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The Structure of O-antigen from Lipopolysaccharide of Rhizobium leguminosarum 128C53 and Its Nod-Fix-Mutant

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The Structrue of O-antigen from Lipopolysaccharide of *Rhizobium leguminosarum* 128C53 and Its Nod-Fix- Mutant

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THE STRUCTURE OF O-ANTIGEN FROM LIPOPOLYSACCHARIDE OF

RHIZOBIUM LEGUMINOSARUM 128C53 AND ITS NOD$^-$ FIX$^-$ MUTANT

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ABSTRACT

The LPS of R. leguminosarum 128C53 sm^r rif^r (a streptomycin and rifampicin resistant strain of wild type 128C53) and its mutant ANU54 (nod^-, fix^-) were isolated from the bacterial pellet by hot phenol/water extraction followed by gel filtration chromatography. The O-antigen was isolated by mild-acid hydrolysis of the LPS and purified by gel filtration chromatography using a Sephadex G-50 or G-25. The following results were the same for both the parent and mutant. The composition and linkage of the O-antigen were determined by gas chromatography (GC), GC-mass spectrometry, ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) techniques. The data indicate that the O-antigen of the LPS from parent R. leguminosarum 128C53 and its mutant ANU54 are identical. The O-antigen contains a tetrasaccharide repeating unit. The backbone consists of one 1,3-linked-rhamnose and two 1,3-linked-fucose residues. A terminal mannose is linked to the 2-position of one of the two fucose residues. The ^1H-NMR analysis indicates that all the glycosyl residues are alpha linked. The exact position of the mannose residue is under further investigation.

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INTRODUCTION

Chemical analyses show that the four most abundant elements present in plant tissues are usually carbon, hydrogen, oxygen and nitrogen. Of these, the first three are available to the plant in ample supply from atmospheric and soil sources, primarily as carbon dioxide, water and oxygen. On the other hand, although nitrogen comprises 80% of the atmosphere, it cannot be directly used by the vast majority of plants. As a result, nitrogen is typically supplied to plants in the form of various commercial fertilizers such as anhydrous ammonia, urea, and other nitrogenous compounds.

However, certain microorganisms are able to convert atmospheric nitrogen into ammonia. **Rhizobium** is one of the organisms that carries out this conversion through a symbiotic relationship with legume plants. Owing to nitrogen's importance as a macronutrient in plant growth and development and the increasing cost of commercial fertilizers, the biological process of nitrogen fixation has currently become an attractive topic for research.

Rhizobium is very specific in its ability to effectively infect the legume host plant. For example,

Rhizobium leguminosarum only nodulates peas, Rhizobium trifolii only nodulates clover and Rhizobium japonicum nodulates soybeans. According to their growth rate in culture, rhizobia are classified into the fast-growing (e.g. R. leguminosarum, R. trifolii) and the slow-growing (e.g. R. japonicum) organisms.

The process of nodulation of the host legume by a species of Rhizobium consists of several steps (2,43). Attachment of Rhizobium cells to legume root hairs is the first step in the required sequence of interactions leading to infection and nodulation (16). It has been hypothesized that the attachment to the host legume root hair is mediated by the host plant lectins (8,15,18). This mechanism is a reasonably common feature of legume/Rhizobium association. After this attachment, Rhizobium induces the curling and branching of root hairs. The subsequent result is the formation of infection threads which are tubular structures that carry Rhizobium cells, often single file, from the root surface into the root cortex. A Rhizobium is released from the end of the infection thread in a host membrane envelope where it undergoes a morphological change, establishing the bacterial symbiont in the host cortical cell cytoplasm. These early steps are followed by the induction of plant cell division in the localized region of infection and subsequent nodule growth and development (19). The nodules

are the location where the nitrogen is converted into ammonia catalyzed by nitrogenase, an enzyme produced by the **Rhizobium.**

Rhizobium is a gram-negative bacterium. In this bacterium, a thin peptidoglycan layer is sandwiched between the cytoplasmic membrane and an outer membrane (40). The outer membrane contains lipopolysaccharide (LPS), phospholipid and lipoprotein. In addition to LPS, the surface polysaccharides also consist of extracelluar polysaccharides (EPS) and capsular polysaccharides (CPS), which exist in the surrounding medium. All of these molecules have been thought to play an important role in the specific attachment of the symbiont bacteria to the legume host (10,16).

There are many reports which indicate a role for the EPS in the symbiotic process of **Rhizobium.** An example is the mutant of **R. leguminosarum** which produces diminished amounts of EPS will not nodulate its host pea, while mutants which produce larger amounts of EPS will produce more nodules (39). The result that diminished production of EPS is related to the inability of the mutant to nodulate its host has been described by Sanders et al (38). Also, they have reported that the exo^- LPS has a very similar structure to the parental LPS (38). These results suggest that **Rhizobium** EPS is important for the initial interaction

between a **Rhizobium** and its host. Moreover, the structural sequences of EPS from several species of **Rhizobium** have been determined (8). These studies have shown that **Rhizobium** EPS can be very complex structures containing a repeating oligosaccharide of more than eight sugars (8).

However, **Rhizobium** LPS has not been investigated as extensively as the EPS. Unlike EPS, the sugar compositions of highly purified LPS from several strains of fast-growing **Rhizobium** indicate that LPS composition varies greatly from strain to strain (7,8,50). The LPS is synthesized in the plasma membrane and is then transferred to the outer membrane (41). The LPS from those of published studies of **Salmonella** and **E. Coli** have been shown to be present in two general forms. One form is a complete molecule which contains three major regions : O-antigen (polysaccharide), core (oligosaccharide), and lipid A (33,48). The other form is the incomplete molecule which lacks the O-antigen (27). The O-antigen is a polysaccharide that consists of a repeating oligosaccharide which is attached to the core oligosaccharide and varies in composition from strain to strain (10,13). The core oligosaccharide is attached to the lipid-A through an acid-labile ketosidic bond with 2-keto-3-deoxyoctonic acid (KDO) (27). The lipid-A moiety is the hydrophobic part of this large molecule. It contains fatty acids which may be linked to glucosamine residues. In

contrast to the fatty acyl part of lipid-A, the O-antigen and core are highly hydrophilic. The polysaccharide region of **Rhizobium** LPS can be separated from the lipid-A by mild acid hydrolysis with acetic acid due to the lability of the ketosidic KDO bond (21). The two LPS structural regions, the core oligosaccharide and O-antigen oligosaccharide, can be separated by using Sephadex G-50 gel-filtrarion chromatography.

Although the LPS compositions vary greatly among different strains, they have some features in common (8). These features are : i) the LPS of all of the strains (**R. leguminosarum, R. trifolii,** and **R. phaseoli**) contain lipid-A, KDO, and polysaccharide, and ii) the lipid-A--polysaccharide bond can be hydrolyzed by mild acid. In addition, more recent studies have shown that the core regions of LPS from **R. phaseoli, R. leguminosarum** and **R. trifolii** all have molecular weights of about 600-700 and are composed largely of galacturonic acid with smaller amounts of mannose, galactose, glucose (10, 13) and KDO (13). Recently, the structure of the major core component from **Rhizobium trifolii** ANU843 LPS has been determined (11). It consists of two terminal galacturonic acid residues linked (alpha) to the 4 and 7 positions of KDO (11). In most cases the O-antigens are likely to be complex oligosaccharides (10,13). For several **R. trifolii** LPS it is known that both

the O-antigen and core have KDO at their reducing end indicating that both are linked to the remainder of the LPS molecule via a KDO residue (11,13). These results are unlike the general types from **Salmonella** and **E. Coli** LPS given above (48).

