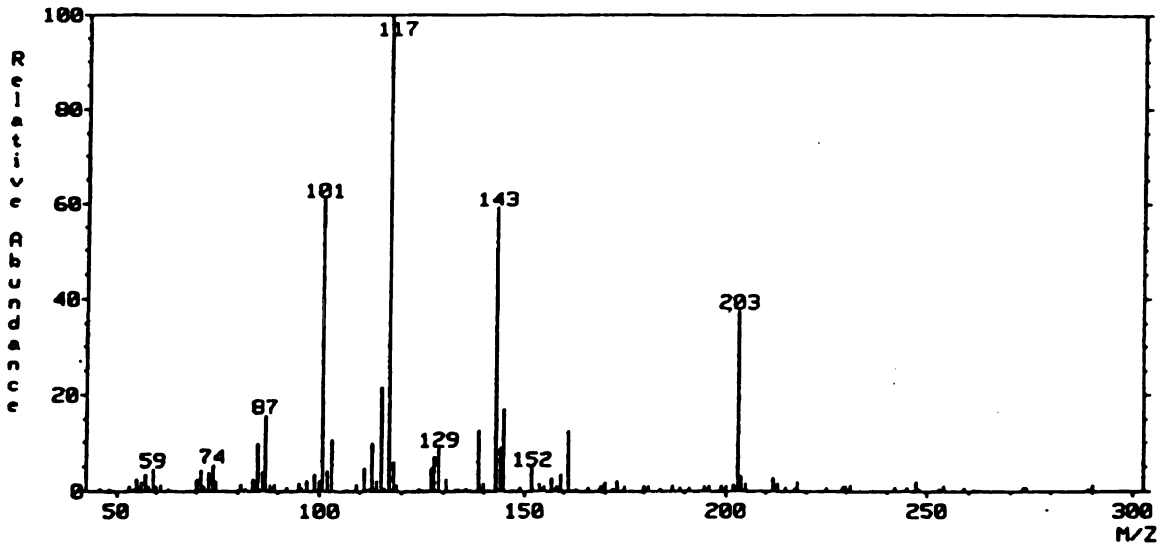
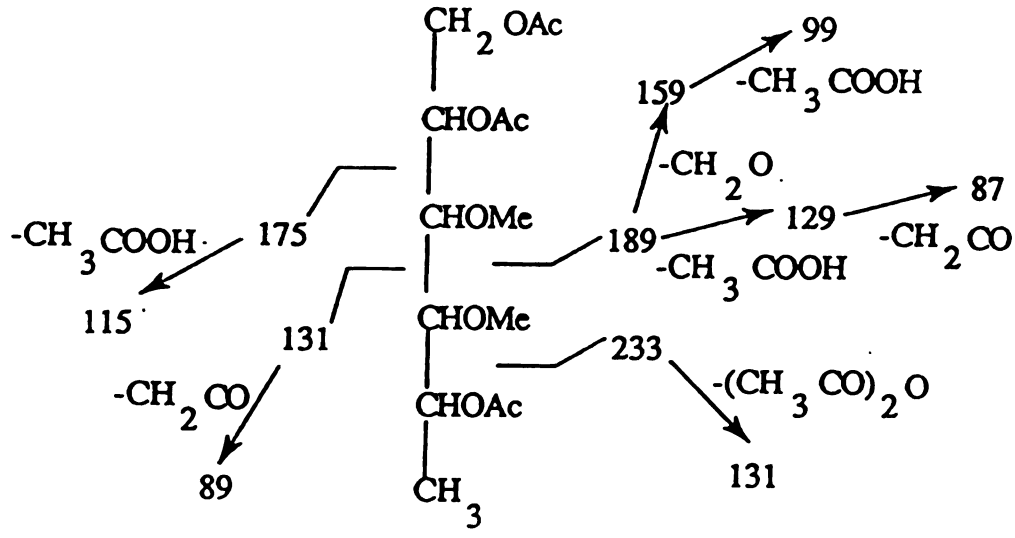

100
80
60
40
20
0
0
100
200
300
M/Z
4
18
28
43
87
89
99
115
129
131
159
175
189
233
*5.0

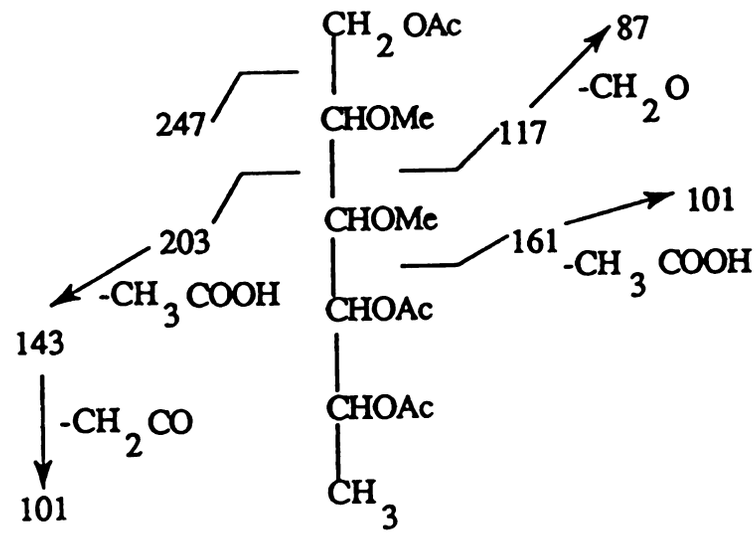
FIG. 3. Electron impact mass spectrum of the di-O-methyl 6-deoxy hexose alditol acetate derivative, which is synthesized by ***R. leguminosarum*** bv. ***phaseoli*** in response to depression of the pH of the growth medium.


Relative Abundance
100
80
60
40
20
0
117
101
143
203
87
59
74
129
152
50
100
150
200
250
300
M/Z

SCHEME 1. Fragmentation scheme of the dimethyl 6-deoxy hexose alditol acetate derivative, which is synthesized by ***R. leguminosarum*** bv. ***viciae*** in response to depression of the pH of the growth medium. This scheme corresponds to the electron impact mass spectrum in Figure 2.


CH2 OAc
CHOAc
CHOMe
CHOMe
CHOAc
CH3
99
159
-CH3COOH
-CH2O
87
129
-CH2CO
189
-CH3COOH
175
-CH3COOH
115
131
-CH2CO
89
233
-(CH3CO)2O
131

SCHEME 2. Fragmentation scheme of the di-O-methyl 6-deoxy hexose alditol acetate derivative, which is synthesized by ***R. leguminosarum*** bv. ***phaseoli*** in response to depression of the pH of the growth medium. This scheme corresponds to the electron impact mass spectrum in Figure 3.


CH2OAc
247
CHOMe
87
-CH2O
117
CHOMe
203
-CH3COOH
161
101
-CH3COOH
CHOAc
143
-CH2CO
CHOAc
101
CH3

The ^{1}H-NMR spectra of the carbohydrate components of peaks 2 and 3 of figure 1A, for strain 300 cells grown under neutral conditions, corresponded to the structures reported (5,6,16,17) for tetra- and trisaccharide core components of the LPS of ***Rhizobium trifolii*** ANU 843, except that in this case, the tetrasaccharide was not acetylated.

For cells grown under acidic conditions, it can be seen that, when the gel permeation profile is compared to the one for cells grown in neutral media (Figure 1A), there was a decrease in production of carbohydrate components of peak 1, relative to an increase in production of carbohydrate components of peak 2 (Figure 1B). The discrepancy in fraction numbers arose because the surface tension of the drops collected in the fractions corresponding to peak 2 (Figure 1B), was greater than the surface tension of the drops collected in the fractions corresponding to peak 1 (Figure 1A). This was caused by the greater carbohydrate concentration in the fractions corresponding to peak 2 (Figure 1B), than in the ones corresponding to peak 2 (Figure 1A). This effect resulted in more fractions collected for peak 2 (Figure 1B), when compared to peak 2 (Figure 1A); however, the total volume collected was similar for both peaks.

^{1}H-NMR spectroscopy analysis of the carbohydrate fragments eluting as peak 2, indicated that this fraction

FIG. 4. Ion-exchange chromatography (DEAE) profile of the ***R. leguminosarum*** bv. ***viciae*** carbohydrate fragments contained in the "tetrasaccharide like" fraction, peak B, eluting from the gel permeation chromatography column (Biogel P2). Peak B was resolved into two major oligosaccharide fragments, only the second one corresponded to the normal tetrasaccharide.

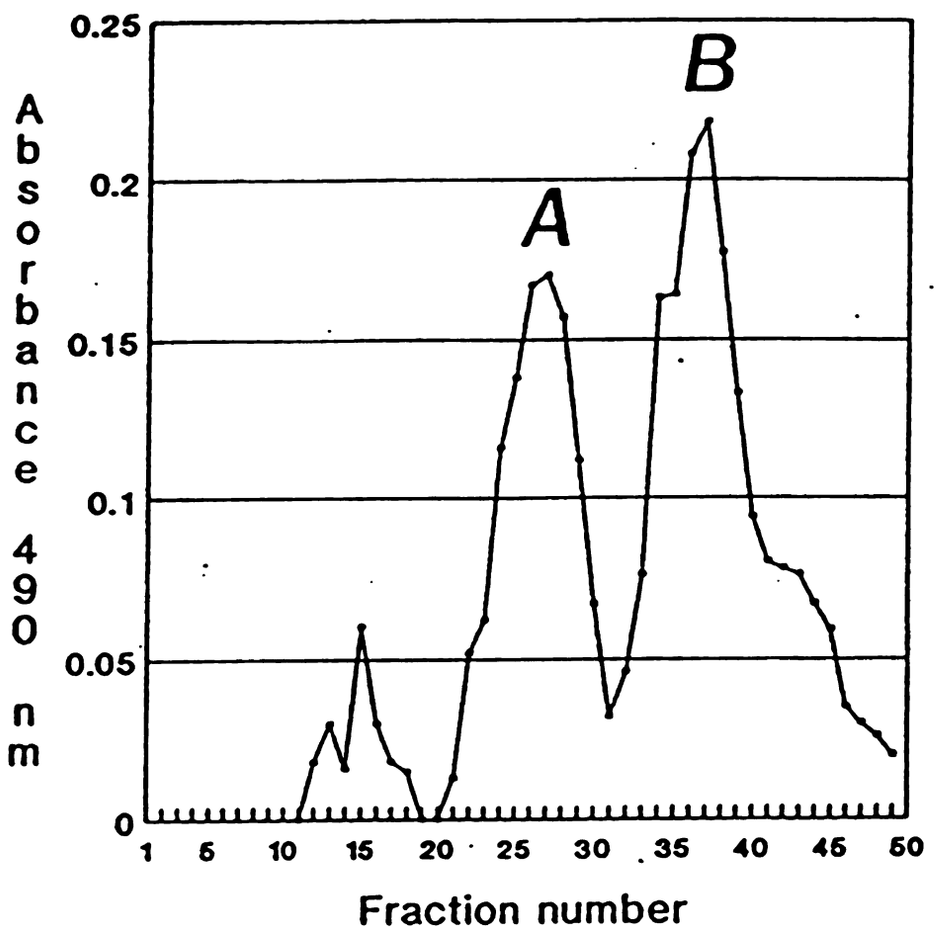
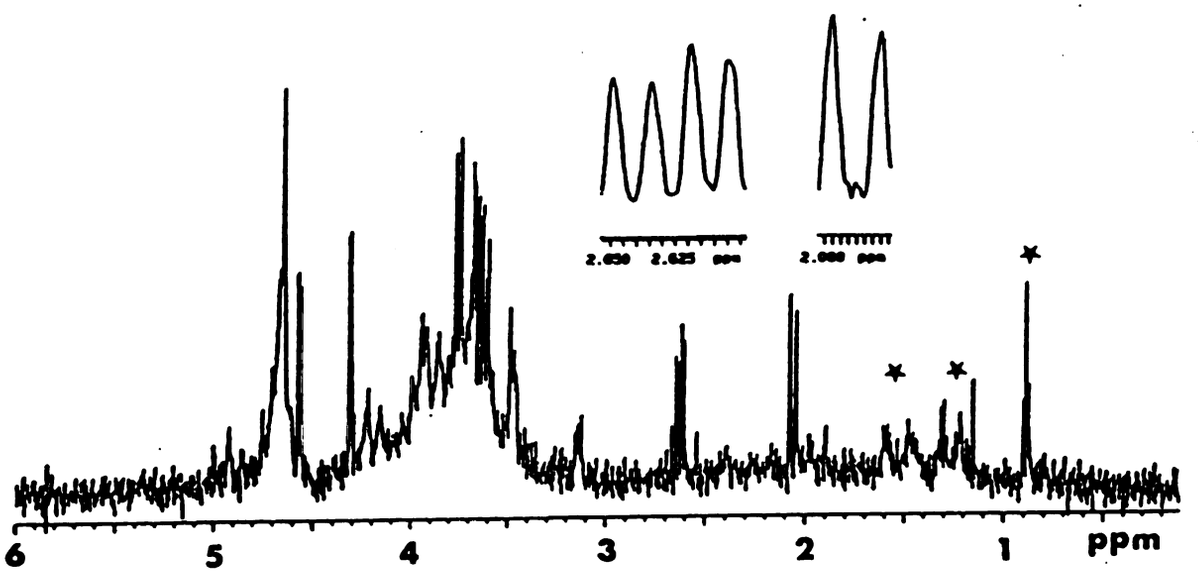
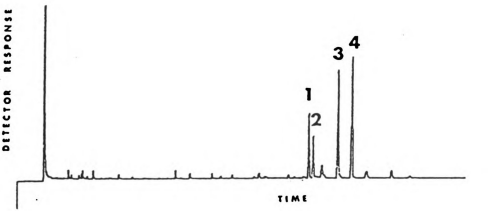

0.25
0.2
0.15
0.1
0.05
0
Absorbance 490 nm
A
B
1
5
10
15
20
25
30
35
40
45
50
Fraction number

FIG. 5. ^{1}H-NMR spectrum of the oligosaccharide eluting as peak 1 on the ion-exchange chromatography (DEAE) column. This peak corresponded to an unusual oligomer, where the presence of a two deoxy sugar component is indicated by the doublet of doublets with resonances at 2.02 ppm, and of another doublet of doublets with resonances at 2.60 ppm relative to external Me_4Si. Peaks marked with an asterisk are impurities.


2.650 2.625 ppm
2.000 ppm
6
5
4
3
2
1
ppm

contained a mixture of oligomers. Further purification by ion-exchange chromatography on DEAE Sephadex resulted in the separation of this material into two major carbohydrate peaks (1 and 2, Figure 4). The ^{1}H-NMR spectrum of the oligosaccharide eluting as peak 2 corresponded to the previously mentioned tetrasaccharide found in cells grown in neutral media; it contains galactose, mannose, galacturonic acid and 3-deoxy-2-octulosonic acid. The ^{1}H-NMR spectrum (Figure 5), of the predominant oligosaccharide eluting as peak 1, was similar to that of an unusual oligomer isolated from a ***Rhizobium leguminosarum*** biovar ***viciae*** mutant which was impaired in the synthesis of the usual tetrasaccharide (39). This oligosaccharide has been tentatively identified as a trisaccharide containing mannose, galactose and an unusual deoxysugar. The deoxy sugar component was indicated by the presence of an upfield doublet at 2.02 ppm and by a doublet of doublets with resonances at 2.60 ppm. The glycosyl composition of this oligosaccharide as determined by GC and GC/MS indicated the presence of mannose, galactose, and equal proportions of two additional peaks, identical to those observed in the mutant strain (39) (Figure 6). Similar analyses on other strains of ***Rhizobium*** indicated that this oligosaccharide was also found in the LPS of ***Rhizobium leguminosarum*** bv ***viciae*** 128C53 and bv ***phaseoli*** strain UMR1632, only when these cells were grown under acidic conditions (pH 4.5).

FIG. 6. Gas chromatography profile of the alditol acetate derivatives from the unusual acid-induced LPS component. Peaks 1 and 2 are from mannose and galactose respectively. Peaks 3 and 4 are from the unidentified deoxy glycosyl component.


DETECTOR RESPONSE
1
2
3
4
TIME

When ***Rhizobium leguminosarum*** bv. ***trifolii*** strains ANU843 and0403 were grown in acidic media, no indication of the production of this unusual oligosaccharide was observed for either of them.

DISCUSSION

The dynamics of the chemistry of the bacterial cell-surface presents a complication and a challenge in the study of its antigenicity, and in defining the role of the cell-surface carbohydrates in symbiosis. Changes in the methylation of glycosyl components of the "O-antigen" chain and extracellular polysaccharide (EPS) of ***Rhizobia***, as a function of growth stage, have been demonstrated in several systems (10,18,21,22). There is also known to be a direct link between culture age and medium pH (25), which demonstrated that fast-growing ***Rhizobia*** produced a reduction in the pH of the medium as culture age progressed. Recently, Bhat and Carlson (1), demonstrated specific changes in the extent of methylation of the "O-antigen" of a strain of ***Rhizobium leguminosarum*** biovar ***phaseoli***. The general nature of these modifications is in agreement with observations in this study. It is evident that when the redox potential in the medium changes directly by adjusting the pH or, indirectly by culture age, one result is that the glycosyl components of the "O-

antigen" of those organisms are methylated.

The capacity of any given strain of ***Rhizobium*** to synthesize more than one extracellular polysaccharide (EPS) type has been demonstrated in at least two ***Rhizobium meliloti*** systems (15,38). In both instances, another EPS type was synthesized in a mutant strain instead of the usual succinoglycan. The capacity of ***Rhizobium meliloti*** to synthesize more than one LPS type was also demonstrated (35,36) by the characterization of a gene that regulates the synthesis of another LPS species, in addition to the predominant type synthesized by the wild type organism. In recent work (39), we demonstrated that an infection-defective mutant strain of ***Rhizobium leguminosarum*** biovar ***viciae*** VF-39 which was incapable of synthesizing a previously described tetrasaccharide component (as was the wild type organism), synthesized, instead, an LPS molecule that contained an unusual deoxy glycosyl component. A trisaccharide fragment from this LPS type was isolated and partially characterized. The nature of the genetic and environmental control of the synthesis of these different LPS species is as much a mystery as is their physiological significance and role in symbiosis.

This study describes a direct link between an important environmental parameter in symbiosis (pH) and the synthesis of

this unusual bacterial cell-surface component. It also supports the observations of Kannenberg and Brewin, and Vanden Bosch et al. (19,33). These investigators found, using monoclonal antibodies, that in ***Rhizobium leguminosarum*** biovar ***viciae***, a cell-surface carbohydrate antigen (not chemically characterized) was only expressed in mature regions of the nodule by membrane-enclosed bacteroids (including immature forms), and by bacteria in infection threads located between bacteroid-containing plant cells in mature nodule tissue where the oxygen tension is expected to be low (33). This same antigen was also recognized by the same monoclonal antibodies in free-living cultures grown under either low oxygen or low pH environments (19).

In this study we have shown that ***Rhizobium leguminosarum*** bv. ***viciae*** and ***phaseoli*** adapt to acidic environmental conditions seemingly by shutting off or down-regulating the production of normally synthesized cell-surface carbohydrates, and by inducing the production of new antigens, in this case a trisaccharide containing an unusual 2-deoxy sugar. These changes in the cell-surface chemistry might explain the observations described by Kannenberg and Brewin, and Van den Bosch et al., (19,33). In early stages of infection, bacteria may produce certain carbohydrate antigens which might be the same ones associated with cells grown in neutral environments

and normal oxygen tension. As bacteria go deeper into the infection thread, and further into mature regions of the nodule, the cell-surface adapts to conditions where oxygen tension is limiting and pH has dropped, by synthesizing new carbohydrate antigens. This scenario, supported by previous findings (19,33), is correlated with the production of new LPS or other cell-surface carbohydrates, such as ones containing the newly described trisaccharide. The synthesis of this LPS, containing the unusual trisaccharide, is induced in ***Rhizobium leguminosarum*** bv. ***viciae*** and ***phaseoli*** under acidic conditions. Its presence in such large amounts in the LPS of a mutant of ***Rhizobium leguminosarum*** bv. ***viciae*** impaired in normal LPS biosynthesis, is most likely because the mutant would not be viable unless a surrogate LPS were synthesized. A more precise role in the infection process needs to be defined for this newly identified molecule. This will be facilitated by the investigation of its exact structure. This work is in progress.

This study clearly demonstrates that a reduction of the pH of the growth culture of strains of ***Rhizobium*** can lead to suppression of the synthesis of a normally abundant cell-surface antigen, and to the induction of the synthesis of a new dominant antigen which is often not even detectable at pH 7. It is interesting that while this new trisaccharide

component has been detected in all of the strains of ***R. leguminosarum*** biovar ***viciae*** studied so far, it has not been observed in the two strains of the ***trifolii*** biovars that we have analyzed. It is therefore possible that it might represent a host range indicator or contributor.

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CHAPTER 4

DYNAMICS OF THE CELL-SURFACE CARBOHYDRATE CHEMISTRY AND IMMUNOCHEMISTRY OF RHIZOBIUM LEGUMINOSARUM BIOVAR VICIAE 300 IN RESPONSE TO CHANGES IN ENVIRONMENTAL PARAMETERS AND IN BACTEROIDS WITHIN THE NODULE.

ABSTRACT

Chemical and immunochemical studies of the bacterial cell-surface carbohydrate antigens were conducted on ***Rhizobium leguminosarum*** bv. ***viciae*** 300 free-living cells grown in liquid media with variations in several nutrient and environmental parameters. The presence and distribution of these carbohydrate epitopes in free-living cells, were correlated with the presence and distribution of carbohydrate epitopes in bacteria inside nodules. Antibodies were generated against immunogens synthesized from the purified tetra- and trisaccharide components of the lipopolysaccharide (LPS), and against the so-called "O-antigen fragment" from vegetative cells. Results indicated that the relative proportions of tetrasaccharide and trisaccharide synthesized by free-living cells were influenced by nutrient and environmental parameters, and by the presence of flavone inducers, indicating that the synthesis of these two oligosaccharides is independent of each other.

Cells grown under different conditions synthesized a wide variety of different "O-antigen" fragments (and hence LPS types), which were components of LPS agregates held together

by hydrophobic and electrostatic interactions. Mild acid hydrolysis of these LPS agregates yielded a family of oligosaccharides one of which was fairly conserved under our growth conditions. Differences were observed, however, in the levels of methylation and acetylation of this conserved oligosaccharide.

^{1}H-NMR observations indicated that small amounts of a capsular polysaccharide (CPS), devoid of its usual negatively charged non-carbohydrate substituents, were recovered from free-living cells grown under low oxygen or low pH.

The specific cell-surface carbohydrates normally found in ***Rhizobium leguminosarum*** vegetative cells were present in bacteria inside nodules. The distribution of these cell-surface antigens in cells grown under low oxygen conditions, or low pH, resembled the distribution of these antigens on the surface of bacteroids inside the nodule. These two growth conditions are potential bacteroid-like models for the study of cell-surface structures.

INTRODUCTION

The possible role of bacterial cell-surface carbohydrates in early stages of the interaction between bacteria of the genus ***Rhizobium*** with legumes, leading ultimately to the formation of nitrogen-fixing organs called nodules, has been the focus of much research. One early theory focused on the role of "carbohydrate receptors" on the bacterial cell-surface which interact with lectins released from the legume roots (2). Since then, numerous studies have been conducted addressing the continuous involvement of cell-surface carbohydrates in all stages of nodule development, including recognition and attachment, nodule development and maintenance, and release of bacteria from the infection thread (2,4,8,11,12,14,28,31,36,43).

Several complications arise in trying to define the role of bacterial cell-surface carbohydrates in symbiosis. These include the capacity of bacterial strains to synthesize more than one extracellular polysaccharide (EPS) and lipopolysaccharide (LPS), with their synthesis regulated in a complicated fashion (18,50,51), and the constant changes of bacterial cell-surface carbohydrate epitopes in response to

environmental conditions (1,25,44,48).

The study of the contribution of cell-surface carbohydrates to the plant/bacterium symbiosis has been facilitated by the elucidation of the complete structure of the capsular polysaccharide (CPS) for some strains (22,35,38), and structures of some components of the lipopolysaccharide (LPS) (7,20,21,23,27), for vegetative cells grown at pH 7 with good aeration. Two LPS components of ***Rhizobium leguminosarum*** bv. ***trifolii*** 843 have been isolated. One was found to be a tetrasaccharide containing galactose, mannose, 2-keto-3-deoxyoctusolonic acid (KDO) and galacturonic acid (20,21). The other one was identified as a trisaccharide containing two galacturonic acid residues and KDO (7,21). These two structures appear to be conserved in the ***Rhizobium leguminosarum*** biovars.