The biological activity of **Rhizobium** LPS in symbiosis is still not clear. Carefully purified LPS has not been tested with any host legume for specific root hair curling activity, nodulation enhancement activity, inhibition of **Rhizobium** attachment to roots, etc.(2). Until now, there have been no reports in the literature which outline the exact structure of any **Rhizobium** LPS. Recently, the chemical compositions of LPS from several strains of **R. trifolii, R. leguminosarum, R. phaseoli,** and **R. meliloti** have been determined (8). Moreover, there are several reports which suggest that the LPS is important in the symbiotic process. LPS from several species of **Rhizobium** can interact specifically with host seed lectins (3,17,26,29-31, 37,49). Some reports present data showing that lectin from the host appears to bind LPS only from the symbiont bacteria. Also, it has been reported that LPS can inhibit the binding of the bacterial symbiont to the host root (24,32,46,47). However, these reports cannot define a role for LPS in symbiosis, since the data concerning the specificity of attachment and the binding of host lectin to

the rhizobial symbiont are ambiguous (12). There are other reports which present data that indicate attachment is not specific.

Tentatively, it appears that the ability to synthesize LPS molecules containing the O-antigen is essential for carrying infection of bean beyond an early stage (12). A recent report describes two mutants of R. phaseoli which form nodules with a characteristic aberration in development (35). These nodules contain infection threads and bacteria, however, the infection threads develop abnormally and abort (35). The mutants have been shown to have an altered LPS. Futhermore, the defect in LPS and the defect in symbiosis are due to a single mutation in each mutant (35). When the mutant LPSs are analyzed by SDS polyacrylamide gel electrophorsis (PAGE), they lack a higher molecular weight band, LPSI, but contain a lower molecular weight band (LPSII) which is present in both the parent and mutant LPSs (35). Some reports have suggested that the LPSI is due to the complete form of LPS which carries the O-antigen while LPSII is due to LPS which lacks th O-antigen (9,10,13). Lately, a report by Carlson et al shows that the component missing in the R. phaseoli mutant LPS is the O-antigen portion (12).

This thesis concentrates mainly on structural determination of the O-antigen from Rhizobium leguminosarum

l28C53 sm^r rif^r (a streptomycin and rifampicin resistant strain of wild type l28C53, hereafter designated **Rhizobium leguminosarum** l28C53) and its mutant ANU54 (nod^-, fix^-).

EXPERIMENTAL SECTION

Organisms

Bacterial strains were obtained from Dr. Peter Albersheim, Carbohydrate Research Center, University of Georgia at Athens.

Growth of The Bacteria

Rhizobium leguminosarum 128C53 and its Exo^{-1} mutant ANU54 were grown in New Modified Bergersen's Medium (NMB), which contains the components listed in Table 1. The pH was adjusted to 6.9 with 6N NaOH prior to autoclaving.

TABLE 1

New Modified Bergersen's Medium

Components	Amount
Potassium phosphate (K_2HPO_4)	250 mg/l
Magnesium sulfate ($MgSO_4.7H_2O$)	100 mg/l
Glutamic acid	1.1 g/l
Mannitol	10 g/l
Calcium pantothenate	4 mg/l

(To be continued...)

Trace elements:*		
Calcium chloride ($CaCl_2.2H_2O$)	6.62	mg/l
Boric acid (H_3BO_3)	0.145	mg/l
Cobalt chloride ($CoCl_2.6H_2O$)	0.059	mg/l
Ferrous sulfate ($FeSO_4.7H_2O$)	0.125	mg/l
Copper sulfate ($CuSO_4.5H_2O$)	0.005	mg/l
Manganese chloride ($MnCl_2.4H_2O$)	0.0043	mg/l
Zinc sulfate ($ZnSO_4.7H_2O$)	0.108	mg/l
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.125	mg/l
Nitrilotriacetic acid, trisodium salt $N(CH_2CO_2)\ Na_3.H_2O$)	7.00	mg/l
Vitamins:**		
Riboflavin	0.02	mg/l
p-Aminobenzoic acid	0.02	mg/l
Nicotinic acid	0.02	mg/l
Biotin	0.02	mg/l
Thiamine-HCl	0.02	mg/l
Pyridoxine-HCl	0.02	mg/l
Inositol	0.12	mg/l

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* Trace elements were prepared as a 1000 X stock solution and the pH was adjusted to 5.0 before adding $N(CH_2CO_2)Na_3.H_2O$ to prevent precipitation of the trace elements.

(To be continued...)

** Vitamins were prepared as a 1000 X stock solution in 0.05 M sodium phosphate buffer at a pH = 7.0.

Bacterial slants were checked by gram stain (22) prior to inoculating three or four 125 ml starter flasks containing 40 ml culture medium. The flasks were placed on a shaker for 3-4 days at 26°C. One day prior to the transfer to a 10L bottle, the following tests were performed on each flask to check for contamination: i) gram stain, ii) streak on nutrient agar, and iii) streak on solid medium containing the appropriate antibiotics (refer to Table 2).

TABLE 2

The Growth Characteristics of R. leguminosarum Strains

Strain	NA	NMB	NMB+Rif.*	NMB+Str.*	NMB+Kan.*
128C53	-	+	+	+	-
ANU54	-	+	+	+	-

(To be continued...)

at 65°C was then added and this mixture was stirred for 15 minutes at 65°C, cooled for 15 minutes in an ice bath, and centrifuged at 8,000 x g for 20 minutes at 5°C. The top water layer containing the LPS was removed and saved. The phenol mixture was mixed with 100 ml of deionized water at 65°C and the procedure repeated. The water layers were combined and dialyzed against deionized H_2O in Spectrapor membrane tubing with a molecular weight cut off at 12,000-14,000.

The nucleic acids of the dialyzed LPS solution were removed by digestion with RNase A and DNase I enzymes. The nuclease treatment is shown in Table 3.

TABLE 3

The Nuclease Treatments

	Concentration	Amount*
DNase	1 mg/ml in 0.1M Tris and 0.01M $MgSO_4$, PH=7.2	0.1 ml
RNase	10 mg/ml in 0.1M Tris and 0.01M $MgSO_4$, PH=7.2	0.1 ml
Buffer	0.1M Tris and 0.01M $MgSO_4$, PH=7.2	10 ml

* Per 100 ml of water layer.

This mixture was allowed to stand at room temperature for 24 hours, and dialyzed as before. The dialyzed LPS-containing solution was then concentrated in a Buchi Rotavapor evaporator to about 50 ml and freeze-dried.

Purification of Lipopolysaccharides

Purification of LPS was accomplished by gel filtration using a Sepharose 4B column. The buffer (pH = 7.0) applied to the column contained 2.922 g/l of EDTA, 4.3 ml/l of triethylamine and 200 mg/l of sodium azide. The flow rate was about 0.2 ml/min. Since the LPSs contain hexose and 2-keto-3-deoxyoctonate, peaks were detected by using hexose (20) and KDO assays (44) (see Appendix I and II). The first peak from the 4B column, which was pure LPS, was collected and dialyzed against deionized H_2O and freeze-dried.

Separation and Purification of O-antigen

The LPSs were weighed and subjected to mild acid hydrolysis. The samples (2 mg/ml) were dissolved in 1% acetic acid, heated at 100^{o}C for one hour, and cooled in an ice bath. Lastly, they were centrifuged to remove lipids and the supernatant was freeze-dried. O-antigen was

separated from the core by using a Sephadex G-50 or G-25 gel-filtration column (10). The peaks were determined by hexose and uronic acid assays (5) (see Appendix III).