Immunological characterization of different cell-surface epitopes of free-living bacteria and bacteroids,' within the infection thread and nodule, has been done using either antibodies raised against whole cell extracts (3,13), or undefined fractions of the LPS (1,15,25,44,48). The most recent of these studies tries to correlate changes in surface chemistry, observed in free-living bacteria grown in acidic environments or low oxygen, with changes known to occur within the nodule (1,25).

The expression of genes involved in nodulation are also thought to be controlled by plant metabolites. These plant factors have been identified as flavones (17,34,37). It is unclear what role (if any) these molecules have on the differential expression of the various carbohydrate cell-surface antigens of the bacterium. Demonstrating that the presence or absence of flavones in the culture medium has a direct effect on bacterial cell-surface chemistry is an important step in defining a link between nod gene function and LPS and CPS structure. The relevance of nod gene expression to bacteria in different stages of bacteroid development has been addressed in the ***Rhizobium meliloti*** system by experiments involving fusion of the relevant nod genes with gus A (41). These experiments indicated that, in alfalfa nodules, the inducible nod genes are not expressed at all in later stages of the symbiosis, and that the levels of expression of nod D1 and nod D3 are reduced in older zones of the nodule. Similar results were obtained in the ***Rhizobium leguminosarum*** bv. ***viciae/Pisum sativum*** system using very different methods (40). Here the strategy was to look for both transcription and translation of nod genes using a combination of Northern (RNA) blot and in situ RNA hybridization, and by immunoblots using antibodies raised to specific nod proteins. Both of these studies underline the dynamics of nod gene expression as the infection process and symbiosis progresses, but a link between these events and bacterial cell-surface

chemistry is missing.

Direct chemical characterization of the changes that occur in the cell-surface carbohydrates of bacteria during nodule development is made difficult by the amount of material needed. It is also complicated by the presence of carbohydrate material of plant origin. Artificial systems where bacteria undergo changes that resemble those found under nodule conditions need, therefore, to be developed to facilitate these studies. Bacterial forms whose shape and morphology resemble in-planta bacteroids have been induced with succinate-enriched media (45,46), and with acidic conditions or microanaerobic environments (25). These models could provide valuable information on the dynamics of bacterial surface chemistry during the infection process, especially if the carbohydrate epitopes characterized in them are also found in actual plant bacteroids.

In this study on ***Rhizobium leguminosarum*** bv. ***viciae*** 300, we chemically characterized several LPS epitopes of cells grown in liquid media with variations in several nutrient and environmental parameters such as succinate enrichment, low pH and low-oxygen tension and the presence of flavone inducers. We also used antibodies raised against synthetic immunogens made with cell-surface components from vegetative cells, and whose exact structures or compositions are known. These structures were a tetrasaccharide and a trisaccharide from the

LPS, purified and characterized O-antigen fragments and a purified, characterized CPS oligomer. This study allows us to answer questions about the presence and spatial distribution of these chemically defined species in the free-living cells, using light microscopy. These same antibodies were then used to probe the cell-surface of bacteroids isolated from nodules, and of bacteroids observed in-situ within the nodules. In an effort to assess the impact of the independent environmental parameters on cell-surface chemistry, correlations were made between the presence and distribution of these carbohydrate epitopes in bacteroids inside nodules, and free-living cells obtained under different growth conditions.

MATERIALS AND METHODS

General methods: Compositional analysis of the O-antigenic fragments was performed by converting the glycosyl components to their alditol acetate derivatives (39). These derivatives were analyzed by gas chromatography (GC), using a capillary DB 225 column and flame ionization detector. The running conditions were the following: initial temperature of 180°C, rate of 2 °C/min, final temperature of 230°C, final hold time 55.00 min, and a run length of 70.00 min. Glycosyl composition was confirmed by combined gas chromatography/mass spectrometry (GC-MS), on a JEOL 505 mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR300 spectrometer operating at 300 MHz for protons. Spectra were obtained in deuterium oxide. Carbohydrate content in fractions from gel filtration columns was monitored by the phenol/sulfuric acid method (16).

Bacterial culture conditions. *Rhizobium leguminosarum* bv. *viciae* 300 was grown aerobically in modified Bergensen's (BIII) liquid medium (pH 6.9) at 30°C, as previously described (9). Briefly 250 ml of cells in stationary phase were inoculated into 4 liter flasks containing 2 liters of BIII medium, and incubated until early stationary phase. The culture medium was

adjusted to pH 4.5 by adding 2 liters of BIII medium at pH 3.2, to 2 liters of cell culture at late exponential growth in neutral BIII medium. The resulting cell suspension was adjusted to pH 4.5 with HCl, and split into batches of 2 liters contained in 4-liter Erlenmeyer flasks. To the culture medium was added a succinate-rich syrup (casamino acids 100 mg/L, yeast extract 100 mg/L, sodium succinate 450 mg/L and glucose 400 mg/L); 20 ml of syrup was added to 2 liters of culture medium contained in a 4-liter Erlenmeyer flask, 4 hours after inoculation with bacteria. Cells were further grown until early stationary phase. Microanaerobic conditions were created by stoppering the 4 liter Erlenmeyer flasks containing 2 liters of culture medium, with butyl rubber stoppers at the time of inoculation, to restrict oxygen exchange with the atmosphere. Cells were grown until early stationary phase.

LPS isolation and purification. LPSs were extracted from cells using a hot phenol/water method (49). After dialyzing, concentrating and treating the aqueous phase with DNAse and RNAse, the LPS was purified by gel permeation chromatography on Sepharose 4-B, with aqueous formic acid (0.05M, adjusted to pH 5.5 with concentrated ammonia) as eluent. After releasing the carbohydrates by mild hydrolysis (1% acetic acid at 100 °C for 2 hours), and extracting the released chloroform-soluble lipids, the aqueous layer was lyophilized and fractionated by size exclusion chromatography on Biogel P-2, with 1% aqueous

formic acid as the mobile phase. The fractions which eluted as a peak were pooled, lyophilized and analyzed by ^{1}H-NMR spectroscopy. Their glycosyl compositions were determined as the alditol acetate derivatives after hydrolysis, reduction and acetylation. Fractions which appeared impure by these two methods were subjected to further chromatography by gel permeation chromatography on Biogel P-10, with 1% aqueous formic acid as the mobile phase. Separation of some of these peaks was further achieved by ion-exchange chromatography on DEAE-Sephadex, in aqueous formic acid (0.01% adjusted to pH 5.5 with ammonia), and eluting with a linear gradient of 0-0.2M ammonium chloride.

Synthesis of the carbohydrate antigens and production of polyclonal antibodies. Allyl glycosides were prepared from the purified tetrasaccharide and trisaccharide, and from the "O-antigen" fragments of the LPS of ***Rhizobium leguminosarum*** bv. ***viciae*** 300 vegetative cells. Polyclonal antibodies were produced as described in Chapter 2 (pages 47-48). Also, Chapter 2 (page 68) shows the results of the ELISA test conducted to assay the specificity and reactivity of these antibodies against specific carbohydrate antigens.

Growth of plants and inoculation with bacteria. Seeds of Freezonian peas were grown following procedures previously described (47). Seeds were sterilized with 10% bleach for half hour, and rinsed with 5 changes of sterile water for 30 minutes each change. Sterilized seeds were placed in petri

dishes containing sterile water for imbibition overnight, and then transferred to Jensen agar plates for germination. Three day old seedlings were inoculated with ***Rhizobium leguminosarum*** bv. ***viciae*** cells and transferred into 500 ml erlenmeyer flasks containing 150 ml of Jensen agar media. Root nodules were harvested 4 weeks after inoculation.

Bacteroid isolation from nodules. Bacteroids were isolated from 4-week old red nodules by squashing them. Nodule contents were collected on a microscope slide, air dried, heat fixed, and washed with water.

Fluorescence light microscopy on nodule sections. Procedures were adapted from Klomparens et al. (26). Red nodules were harvested from pea roots and fixed overnight in 2% paraformaldehyde. Specimens were dehydrated in ethanol series (25,50,75,95,100%; 30 minutes each), and infiltrated in epon-spurr resin by successive 8 hour incubations with mixtures of epon-spurr and 100% ethanol in 1:2 and 2:1 ratios, followed by two incubations in 100% epon-spurr for 16 hours, with a change of resin at 8 hours. Specimens were transferred to gelatin capsules containing epon-spurr resin, and allowed to polymerize for 16 hours at 60°C. Semi thick sections (0.5 µm) were cut with a microtome, equipped with a glass knife. Sections were mounted on a microscope slide and heat fixed, covered with 3% bovine serum albumin, and incubated at room temperature for 1 hour. Sections were then prepared for immunofluorescence microscopy using the antibodies raised

against the different LPS substructures, and stained with acridine orange (100 ppm in water), for 5 minutes.

Immunofluorescence microscopy: Procedures used were adapted from (42) and are described in Chapter 2 (page 49).

RESULTS

Quantitation of the relative proportions of tetrasaccharide and trisaccharide synthesized in response to variation of environmental parameters. Size exclusion chromatography on Biogel P-2 (with 1% formic acid as the mobile phase), of the aqueous fraction of the acetic acid hydrolysate of the LPS from cells grown under normal conditions (pH 6.9), resulted in the separation of the mixture into three components. The first eluting peak, which voided the column, corresponded to the so called "O-antigen" fragment. The second and third eluting peaks corresponded, respectively, to a tetrasaccharide and a trisaccharide (7,20,21) normally found in wild type strains of *Rhizobium leguminosarum* biovars.

The tetrasaccharide had a structure identical to one first characterized in *Rhizobium leguminosarum* bv. *trifolii* ANU843 (20,21), except that it lacked an acetyl group. It contained mannose, galactose and galacturonic acid all in the pyranose form and all α-linked. The fourth glycosyl component was 3-deoxy-2-octusolonic acid. The trisaccharide was also the same as that characterized in ANU 843 (7,21), and contained two α-linked residues of galacturonic acid in the pyranose form, and

one 3-deoxy-2-octusolonic acid residue.

The anomeric proton resonances in the ^{1}H-NMR spectra of these tetra- and trisaccharide molecules were well separated, and it was possible to quantitate their relative amounts by integration of these signals. We compared the relative proportions of tetrasaccharide versus trisaccharide synthesized by cells grown under different environmental conditions, by measuring the intensity of the signals belonging to the anomeric protons in a mixture that contained all of the tetrasaccharide and trisaccharide extracted. The proportion of tetrasaccharide to trisaccharide synthesized in cells grown under neutral or low-oxygen conditions in the absence of flavone inducers was 1:2.5 and 1:1.2, respectively. This proportion, which reflected a greater amount of trisaccharide, was reversed for cells grown in acidic (pH 4.5) or succinate-enriched medium in the absence of flavone inducers. In the latter case, the proportion of tetrasaccharide synthesized increased over the proportion of trisaccharide in a ratio of 2.3:1 and 1.23:1, respectively. Except for cells grown under normal conditions, the amount of tetrasaccharide synthesized was further increased when the flavone inducer naringenin was added to the medium. The proportions of tetrasaccharide to trisaccharide synthesized were 1.5:1 and 1.7:1, respectively, for cells grown in succinate-enriched medium or under low-oxygen conditions in

the presence of naringenin. Trisaccharide production was not detected by ^{1}H-NMR spectroscopy for cells grown in acid medium (pH 4.5) in the presence of naringenin.

These observations partially explain the increase in intensity of the second peak that eluted from the P-2 column, that usually corresponded to the tetrasaccharide. In addition to the usual tetrasaccharide found in this second peak, we also found coeluting with it, only under acidic conditions an unusual trisaccharide previously reported (Chapter 3). This unusual trisaccharide was not detected by ^{1}H-NMR spectroscopy when naringenin was added to the medium. Cells grown under low-oxygen or in succinate-enriched medium synthesized unusual unidentified oligomers, which are currently under investigation.

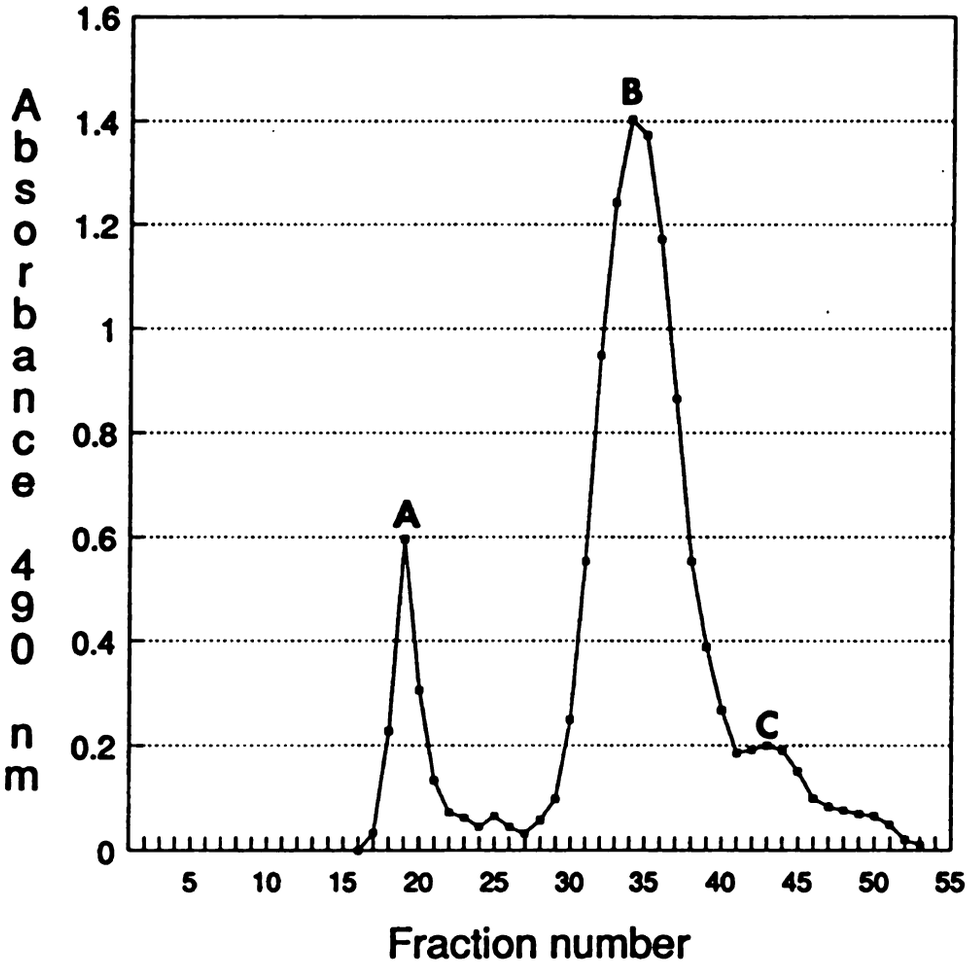
Chemical characterization of the "O-antigen" fragment of cells grown under different environmental conditions. The "O-antigen" fragment, the first eluting peak from the Biogel P-2 column, of cells grown under normal conditions contained 6-deoxyhexoses (rhamnose and fucose), glucose and 2-amino-2,6-dideoxyhexose in an approximate ratio of 2:2:1. In addition, there were smaller amounts of O-methyl-6-deoxyhexoses and traces of mannose and galactose. The purity of this fragment was further assessed by gel permeation chromatography over Biogel P-10, with 1% formic acid as the mobile phase. Two

peaks were recovered. The first one voided the Biogel P-10 column, and had a ^{1}H-NMR spectrum which corresponded to the CPS previously reported for ***Rhizobium leguminosarum*** bv. ***trifolii*** ANU843 (22). Fractions eluting in the second peak, already known to be a mixture of oligosaccharides normally referred to as "O-antigen" fragment (Chapter 2), were pooled, lyophilized and saved for further analysis. The "O-antigen" fraction recovered from cells grown under normal conditions in the presence of naringenin showed similar characteristics, by ^{1}H-NMR spectroscopy and GC/MS analysis, to those of the fraction synthesized by the cells in the absence of the flavone inducer.

The fraction that voided the Biogel P-2 column was also recovered from cells grown under different environmental conditions and purified on Biogel P-10, with 1% ammonium formate as the mobile phase. For cells grown in acid medium or acid medium with naringenin, only one peak was recovered, indicating that no further fractionation was achievable by size exclusion chromatography. This peak did not void the column, indicating that the "O-antigen" was devoid of the usual capsule.

For cells grown with succinate-enriched medium in the absence of naringenin, the "O-antigen" was resolved into three peaks (Figure 1). Their ^{1}H-NMR spectra and glycosyl

FIG. 1. Gel permeation chromatography (Biogel P-2) profile of the "O-antigen" fragment of the LPS of cells grown in succinate-enriched media. Carbohydrate content was monitored by the phenol/sulfuric acid assay.


Absorbance 490 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A
B
C
5
10
15
20
25
30
35
40
45
50
55
Fraction number

composition analyses revealed that these were three distinct fragments. Peak A corresponded to a high molecular weight acetylated glucan (Figure 2A), where the signal at 1.8 ppm corresponded to acetate released after the hydrolysis of the glucan with 0.1M NaOD. Both the glycosyl composition and the ^{1}H-NMR spectrum of peak B were very similar to the "O-antigen"fragment obtained from the LPS of cells grown under normal conditions, except that there was an increase in the methylation levels. In the ^{1}H-NMR spectrum of this fraction (Figure 2B), resonances between 1.0 and 1.25 ppm (relative to external Me_4Si), corresponded to methyl protons of 6-deoxy hexoses and signals between 1.75 and 2.25 ppm to methyls of O- and N-attached acetyl groups. The signal at 2.6 ppm corresponded to N-methyl and intense singlets between 3.25 and 3.4 ppm to O-methyls. The cluster of signals around 5.1 ppm was assigned to anomeric protons. The solvent line appeared as a broad singlet at 4.65 and the rest of the signals corresponded to carbohydrate ring protons. These observations were confirmed by analyzing the glycosyl composition of the peak B fraction by GC/MS (Figure 3B). This revealed the presence of 3,4-O-methyl-6-deoxyhexose, 3-O-methyl-6-deoxyhexose, fucose and 2-amino-2,6-dideoxyhexose in an approximate ratio (as determined by the detector response) of 1:2:1:2. Rhamnose, mannose, galactose and glucose were present in trace amounts. This suggests that enrichment of the media with succinate causes the "neutral O-antigen" to undergo side

FIG. 2. (A): ^{1}H-NMR spectrum of the high molecular weight acetylated glucan eluting as peak A in Figure 1. The signal at 1.8 ppm corresponds to acetate released after hydrolysis with 0.1M NaOD. (B): ^{1}H-NMR spectrum of the fraction eluting as peak B in Figure 1. The spectrum of this fraction was very similar to the spectrum of the "O-antigen" fraction obtained from the LPS of cells grown under normal conditions except that there was an increase in the methylation levels. In this figure resonances between 1.0 and 1.25 ppm (relative to external Me_4Si) correspond to methyl protons of 6-deoxyhexoses and signals between 1.75 and 2.25 to methyls of O- and N-attached acetyl groups. The signal at 2.6 ppm corresponded to N-methyl and the intense singlets between 3.25 and 3.4 ppm to O-methyls. The cluster of signals at around 5.1 ppm was assigned to anomeric protons. (C): ^{1}H-NMR spectrum of the fraction eluting as peak C in Figure 1. Note the decrease in acetylation and methylation with respect to peak B judged by the decrease in the intensity of the corresponding signals at 1.75-2.25 ppm and 3.25-3.4 ppm with respect to those of the methyl protons of the 6-deoxyhexoses between 1.0 and 1.25 ppm.

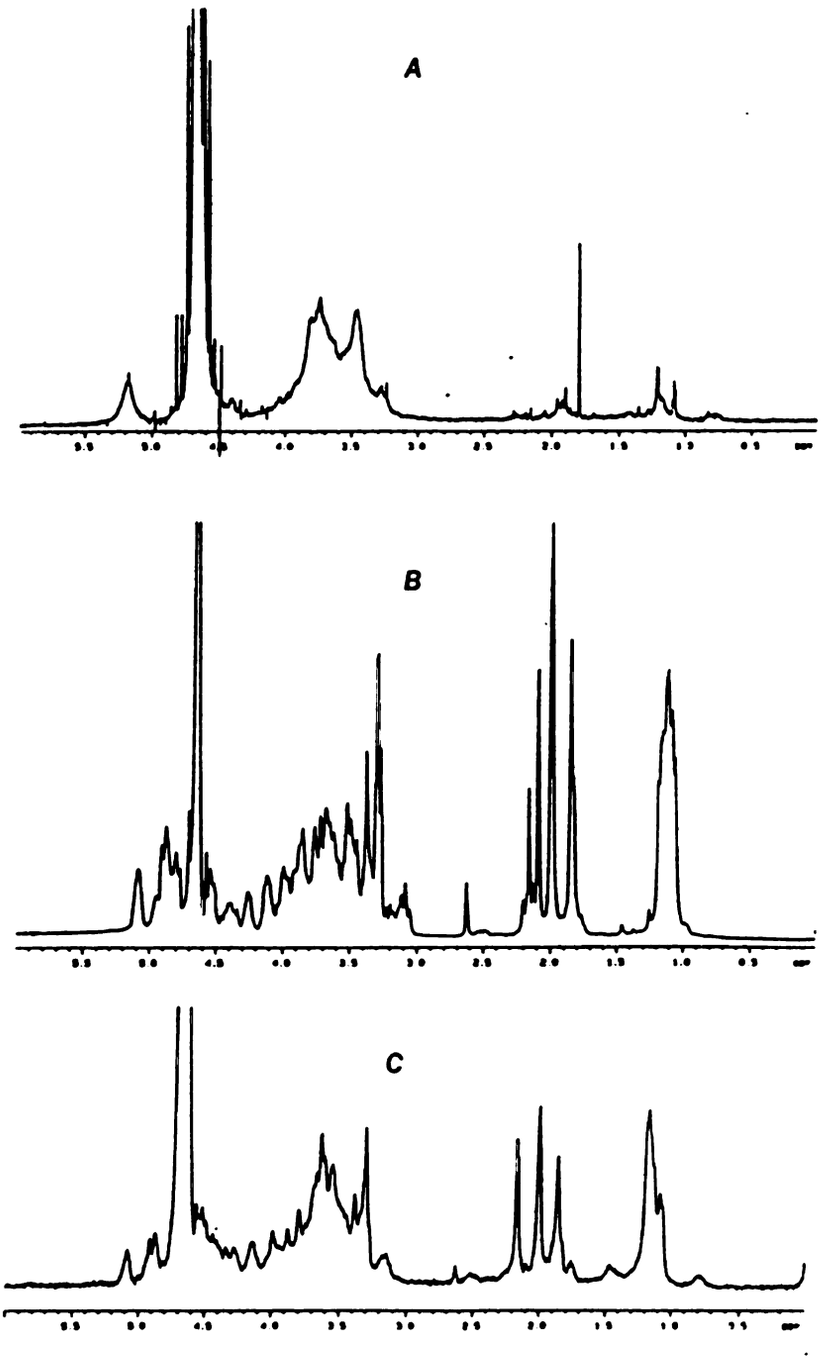
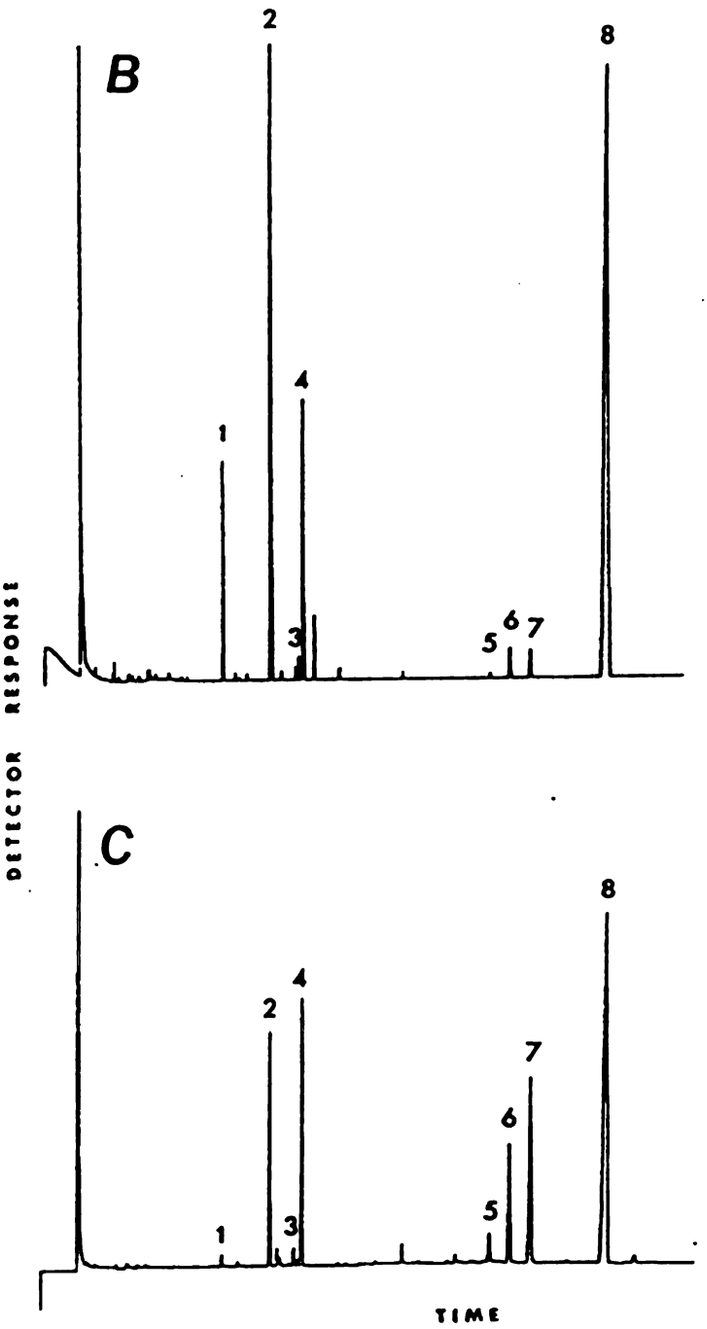

A
B
C

FIG. 3. Gas chromatography profile of the alditol acetate derivatives of the sugar components of the oligosaccharides eluting as peak B, and C respectively from the size exclusion chromatography over Biogel P-10. In each chromatography profile peaks labeled 1 through 8 correspond respectively to 3,4-O-methyl-6-deoxyhexose, 3-O-methyl-6-deoxyhexose, rhamnose, fucose, mannose, galactose, glucose and 2-amino-2,6-dideoxyhexose.