Smith Degradation

The O-antigen was further analyzed by the Smith degradation method (28). The lipopolysaccharides from 128C53 and ANU54 (150 mg) were each dissolved in 50ml 0.1 M sodium acetate buffer at pH 4.9 (1.36 g NaAc/100 ml H_2O, adjust pH to 4.9 with HAc). Sodium periodate (0.2 M) in the same buffer (12.5 ml) was added to a final concentration of 0.04 M. The oxidation was carried out in the dark at $4^{o}C$ for 3 days, and was stopped by the addition of ethylene glycol (280 ul). The solution was dialyzed against deionized H_2O and concentrated to about 20 ml. Sodium borohydride (500 mg) was added and the solution was kept at room temperature overnight. Neutralization, dialysis and freeze-drying yielded the polyalcohol. Delipidation and cleavage were performed in one step by hydrolysis with 1% acetic acid at $100^{o}C$ for one hour. Centrifugation to remove lipid followed by gel-filtration with G-50 column gave the Smith degraded O-antigen (O-anti-SD).

Analytical Techniques

The hexose composition of the O-antigen (before and after Smith degradation) was quantitatively determined by gas chromatography (GC) (1) of the alditol acetate derivatives performed on a Hewlett-Packard instrument (Model 5890) equipped with a 0.2 um df by 15 m Fused Silica Capillary SP-2330 column. The O-antigen and O-anti-SD samples were hydrolyzed in 2M trifluoroacetic acid for 2 hours at 121^{o}C. The resulting monosaccharides were then converted into their corresponding alditols with sodium borohydride and then into acetates by heating with acetic anhydride in pyridine (see Appendix IV). The alditol acetates were identified by comparision of their GC retention times and their peak areas to the standard sugars. The amount of each sugar was determined by integrating the curve generated by the flame ionization of the gas chromatographic effluent. The integration was accomplished with a computer data system, chromatochart by IMI, State College, Pa. The amount of each alditol acetate has been corrected for differences in the flame ionization detector responses using a correction factor. This correction factor was calculated from the ratio of the area obtained for a known amount of the standard sugar to a known amount of inositol, the internal standard.

The linkages of the O-antigen were determined by methylation and GC-mass spectrometry technique (4). The polysaccharides were methylated by a modification of Hakimori's procedure (25,42). Prior to methylation, dimethyl sulfoxide (DMSO) anion was prepared as follows: KH (1 g) in oil was washed with hexane three times, and kept in 2 ml of hexane. Four ml of DMSO was added dropwise slowly to the KH. This reaction was allowed to proceed for 3 hours at room temperature with stirring. The molarity of the DMSO anion was determined by titration with 0.1 M HCL and found to be about 2 M. One mg (or less) of sample was dissolved in 500 ul of DMSO and then 100 ul of DMSO anion was added and the mixture was shaken for one hour. Next, an equivalent mole of CH_3I was added and this mixture was shaken for one hour. After the above procedures had been repeated one or two more times, 500 ul of DMSO anion was added and shaken for one hour. An excess of CH_3I (about 1 ml) was added and the mixture allowed to stand at room temperature overnight. The methylated polysaccharides were purified by a Sep-Pak C_{18} column (42) (see Appendix 5) and then acetylation was performed as described earlier. The linkages can be determined from the GC-mass spectrometry data. This was done at the Regional NIH GC/MS facility at Washington University, St. Louis, Missouri. Comparison of retention times to those of authentic standards was done by

Dr. Mike McNeil at the University of Colorado, Department of Chemistry.

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) were also performed on the samples. The proton spectra were recorded on Nicolet T360 spectrometer in deuterium oxide (D_2O) at $80^{o}C$ operating at 360 MHz and ^{13}C spectra were recorded on T 360 spectrometer in D_2O at $25^{o}C$ operating at 90 MHz for 17 hrs. All chemical shifts are referred to TMS.

RESULTS

Purification of O-antigen. LPSs of R. leguminosarum 128C53 and ANU54 mutant isolated by the phenol-water extraction procedure were purified by gel filtration on a Sepharose 4B column eluting with an EDTA- and triethylamine-containing buffer. It has been found that the LPS can be cleanly separated from the glucose-rich small molecular weight components in this column (7). About 112 mg of purified LPS from 128C53 and 200 mg of purified LPS from ANU54 were obtained from 10 L of bacteria which were grown to stationary phase. The polysaccharide regions (O-antigen and core) of LPS were separated from the lipid by mild acid hydrolysis. The O-antigen and the core were then separated and identified by Sephadex G-50 gel-filtration column chromatography eluting with de-gassed deionized water. About 3 mg (some material was lost) of O-antigen from 40 mg 128C53 LPS and 30 mg of O-antigen from 150 mg ANU54 LPS were obtained.

Smith degradation is an important method used in the structural sequence analysis of polysaccharides (36). The method involves sodium periodate oxidation which only cleaves the 1,2-diol bond (-CHOH-CHOH), followed by sodium

borohydride reduction and controlled acid hydrolysis of acyclic acetal linkages. The purposes of Smith degradation are to identify the terminal sugar and to give information about the arrangement of the polysaccharide. After Smith degradation, about 10 mg of O-anti-SD from 200 mg ANU54 LPS and 3.5 mg of O-anti-SD from 75 mg 128C53 LPS are obtained. All of these samples are then subjected to gas chromatography, GC-mass spectrometry, ^{1}H-NMR and ^{13}C-NMR analysis.

Composition analysis. The gas chromatography analysis gives the relative compositions of O-antigen in Table 4 and the relative compositions of O-anti-SD in Table 5.

The data in Table 4 show that the ratio of Rha:Fuc:Man is roughly 1:2:1. The rhamnose residues in both parent and mutant are somewhat higher.

After Smith degradation (Table 5), rhamnose and fucose are retained with a ratio of Rha:Fuc = 1:2.

Table 4

The Relative Sugar Compositions of the O-antigen from **R. leguminosarum** 128C53 and mutant ANU54.

GC condition: 190-10-240 hold 10 min.

Components	Relative % of Mass	
	128C53*	ANU54**
Rhamnose	29	29
Fucose	43	47
Mannose	22	24
Glucose	6	0

* These values account for 99% of 250 ug samples.
** These values account for 88% of 250 ug samples.

Table 5

The Relative Sugar Compositions of O-anti-SD from **R. leguminosarum** 128C53 and mutant ANU54

GC condition: 190-10-240 hold 10 min.

Components	Relative % of Mass	
	128C53	ANU54
Rhamnose	33	34
Fucose	67	65
Glucose	trace	trace

The linkage analysis. The O-antigens and O-anti-SD were methylated and acetylated to determine their linkages. The aim of methylation is to methylate all of the free hydroxyl groups in these polysaccharides. This was achieved by repeating the reaction with DMSO anion and methyl iodide. The methylated O-antigen and O-anti-SD were then hydrolyzed, reduced and acetylated. Sodium borodeuteride was used to label the C-1 carbon in the reduction.

It has been shown that there are four peaks in the gas chromatographic trace (capillary column) for the methylated O-antigen of 128C53 and ANU54 (Figure 1) and two peaks in the GC trace for the methylated O-anti-SD of 128C53 and ANU54 (Figure 2). The relative area percentages of these peaks are listed in Table 6. The linkage analysis was done by using the combined application of gas chromatography and mass spectrometry. These procedures were performed at the regional NIH GC/MS facility at Washington University. The mass spectra and the primary fragments of methylated O-antigen and O-anti-SD from ANU54 are displayed in Figures 3 to 7.

Table 6

The Relative Area Percentages of the Methylated O-antigen and O-anti-SD from **R. leguminosarum** 128C53 and ANU54

GC condition: 150-4-240 No hold.

Components	Area Percentages			
	O-antigen		O-anti-SD	
	128C53[1]	ANU54[2]	128C53[3]	ANU54[4]
1,3-L-Rhamnose	31	26	38	39
1,3-L-Fucose	24	27	62	60
1,2,3-L-Fucose	24	24	0	0
t-Mannose	21	23	0	0

1. These values account for 83% of 100% area.
2. These values account for 78% of 100% area.
3. These values account for 84% of 100% area.
4. These values account for 85% of 100% area.