B
C
1
2
3
4
5
6
7
8
DETECTOR RESPONSE
TIME

chain methylation at the 6-deoxyhexose sites, causing an increase in the proportion of the O-methyl-6-deoxy hexoses at the expense of the 6-deoxy hexoses. The ^{1}H-NMR spectrum of peak C is shown in Figure 2C. A decrease in acetylation and methylation was observed, judged by the decrease of the intensity of the corresponding signals at 1.75-2.25 ppm and at 3.25-3.4 ppm, with respect to those of the methyl protons of the 6-deoxyhexoses between 1.0 and 1.25 ppm. Results of the glycosyl analysis of the peak C fraction supported these observations (Figure 3C), where the 3,4-O-methyl,6-deoxy hexose was not present and the 3-O-methyl-6-deoxyhexose was present in the same proportion. The rest of the glycosyl components, fucose, galactose, glucose and 2-amino-2,6-dideoxyhexose were present in an approximate ratio of 2:1:1:2. Traces of mannose were present. Cells grown in succinate-enriched media in the presence of the flavone inducer synthesized an "O-antigen" fraction that contained primarily glucose.

Separation of the "O-antigen" fragment by gel permeation chromatography on Biogel P-10 was also observed for cells grown under low-oxygen conditions in the absence and presence of naringenin. In these cases two different peaks were observed. For cells grown in the absence of the flavone inducer, this first peak corresponded to the CPS of normal cells, but ^{1}H-NMR spectroscopy indicated that it lacked the

negatively charged non-carbohydrate pyruvyl substituents as well as 3-hydroxybutanoyl groups. The ^{1}H-NMR spectrum of the second peak had features similar to those of "O-antigen" of cells grown under normal conditions, however, the levels of acetylation and methylation were less. This was confirmed by analysis of its glycosyl composition. This "O-antigen" fragment was also similar to the one produced by cells grown under low-oxygen conditions in the presence of naringenin which was also devoid of methylated sugars. Only traces of the typical methylated sugars (3,4-O-methyl and 3-O-methyl-6-deoxyhexoses), were detected during analyses by GC/MS. Traces of rhamnose, mannose and galactose were also found. The major glycosyl components: fucose, glucose and 2-amino-2,6-dideoxyhexose, were present in an approximate ratio of 1:2:2. Low-oxygen conditions, therefore, prevented methylation of the side chain.

Cells grown under a combination of low-oxygen, low pH (pH 4.5), succinate enrichment and in the presence of naringenin, yielded two peaks on the Biogel P-10 column. The first one was a fraction that resembled the "O-antigen" fragment obtained from normal cells. ^{1}H-NMR analysis showed a slight increase in the amount of acetylation and methylation in this first fraction, compared to the corresponding one obtained from normal cells. These results were consistent with those obtained from the glycosyl compositional analyses by GC/MS.

These analyses indicated that 3-O-methyl-6-dideoxyhexose, rhamnose, fucose, glucose and 2-amino-2,6-dideoxyhexose were present in an approximate ratio of 1:1:2:2:2, and that mannose and galactose were present in trace amounts. The second eluting peak from the P-10 size exclusion column was a high molecular weight galacto-glucan, which contained galactose and glucose in an approximate 1:2 ratio. This galacto-glucan was smaller than the high molecular weight glucan described earlier and obtained from cells grown in succinate-enriched media, where the glucan molecule eluted first and contained mannose and glucose in a 1:1 ratio.

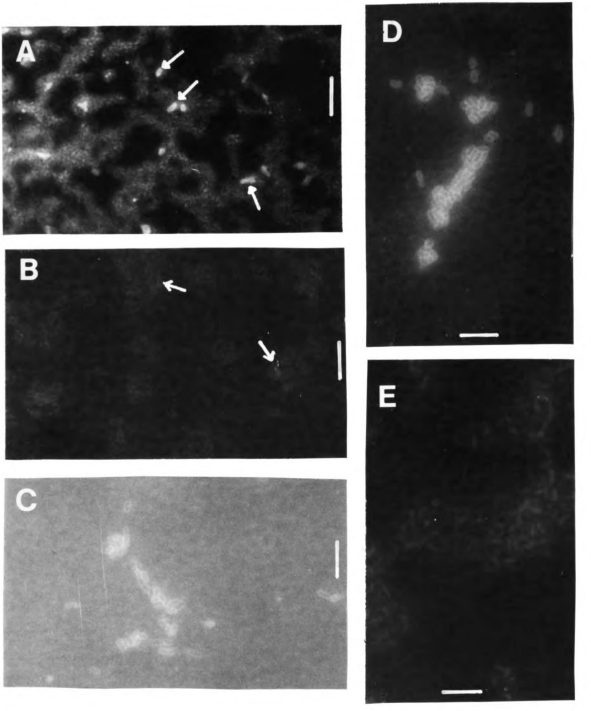
Further separation of the peaks that eluted from the P-10 column was achieved using ion-exchange chromatography (DEAE Sephadex), indicating that the so-called "O-antigen" is a family of distinct oligosaccharides.

Immunocytochemical characterization of cell-surface carbohydrates. Antibodies were raised specifically against the conserved tetrasaccharide and trisaccharide, against two oligosaccharide fragments obtained from the "O-antigen" chain of cells grown under normal conditions (which we will refer to as O-antigen 1 and O-antigen 2), and against the CPS. The antibodies were used to determine by indirect immunofluorescence microscopy the spatial distribution of these epitopes on cells grown under the different

environmental conditions. As described earlier in this dissertation, cells grown under normal conditions in BIII medium exposed epitopes corresponding to the LPS substituents only in a few localized areas of the cell-surface, while the CPS was available for reaction with its respective antibody all around the cell-surface (Chapter 2).

Cells grown in succinate-enriched medium. When cells were grown in succinate-enriched medium in order to induce bacteroid forms (46,47), most cells swelled and were larger in size than cells grown under normal conditions. Some of them were more refractile under the light microscope using phase contrast optics. A large variability in the resulting cell sizes was observed. When these cells were incubated with antibodies raised against the capsule, few cells reacted (Figure 4A, see arrows). This is consistent with what we had previously observed by ^{1}H-NMR spectroscopy and glycosyl compositional analysis. These techniques indicated that these cells synthesized a high molecular weight glucan that caused them to "clump" together inside of a matrix. This matrix probably also contained some normal CPS. Fluorescent dye was observed in the background distributed in a pattern that followed the matrix. When cells were incubated with either the antibodies against the O-antigen 1 or the O-antigen 2 (Figure 4B and 4C respectively), cells showed an uneven distribution of fluorescence intensity on their surface. Arrows in Figure 4B point at some of the few cells where the dye was found

FIG. 4. Spatial distribution of the CPS (A), O-antigen 1 fraction (B), O-antigen 2 fraction (C), tetrasaccharide and trisaccharide on the cell-surface of free-living ***Rhizobium leguminosarum*** bv. ***viciae*** cells grown in succinate-enriched medium. These cells synthesized a high molecular weight glucan that caused them to "clump" together. The glucan formed a matrix which probably contained some normal CPS. Only few cells reacted with antibodies against the CPS (A). An uneven distribution of fluorescence intensity around the cell-surface was observed in cells incubated with antibodies against the O-antigen 1 or O-antigen 2 (B,C). Most cells reacted evenly with antibodies against the tetrasaccharide (D). The larger and more refractile cells under the light microscope using phase contrast reacted with the anti-trisaccharide antibody (E). Notice the variability in sizes and morphologies. (Bar = 5 μm)


A
B
C
D
E

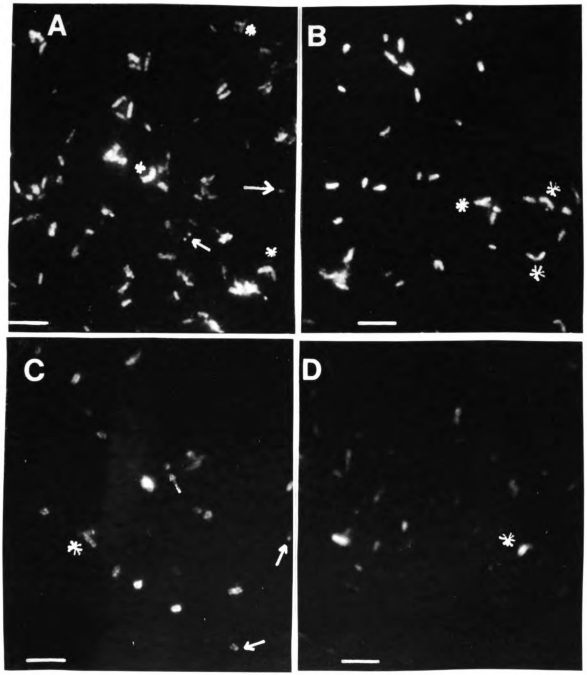
evenly distributed on their surface. Most cells reacted evenly with antibodies against the tetrasaccharide (Figure 4D), few of them (most of them larger and darker in phase contrast), reacted with antibodies against the trisaccharide (Figure 4E). Reaction of these antibodies with the cell-surface was expected since, as we discussed in previous sections, all of the epitopes to which the antibodies were raised were synthesized by cells grown in succinate-rich medium.

Cells grown under low-oxygen conditions. Most cells grown under these conditions were darker (more refractile), in phase contrast light microscopy, than cells grown in neutral conditions, and displayed a large variability in sizes. As opposed to cells grown in succinate-enriched media, very few cells swelled. Some "Y" and "V" shaped cells were observed (see stars in Figures 5A-5D).

These cells did not react with antibodies against the capsule. This was expected because only small amounts of a modified capsule were synthesized. In this capsular material some of the negatively charged non-carbohydrate substituents (pyruvate and 3-OH butyrate), normally present in vegetative cells, were not present, giving it a much less negatively charged character.

Cells incubated with antibodies raised against the O-antigen 1 (Figure 5A) reacted differently according to their

FIG. 5. Spatial distribution of the O-antigen 1 fraction (A), O-antigen 2 fraction (B), tetrasaccharide (C) and trisaccharide (D) on the cell-surface of free-living ***Rhizobium leguminosarum*** bv. ***viciae*** cells grown under low-oxygen conditions. These cells which only synthesized small amounts of a modified capsule did not react with anti-CPS antibodies. Cells of different sizes reacted differently with anti-O-antigen 1 antibodies (A), in some smaller cells fluorescence was more intense in localized areas (see arrows) giving the impression that these were vegetative cells in the process of transformation. Stronger reactivity with anti-O-antigen 2 antibodies (B) was observed with larger cells and with "Y" and "V" shaped ones. Of the cells that reacted with anti-tetrasaccharide antibodies (C), the larger ones fluoresced more intensely than the smaller cells where the fluorescence was mostly distributed in a polar fashion (see arrows). Reaction with antibodies against the trisaccharide (D) was weak and only the largest cells (see arrows) fluoresced intensely. Notice the large variability in sizes and morphologies. (Bar = 5 μm)


A
B
C
D

sizes. Larger cells fluoresced more intensely than smaller cells. In some of the smaller cells, fluorescence was more intense in localized areas (see arrows), giving the impression that these were vegetative cells in the process of transformation. Antibodies raised against the O-antigen 2 (Figure 5B), reacted with most cells. Stronger reactivity was observed with larger cells and with "Y" and "V" shaped ones. Cells incubated with antibodies raised against the tetrasaccharide showed weaker fluorescence than that observed with antibodies against either of the O-antigens (Figure 5C). Of all the cells that reacted, larger cells fluoresced more intensely than smaller cells, where the fluorescence was mostly distributed in a polar fashion on the cell-surface (see arrows). Reaction with antibodies against the trisaccharide (Figure 5D) was very weak, and only the largest cells fluoresced intensely. These results indicated that smaller cells still resembled the vegetative state, while larger cells or "Y" and "V" shaped ones, had been transformed, so as to expose the carbohydrate epitopes in a more homogeneous fashion on their surface. The lower reactivity found with antibodies against the trisaccharide was consistent with the NMR observations discussed in the previous section, where cells grown under low-oxygen conditions synthesized less trisaccharide than tetrasaccharide.

Cells grown in acid medium (pH 4.5). A large variability in cell size and refractivity under the light

microscope using phase contrast optics was observed for this population. Antibodies against the capsule did not react with the cell-surface epitopes present on these cells. This was expected since almost no capsule was recovered from these cell cultures. Antibodies against the O-antigen 1 and O-antigen 2 reacted with most cells, though fluorescence distribution was not homogeneous. Differences in intensity could not be associated with a specific cell size or morphology. Epitopes reacting with antibodies raised against the tetrasaccharide or trisaccharide (Figure 6) were also unevenly distributed on the cell-surface. Most cells displayed stronger reactivity at a localized "pole" (see arrows). In cells grown in acid medium (pH 4.5), the LPS carbohydrate epitopes studied were exposed to react with their respective antibodies on the cell-surface. The distribution of these epitopes,however, was not homogeneous and sometimes the fluorescent label was found to be more concentrated at the poles of the cells.

Bacteroids isolated from the nodules. Bacteria isolated from nodules had specific types of morphologies (rods, elongated rods, pear shaped, "Y"-shaped and spheroidal bacteroids), that reflected different stages of differentiation: from vegetative cells, to nitrogen fixing symbiotic bacteroids, to senescent bacteroids. At least one LPS domain was found on one or more of these forms by the use of immunofluorescence microscopy (Figure 7A-D). The specific LPS carbohydrate domains, to which antibodies were generated,

FIG. 6. Spatial distribution of the trisaccharide on the cell-surface of free-living ***Rhizobium leguminosarum*** bv. ***viciae*** cells grown in acid medium (pH 4.5). Reactivity with antibodies against the respective epitopes was uneven around the cell-surface. (Bar = 5 µm)

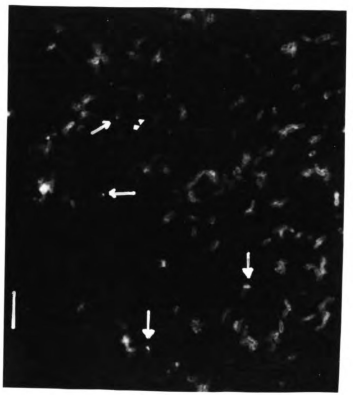
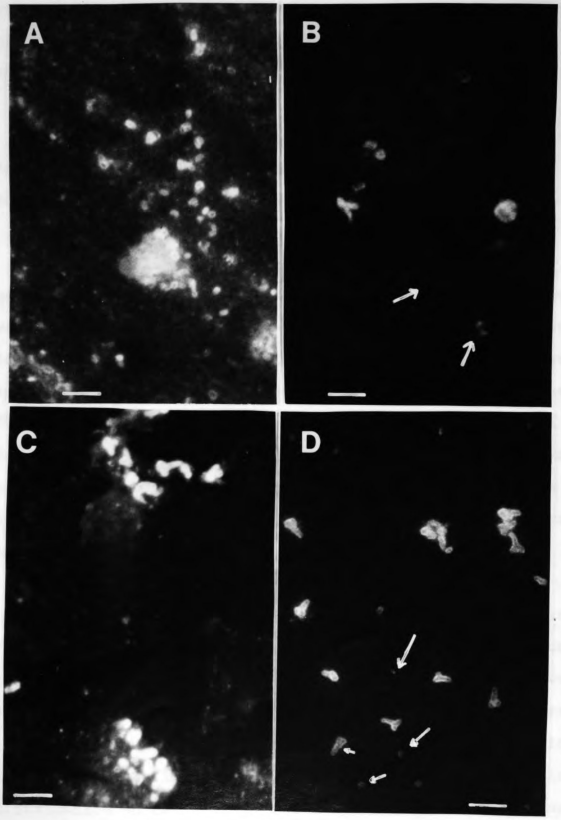


FIG. 7. Spatial distribution of the O-antigen 1 fraction (A), O-antigen 2 fraction (B), tetrasaccharide (C) and trisaccharide (D) on the cell-surface of ***Rhizobium leguminosarum*** bv. ***viciae*** cells isolated from nodules. Epitopes that reacted with antibodies against the O-antigen 1 were only detected on small rods (A). Antibodies against the O-antigen 2 reacted with a small percentage of the total cells present and reactivity was not associated with a specific morphology (B). Some "Y"-shaped bacteroids barely reacted (see arrows) while others showed intense fluorescence. Some short rods did not show much reactivity, however in others intense fluorescence was located at the poles (short arrows). Only the bacteroid forms, enlarged rods and large pear- and "Y"-shaped cells reacted strongly with antibodies against the tetrasaccharide (C). Little reactivity, concentrated at the poles, was observed with this antibody with the small vegetative-like rods (see arrows). All cells isolated from nodules reacted with antibodies against the trisaccharide (D). While these epitopes showed an uneven polar distribution in small vegetative rods (see long arrows), the polarity gradually disappeared as the cell size increased resulting in "Y"-shaped bacteroids with fairly homogeneous fluorescence distribution (arrows in decreasing length indicate gradual decrease in polar location of the epitope). (Bar = 5 μm)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were present on cells isolated from plant nodules. The actual epitope expressed by a given cell was dependent on its morphology.

Epitopes that reacted with antibodies against the O-antigen 1 were only detected on small rods and were not detected on larger or "Y"-type shape bacteroids (Figure 7A).

Antibodies against the O-antigen 2 reacted with a small percentage of the total cells present, and their reactivity was not associated with a specific morphology (Figure 7B). Some "Y"-shaped bacteroids barely reacted with these antibodies (see arrows), while others showed intense fluorescence. Short rods did not show much reactivity; in a few of them fluorescence was located at the poles. We still need to determine if these antibodies against the O-antigen 2 LPS epitope (which contains large proportions of glucose) can cross-react with material of plant origin.

Only the bacteroid forms, enlarged rods, large pear- and "Y"-shaped cells, reacted strongly with antibodies against the tetrasaccharide (Figure 7C). Little reactivity was observed with the small vegetative-like rods. All cells isolated from nodules reacted with antibodies against the trisaccharide (Figure 7D). While these epitopes showed an uneven polar distribution on the small vegetative rods (see long arrows),

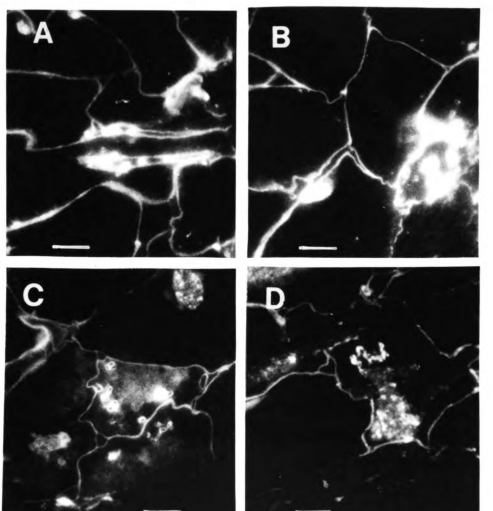
the "polarity" gradually disappeared as the cell size increased, resulting in "Y"-shaped bacteroids with fairly homogeneous fluorescence distribution (arrows in decreasing length indicate gradual decrease in polar location of the epitope). These microscopy observations indicate that the extent of antibody reactivity against the tetrasaccharide epitopes increased drastically in cells of larger size (pear- and "Y"-shaped bacteroids). Only a slight difference in reactivity was found among cells of different sizes, with antibodies against the trisaccharide.

These observations can be correlated with what was observed in free-living cells grown under low-oxygen or pH 4.5. In the previous section we reported that free-living cells grown under neutral conditions synthesized more trisaccharide than tetrasaccharide. Upon exposure to conditions where oxygen is limiting or pH is low, the proportion of tetrasaccharide produced was greater than the proportion of trisaccharide produced by these *Rhizobium* free-living cells.

The fate of these LPS epitopes was also followed by tracing their degree of expression within different zones of the nodules, using immunofluorescence microscopy. It was observed that cells which reacted more strongly with antibodies against the O-antigen 1 were still present in the

infection thread and had not yet been released into the plant cell (Figure 8A). Reactivity with antibodies against the O-antigen 2 was found mostly in bacteroids present in deeper parts of the nodule (Figure 8B). Once the bacteroids had been released into the plant cell, the greatest reactivity was observed with antibodies raised against the tetrasaccharide (Figure 8C). The reaction to the anti-trisaccharide antibody (Figure 8D) was also strong. No reactivity with antibodies against the capsule was observed with bacteria inside the nodule. However, in the same preparations, small vegetative cells sitting outside the nodule were observed to fluoresce.

FIG. 8. Spatial distribution of the O-antigen 1 fraction (A), O-antigen 2 fraction (B), tetrasaccharide (C) and trisaccharide (D) on the cell-surface of ***Rhizobium leguminosarum*** bv. ***viciae*** cells within different zones of the nodules. Cells which reacted better with anti-O-antigen 1 antibodies were still present in the infection thread (A). Reactivity with anti-O-antigen 2 antibodies was found mostly in bacteroids present in deeper parts of the nodule (B). Reactivity with anti-tetrasaccharide antibodies (C) or anti-trisaccharide antibodies (D) was mainly observed in bacteria which had been released into the plant cell. (Bar = 5 μm)


A
B
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DISCUSSION

In this study we defined some of the changes that occur in *Rhizobium* cell-surface carbohydrates as a function of environmental conditions. The structural information available on carbohydrate components of the LPS describes the conserved tetrasaccharide (20,21) and trisaccharide (7,21) "core" components of the LPS. One study (6) suggests that these two "core" components form part of the same LPS molecule. Discrepancies exist between this and other studies (20,21), which suggest the possibility that the synthesis of these two molecules is independent of each other, thus raising the possibility that they could be part of different LPS molecules.

In our previous work (Chapter 2), we reported that antibodies raised against the tetrasaccharide and trisaccharide showed similar reactivity in ELISA inhibition assays. However, vegetative cells incubated with antibodies raised against the trisaccharide showed more reactivity than cells incubated with antibodies raised against the tetrasaccharide. These observations suggested that these two oligosaccharides were not synthesized in a 1:1 ratio and,

therefore, their synthesis could be independent of each other.