By comparing the retention times to those of authentic standards, done by Dr. Mike McNeil at the University of Colorado, we find that the four peaks are attributable to t-mannose, 1,3-linked-rhamnose, 1,3-linked-fucose and 1,2,3-linked-fucose in the O-antigen GC trace (Figure 1), respectively. The area percent ratio of 1,3-L-Rha : 1,3-L-Fuc : 1,2,3-L-Fuc : t-Man is 1:1:1:1. In the O-anti-SD GC trace (Figure 2), the peaks of t-mannose and 1,2,3-linked-fucose disappear, while the peaks of 1,3-linked-rhamnose and 1,3-linked-fucose are retained. Meanwhile, the peak of 1,3-linked-fucose increases. The area percent ratio of 1,3-L-Rha:1,3-L-Fuc is about 1:2. Also, from the GC/MS fragment data of the O-antigen (Figure 3), we can deduce that peak #1 is a mixture of t-mannose and 1,3-linked-rhamnose since the packed column used in the regional NIH GC/MS facility cannot separate these two sugars completely. Peak #2 (Figure 4) is 1,3-linked-fucose and peak #3 (Figure 5) is 1,2,3-linked-fucose. For the O-anti-SD, peak #1 is 1,3-linked-rhamnose and peak #2 is 1,3-linked-fucose.

Samples are subjected to further analysis using ^{1}H and ^{13}C nuclear magnetic resonance (NMR). ^{1}H-NMR spectra of O-antigen from 128C53 (Figure 8) and ANU54 (Figure 9) present the signals listed in Table 7.

Table 7

The ^{1}H-NMR chemical shifts, assignments* and integral of O-antigen from **R. leguminosarum** 128C53 and ANU54.

δ (ppm)		^{1}H		Integral	
128C53	ANU54	128C53	ANU54	128C53	ANU54
5.40	5.37	H-1(eq) Man	H-1(eq) Man	11	10
5.18 5.14	5.15 5.10	H-1(eq) Rha Fuc	H-1(eq) Rha Fuc	32	32
1.35 1.29	1.32 1.26	$-CH_3$ Rha Fuc	$-CH_3$ Rha Fuc	104	94

* Refer to reference (36).

The chemical shifts of the anomeric protons on the pyranose rings indicate that all anomeric linkages are alpha (36). The integral of these peaks from both parent and mutant strains reveal that the ratio of anomeric mannose proton to the anomeric proton of 6-deoxyhexoses and methyl proton of the 6-deoxyhexoses is 1:3:9. This is consistent with both the composition and methylation data given above, all of which support a tetrasaccharide repeating unit.

The ^{1}H-NMR spectra of O-anti-SD from 128C53 (Figure 10) and ANU54 (Figure 11) give further evidence. Their chemical shifts, assignments and integral are listed in Table 8. The ^{1}H-NMR spectra from 128C53 and ANU54 of O-anti-SD show that the `-anomeric mannose proton signals disappear and the signals of anomeric and methyl group protons assigned to the 6-deoxyhexose residues remain (Figures 8 and 9). The integral of these peaks indicate that the ratio of anomeric 6-deoxyhexose protons to methyl group protons of 6-deoxyhexose is 1:3.

The ^{13}C-NMR spectra of O-antigen (Figure 12) and O-anti-SD (Figure 13) from ANU54 give more confirmative information. The assignments of some special signals are given in Table 9.

Table 8

The ^{1}H-NMR chemical shifts, assignments and integral of O-anti-SD from **R. leguminosarum** 128C53 and ANU54.

δ (ppm)		^{1}H		Integral	
128C53	ANU54	128C53	ANU54	128C53	ANU54
5.09	5.09	H-1(eq) Rha Fuc	H-1(eq) Rha Fuc	663	341
1.30 1.21	1.25 1.20	$-CH_3$ Rha Fuc	$-CH_3$ Rha Fuc	2,084	1,033

Table 9

The ^{13}C-NMR chemical shifts and assignments* of O-antigen and O-anti-SD from ANU54.

δ (ppm)		^{13}C	
O-antigen	O-anti-SD	O-antigen	O-anti-SD
105.2	104.7	C-1(α-Rha)	C-1(α-Rha)
99.1		C-1(α-Man)	
97.5	98.8	C-1(α-Fuc)	C-1(α-Fuc)
95.3	98.5	C-1(α-Fuc)	C-1(α-Fuc)
64.2		C-6(Man)	
19.6	19.6	C-6(Rha)	C-6(Rha)
18.1	18.1	C-6(Fuc)	C-6(Fuc)
18.0	18.0	C-6(Fuc)	C-6(Fuc)

* Refer to reference (6,23).

The data show that there are four anomeric carbon atoms (C-1), three methyl carbon atoms (C-6) assigned to three 6-deoxyhexose residues and one $-CH_2OH$ group carbon atom (C-6) assigned to the mannose residue in the spectra prior to Smith degradation. The signals of the C-1 and C-6 of mannose disappear after Smith degradation. Also, in these spectra at least 23 of the possible 24 tetrasaccharide carbon resonances for the O-antigen and 16 of the possible 18 trisaccharide carbon resonances for the O-anti-SD are detected.

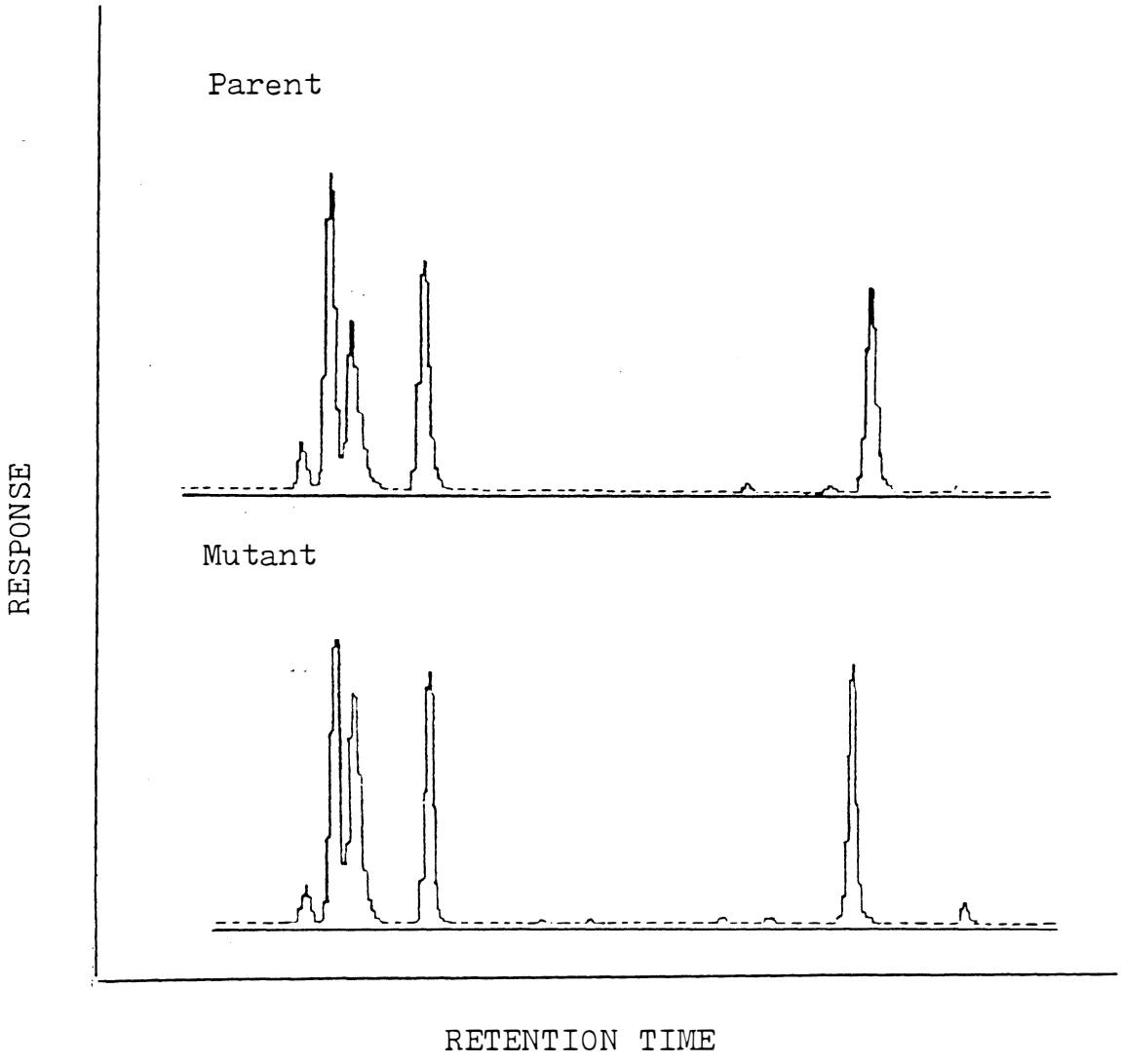


Figure 1. The GC trace of methylated O-antigen from R. leguminosarum 128C53 and ANU54 mutant.

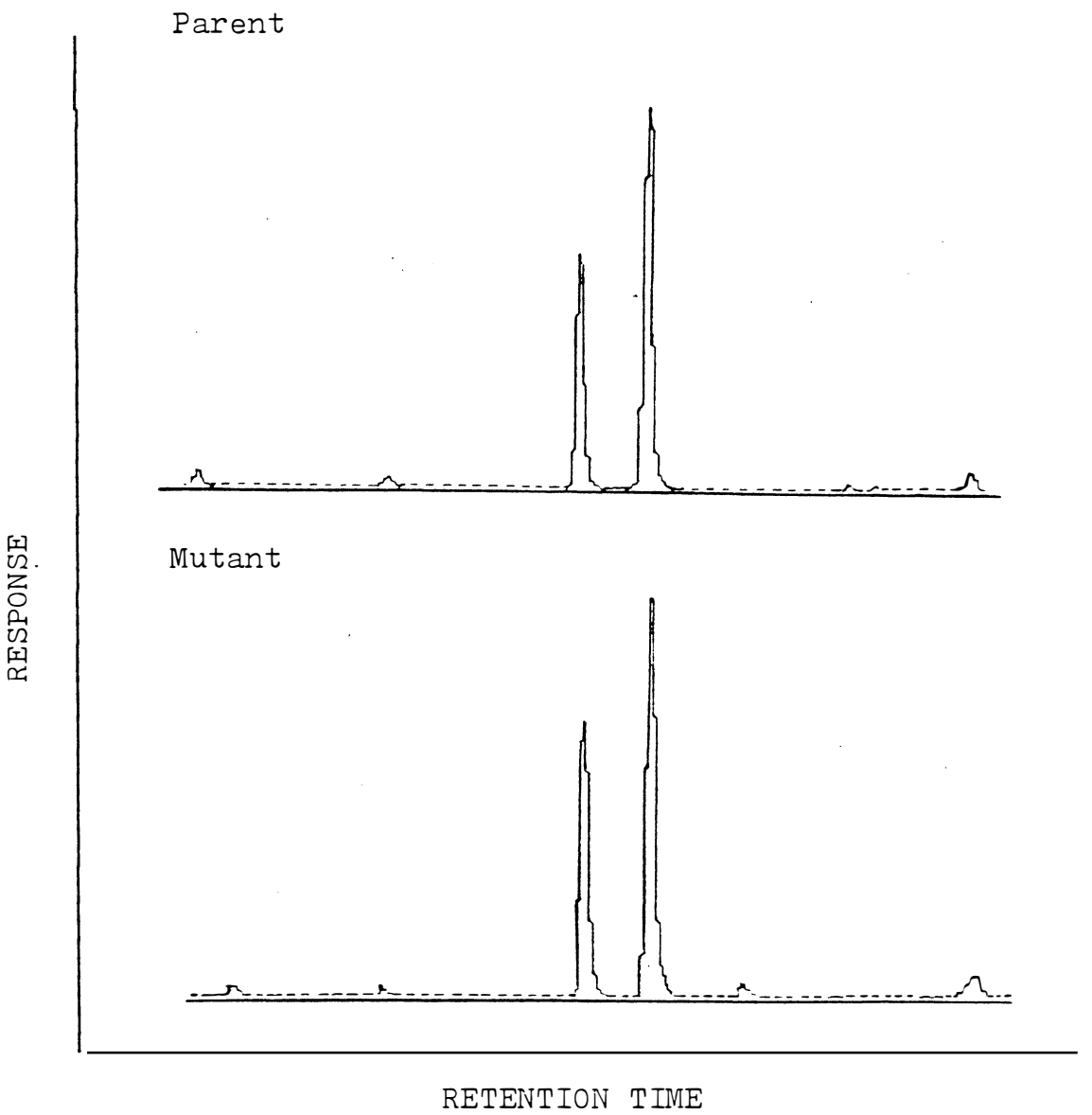


Figure 2. The GC trace of methylated O-anti-SD from R. leguminosarum 128C53 and ANU54 mutant.