This present study shows that the relative proportions of tetrasaccharide and trisaccharide synthesized by free-living ***Rhizobium leguminosarum*** cells were influenced by nutrient and environmental parameters, and by the presence of flavone inducers. These results confirm our previous observations that the synthesis of these two oligosaccharides is not clearly coupled and that it could be independent of each other.

The changes in nutrient and environmental parameters described in this study also altered the acetylation and methylation patterns of the "O-antigen" fragments, and induced the synthesis of unusual oligomers. The type and amounts of oligosaccharides synthesized were influenced by the presence of the flavone inducer naringenin. Further, antibodies raised against the conserved tetrasaccharide were more reactive towards mature bacteroids than antibodies against the trisaccharide. These observations also include the possibility that these oligomers could be part of two different LPS molecules.

The idea that ***Rhizobium*** strains are capable of synthesizing more than one EPS and LPS type has been supported in several recent studies (18,39,40,52,53). In-planta observations using monoclonal antibodies against

uncharacterized fragments of the LPS showed that carbohydrate antigens recognized by these antibodies in free-living cultures grown under low pH or low-oxygen environments (25) are only expressed in mature regions of the nodule and in other regions of the nodule where the oxygen tension is expected to be low (25,48).

In this study we showed that ***Rhizobium leguminosarum*** free-living cells grown under different environmental conditions synthesize a wide variety of different "O-antigen" fragments. For each specific growth condition we found that the so-called "O-antigen" fragment is composed of a family of oligosaccharides. The nature of the oligosaccharide mixture is specific of each growth environment. Within these oligosaccharides, one of them is fairly conserved under our growth environments, and resembles an oligosaccharide synthesized by cells grown under conditions which represent the vegetative state. Differences in the "conserved" oligosaccharide were found at the levels of methylation and acetylation. Recently (1), it was reported that changes in methylation levels of the "O-antigen" occur in a strain of ***Rhizobium leguminosarum*** bv. ***phaseoli*** when grown in acidic conditions. It is a well documented phenomenon that changes in the methylation of the glycosyl components of the "O-antigen" chain and EPS of ***Rhizobia*** occur as a function of growth state (12,24,29,30). The direct link between culture age and medium

pH (33) suggest that changes in the redox potential of the medium cause the glycosyl components of the "O-antigen" to be methylated by bacteria. Similarly, these changes observed in the levels of methylation and acetylation of the "conserved" fragment would indicate that, as a response to different growth environments, bacteria acetylate and methylate the existing oligosaccharides at different rates. Also, we have observed that under these different conditions, cells induce the production of short oligosaccharides characteristic of a specific environment. One of them is a previously reported novel trisaccharide containing an unusual 6-deoxyglycosyl component induced under acidic environments in the absence of the flavone inducer naringenin and found in ***viciae*** and ***phaseoli*** biovars but not in ***meliloti*** (Chapter 3). The others are still under investigation.

While several studies have tried to define the possible roles that small changes in side groups and non-carbohydrate substituents of conserved structures can have in defining in host specificity and its relevance in the infection process (5,32,33), no studies have addressed the correlation (if any) between the carbohydrates found in free-living cells and the ones found in bacteria within the nodule. In previous work (Chapter 2) we were able to map the cell-surface distribution of the conserved tetrasaccharide and trisaccharide molecules, of two chemically characterized fragments of the "O-antigen"

and of the CPS in vegetative cells. We found that the CPS is uniformly distributed on the cell-surface and that the defined components of the LPS are polarly exposed. In the present study, using the same antibodies raised against immunogens made from chemically characterized cell-surface carbohydrates, we found that the conserved tetrasaccharide and trisaccharide were available to react with their respective antibodies all around the cell-surface when free-living cells were grown under environmental conditions meant to simulate actual nodule conditions (low-oxygen or low pH). These antibodies reacted in the same fashion with mature bacteroids extracted from the nodule and with bacteroids that were still present in the nodule indicating that the usual tetrasaccharide and trisaccharide components of the LPS isolated from vegetative cells are present in bacteroids and their distribution on the cell-surface is best represented by free-living cells grown under low-oxygen or acidic conditions. Using antibodies raised against characterized fragments of the "O-antigen" of vegetative cells, we found reactivity in cells grown under low-oxygen or acidic conditions. This was expected since the large heterogeneity in size and morphology found in these free-living cells indicated that not all cells within a population adapt to the environment at the same rate. The possibility is not excluded that cells closer in development to the vegetative stage were synthesizing an "O-antigen" similar to the one obtained from cells grown under neutral

conditions and against which the antibodies were generated. These results could also explain observations reported in previous studies where it was found that some antibodies raised against uncharacterized fragments of the LPS of vegetative cells reacted with free-living cells grown at low pH or in low-oxygen conditions (1,44). Only the small rods from the bacteroids extracted from the nodule and small bacteroids still present in the infection thread reacted with antibodies against the O-antigen 1. Only random reactivity was observed with antibodies against the O-antigen 2; the randomness of the observed reactivity does not exclude the possibility that antibodies against this glucan-like oligosaccharide could cross-react with oligosaccharides of plant origin. The general nature of our results supports observations from previous work (25,48) where it was demonstrated that antibodies against undefined carbohydrate cell-surface antigens extracted from free-living cells grown under low pH or low-oxygen conditions only react in bacteroids located in mature regions of the nodule.

Our NMR observations of the CPS indicate that small amounts are recovered from free-living cells grown under low-oxygen or low pH, and that the small amount recovered is devoid of the negatively charged non-carbohydrate substituents normally found in vegetative cells. These results were in agreement with our immunochemical studies where no reactivity

was found between antibodies raised against the CPS and free-living bacteria under any of the conditions investigated. While these antibodies reacted with vegetative cells outside the nodule, no reactivity was found with bacteroids inside the nodule indicating that bacteroids were also devoid of capsule. Cells grown in succinate-rich media did not react with antibodies raised against the CPS. While these cells did not produce the normal CPS, they synthesized a high molecular weight glucan for which the potential functions in host recognition and nodule development need to be investigated.

In this study we have demonstrated that the specific cell-surface carbohydrates normally found in ***Rhizobium*** cells grown in conditions that resemble the vegetative state are present in bacteroids within the nodule. The distribution of these cell-surface antigens in cells grown under low-oxygen conditions or low pH resembles the distribution of these antigens on the surface of bacteroids, making these two growth conditions potential bacteroid-like models for the study of cell-surface structures. The distribution of these cell-surface antigens changes between vegetative cells and bacteroids; the sequence involves losing the CPS, exposing conserved fragments of the LPS and synthesizing new carbohydrate antigens. A wide variety of new, distinct oligosaccharide fragments are synthesized by free-living cells grown under different environmental conditions and in the

presence or absence of flavone inducers. Some of these oligosaccharides are held together by electrostatic interactions, indicating that the traditional "O-antigen" of ***Rhizobium*** species is an heterogeneous fraction that contains more than one carbohydrate entity. Methods that destroy these electrostatic interactions need to be used in the study of the typical "O-antigen" fragment. Finally, we have shown that the ratio of the conserved tetrasaccharide and trisaccharide fragments, which have been thought to form part of a "core" component, change in response to different growth environments indicating that their synthesis is not tightly coupled thus allowing the possibility that these two fragments form part of two different LPS molecules.

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CHAPTER 5

INFECTION PROCESS: A MECHANISTIC MODEL FOR THE RELEASE OF RHIZOBIUM FROM THE INFECTION THREAD INTO NODULES.

ABSTRACT

Changes in cell-surface charges were induced in free-living ***Rhizobium leguminosarum*** biovar ***viciae*** 300 cells grown under environmental conditions meant to simulate environments inside the nodule. These changes were correlated with changes observed in the cell-surface carbohydrates of bacteria inside of actual nodules. Cells grown at pH 7 and normal oxygen concentration were surrounded by a negatively charged capsule and were more mobile towards the anode than free-living rhizobial cells grown under nodule-like conditions. These were devoid of capsule or produced small amounts of capsular material devoid of negatively charged non-carbohydrate substituents. Paper electrophoresis revealed that when compared to cells grown under normal conditions, cells grown under nodule-like conditions synthesized lipopolysaccharides containing substantially greater amounts of positively charged carbohydrate fragments.

These changes in electrophoretic mobility were correlated with changes in O-antigen fragments exposed to the surface, changes in production of negatively charged capsular material, and induction of the synthesis of new positively charged

carbohydrate species. The latter change was observed in cells grown under conditions meant to simulate environments inside the nodule and in bacteroids inside the nodule. Our observations suggested that as bacteria move deeper towards the inside of the nodule, their cell-surfaces gradually become more positively charged. Based on our findings and supported by our previous studies (Chapter 2, Chapter 3 and Chapter 4), we propose a mechanistic model which explains bacterial release from the infection thread.

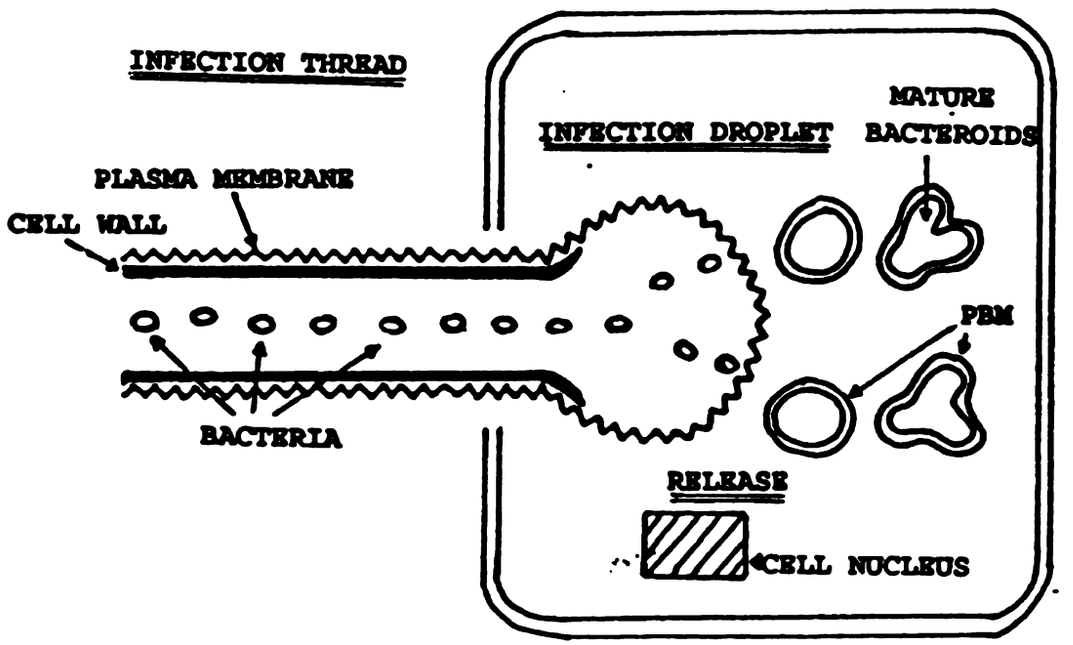
INTRODUCTION

In most infection processes, numerous interactions occur between the host cells and the invasive microorganisms. These ultimately lead to the uptake, by host cells, of the infective microorganism. Communication between both cells takes place before and after the initial steps of infection. In the case of ***Rhizobium***, signal exchange is thought to start in the rhizosphere, before adhesion and attachment of the bacterium to the host root occur. Root exudate factors, identified as flavonoids, induce nodulation (nod) genes in ***Rhizobium***, which confer on the bacterium the ability to nodulate the host-plant. These nod genes are conserved in a wide range of ***Rhizobia*** (for a review see 6). The bacteria are thought to produce a specific signal to the plant. In the case of ***Rhizobium meliloti*** and ***Rhizobium leguminosarum***, these signals have been characterized as glucosamine-containing lipo-oligosaccharides, which are sulfated in the case of ***Rhizobium meliloti*** (17,18). Cell to cell signal exchange continues as the bacteria attach to the host root causing curling and marked, characteristic deformations (8). Bacteria begin penetration of the host plant through an inward-growing plant structure known

as the "infection thread". This resembles an "inside-out" root hair. In this infection thread, the bacteria are separated from the cytoplasm by the host plasmalemma and by a layer of wall material which appears similar or identical to the normal inner layer of the root hair cell wall (4,29). To ensure its viability, the bacterial cell-surface has to adapt to the gradually changing environment of this infection thread. The infection thread carries bacteria from the root hair to the nodule being formed in the root cortex, where it ramifies. Cortical cells which are undergoing morphological changes, probably induced by bacterial signals, are penetrated by infection thread branches which eventually give origin to infection droplets. These are shapeless vesicles bounded by plasma membrane, and are not bounded by cell wall cellulose, pectic and xyloglucan components, or constrained by the plant cytoskeleton (4). Subsequently, bacteria are released into the cytoplasm of the host cell (3) (Scheme 1), surrounded by a membrane of plant origin, the peribacteroid membrane.

Numerous studies conducted on the ***Rhizobium***/legume system have addressed the importance of the constant changes that bacteria undergo during the infection process: from changes in size and morphology, to chemical changes in their antigenicity. An early study (14) demonstrated that after bacteria invade the leguminous cell cytoplasm, they undergo distinct morphological modifications. These include increase

SCHEME 1. Diagrammatical longitudinal section of an infection thread in a pea nodule. Represents tissue invasion by ***Rhizobia,*** formation of an infection droplet and bacteroid release from the infection thread into the host cell surrounded by the peribacteroid membrane (PBM), a membrane of plant origin.


INFECTION THREAD
INFECTION DROPLET
MATURE BACTEROIDS
PLASMA MEMBRANE
CELL WALL
PBM
BACTERIA
RELEASE
CELL NUCLEUS
HOST PLANT CELL

in volume and progressive modification of the shape from short rods to filamentous, pear shaped, then spherical and sometimes polyhedric morphologies. Recently, Vasse et al. (32) observed that changes in bacterial morphology were correlated with the redox potential of the nodule. It was, therefore, proposed that changes in oxygen concentration and/or pH that occur within the nodule might have regulatory roles in triggering bacteroid differentiation. This is consistent with the idea that bacterial cell-surfaces need to adapt to the changing conditions in the nodule to facilitate nutrient exchange and to communicate with the host cell.

In the ***Rhizobium***/legume system, bacterial cell-surface carbohydrates have been implicated in several key aspects of the symbiotic relationship. Recognition and attachment of the bacterium to the host plant is thought to be mediated by potential carbohydrate receptors on the bacterial cell-surface (2,7,10,36). Bacterial cell-surface carbohydrates are also required during host root infection, nodule development and maintenance, and control and release of bacteria from the infection thread (5,9,11,13,21-24,26). One factor that complicates the identification of specific cell-surface carbohydrates during a certain stage of bacterial development, and that complicates defining their role in symbiosis, is the constant changes in antigenicity that the bacterial cell-surface undergoes in response to changes in environmental

parameters (Chapter 3, Chapter 4,1,16,28). It is also made difficult by the ability of any given strain of ***Rhizobium*** to synthesize alternate carbohydrate structures that restore the wild type phenotype (12,34,35,37,38). The fact that completely different alternate carbohydrate structures can have the same function in symbiosis raises the question of the relevance of a specific structure in defining the bacterial-host interaction. If host cells possess specific receptors for certain carbohydrate epitopes, then do these epitopes remain unchanged during the environmentally induced changes of the bacterial cell-surface carbohydrates? It would not be unreasonable to think that, as in other processes, several molecules have the same biological effect provided they contribute with the same charges, charge distribution, geometries and electrostatic interactions to the system. In this study, we correlated the changes in cell-surface charges that occur in free-living ***Rhizobium leguminosarum*** biovar ***viciae*** cells grown in nodule-like environments, with cell-surface carbohydrate changes observed in bacteria inside the nodule. A mechanistic model that explains bacterial release from the infection thread is proposed.

MATERIALS AND METHODS

Bacterial culture conditions. ***Rhizobium leguminosarum*** bv. ***viciae*** 300 was grown aerobically in modified Bergensen's (BIII) liquid medium (pH 6.9) at 30°C as previously described (6). Briefly 250 ml of cells in stationary phase were inoculated into 4 liter Erlenmeyer flasks containing 2 liters of BIII medium and incubated until early stationary phase. Acid conditions of the culture medium (pH 4.5) were induced by adding 2 liters of BIII medium at pH 3.2 to 2 liters of a cell culture at late exponential growth in neutral BIII medium. The resulting cell suspension was adjusted to pH 4.5 with HCl and split into batches of 2 liters contained in 4 liter Erlenmeyer flasks. The culture medium was enriched with a succinate-rich syrup (casamino acids 100 mg/L, yeast extract 100 mg/L, sodium succinate 450 mg/L and glucose 400 mg/L) by adding 20 ml of syrup in 2 liters of culture medium contained in 4 liter Erlenmeyer flasks 4 hours after incubation had started, then growing the cells until early stationary phase. Microanaerobic environment was created by stoppering the 4 liter Erlenmeyer flasks containing 2 liters of culture medium with butyl rubber stoppers at the time of inoculation to restrict oxygen exchange with the atmosphere. Cells were grown until early

stationary phase.

LPS isolation and purification. LPS were extracted from cells using a hot phenol/water method (33). After dialyzing, concentrating and treating the aqueous phase with DNAse and RNAse, the LPS was purified by gel permeation chromatography on Sepharose 4-B with aqueous formic acid (0.05M, adjusted to pH 5.5 with concentrated ammonia). After releasing the carbohydrates by mild hydrolysis (1% acetic acid at 100 °C for 2 hours) the chloroform soluble impurities were extracted. The water soluble fraction was lyophilized and saved for paper electrophoresis.

Paper electrophoresis. Carbohydrate fractions were spotted on 3MM Whatman paper (35 cm long) for high voltage paper electrophoresis, and run at 3000 V/75 mA for 15 minutes in 6% acetic acid/3% formic acid in water. (Approximately the same amount of material was spotted from each fraction. However, comparisons were only made of the relative intensities between spots in a given lane). Resulting carbohydrate spots were stained with 3% $AgNO_3$ and developed with 2% NaOH in ethanol until brown spots appeared. The paper was then dipped in commercial Kodak fixative and rinsed with water for several hours.

Electrophoretic mobility measurements. The electrophoretic mobility measurement was determined in a model 501, LASER ZEE METER^{tm} (Pen Kem, INC) as follows. The instrument has a prism located inside the microscope

interposed between the objective lens and the eyepiece. The prism is mounted on a galvanometer which caused the prism to repeatedly rotate a few degrees and then flip back to start another cycle. The effect of this motion is to cause the image viewed through the microscope to be repeatedly scanned in one direction and then reset. The rate and direction of the prism motion can be adjusted by the operator. An electrophoretic mobility measurement is made by adjusting the prism control unit until the apparent motion caused by the prism exactly cancels the particle velocity caused by the applied field. At this point, the particles appear stationary in the field of view, and the electrophoretic mobility is displayed on a digital readout on the front panel. The electrophoretic mobility determines the rate at which the particles move in the known electric field. Measurements were made in phosphate buffered saline at room temperature.

RESULTS

For bacteria grown in liquid media with variations in environmental parameters, a diversity of electrophoretic mobilities was measured (Table 1). Cells grown under normal conditions, or under normal conditions in the presence of flavones, had the fastest mobility towards the anode (-1.67 and -1.57 10^{-8} m $volt^{-1}$ sec^{-1}, respectively). Cells grown under conditions which simulated the environment inside the nodule, migrated more slowly towards the same pole. Values ranged from -1.45 10^{-8} m $volt^{-1}$ sec^{-1} for cells grown under low oxygen conditions in the presence of the flavone inducer, to -0.11 10^{-8} m $volt^{-1}$ sec^{-1} for cells grown under a combination of low oxygen, succinate-enriched acid medium and in the presence of the flavone inducer. Values obtained for cells grown in succinate rich media (+0.28 10^{-8} m $volt^{-1}$ sec^{-1}), indicated that these cells migrated towards the anode. Mobility values obtained for these cells were affected by the "clumping" caused by a high molecular weight glucan that they synthesized under this growth condition (Chapter 4). However, the "clumping" observed would affect the rate of motion, not its direction. Mobility values could be affected by cell size, however for these measurements, size effect was expected to be

TABLE 1. Electrophoretic mobilities of free-living ***Rhizobium leguminosarum*** cells grown in liquid media with variations in environmental parameters. Mobility measurements were determined in phosphate buffered saline (pH 7.2).

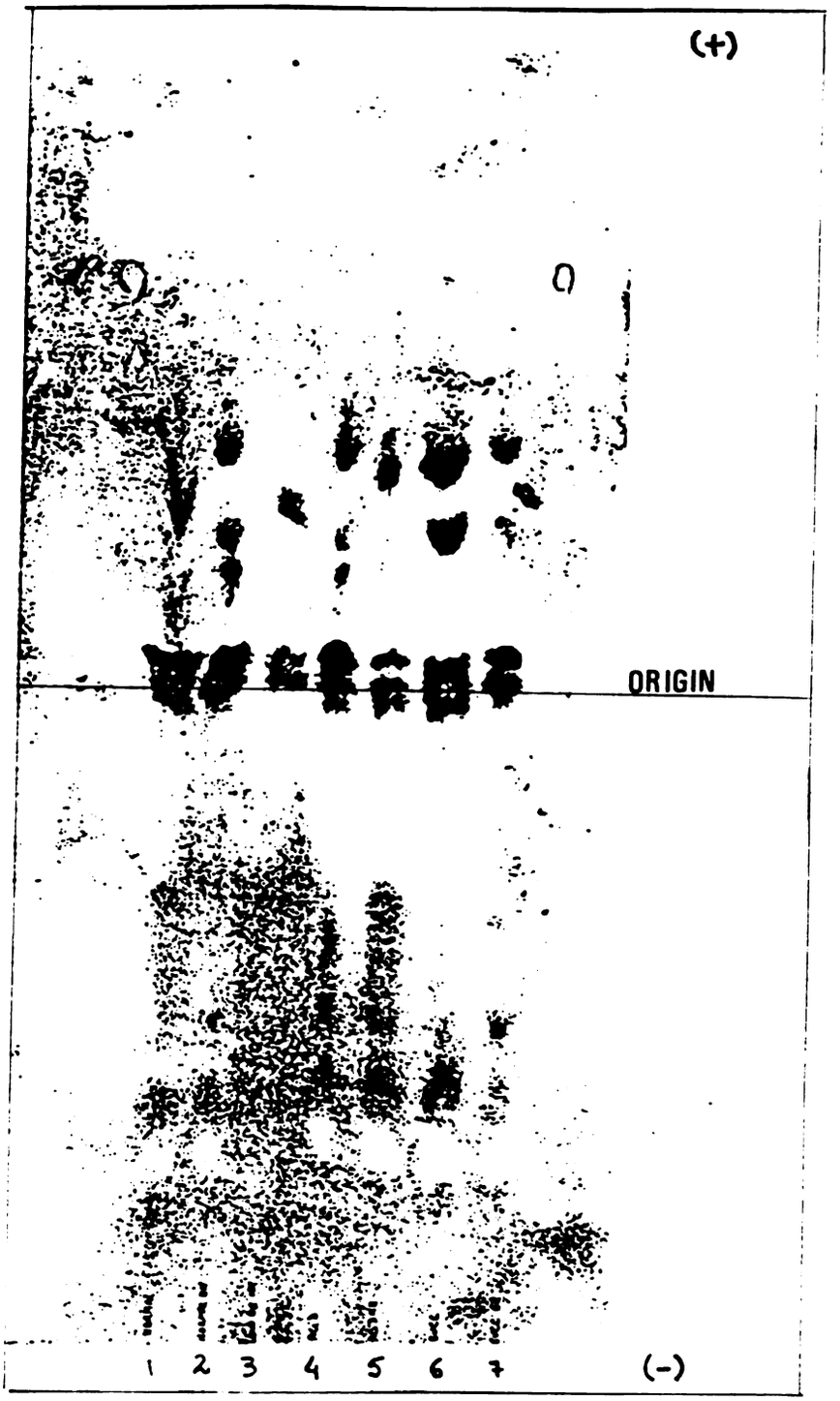
Growth medium	Mobility	(S.D.)
	10^{-8} m volt^{-1} sec^{-1}	
Normal (pH 6.9)	-1.67	0.12
Normal w/naringenin	-1.57	0.11
Acid medium (pH 4.5)	-0.91	0.07
Acid medium w/naringenin	-0.71	0.20
Low oxygen	-1.03	0.06
Low oxygen w/naringenin	-1.45	0.05
Succinate enriched	+0.28	0.39
Succinate enriched w/naringenin	-0.84	0.09
Low oxygen and acid (pH 4.5)	-0.52	0.06
Combination of factors*	-0.11	0.04

* Low oxygen conditions, succinate enriched acid medium (pH 4.5) in the presence of naringenin.

negligible over the charge effect, because each culture population had a large variability in cell sizes, and an average value which included all cell sizes was obtained. However, the cell electrophoretic mobilities were determined by the interaction of cell size and cell charge, and both effects combined should be kept in mind when extrapolating these results. Our observations indicated that when cells were grown under nodule-like environments, their surfaces adapted to the environment by modifying their surface charge, rendering it more positive than in cells grown in normal conditions. Addition of flavone inducers did not cause changes that could be considered significant.

Paper electrophoresis results of the carbohydrate components of the LPS for cells grown under different environmental conditions are shown in Figure 1. Most carbohydrates extracted from cells grown under normal conditions migrated towards the anode, indicating that they were negatively charged. Comparing the intensity of the spots that migrated, it can be observed that small amounts of material remained at the origin, and thus, were neutral in net charge. Only neutral and negatively charged carbohydrates were observed when flavone inducers were added to the media of cells grown under normal conditions. The number of carbohydrate spots observed for cells grown under neutral conditions were different, and had different retention times,

FIG. 1. Paper electrophoresis chromatograph of the LPS carbohydrate components that are released under mild acid hydrolysis (1% acetic acid at 100 °C for 2 hours) for ***Rhizobium leguminosarum*** biovar ***viciae*** free-living cells grown in liquid media with variations in several nutrients and environmental parameters. Cells were grown under (1) normal conditions (pH 6.9), (2) normal conditions in the presence of flavones, (3) low oxygen in the presence of flavones, (4) acid medium (pH 4.5), (5) acid medium in the presence of flavones, (6) succinate enriched medium and (7) succinate enriched medium in the presence of flavones).


(+)
ORIGIN
1 2 3 4 5 6 7
(-)

than the ones for cells grown under normal conditions in the absence of flavones. This indicated that the flavone inducers affected the type of carbohydrates produced. Cells grown under low-oxygen in the presence of flavones synthesized only carbohydrates that were negatively charged or neutral. Comparing the intensity of the spot that remained at the origin with the intensities of the spots that moved slightly towards the anode, and the one that moved faster, it could be noticed that the proportion of material that remained at the origin was greater. This would be consistent with what is observed in the electrophoretic mobilities results, were cells grown under low-oxygen conditions in the presence of flavone inducers, had only a slight difference in mobility compared to cells grown under normal conditions (Table 1). For cells grown in acid media, or in acid media in the presence of flavone inducers, carbohydrate fragments that migrated towards the cathode and thus, positively charged, were observed. The same trend was observed for cells grown under low-oxygen conditions in the absence of flavone inducers (not shown). Cells grown under succinate-rich media also synthesized carbohydrate fragments that were positively charged.

DISCUSSION

There is a critical interaction between the bacteria in the infection thread and the host plant membrane, which eventually leads to the invading bacteria being completely surrounded by the plant membrane inside of the plant cell, through an endocytic-like process. In the ***Rhizobium***/legume system, the mechanisms responsible for the release of the bacterium into the plant cell are unknown.

Several mechanisms of bacterial entry into host cells have been suggested for general infectious processes. Isberg, 1991 (15), proposed a potential general principle, where host cells need to have a large surface area that can be recognized by the infective microorganism, for microorganism internalization and host infection to occur. According to this principle, host cells must encode and be able to mobilize receptors on their cell-surface that are critical for the uptake process. Singer, 1992 (25) proposed a model of mutual co-capping, where the specific ligand-receptor pair responsible for binary cell-cell communications would be in low concentrations, or their bonds would be too weak to allow stable cell to cell contact. Therefore, bond stabilization

would be provided by an independent ligand-receptor species, which would be present in higher concentrations, and/or with bonds strong enough to stabilize the complex. Both models assume the existence of conserved ligands in the invading microorganism that will bind specific receptors on the host cell.

In the ***Rhizobium***/legume system, it has been demonstrated between symbiotic partners, that if the bacterium lacks a complete O-antigen on its cell-surface, it is incapable of entering the host cell. These observations would mandate that the LPS O-antigen are the bacterial receptors addressed by Isberg (15). The paradox here is that in pure culture, different strains of the same species have completely different O-antigen structures, but easily infect the same host. One obvious interpretation of this effect is that the plant membrane has several receptor states which are different from each other, and each recognizes a specific type of bacterial receptor/ligand. The second interpretation, is that the different bacterial O-antigens all have some common critical physicochemical property, which ensures entry into the host cell and which does not require a specific chemical structure.

The variation in cell-surface charges, that occur during the bacterial movement inside the infection thread, is a major

contributor towards the successful association between bacteria and the plant membrane. Indications of the influences of the bacterial cell-surface charges in adhesion processes have been considered in several studies. Electrophoretic mobility and hydrophobicity have been used as a measure to predict bacterial adhesion (30). It has also been shown that hydrophobic bacterial cells adhere to sulfated polystyrene to a greater extent than hydrophilic cells (31). Bacterial electrophoretic mobilities and cell-surface hydrophobicity in ***Rhizobium*** were shown to be dependent on the presence of an O-antigenic structure (11). In the cited study, LPS-defective ***Rhizobium*** mutants which completely or almost completely lacked an O-antigen-containing LPS were more hydrophobic, and had increased electrophoretic mobility. In these mutants, nodulation was blocked in the stage of bacterial release from the infection thread. LPS mutants which synthesized normal amounts of an antigenically altered O-antigen-containing LPS, were more hydrophilic, had lower electrophoretic mobilities, and showed normal nodule development. Changes observed in physicochemical properties of the cell-surface of bacteria might not be specific to the ***Rhizobium*** system. Changes of similar characteristics were observed with ***Salmonella typhimurium*** LPS-defective mutants, when compared with the respective wild type cells (20,27). These observations provide further evidence for questioning the relevance of the existence of a

specific structure, for the occurrence of bacterial release from the infection thread.

In previous work (Chapter 2), we have shown that a negatively charged capsule is uniformly distributed on the cell surface of free-living ***Rhizobium leguminosarum*** cells in the vegetative state. The O-antigenic fragments, which are more positively charged than the capsule (Chapter 2), are only exposed in a few localized areas of the cell-surface. In the present study, we showed that these same vegetative cells moved towards the anode in an electrophoresis field and only showed negatively charged and neutral carbohydrate species on paper electrophoresis. Using antibodies raised against a purified CPS oligomer, encapsulated cells were only observed outside nodules (Chapter 4).

Free-living rhizobial cells grown under nodule-like conditions had decreased electrophoretic mobilities towards the anode when compared to cells grown under normal conditions and synthesized positively charged carbohydrate fragments, as revealed by paper electrophoresis. Some of these induced positively charged carbohydrate species, could be the unusual oligosaccharides synthesized by ***Rhizobium leguminosarum*** grown under nodule-like conditions (Chapter 3, Chapter 4). Results of this study were consistent with our previous observations (Chapter 4) where free-living ***Rhizobial*** cells grown under

nodule-like conditions, exposed O-antigenic fragments all around their cell-surface. These cells were either devoid of negatively charged capsule, or synthesized small amounts of capsular material devoid of the negatively charged non-carbohydrate substituents found in vegetative cells. Using antibodies raised against chemically characterized O-antigenic fragments, it was found that cells which exposed O-antigen fragments homogeneously on the cell-surface were located inside infection threads. After being released from the infection thread, the O-antigen fragments analyzed no longer reacted with their respective antibodies.

The decreased electrophoretic mobility towards the cathode observed in cells grown under nodule-like conditions, when compared to those grown under normal conditions, were correlated with the increase in surface O-antigen fragments, the decrease in production of negatively charged capsular material, and the induction of the synthesis of positively charged carbohydrate species. A gradual increase in the exposure of O-antigen fragments to the surface and decrease in negatively charged capsular material synthesized, was also observed for bacteria inside the nodule. These observations suggested that, as the cells moved deeper inside the nodule, their surfaces became gradually more positively charged. It is important to note that in the electrophoretic mobility measurements on the bacterial cells an average value

corresponding to movement of a cloud of bacteria was observed. Therefore, some bacteria might have had a substantially more positive charge than the mean measured value, in instances where the decrease in mobility towards the cathode was observed. In other words, there are differences in charge populations, and an appreciable subset of bacteria might show an increase in positive character. This change, however, might be attenuated by a non-responsive, still highly negatively charged, subpopulation.

Based on our findings, and supported by the studies previously described, we propose the following mechanistic model, depicted in Scheme 2, which explains bacterial release from the infection thread. In the vegetative state, bacteria are surrounded by a negatively charged capsule and, therefore, their cell-surface charge is similar to those of the plant meristematic cell wall, which constitutes the infection thread. As the bacteria advance within the infection thread towards the inner portions of the nodule, the environment gradually changes, oxygen and nutrients become limiting, and the pH decreases. The bacteria adapt to these new conditions by modifying their size and morphology, and also by changing the cell-surface carbohydrate components, so as to adopt a more positive charge. Cells are no longer encapsulated, and the small amount of tightly bound capsular material is now completely devoid of the negatively charged pyruvyl residues.

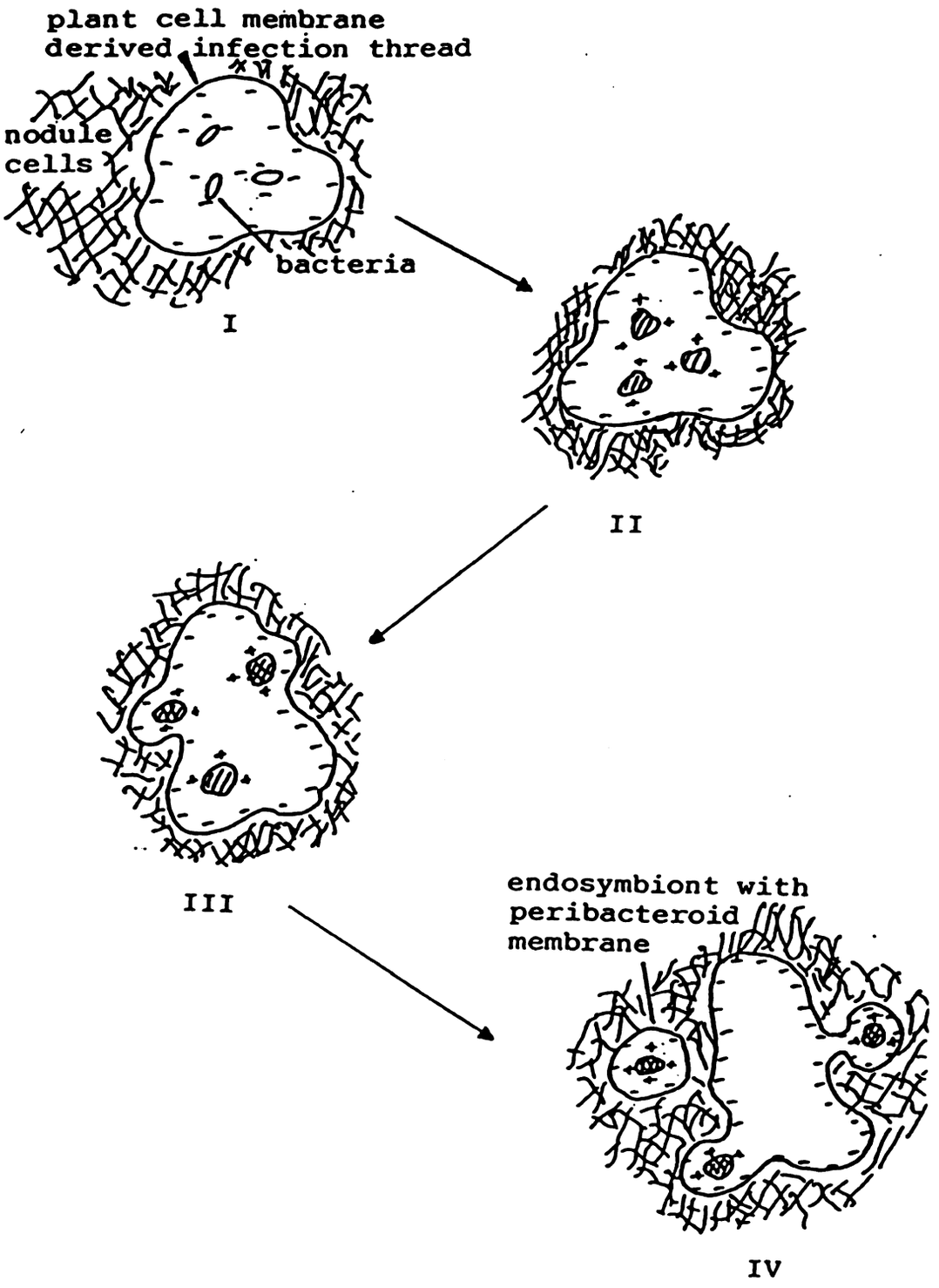
SCHEME 2. Mechanistic model proposed to explain bacteroid release from the infection thread into the nodule.

Stage I. Bacteria are in the vegetative state. Their cell-surface charge is similar to those of the plant cell wall which constitutes the infection thread.

Stage II. Bacteria adapt to the environment inside the infection thread by modifying their size, morphology and by changing the cell-surface carbohydrate components so as to adopt a more positive charge.

Stage III. Cell-surface charges now favor bacterial repulsion from one another and interaction with the negatively charged plant cell membrane.

Stage IV. The process of endocytosis begins and the bacteria are released surrounded by a concentric sphere of plant cell membrane, which becomes the peribacteroid membrane. Differentiation to bacteroids begins.


plant cell membrane
derived infection thread
nodule
cells
bacteria
I
II
III
endosymbiont with
peribacteroid
membrane
IV

The characterized O-antigenic fragments which contain amino sugars, bearing positive charges, are now exposed all around the cell-surface. The synthesis of other uncharacterized, positively charged, carbohydrate species is induced. Cell-surface charges now favor bacterial repulsion from one another, and interaction with the negatively charged plant cell membrane. The process of endocytosis begins, and the bacteroids are released surrounded by a concentric sphere of plant cell membrane, which becomes the peribacteroid membrane.

This model explains the importance of the dynamics of the bacterial cell-surface chemistry, which lead to changes in cell-surface charges, thus, facilitating the interaction with the host membrane of the opposite charge. Following the classical electrostatic theory, positively charged bacteria will be attracted towards the negatively charged plant cell membrane, and interact to create a situation where the net electrostatic field is minimum. This field is minimized when bacteria are completely surrounded by the host membrane. We are currently working on a mathematical model that will explain this process, using a combination of the classical electrostatic theory, statistical random walk theory and already existing models.

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CHAPTER 6

SUMMARY AND PERSPECTIVES

SUMMARY

In these studies we evaluated the implications of the dynamics of the bacterial cell surface carbohydrate chemistry for the infection process between eucaryotes and bacteria. The system analyzed was the ***Rhizobium***/legume symbiosis, using primarily the ***Rhizobium leguminosarum*** biovar ***viciae*** 300 strain.

Changes in cell surface carbohydrate antigenicity occur in response to changes in the environment to which bacteria are exposed during the infection process. Direct chemical characterization of the changes that occur in these cell surface carbohydrate epitopes during the infection process is made difficult by the amount of material needed and by the presence of carbohydrate of plant origin, presenting therefore, a major complication in defining the role of specific carbohydrate structures in symbiosis.

To assess the impact of changes in the environment on the cell surface carbohydrates, we used artificial systems that simulated nodule-like conditions. These conditions included changes in pH, oxygen tension, carbon source and flavone inducers. Cell surface carbohydrate antigens, isolated and

purified from free-living cells grown under these different environmental conditions, were chemically characterized using a combination of ^{1}H- and ^{13}C-NMR spectroscopy and gas chromatography/mass spectrometry (GC-MS).

Immunochemical studies were conducted to characterize the spatial and clonal distribution of chemically characterized carbohydrate antigens on the bacterial cell surface. They also allowed correlation of changes that occur in the cell surface carbohydrates of free-living cells grown under different environmental conditions, with changes that occur in the cell surface antigenicity of bacteria inside the nodule. Antibodies were generated against immunogens synthesized from the previously characterized tetrasaccharide and trisaccharide "core"; from the "O-antigen" fragment components of the lipopolysaccharide (LPS); and against an octasaccharide fragment isolated and purified by enzymatic degradation from the capsular polysaccharide (CPS).

In vegetative cells the CPS surrounds the cell surface, the "O-antigen" fragment, the tetrasaccharide and the trisaccharide are polarly exposed to the cell surface having therefore a potential role in the cell "polar attachment" to the host root.

When cells were grown in liquid media with variations in

carbon source, oxygen and pH availability and presence of flavone inducers, a wide variety of different LPS molecules were synthesized. These LPS molecules are held together in the bacterial outer membrane by the hydrophobic effect of the lipid chains of the lipid A domain, and by electrostatic interactions of the LPS head groups which are the "O-antigen" fragments. These "O-antigen" fragments are composed of chemically distinct oligosaccharides. Within these oligosaccharides, one of them is fairly conserved under our growth environments. Differences in this conserved oligosaccharide are found at the level of methylation and acetylation. The synthesis of other smaller alternate oligosaccharides is also induced under these conditions. Specifically when cells are grown in acid medium (pH 4.5), the synthesis of an unusual trisaccharide which contains mannose, galactose and an unidentified deoxy-sugar is induced. This unusual trisaccharide was only observed in the ***Rhizobium leguminosarum*** and ***phaseoli*** strains analyzed, when grown in acid conditions in the absence of flavone inducers. It was not synthesized by the two strains of the ***Rhizobium trifolii*** analyzed. This makes the lipopolysaccharide containing the unusual trisaccharide a possible candidate for a species-dependent surface carbohydrate with special significance for the infection process.

Cells grown under low oxygen conditions or in acid medium

either did not synthesize tightly bound CPS material or synthesized small amounts of a capsule which was devoid of the negatively charged non-carbohydrate substituents normally present in the CPS of vegetative cells. Cells grown in succinate-enriched medium synthesized a high molecular weight glucan which surrounded the cells and caused them to "clump" together.

Nutrient, environmental parameters and flavone inducers also influence the relative proportions of the usual tetrasaccharide and trisaccharide components of the LPS synthesized. This indicates that the synthesis of these two oligosaccharides may be separately regulated and, therefore, would not be "core components" of the same LPS molecule as usually believed.

The specific cell surface carbohydrates normally found in ***Rhizobium leguminosarum*** vegetative cells are present in bacteria inside nodules. The distribution of these cell surface antigens in cells grown under low oxygen conditions or low pH resembles the distribution of these carbohydrate antigens in the surface of bacteria inside the nodule. These two growth conditions offer potential bacteroid-like models for the study of cell surface structures.

Changes in cell surface charge of free-living cells grown

under different environmental parameters were correlated with changes observed in the cell surface carbohydrates of bacteria. Vegetative-like cells, which are surrounded by a negatively charged CPS, are more mobile towards the anode than free-living rhizobial cells grown under nodule-like conditions, which are devoid of capsule or produce small amounts of capsular material devoid of negatively charged non-carbohydrate substituents. Paper electrophoresis indicated that, when compared to vegetative-like cells, cells grown under environments that represent the nodule synthesized positively charged carbohydrate fragments.

Studies on the ***Rhizobium tropici*** and ***Rhizobium leguminosarum*** biovar ***phaseoli*** systems indicated that, while these two bacteria species share a common host, no similarities exist in the chemistry of their cell surface carbohydrate components. This is consistent with our theory that in biology different molecules can have the same function provided they contribute with the same charges and geometric components to the system.

Based on our findings, we proposed a model that explains bacterial release from the infection thread into the nodule. This model emphasizes the importance of the dynamics of bacterial cell surface carbohydrate chemistry to the infection process. In the model, changes in environmental parameters lead to changes in cell surface charge. These changes

facilitate the interaction of the bacterial cell with the host membrane leading, eventually, to bacterial release.

PERSPECTIVES

Studies on the clonality and spatial distribution of cell surface carbohydrate antigens in bacteria within the nodule. In Chapter 4 we demonstrated that the specific cell surface carbohydrates normally found in ***Rhizobium*** cells grown under conditions that resemble the vegetative state are present in bacteroids within the nodule. The clonality and spatial distribution of these cell surface antigens in bacteria within the nodule need to be examined further. This can be done using immunogold labeling transmission electron microscopy on nodule thin sections.

Are all carbohydrate antigens synthesized by free-living bacteria grown under nodule-like conditions also synthesized by bacteria inside the nodule? In Chapter 4, we showed that a wide variety of new, distinct oligosaccharide fragments are synthesized by free-living cells grown under different environmental conditions. The question that now follows is: which one of these oligosaccharides are synthesized by bacteria within the nodule? Immunochemical studies would be impractical due to the extensive number of distinct oligosaccharides synthesized by cells grown in different

environmental conditions. Therefore, generating antibodies against immunogens synthesized from all these possible carbohydrate antigens would be extremely costly and time consuming.

A rapid method to check for the existence and proportions of these oligosaccharides present in bacteria within the nodule is being developed. Briefly, a chromophore molecule, 4-chloro-7-nitrobenzofurazan (NBD), was attached to the reducing end of the purified oligosaccharides through reductive amination[1]. The purity of the NBD-linked oligosaccharides was assessed by monitoring for the presence of linked chromophore by reverse-phase high performance liquid chromatography (HPLC)[2]. (Linked NBD absorbs at 475 nm, while free NBD absorbs at 360 nm). Peaks which corresponded to chromophore-linked oligosaccharides were collected. The peaks which contained pure chromophore-linked oligosaccharides are now being

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[1]100µg of oligosaccharide were dissolved in minimum volume of 5% acetic acid in 3:1 methanol:water. Octylamine (1.2 mole equivalents) and sodium cyanoborohydride (1.5 mole equivalents), were added. The mixture was heated at 70°C for 1 hour and then concentrated to dryness. The chromophore was attached as follows. NBD (1.2 mole equivalent) was dissolved in minimum volume of a 1:1 mixture of 0.1M sodium bicarbonate in water and propanol and added to the previous concentrate. The mixture was heated at 40°C for 1 hour and submitted to HPLC.

[2] A Waters 600 multisolvent delivery system linked to a Spectroflow 783 programmable absorbance detector was used for chromophore detection at wavelength of 475 nm and for peak separation. The column used was a Beckman ultrasphere ODS (5 µ). The following solvent system was used: an isocratic gradient of 90% water and 10% acetonitrile for 10 minutes, followed by the gradient curve shape #5 specified by the manufacturers, to give 100% acetonitrile in a total of 70 minutes. The column was washed with acetonitrile for 30 minutes and equilibrated with the starting solvent system for 1 hour. The column temperature was kept at 50°C during the run.

characterized by infrared spectroscopy (IR) and fast atom bombardment mass spectrometry (FAB-MS) and will be used as standards.

Bacteria can be isolated from the nodule using a sucrose gradient. Carbohydrates extracted using the hot water/phenol method described in Chapter 1 can then be linked to the chromophore through reductive amination as described above. The mixture of oligosaccharides can be separated by HPLC using the conditions described in Footnote 2. An IR spectrum and a FAB spectrum is then obtained for each oligosaccharide peak collected. Spectra can be matched with the ones obtained for the oligosaccharide standards to determine the presence of those specific oligosaccharides in bacteria within the nodule. The intensity of the peaks obtained from the HPLC and that correspond to these oligosaccharides can then be used to asses the relative proportions of oligosaccharides synthesized.

Total cell fatty acid analysis. No information is available on the fate of rhizobial total cell fatty acids during the infection process. Our preliminary data on total cell fatty acid composition[3] indicate that when free-living

[3] Total cell fatty acids were determined as their methyl ester derivatives as follows. For each growth condition, 3 samples with approximately 100 mg of wet bacterial cells each, were sonicated for 15 minutes in 0.1 ml of chloroform and 0.4 ml of methanol containing 5% of hydrochloric acid by volume. The cell suspensions were heated at 70°C for 24, 30 and 40 hours respectively, during that time cells were sonicated for 15 minutes every 6 hours. Suspensions were then cooled to room temperature and concentrated to dryness under nitrogen. The dry extracts

cells are grown in succinate-rich medium, low oxygen conditions or acid medium, the synthesis of short chain fatty acids is induced. These short chain fatty acids could be responsible for the greater fragility observed in bacteroids when compared to vegetative cells. The ability of these cells to synthesize different fatty acids under different conditions, thus adapting their cell membrane fluidity, could be a factor determining bacterial survival. Further studies need to be conducted to completely characterize the total cell fatty acids composition of the vegetative cell and the changes that occur in fatty acid composition and proportions when cells are grown in different environmental conditions. Studies need to be undertaken to evaluate specifically what is the importance of these changes in determining bacterial fragility and bacteria adaptation to new environmental conditions.