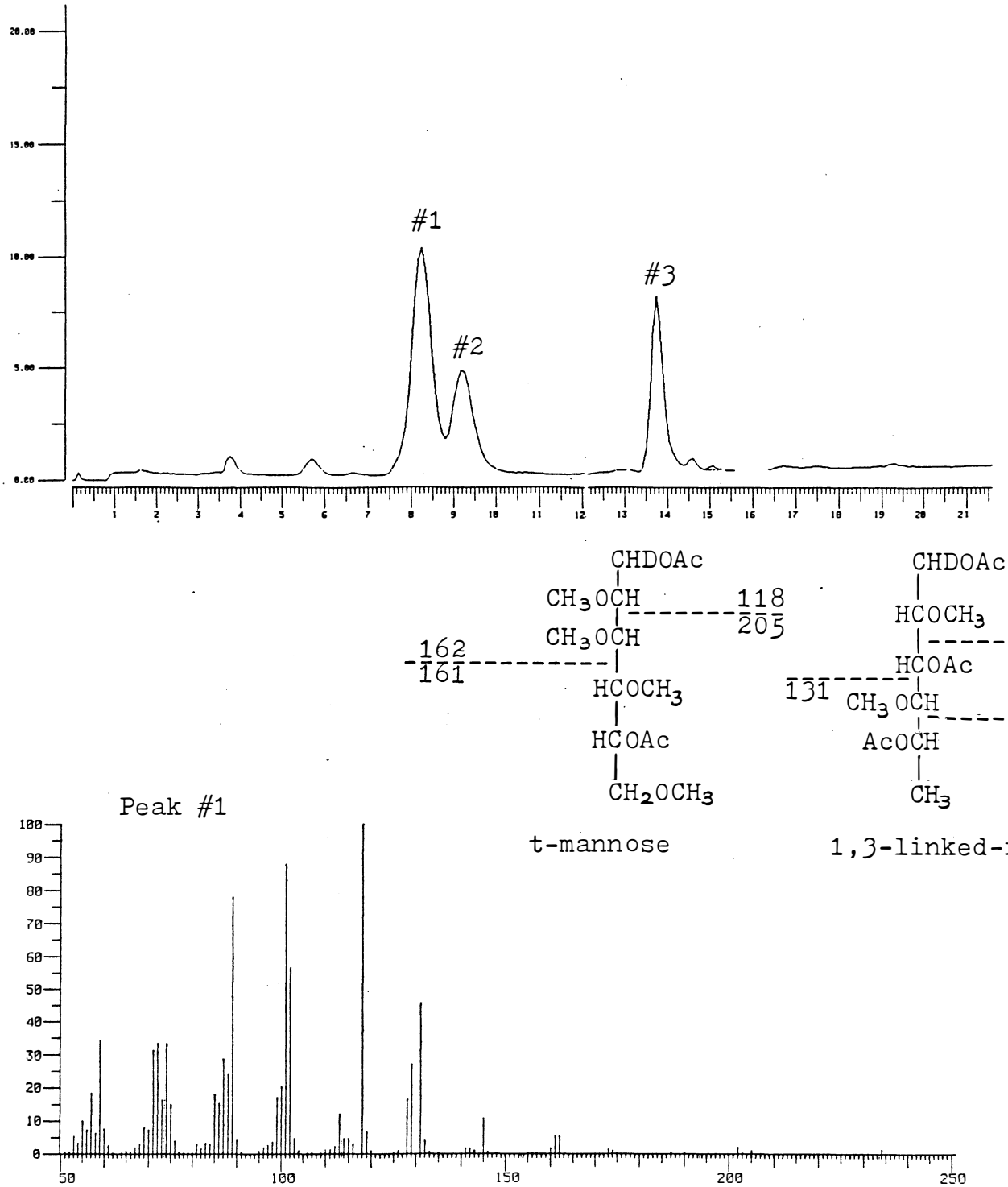


Figure 3. GC-mass spectra of O-antigen (peak #1) from ANU54.

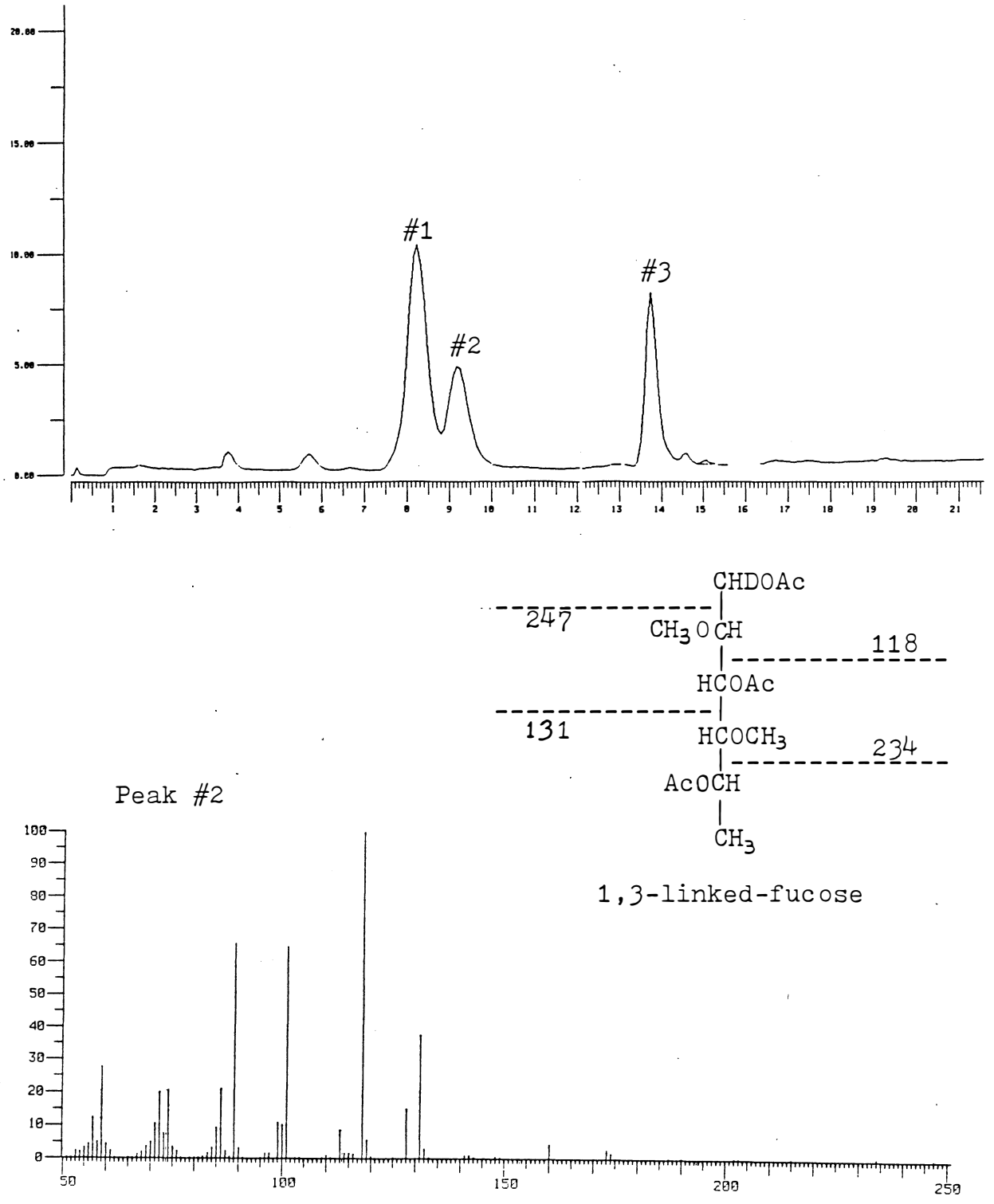
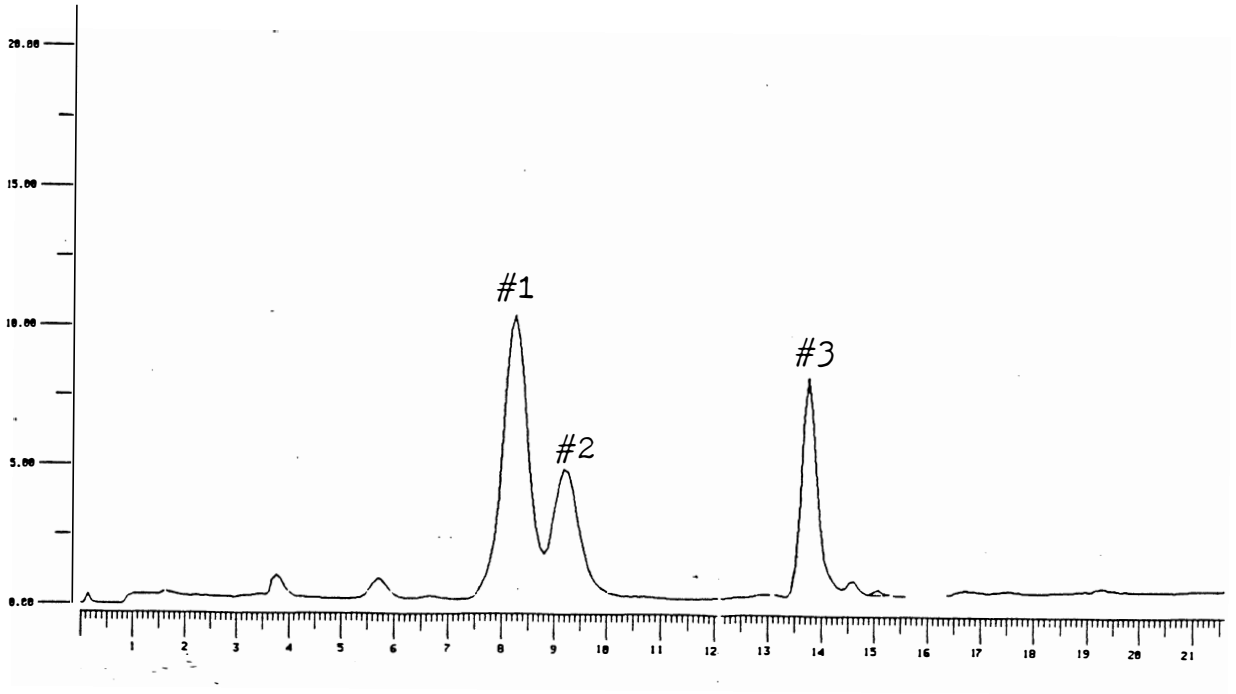


Figure 4. GC-mass Spectra of O-antigen (peak #2) from ANU54.



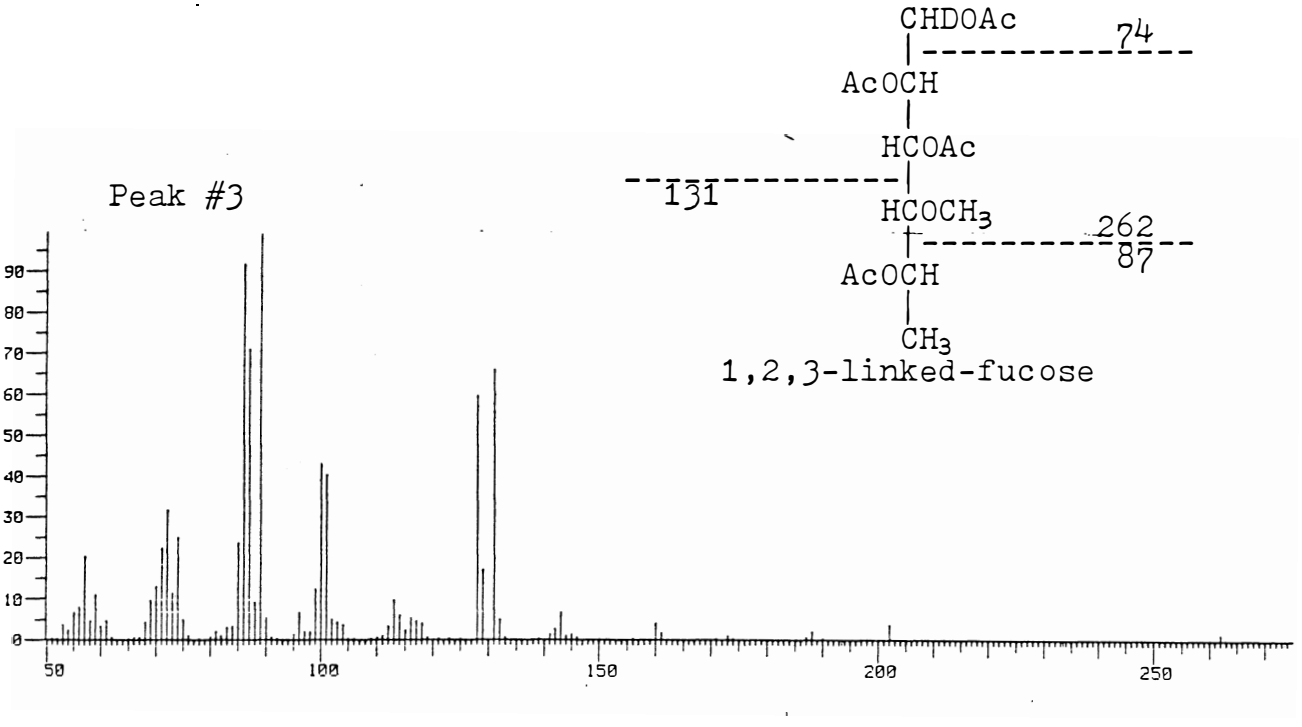
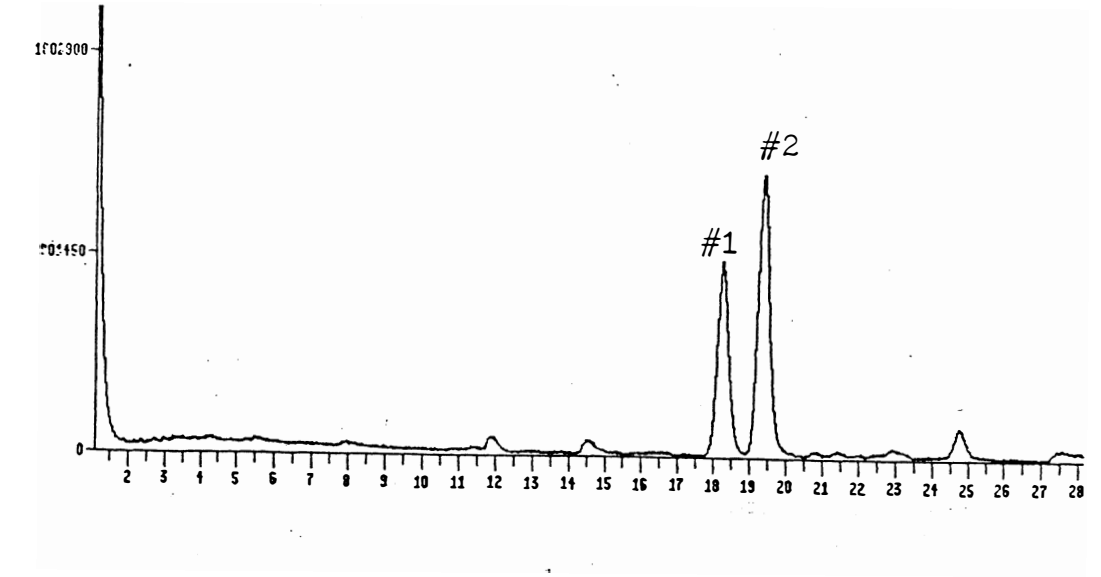


Figure 5. GC-mass spectra of O-antigen (peak #3) from ANU54.