Phospholipid analysis[4]. The dynamics of the chemistry of bacterial phospholipids when cells are exposed to different environmental conditions need to be analyzed. Our preliminary data indicate that ***Rhizobium leguminosarum*** cells grown under

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were dissolved in 1:1 water:hexanes. The hexanes layers were recovered and concentrated to dryness under nitrogen. These derivatives were analyzed by gas chromatography on a DB-1 column with an initial temperature of 150°C, rate 3°C/minute, final temperature 330°C and final hold time of 20 minutes. Their composition was confirmed by gas chromatography-mass spectrometry.

[4]Total phospholipid analysis was conducted as follows. Approximately 100 mg of dry cells were dissolved in a few drops of water. 0.5 ml of chloroform:methanol in a 2:1 proportion were added. After stirring the cell suspension at 45°C for 6 hours, the organic layer was recovered, concentrated under nitrogen and used for thin layer chromatography.

different environmental conditions synthesize phospholipids with same retention times on thin layer chromatography plates. Studies need to be conducted to determine what these phospholipids are and to evaluate if there are any differences in the amounts synthesized by cells grown in different environmental conditions.

Mathematical model that explains bacteria release from the infection thread into the nodule. Based on our observations, in Chapter 5 we proposed a mechanistic model to explain bacterial release into the infection thread. We believe that this infection process can be generalized to other systems through a mathematical model, which we are currently working on. This model, based on a combination of the classical electrostatic theory, statistical random walk theory and already existing epidemiological models, will explain the process of bacteria release from an electrostatic point of view. Following the classical electrostatic theory, positively charged bacteria will be attracted towards the negatively charged plant cell membrane and interact to create a situation where the net electrostatic field is minimum. This field is minimized when bacteria are completely surrounded by the host membrane.

APPENDIX I

CHEMICAL CHARACTERIZATION OF THE CELL SURFACE CARBOHYDRATES OF TWO DIFFERENT RHIZOBIUM STRAINS, TROPICI AND LEGUMINOSARUM BIOVAR PHASEOLI, WHICH SHARE A COMMON HOST.

ABSTRACT

Rhizobium tropici UMR 1899 and ***Rhizobium leguminosarum*** biovar ***phaseoli*** UMR 1632 belong to two different species but have a common host, ***Phaseolus vulgaris*** L. beans. While both strains are highly effective in nodulation, ***Rhizobium tropici*** is more competitive under strongly acid situations. In this study we chemically characterized the cell-surface carbohydrate components of these two strains to try to define the chemical basis for the difference in competitiveness under acid conditions. ***Rhizobium leguminosarum*** biovar ***phaseoli*** synthesized the previously reported tetra- and trisaccharide components of the LPS which are common to several ***Rhizobium*** species. Under acidic conditions, the synthesis of the unusual trisaccharide found only in the ***viciae*** and ***phaseoli*** biovars was induced. The so called "O-antigen" fragment was composed of several highly methylated oligosaccharide fragments held together by electrostatic interactions. The synthesis of the usual trisaccharide and tetrasaccharide was not observed for ***Rhizobium tropici*** cells, nor was the synthesis of the novel trisaccharide induced. This strain, however, synthesized an unusual trisaccharide and a short oligomer which were devoid

of uronic acids. Their structures are under investigation. The "O-antigen" fragment was composed of several oligosaccharides, some of them with a wide variety of methylated glycosyl components. The structure of one of them, a trisaccharide repeating unit, is described in Appendix II. Our studies suggested that the carbohydrate differences found at the cell-surface level could render bacteria more competitive under acid conditions. Further, it is consistent with our theory that, during the symbiotic process, several molecules can have the same biological effect provided they contribute with the same charges, charge distribution, geometries and electrostatic interactions to the system.

INTRODUCTION

The nitrogen-fixing symbiotic relationship between bacteria of the genus ***Rhizobium*** and their host legume plants is host-specific. The ***Rhizobium leguminosarum*** species, which infects peas, clovers and beans, has three biovars. These biovars contain different symbiotic plasmids that encode distinct nodulation specificities. ***Rhizobium leguminosarum*** biovar ***phaseoli*** specifically nodulates beans (***Phaseolus vulgaris L.***). Recently, Martinez-Romero et al. (25), have proposed a new species, ***Rhizobium tropici***, that also has nodulation specificity for ***Phaseolus vulgaris L.*** and for ***Leucaena spp***. ***Rhizobium tropici***, which was previously named ***Rhizobium leguminosarum*** biovar ***phaseoli***, was proposed as a new species on the basis of the results of the usual differentiation criteria used: DNA-DNA hybridization, analysis of ribosomal DNA organization, sequence analysis of 16S rDNA, and analysis of phenotypic characteristics. ***Rhizobium tropici*** strains were found to be more tolerant of high temperatures and low pH in laboratory conditions, and to be symbiotically more stable than ***Rhizobium leguminosarum*** biovar ***viciae*** (25).

The nature of the interactions between the infecting bacteria and the host plant that lead to this host-specificity are not well understood. While ***Rhizobium*** biovars require a specific host to nodulate, under laboratory conditions legumes can nodulate with a wide range of ***Rhizobia*** (11,13,16,20,24,29). Extracellular signals are thought to be involved in eliciting this host-specific response. Specifically, in the ***Rhizobium meliloti*** system, a sulfated lipo-oligosaccharide is thought to be the critical determinant of host-specificity (21,22). However, the mechanism of action of these "signals" is unknown. It is a well known fact that in most infective systems, direct cell-to-cell interactions are critical for determining the uptake of the infective microorganism by the host cell (19,31). In the ***Rhizobium***/legume system, bacterial cell-surface carbohydrates, which are the most abundant components of the bacterial cell-surface, have been implicated in several key aspects of the symbiotic relationship. Carbohydrate receptors on the cell-surface are thought to mediate recognition and attachment of the bacterium to the host plant (3,7,9,37). Bacterial cell-surface carbohydrates are also required during host root infection, nodule development and maintenance, and for the control and release of bacteria from the infection thread (4,8,10,15,23,26,27,28,32).

To contribute to the understanding of the role of cell-surface carbohydrates in symbiosis, we chemically characterized the cell-surface carbohydrates produced by ***Rhizobium tropici*** UMR 1899, an acid tolerant strain, and by ***Rhizobium leguminosarum*** biovar phaseoli UMR 1632, an acid sensitive strain. Both strains are highly effective but ***Rhizobium tropici*** is more competitive than this ***Rhizobium leguminosarum*** strain in all but strongly-acid conditions.

MATERIALS AND METHODS

General methods. Compositional analysis of the LPS carbohydrate fragments was determined as their alditol acetate derivatives (30) and analyzed by gas chromatography (GC) using a capillary DB225 column and flame ionization detector. Glycosyl composition was confirmed by gas chromatography/mass spectrometry (GC/MS) analysis on a JEOL 505 mass spectrometer in the electron impact mode. Tests for uronic acids and 3-deoxy-D-manno-2-octulosonic acid were done using the m-phenylphenol (2) and the periodate/thiobarbituric acid (33) assays respectively. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR300 spectrometer operating at 300 MHz for protons and 75.43 MHz for ^{13}C. Spectra were obtained in D_2O solutions and chemical shifts were referenced relative to external TMS. Carbohydrate content in fractions from gel filtration columns was monitored by the phenol-sulfuric acid method (12).

LPS and LPS fragments isolation and purification. Bacteria were inoculated on B-III agar plates and transferred after 3 days to 500 ml Erlenmeyer flasks containing 250 ml of B-III liquid medium (pH 6.9) (6). At late log phase each 250 ml inoculum was transferred to separate 4 liter Erlenmeyer flasks containing 2 liters of B-III medium (pH 6.9). Once the

cells reached mid to late exponential growth, 2 liters of B-III medium at a pH of 3.2 (obtained using free glutamic acid in place of sodium glutamate and adjusting the pH with HCl) were added into each flask and the pH of each adjusted to give a final value of 4.5. The contents of each flask was then split into two 4 liter Erlenmeyer flasks each containing about two liters of culture. Cells were grown until the beginning of stationary phase and harvested by centrifugation. Cultures were constantly shaken at 265 rpm.

LPS was extracted by the hot phenol-water method (34), treated with DNAse-RNAse and purified by gel permeation chromatography on Sepharose 4B with aqueous formic acid (0.05M, adjusted to pH 5.5 with ammonia) as the mobile phase. Carbohydrate fragments were released by hydrolyzing the LPS with 1% acetic acid at 100°C for 2h and partitioned between water and chloroform. The aqueous layer was lyophilized and fractionated by size exclusion chromatography on Biogel P2 with aqueous formic acid (0.1%) as the mobile phase. The peak that voided the P2 column was further fractionated by size exclusion chromatography on Biogel P10 with aqueous formic acid (0.1%) as the mobile phase. Fractions eluting as a peak were pooled, lyophilized and analyzed by ^{1}H-NMR spectroscopy and by GC and GC/MS after derivatization into alditol acetates as described in the general methods section. Fractions which appeared impure by these two analyses were subjected to

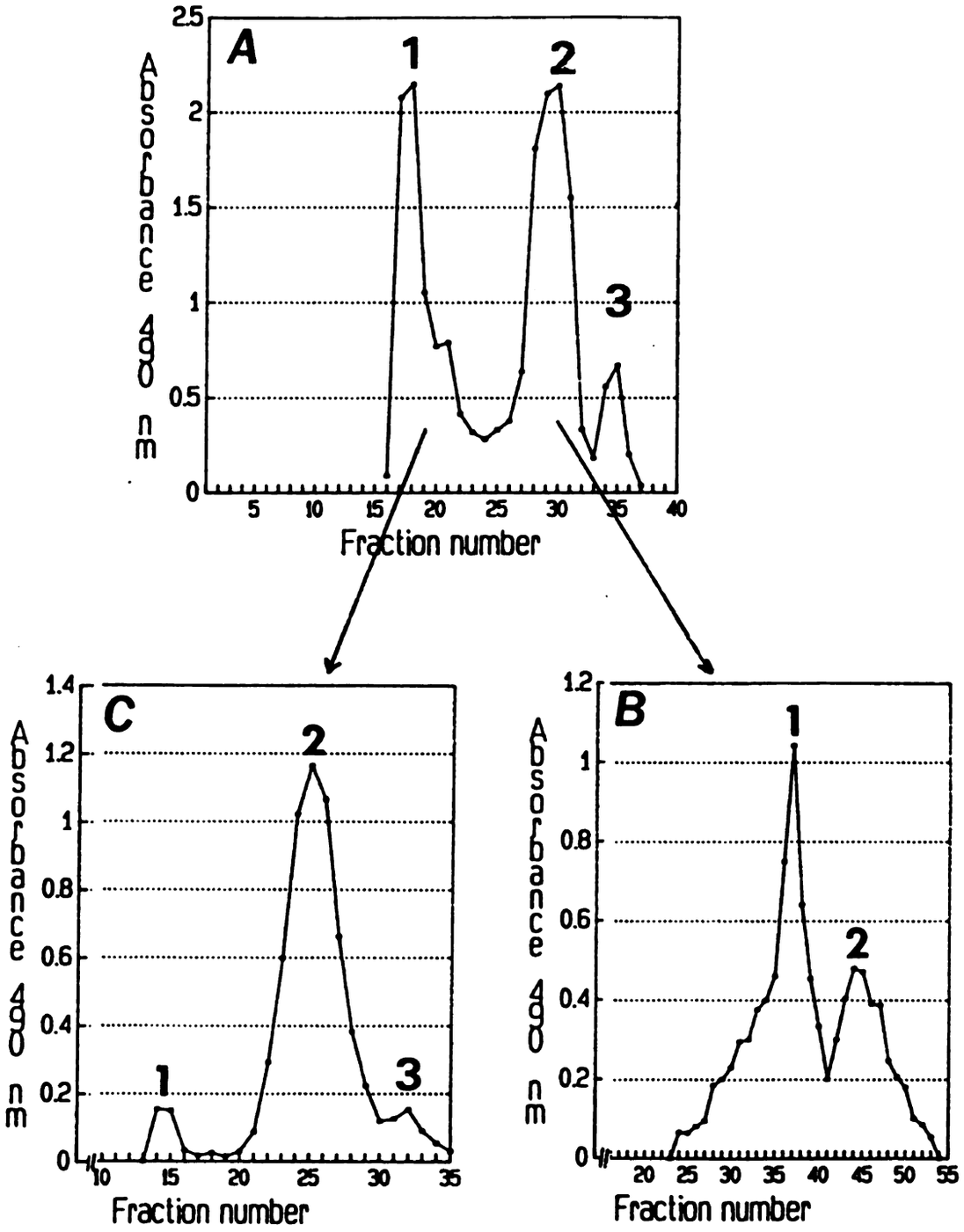
further purification by ion-exchange chromatography over DEAE Sephadex in aqueous formic acid (0.01%, adjusted to pH 5.5 with ammonia) and eluted with a linear gradient of 0-0.2M ammonium chloride.

RESULTS AND DISCUSSION

Gel filtration chromatography (Biogel P-2) of the aqueous fraction of the LPS from ***Rhizobium leguminosarum*** biovar ***phaseoli*** UMR 1632 cells grown in acid media (pH 4.5) resulted in the separation of three peaks (Figure 1A). Peak 3 contained the trisaccharide fragment first identified in the biovar ***trifolii*** (3,4) and also observed in the viciae biovar (Chapter 2,3 and 4).

^{1}H-NMR spectroscopy of the carbohydrate fraction eluting as peak 2, indicated that this fraction contained a mixture of oligomers. Further purification by ion-exchange chromatography on DEAE-Sephadex resulted in the separation of this material into two major carbohydrate peaks (Figure 1B). The ^{1}H-NMR spectrum of the oligosaccharide eluting as peak 1 and compositional analysis were similar to those of the unusual oligomer produced by ***Rhizobium leguminosarum*** biovar ***viciae*** and ***phaseoli*** cells grown in acid medium (Chapter 3) and that has been isolated from a ***Rhizobium leguminosarum*** biovar ***viciae*** mutant which was impaired in the synthesis of the usual tetrasaccharide (39). This unusual trisaccharide was not

FIG. 1. Gel permeation chromatography profile of the water soluble components of the LPS. (A): Biogel P-2 profile of the carbohydrate components of the LPS after hydrolysis with 1% acetic acid, for ***Rhizobium leguminosarum*** biovar ***phaseoli*** cells grown in acid medium (pH 4.5). (B): Ion-exchange chromatography (DEAE) profile of the carbohydrate fragments contained in the tetrasaccharide-like fraction (corresponds to peak 2 in Figure 1A). (C): Gel permeation chromatography (Biogel P-10) profile of the "O-antigen" fragment of the LPS (corresponds to peak 1 in Figure 1A). Carbohydrate content was monitored with the phenol-sulfuric acid assay.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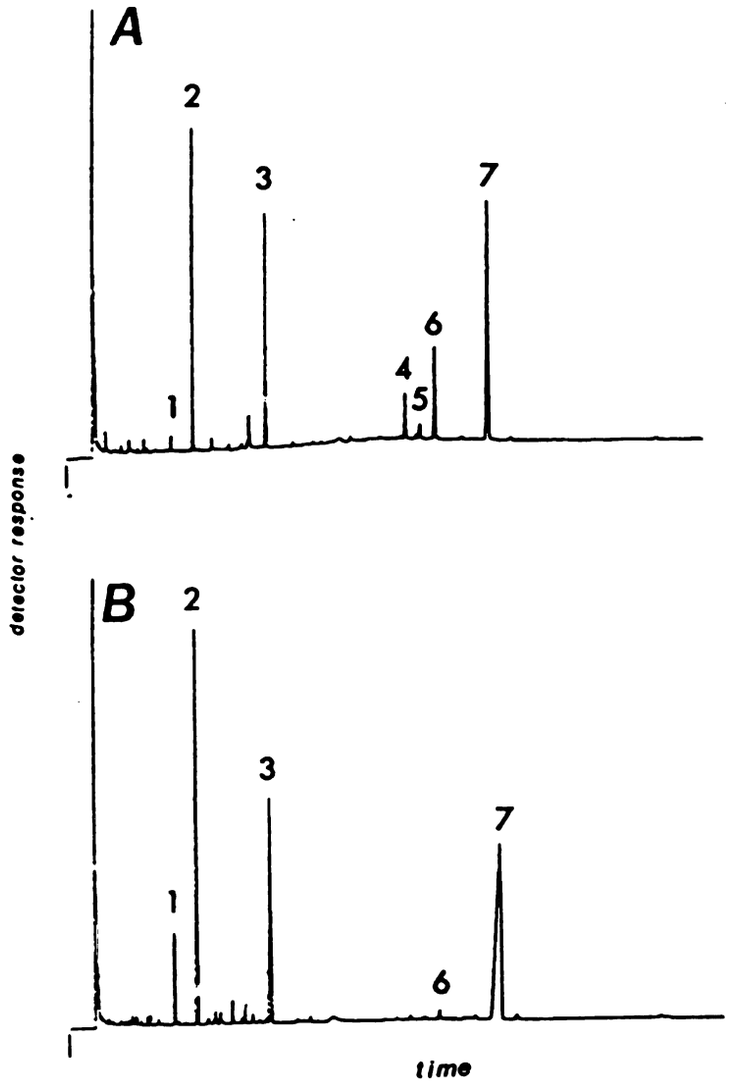
synthesized when this biovar was grown under normal conditions (pH 6.9). The ^{1}H-NMR spectrum of the oligosaccharide eluting as peak 2 (Figure 1B), corresponded to the previously characterized tetrasaccharide (4,17,18), which contains galactose, mannose, galacturonic acid and 3-deoxy-2-octulosonic acid.

The first eluting peak from the gel permeation chromatography over Biogel P-2 (Figure 1A, peak 1), corresponded to the so called "O-antigen". This fraction was further purified over Biogel P-10, where three peaks were recovered (Figure 1C). The first one, peak 1, corresponded to a tightly bound capsular polysaccharide similar to the one observed for ***Rhizobium leguminosarum*** biovar ***viciae*** cells grown in normal BIII medium (Chapter 4). Peak 2 (Figure 1C), consisted of a repeating unit of 2-O-methyl,6-deoxyhexose, fucose and 2-amino,2,6-dideoxyhexose in an approximate ratio of 1.5:1:1 (Figure 2A). The presence of 2,3-O-methyl,6-deoxyhexose indicated that this residue was a side chain substituent or, more likely, it was present due to the fact that under acid conditions bacteria were further methylating the already existing 2-O-methyl,6-deoxyhexose residue. It has previously been demonstrated that upon exposure to acidic conditions, bacteria methylate the already existing "O-antigenic" side chain (Chapter 3 and 4). It remains to be shown if under normal growth conditions (pH 6.9), the repeating unit of this

FIG. 2. Gas chromatography profile of the alditol acetates derivatives of the glycosyl components of the oligosaccharide fractions eluting as peaks 2 (chromatograph A) and 3 (chromatograph B), respectively, from the gel permeation chromatography (Biogel P-10) profile shown in Figure 1C. In each chromatography, peaks correspond to (1) 2,3-O-methyl,6-deoxyhexose, (2) 2-O-methyl,6-deoxyhexose, (3) fucose, (4) mannose, (5) galactose, (6) glucose and (7) 2-amino,2,6-dideoxyhexose, respectively.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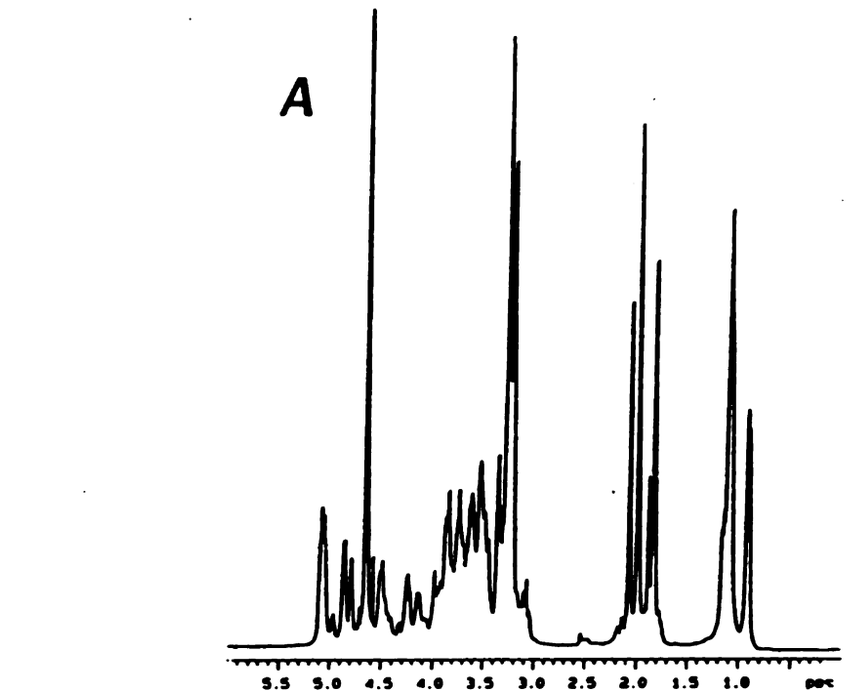
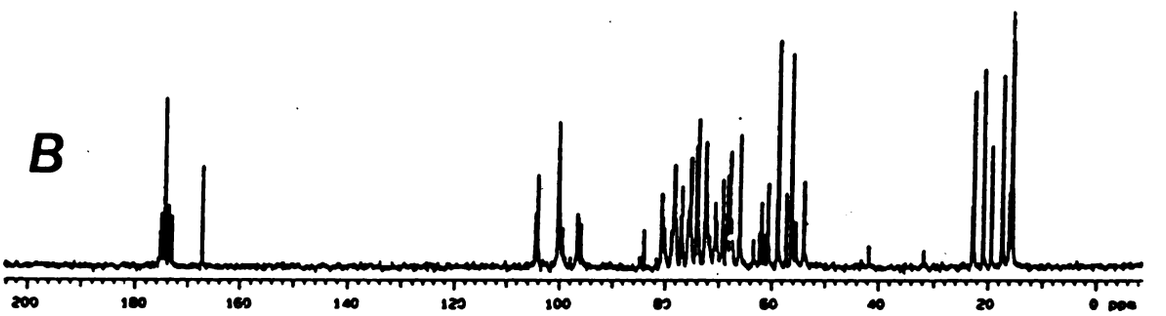
6
time

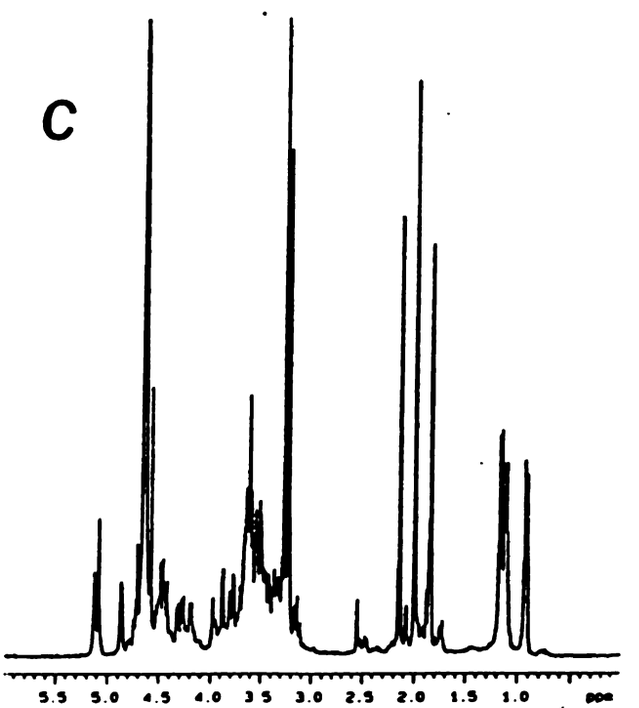
oligosaccharide would consist of 6-deoxyhexose:2-amino,2,6-dideoxyhexose in an approximate ratio of 2:1, and that the 6-deoxyhexose would be methylated when bacteria are exposed to acid conditions. ^{1}H-NMR spectrum of this oligosaccharide (Figure 3A), showed resonances between 0.85 and 1.35 ppm (relative to external Me_4Si), that corresponded to the methyl protons of the 6-deoxy sugars. Signals between 1.75 and 2.25 ppm corresponded to methyl protons of the O- and N- attached acetyl groups. The two intense singlets at 3.15 and 3.25 ppm corresponded to the O-methyl groups of the 6-deoxysugars. The cluster of signals between 4.75 and 5.15 ppm were assigned to anomeric protons. The rest of the signals corresponded to ring protons. The ^{13}C-NMR spectrum of this oligosaccharide is shown in Figure 3B, where the eight resonances between 15 and 22 ppm corresponded to the methyl carbons of the 6-deoxy-hexoses and of acetate groups. Carbonyl carbons belonging to O-acetyl groups appeared as eight resonances between 172 and 178 ppm, ring carbons attached to nitrogen appeared at 56.5 ppm and the six anomeric carbon resonances appeared between 95 and 104 ppm. The rest of the signals corresponded to carbons of the sugar rings.

Peak 3 (Figure 1C), consisted of a repeating unit of 2-O-methyl,6-deoxyhexose, fucose and 2-amino,2,6-dideoxyhexose in an approximate ratio of 1:1:1 (Figure 2B). The small amounts of mannose, galactose and glucose found were either side chain

FIG. 3. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of the oligosaccharide fraction eluting as peak 2 in Figure 1C, correspond to the alditol acetate gas chromatograph shown in Figure 2A. ^{1}H-NMR (C) spectrum of the oligosaccharide fraction eluting as peak 3 in Figure 1C, corresponds to the alditol acetates gas chromatograph shown in Figure 2B. In this figures ^{1}H-NMR resonances between 0.85 and 1.35 ppm (relative to external Me_4Si), corresponded to the methyl protons of 6-deoxy sugars. Signals between 1.75 and 2.25 ppm corresponded to methyl protons of O- and N- attached acetyl groups. The two intense singlets at 3.15 and 3.25 ppm corresponded to O-methyl groups of 6-deoxysugars. The cluster of signals between 4.75 and 5.15 ppm were assigned to anomeric protons. The rest of the signals corresponded to ring protons. Notice the main differences between ^{1}H-NMR spectra at the level of protons of the sugar rings, attributed to the fact that the second one (B) contained mannose, galactose and glucose which were not present in the first one (A). The ^{13}C-NMR spectrum shows the eight resonances between 15 and 22 ppm that corresponded to the methyl carbons of the 6-deoxy-hexoses and of acetate groups. Carbonyl carbons belonging to O-acetyl groups appeared as eight resonances between 172 and 178 ppm, ring carbons attached to nitrogen appeared at 56.5 ppm and the six anomeric carbon resonances appeared between 95 and 104 ppm. The rest of the signals corresponded to carbons of the sugar rings.


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

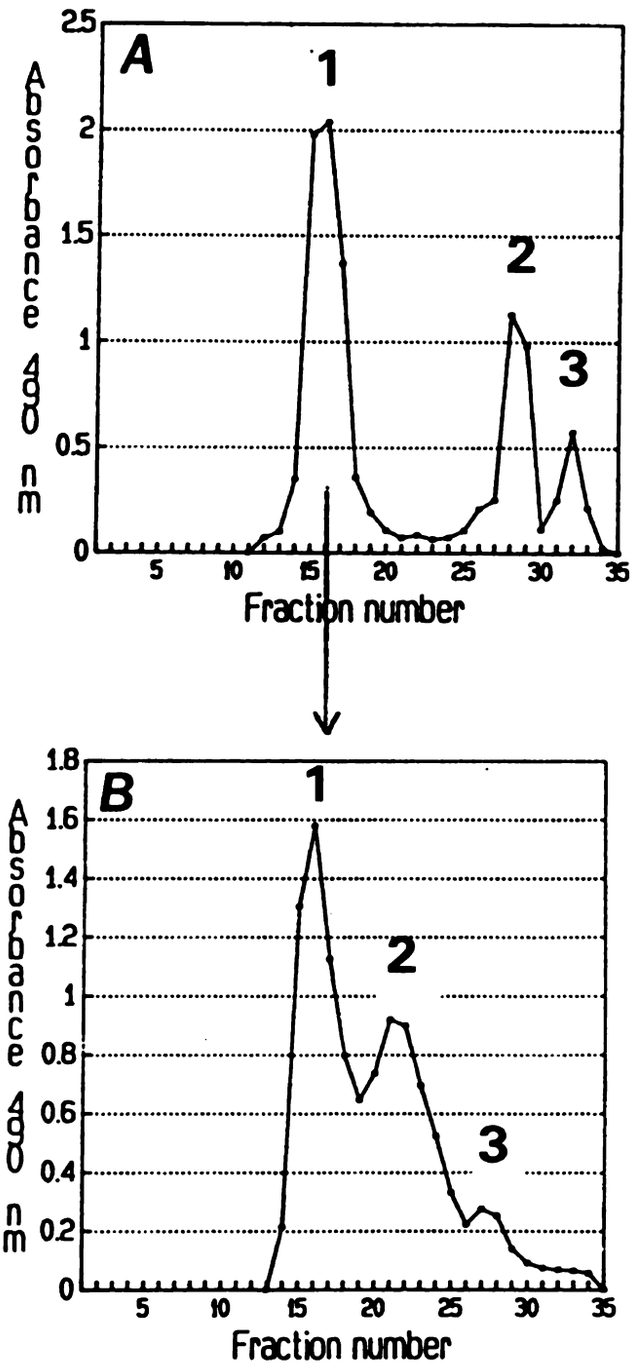

B
200 180 160 140 120 100 80 60 40 20 0 ppm


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

substituents or end of this oligosaccharide fragment. The presence of 2,3-O-methyl,6-deoxyhexose was again attributed to a side chain substituent, or to the methylation of the already existing oligosaccharide at the 2-O-methyl,6-deoxyhexose residue due to the acid nature of the growth medium. The ^{1}H-NMR spectrum of this oligosaccharide (Figure 3C), indicated that the main differences between both oligosaccharides were at the level of protons of the sugar ring signals. This was attributed to the fact this oligosaccharide contained mannose, galactose and glucose that were not present in the former one. Further purification of both oligosaccharides was achieved by ion-exchange chromatography over DEAE-sephadex. The resulting fractions are under investigation.

Gel filtration chromatography (Biogel P-2) of the aqueous fraction of the LPS from ***Rhizobium tropici*** cells grown in acid medium (pH 4.5) resulted in the separation of three peaks (Figure 4A). Peak 3, corresponded to an unusual non-acetylated trisaccharide which contained KDO, galactose and fructose. The presence of KDO was determined by the periodate/thiobarbituric assay described in the materials and methods section. The m-phenylphenol method was negative and therefore no uronic acids were present. The glycosyl compositional analyses determined after derivatizing the oligosaccharide to alditol acetates directly or on a fragment that had been prereduced for three

FIG. 4. Gel permeation chromatography profile of the water soluble components of the LPS. (A): Biogel P-2 profile of the carbohydrate components of the LPS after hydrolysis with 1% acetic acid, for ***Rhizobium tropici*** cells grown in acid medium (pH 4.5). (B): Gel permeation chromatography (Biogel P-10) profile of the "O-antigen" fragment of the LPS (corresponds to peak 1 in Figure 4A). Carbohydrate content was monitored with the phenol-sulfuric acid assay.


A
1
2
3
Absorbance 490 nm
Fraction number
B
1
2
3
Absorbance 490 nm
Fraction number

hours were not different, thus confirming the absence of uronic acids. The presence of fructose at the reducing end of this trisaccharide was determined by reducing the fragment with sodium borodeuteride before performing the usual alditol acetate derivatization, described in the materials and methods section. The uptake of one deuterium atom by the fructose molecule during the prereduction step was determined by observing the presence of deuterium-labelled mannose and glucose in the GC-MS analysis. Mannose and glucose, which result from the isomerization of fructose during the alditol acetates derivatization, cannot be liberated at the reducing end by the mild acid hydrolysis treatment that the LPS was exposed to.

Peak 2 (Figure 4A), corresponded to an unusual non-acetylated oligosaccharide which contained KDO, mannose, galactose and glucose in an 1:1:2:1 ratio. The presence of KDO was determined by the periodate/thiobarbituric assay described in the materials and methods section. The m-phenylphenol method was negative and therefore no uronic acids were present.

Peak 1 (Figure 4A), which corresponded to the so called "O-antigen" fragment, was further purified by gel permeation chromatography over Biogel P-10. Three major carbohydrate peaks were recovered (Figure 4B). The first one contained a

wide variety of methylated sugars (Figure 5A), including 2,3-O-methyl,6-deoxyhexose, 2-O-methyl,6-deoxyhexose, 1,3-O-methylhexose, 1-O-methylhexose, 2-O-methylhexose and 3-O-methylhexose. The major glycosyl components, mannose and galactose, were present in an approximately 1:1 ratio. Fucose, glucose and 2-amino,2,6-dideoxyhexose were also present. The ^{1}H-NMR spectrum of this oligosaccharide is shown in Figure 6A, where resonances between 1.00 and 1.20 ppm corresponded to the methyl protons of the 6-deoxy sugars. The signals between 1.80 and 2.10 ppm were assigned to methyl protons of the O- and N-attached acetyl groups. The intense singlet at 3.25 ppm corresponded to the O-methyl groups of the 6-deoxysugars. The cluster of signals between 4.70 and 5.20 ppm were assigned to anomeric protons. The rest of the signals corresponded to ring protons. The ^{13}C-NMR spectrum of this oligosaccharide (Figure 6B, showed resonances between 14 and 24 ppm which corresponded to the methyl carbons of the 6-deoxy-hexoses and of acetate groups. Resonances between 168 and 176 ppm corresponded to carbonyl carbons of O-acetyl groups. Ring carbons attached to nitrogen appeared at 60 ppm and the anomeric carbon resonances were observed between 98 and 105 ppm. The rest of the signals corresponded to carbons of the sugar rings. It still needs to be determined if this oligosaccharide can be further fractionated by ion-exchange chromatography.

FIG. 5. Gas chromatography profile of the alditol acetate derivatives of the glycosyl components of the oligosaccharide fractions eluting as peaks 1 (chromatograph A) and 3 (chromatograph B), respectively, from the gel permeation chromatography (Biogel P-10) profile shown in Figure 4B. In each chromatography, peaks correspond to (1) 2,3-O-methyl,6-deoxyhexose, (2) 2-O-methyl,6-deoxyhexose, (3) rhamnose, (4) quinivose, (5) fucose, (6) 1,3-O-methylhexose, (7) 1-O-methylhexose, (8) 2-O-methylhexose, (9) 3-O-methylhexose, (10) mannose, (11) galactose, (12) glucose and (13) 2-amino,2,6-dideoxyhexose.

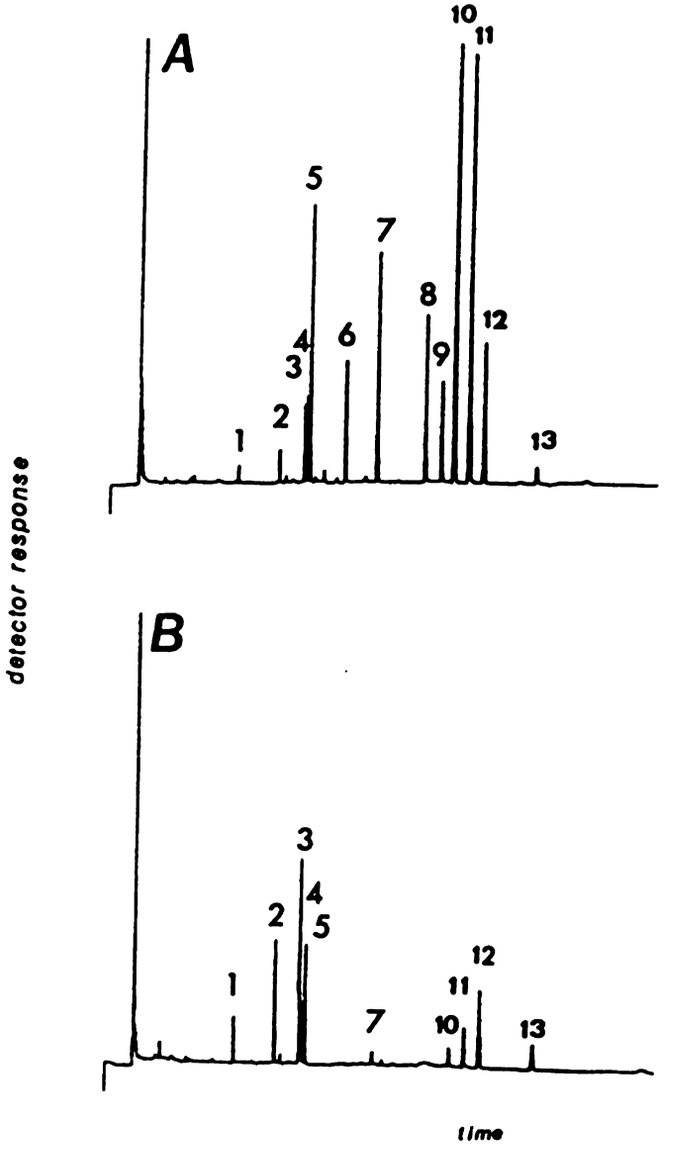
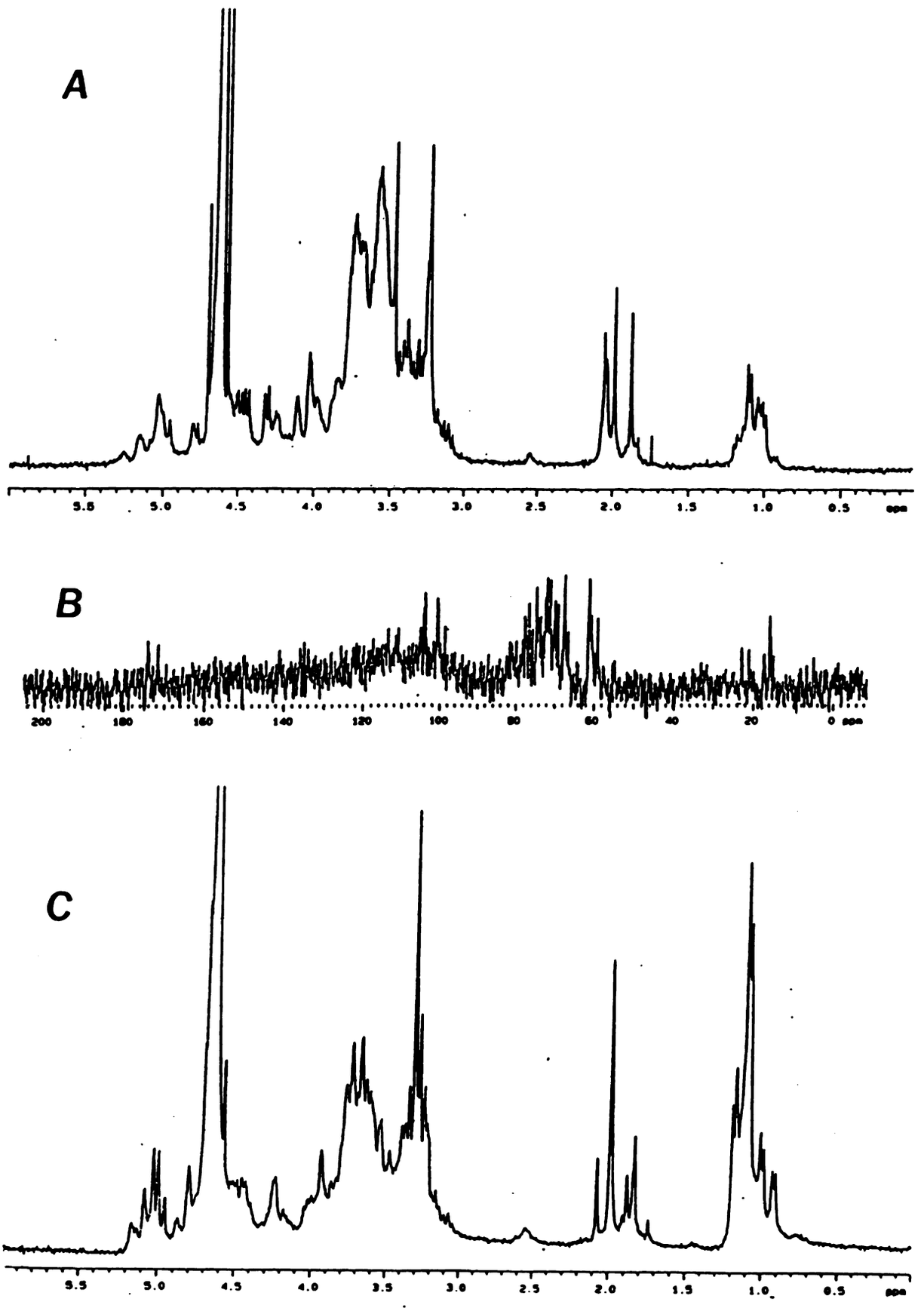

A
B
1
2
3
4
5
6
7
8
9
10
11
12
13
detector response
time

FIG. 6. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of the oligosaccharide fraction eluting as peak 1 in Figure 4B, correspond to the alditol acetates gas chromatograph shown in Figure 5A. ^{1}H-NMR (C) spectrum of the oligosaccharide fraction eluting as peak 3 in Figure 4B, corresponds to the alditol acetates gas chromatograph shown in Figure 5B. In these figures ^{1}H-NMR resonances between 0.85 and 1.35 ppm (relative to external Me_4Si), corresponded to the methyl protons of 6-deoxy sugars. Signals between 1.75 and 2.25 ppm corresponded to methyl protons of O- and N- attached acetyl groups. The two intense singlets at 3.15 and 3.25 ppm corresponded to O-methyl groups of 6-deoxysugars. The cluster of signals between 4.75 and 5.15 ppm were assigned to anomeric protons. The rest of the signals corresponded to ring protons. Notice the intensity of the signals corresponding to the protons of methyl groups from 6-deoxysugars and the intensity of the ones corresponding to O- and N-attached acetyl groups in the second ^{1}H-NMR spectrum (B) relative to the intensities observed in the first one (A). The ^{13}C-NMR spectrum shows resonances between 15 and 22 ppm that corresponded to the methyl carbons of the 6-deoxy-hexoses and of acetate groups. Carbonyl carbons belonging to O-acetyl groups appeared as resonances between 172 and 178 ppm, ring carbons attached to nitrogen appeared at 56.5 ppm and the anomeric carbon resonances appeared between 95 and 104 ppm. The rest of the signals corresponded to carbons of the sugar rings.


A
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm
B
200
180
160
140
120
100
80
60
40
20
0 ppm
C
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

The second peak eluting from the gel permeation chromatography over Biogel P-10 (peak 2, Figure 4B) corresponded to an oligomer formed by a trisaccharide repeating unit whose structure is described in Appendix II. This is the first structure ever reported for an O-antigen fragment of a ***Rhizobium*** species.

The third peak eluting from the gel permeation chromatography over Bio-gel P-10 (peak 3, Figure 4B), contained primarily 2-O-methyl,6-deoxyhexose, rhamnose and fucose (Figure 5B). Mannose, galactose, glucose, 2-amino,2,6-dideoxyhexose, 1,3-O-methylhexose and 2,3-O-methyl,6-deoxyhexose were also present. As expected from the predominant presence of 6-deoxyhexoses and methylated 6-deoxyhexoses in this oligosaccharide, its ^{1}H-NMR spectrum revealed strong signals for the methyl protons of the 6-deoxy hexoses between 0.9 and 1.25 ppm and a sharp singlet due to the protons of the O-methyl groups at 3.25 ppm (Figure 6C). Signals between 1.80 and 2.10 ppm indicated that this oligomer was acetylated.

Our results indicate that under acid conditions the synthesis of the previously identified unusual trisaccharide (Chapter 3) was induced in ***Rhizobium leguminosarum*** biovar ***phaseoli*** UMR 1632. Under these conditions, this biovar still synthesized the tetrasaccharide and trisaccharide components

of the LPS that are common to several ***Rhizobium*** species (4,5,17,18). The "O-antigen" fragment was composed of several fragments that were separated by size exclusion chromatography. The glycosyl composition of the fractions resulting from the size exclusion chromatography was similar, differing primarily at the level of side chain substituents and methylation pattern. The high level of methylation observed was consistent with previous studies that indicated that methylation is induced at the O-antigen level in free-living ***Rhizobium*** cells grown under acid conditions (Chapter 3, 1). Within the fractions, some of them contained several oligosaccharides held together by electrostatic interactions and separation was achieved by ion-exchange chromatography. Further investigation on the composition of these oligosaccharides is in progress.

In contrast, ***Rhizobium tropici*** UMR 1899, did not synthesize the usual galacturonic acid containing tetrasaccharide and trisaccharide components of the LPS common to several ***Rhizobium*** species (4,5,17,18). Eluting with similar retention time to these two oligosaccharides, the presence of a new trisaccharide and a short oligosaccharide were observed. The structures of these two new molecules, which were devoid of galacturonic acid, are under investigation. The "O-antigen" fragment of this strain contained three major fragments that

could be separated by size exclusion chromatography. Two of them were methylated, acetylated and contained a large number of distinct glycosyl components. It still needs to be determined if these fragments are composed of several oligosaccharides held together by electrostatic interactions. The third fraction recovered corresponded to a trisaccharide repeating unit. Its structure is described in Appendix II.

Based on these analyses, no similarities were found at the level of cell-surface carbohydrates between the two species analyzed. Cell-surface carbohydrates could therefore play a role in determining resistance to acid conditions and symbiotic stability. The lack of similarity found between their carbohydrate components is consistent with previous findings from previous studies, where completely different alternate carbohydrate structures had the same function in symbiosis (14,35,36,38,39). Further, it strengthens our theory (Chapter 5), that during the infection process, several molecules can have the same biological effect provided they contribute with the same charges, charge distribution, geometries and electrostatic interactions to the system.

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APPENDIX II

THE STRUCTURE OF AN O-ANTIGEN FRAGMENT ISOLATED FROM A LIPOPOLYSACCHARIDE SYNTHESIZED BY RHIZOBIUM TROPICI CELLS GROWN IN ACID MEDIUM.

ABSTRACT

The structure of one of the O-antigen fragments of the lipopolysaccharides (LPS) produced by ***Rhizobium tropici*** UMR 1899 free-living cells grown in acid medium (pH 4.5) was isolated and characterized. This fragment is an oligosaccharide consisting of an acetylated trisaccharide repeating unit with a glucose molecule at the nonreducing end and a KDO molecule at the reducing end. The glycosyl components of the repeating unit are a 1,4-linked glucose, a 1,3-linked fucose and an unusual 1,4-linked 6-deoxyhexose. The sequence of the fucose and unusual 6-deoxyhexose in the linkage still needs to be determined. All of the aldoses are in the pyranose form, and all of the glycosidic linkages are of the α-form.

The composition of this trisaccharide repeating unit was determined by gas-liquid chromatography of the alditol acetate derivatives of the glycosyl components. The presence of the two methyl groups corresponding to the 6-deoxy sugars were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. These same spectra was used to determine the configuration of the anomeric linkages. Linkage analysis was obtained from the methylation analysis.

INTRODUCTION

Among the carbohydrate components of the cell-surface of ***Rhizobium*** cells, the lipopolysaccharides (LPS), are thought to play a key role in the nitrogen-fixing symbiotic relationship between these bacteria and their host legume plants. Specifically, the O-antigen component of the LPS is a critical requirement for host root infection, nodule development and maintenance (12). Bacterial phenotypes with O-antigen defective LPS are associated defective infection thread development, failure of bacteria to be released from infection threads and the formation of empty, ineffective nodules (2,5,6,8,14,15,16,18). One of the major complications in defining the role of the O-antigen fragment in symbiosis is the lack of structural information available on these molecules.

Structural information on carbohydrate fragments of the LPS has only been obtained for a tetrasaccharide and trisaccharide components of the LPS of several ***Rhizobium leguminosarum*** strains (3,9,10,11). In this earlier work, the tetrasaccharide and trisaccharide were referred to as "core components" of the same LPS molecule. In our recent studies

(Chapter 2 and Chapter 3), we have shown that synthesis of these two molecules is not tightly coupled and that the extent of their syntheses may be separately regulated by the environmental conditions to which the cells are exposed. If this is correct, these two molecules cannot be part of the same "core". These two oligosaccharides, which are conserved between ***Rhizobium*** of related strains (3,9,10,11), were not found in the LPS of the ***Rhizobium tropici*** strain (Appendix I).

The O-antigen fragment is known to be variable from strain to strain; however, it has traditionally been thought that each bacterial strain contains one or at most two different O-antigen containing LPS. In our recent studies (Chapter 2,3 and 4), we have shown that, free-living ***Rhizobium*** cells grown under the same culture conditions synthesized a wide variety of chemically distinct O-antigen fragments some of which were held together by electrostatic interactions. The synthesis of new and chemically distinct oligosaccharides was induced when cells were grown under different environmental conditions which included low oxygen tension, low pH, succinate enriched medium and presence of flavone inducers.

In this study we determined the structure of an O-antigen fragment extracted from the LPS of ***Rhizobium tropici*** UMR 1899. ***Rhizobium tropici***, which as ***Rhizobium leguminosarum*** biovar ***phaseoli***,

nodulates ***Phaseolus vulgaris*** L. and ***Leucaena*** sp. trees was recently proposed as a new strain by Martinez-Romero et al. (13). Elucidating the structure of one of the O-antigen components of the LPS of these bacteria is the first step for determining if this carbohydrate fragment plays a role in defining the favorable competitiveness of the ***Rhizobium tropici*** strain under acid conditions. It is also the first step for understanding the molecular basis of the role of this carbohydrate fragment in the symbiotic process. This is the first study in which the chemical structure of an O-antigen fragment has been determined.

MATERIALS AND METHODS

General methods. Compositional analysis of the O-antigen fragment was determined on the carbohydrate alditol acetate derivatives (17) by gas chromatography (GC) using a capillary DB225 column and flame ionization detector. Glycosyl composition was confirmed by gas chromatography/mass spectrometry (GC/MS) analysis on a JEOL 505 mass spectrometer in the electron impact mode. The glycosyl linkages of the O-antigen oligosaccharide were determined by the Hakamori methylation procedure according to York et al. (21). The methylated products were hydrolyzed with 2M TFA, for 2 hours at 120°C, before converting to alditol acetate derivatives. The partially methylated alditol acetate derivatives were separated and identified by GC-MS. Tests for uronic acids and 3-deoxy-D-manno-2-octulosonic acid (KDO) were done using the m-phenylphenol (1) and the periodate/thiobarbituric acid (19) assays respectively. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR300 spectrometer operating at 300 MHz for protons and 75.43 MHz for ^{13}C. Spectra were obtained in D_2O solutions and chemical shifts were referenced relative to external TMS. Carbohydrate content in fractions from gel filtration columns was monitored by the phenol-sulfuric acid

method (7).

LPS and LPS fragments isolation and purification. Bacteria were inoculated on B-III agar plates and transferred after 3 days to 500 ml Erlenmeyer flasks containing 250 ml of B-III liquid medium (pH 6.9) (4). At late log phase each 250 ml inoculum was transferred to separate 4 liter Erlenmeyer flasks containing 2 liters of B-III medium (pH 6.9). Once the cells reached mid-to-late exponential growth, 2 liters of B-III medium at a pH of 3.2 (obtained using free glutamic acid in place of sodium glutamate and adjusting the pH with HCl) were added into each flask and the pH of each adjusted to give a final value of 4.5. The contents of each flask were then split into two 4 liter Erlenmeyer flasks each containing about two liters of culture. Cells were grown until the beginning of stationary phase and harvested by centrifugation. Cultures were constantly shaken at 265 rpm.

LPS was extracted by the hot phenol-water method (20), treated with DNAse-RNAse and purified by gel permeation chromatography over Sepharose 4B with aqueous formic acid (0.05M, adjusted to pH 5.5 with ammonia) as the mobile phase. Carbohydrate fragments were released by hydrolyzing the LPS with 1% acetic acid at 100°C for 2h and partitioned between water and chloroform. The aqueous layer was lyophilized and fractionated by size exclusion chromatography over Biogel P2 with aqueous formic acid (0.1%) as the mobile phase. The peak

that voided the P2 column was further fractionated by size exclusion chromatography over Biogel P10 with aqueous formic acid (0.1%) as the mobile phase. Fractions eluting as a peak were pooled, lyophilized and analyzed by ^{1}H-NMR spectroscopy and by GC and GC/MS after derivatization into alditol acetates as described in the general methods section.

RESULTS AND DISCUSSION

The aqueous portion of the LPS from ***Rhizobium tropici*** UMR 1899 was separated by gel permeation chromatography over Biogel P-2 with 1% ammonium formate as the mobile phase. Three major carbohydrate peaks were recovered. The first eluting carbohydrate peak, which voided the column and is normally referred to as the "O-antigen" fragment, was further fractionated by gel permeation chromatography over Biogel P-10 with 1% formic acid as the mobile phase. Three major carbohydrate peaks were recovered from this column which corresponded to the different O-antigenic fragments previously described (Appendix I).

Further chemical analyses were performed on the second eluting peak from the gel permeation chromatography over Biogel P-10. Glycosyl composition of this fragment indicated the presence of three major components which corresponded to fucose, glucose and an unidentified peak. The m-phenylphenol and periodate/thiobarbituric acid tests indicated that this fragment was devoid of uronic acids but that it contained KDO.

The ^{1}H-NMR spectrum of this peak confirmed the presence

of KDO with the low intensity multiplet between 1.8 and 2.1 ppm (Figure 1). This same spectrum revealed the presence of three anomeric protons with resonances at 4.82, 4.96 and 5.17 ppm, respectively, indicating that the linkages were α. In this figure, one of the two doublets at 0.98 and 1.08 ppm corresponded to the protons of the methyl group from fucose, the other one to a methyl group from a different 6-deoxyhexoses. The sharp singlet at 1.98 ppm was assigned to protons of O-attached methyl groups from acetate. Consistent with the absence of methylated sugars in the glycosyl composition analysis, no sharp singlets were observed in the region between 2.2 and 3.0 ppm. The rest of the signals corresponded to protons in the sugar rings. Three anomeric carbon signals were observed in the ^{13}C-NMR spectrum (Figure 2) with three resonances between 98 and 102 ppm. One of the two resonances at around 16 ppm corresponded to the methyl carbon of fucose, the other one to a second 6-deoxysugar. The resonance at 21 ppm corresponded to the methyl carbons of acetate residues. The signal at 60 ppm was assigned to the ring proton corresponding to a free primary carbon and hydroxyl group from glucose. The resonance for the carbonyl carbon of acetate was observed at 174 ppm. The rest of the signals corresponded to carbons from the sugar ring.

The gas chromatography profile of the partially methylated sugars from this oligosaccharide indicated the

FIG. 1. ^{1}H-NMR spectrum of the O-antigen fragment isolated and purified from one of the lipopolysaccharides synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5). The low intensity multiplet between 1.8 and 2.1 ppm confirmed the presence of KDO in this fragment. Three resonances corresponding to three anomeric protons were observed at 4.82, 4.96 and 5.17 ppm, respectively, indicating that the linkages were α. One of the two doublets at 0.98 and 1.08 ppm corresponded to the protons of the methyl group from fucose, the other one to a methyl group from a different 6-deoxyhexose. The sharp singlet at 1.98 ppm was assigned to protons of O-attached methyl groups from acetate. The rest of the signals corresponded to protons in the sugar rings.

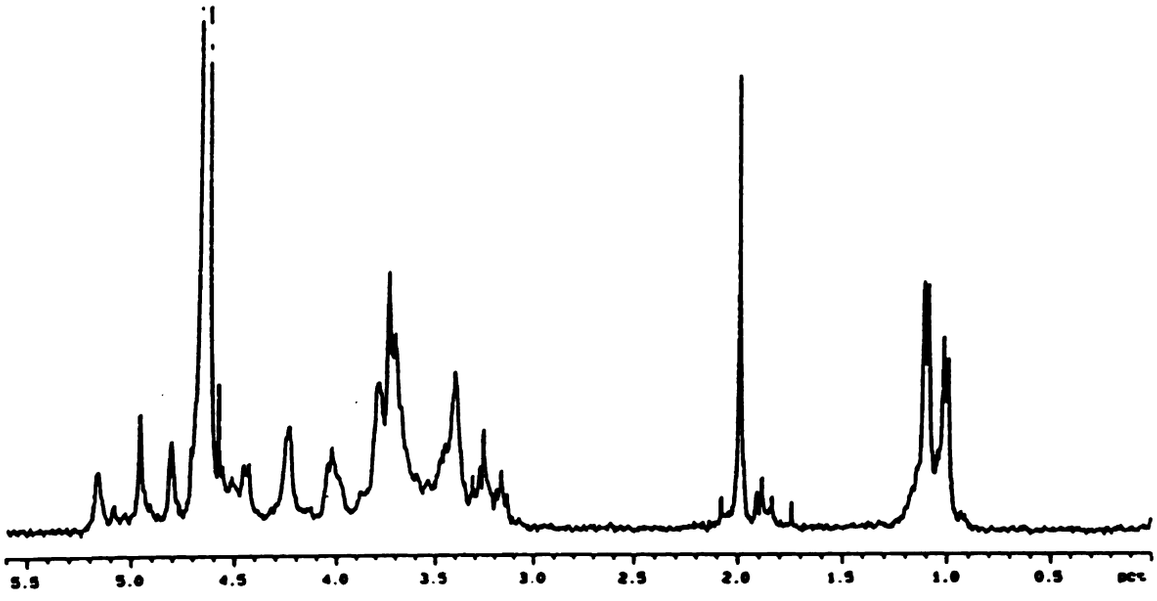

5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
PPM

FIG. 2. ^{13}C-NMR spectrum of the O-antigen fragment isolated and purified from one of the lipopolysaccharides synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5). The three resonances between 98 and 102 ppm corresponded to the three anomeric carbons of this oligosaccharide. One of the two resonances at around 16 ppm corresponded to the methyl carbon of fucose, the other one to a second 6-deoxysugar. The resonance at 21 ppm corresponded to the methyl carbons of acetate residues. The signal at 60 ppm was assigned to the ring proton corresponding to a free primary carbon and hydroxyl group from glucose. The resonance for the carbonyl carbon of acetate was observed at 174 ppm. The rest of the signals corresponded to carbons from the sugar ring.

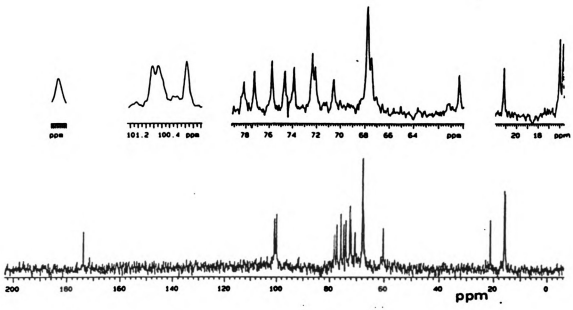
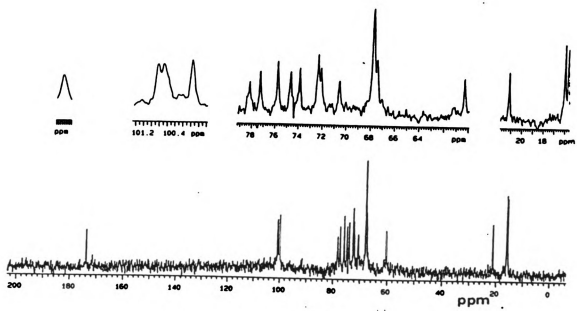

ppm
101.2 100.4 ppm
78 76 74 72 70 68 66 64 ppm
20 18 ppm
200 180 160 140 120 100 80 60 40 20 0
ppm

FIG. 2. ^{13}C-NMR spectrum of the O-antigen fragment isolated and purified from one of the lipopolysaccharides synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5). The three resonances between 98 and 102 ppm corresponded to the three anomeric carbons of this oligosaccharide. One of the two resonances at around 16 ppm corresponded to the methyl carbon of fucose, the other one to a second 6-deoxysugar. The resonance at 21 ppm corresponded to the methyl carbons of acetate residues. The signal at 60 ppm was assigned to the ring proton corresponding to a free primary carbon and hydroxyl group from glucose. The resonance for the carbonyl carbon of acetate was observed at 174 ppm. The rest of the signals corresponded to carbons from the sugar ring.


ppm
101.2 100.4 ppm
78 76 74 72 70 68 66 64 ppm
20 18 ppm
200 180 160 140 120 100 80 60 40 20 0
ppm

presence of three major peaks. The carbohydrate peak that eluted first corresponded to 1,3-linked fucose (Figure 3); the second peak to 1,4-linked glucose (Figure 4) and the third one to a 1,4-linked 6-deoxyhexose which had a mass spectrum showed in Figure 5. This same figure also shows the proposed tentative structure for this unusual sugar. The presence of a minor peak corresponding to a 1-linked glucose residue was also detected in the gas chromatography profile from the methylation analysis. This glucose residue was therefore present at the nonreducing end of the oligosaccharide. Because of the nature of the hydrolysis through which this oligosaccharide fragment was released, the KDO fragment was assigned to the reducing end. All of the aldoses were in the pyranose form.

Based on these analyses we propose that this O-antigen fragment is an oligosaccharide consisting of an acetylated trisaccharide repeating unit which starts with glucose at the nonreducing end, followed by a 1,3-linked fucose and the 1,4-linked unusual 6-deoxysugar in a sequence that still needs to be determined. The repeating unit starts again with glucose which is now 1,4-linked to either fucose or the unusual 6-deoxyhexose. A KDO residue is present at the reducing end, linking this molecule to a core component or to the lipid A fragment of the LPS from which it was isolated. All glycosyl components were in the pyranose form and were attached by

FIG. 3. Electron impact mass spectrum and fragmentation scheme of the 1,3-linked fucose component of the O-antigen fragment isolated and purified from one of the LPS synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5).

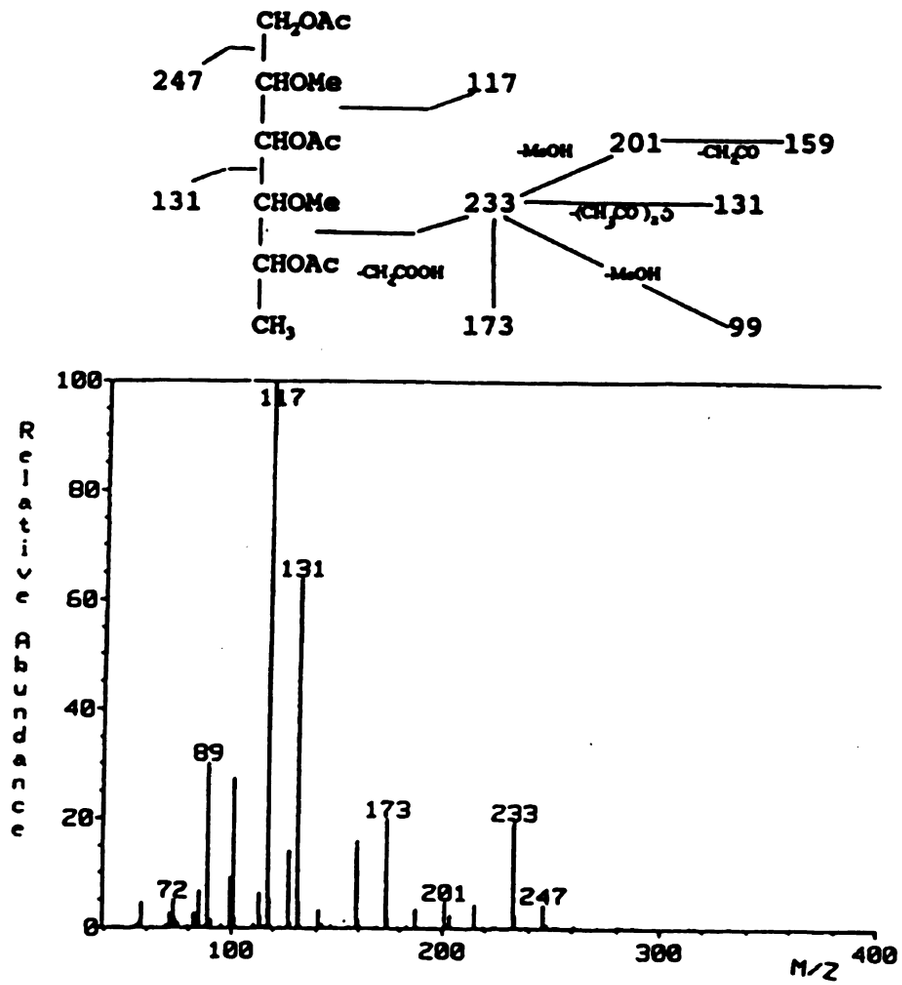
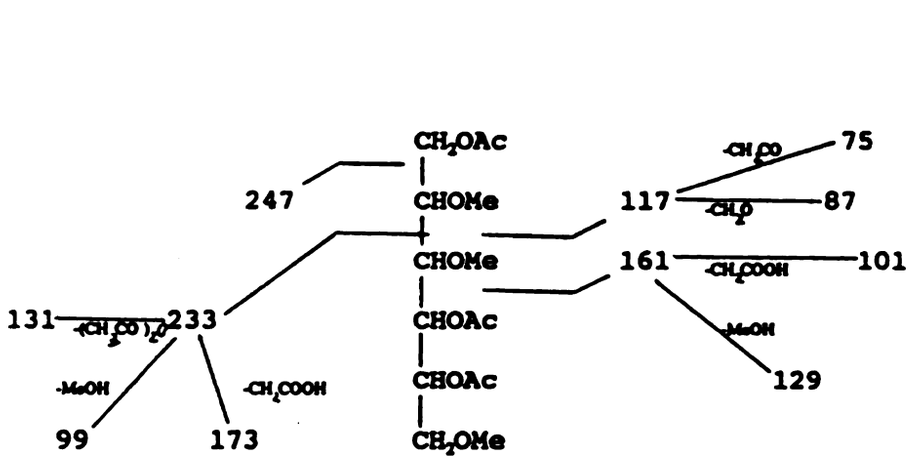

CH2OAc
247
CHOMe
117
CHOAc
-MeOH
201
-CH2CO
159
131
CHOMe
233
-(CH2CO)2O
131
CHOAc
-CH2COOH
-MeOH
CH3
173
99
Relative Abundance
100
80
60
40
20
0
117
131
89
173
233
72
201
247
100
200
300
400
M/Z

FIG. 4. Electron impact mass spectrum and fragmentation scheme of the 1,4-linked glucose component of the O-antigen fragment isolated and purified from one of the LPS synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5).


CH2OAc
CHOMe
CHOMe
CHOAc
CHOAc
CH2OMe
247
131
233
99
173
117
75
87
161
101
129
-(CH3CO)2O
-MeOH
-CH2COOH
-CH2CO
-CH2O
-CH2COOH
-MeOH

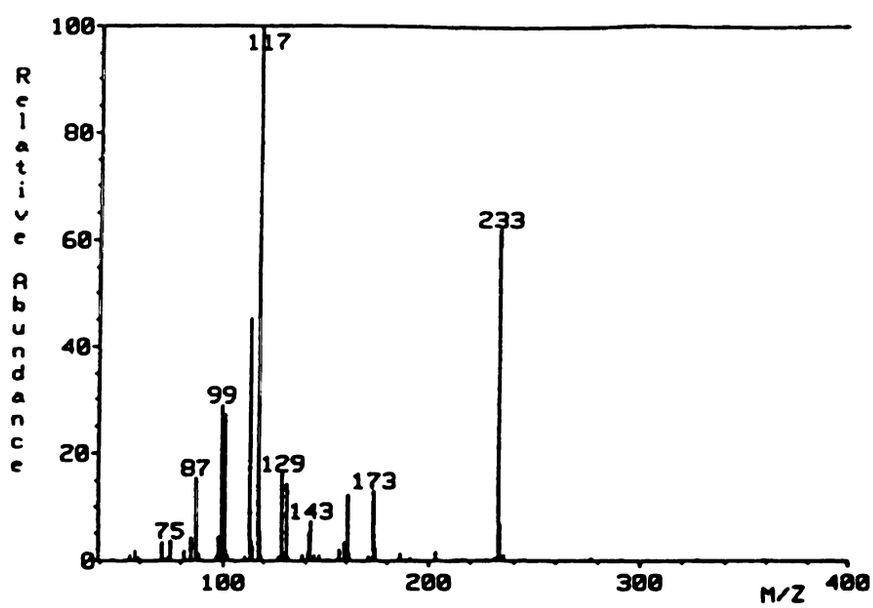
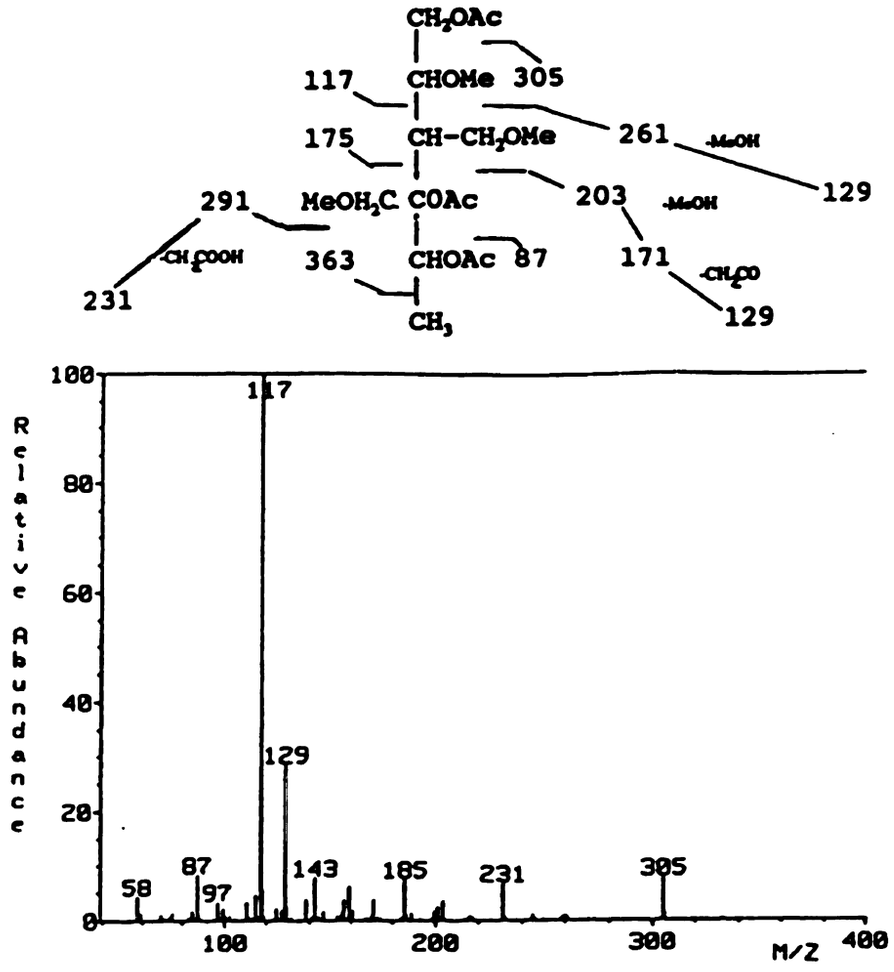

Relative Abundance
M/Z
100
80
60
40
20
0
100
200
300
400
117
233
99
87
129
173
143
75

FIG. 5. Electron impact mass spectrum, proposed tentative structure and fragmentation scheme of the unusual 1,4-linked 6-deoxyhexose component of the O-antigen fragment isolated and purified from one of the LPSs synthesized by ***Rhizobium tropici*** cells grown in acid medium (pH 4.5).


CH2OAc
117
CHOMe
305
175
CH-CH2OMe
261
-MeOH
129
291
MeOH2C
COAc
203
-MeOH
-CH2COOH
363
CHOAc
87
171
-CH2CO
231
CH3
129
Relative Abundance
100
80
60
40
20
0
117
129
87
58
97
143
185
231
305
100
200
300
400
M/Z

α-linkages. The presence of glycosyl side chain substituents is under investigation.

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