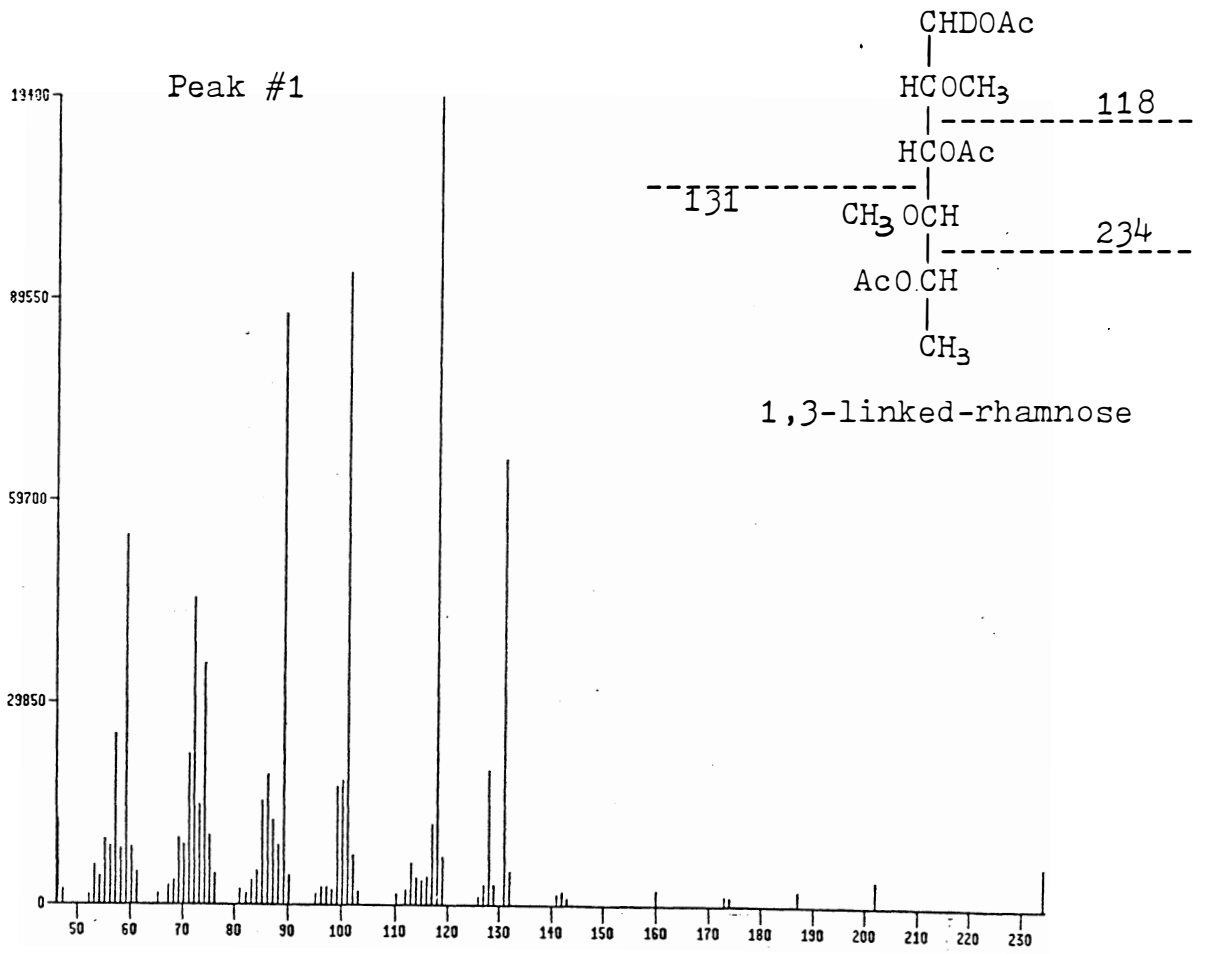
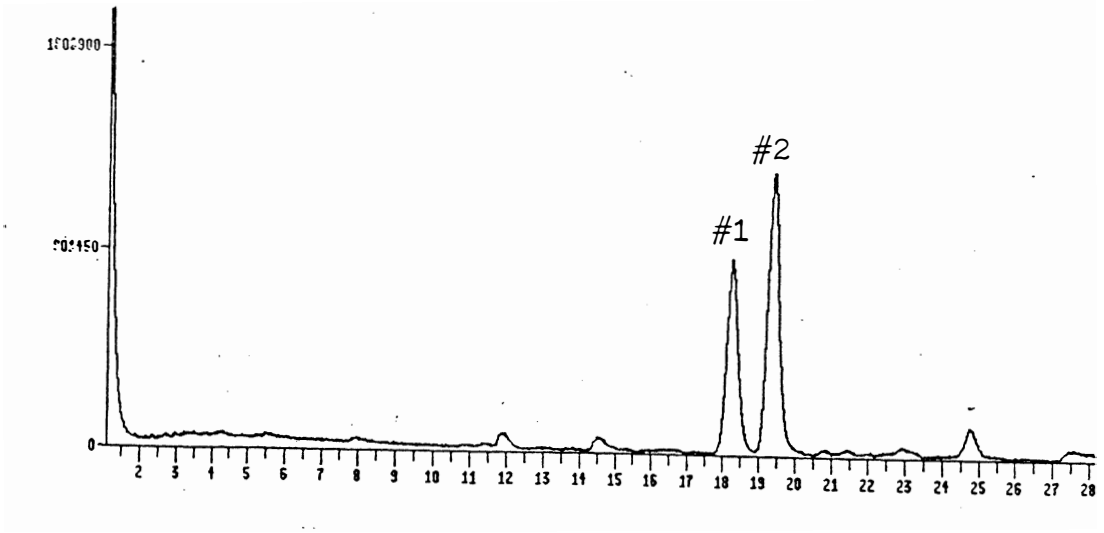


Figure 6. GC-mass spectra of O-anti-SD (peak #1) from ANU54.



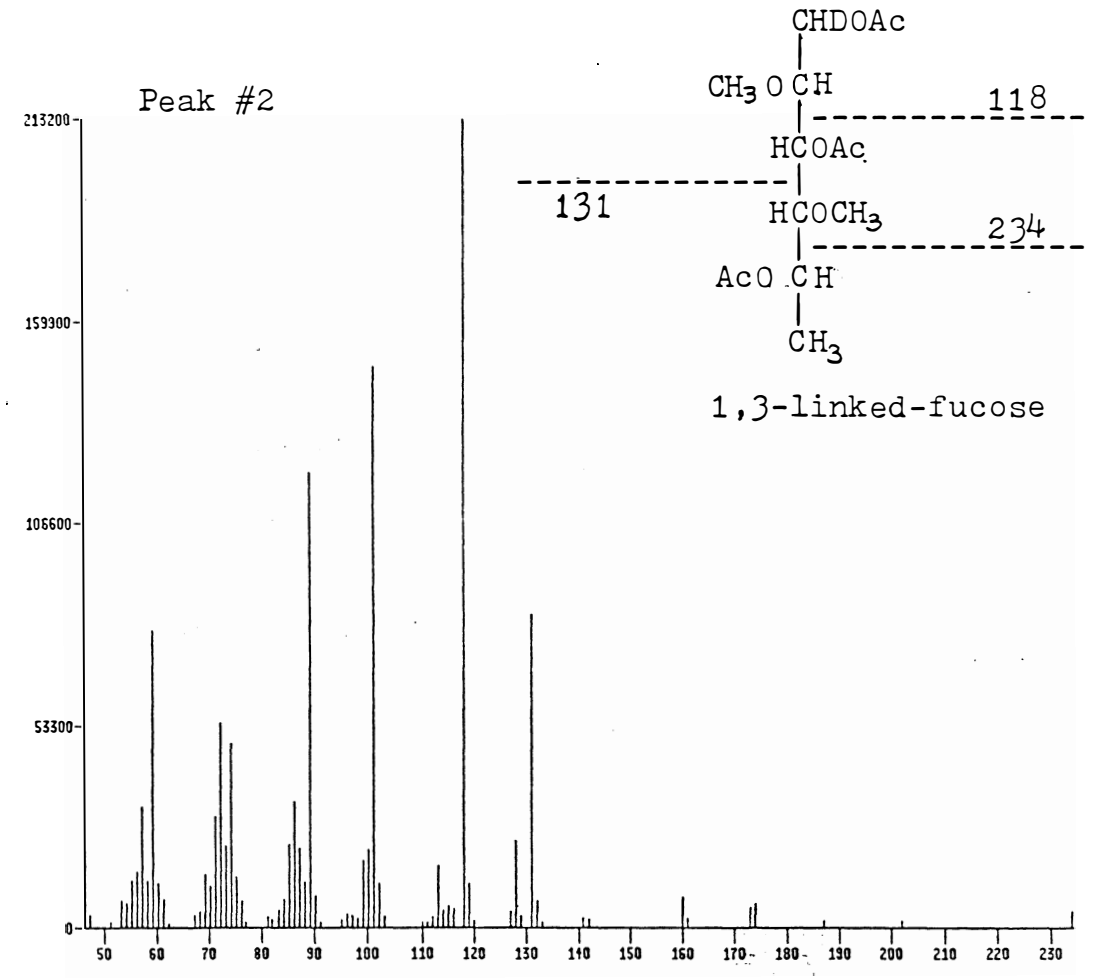


Figure 7. GC-mass spectra of O-anti-SD (peak #2) from ANU54.

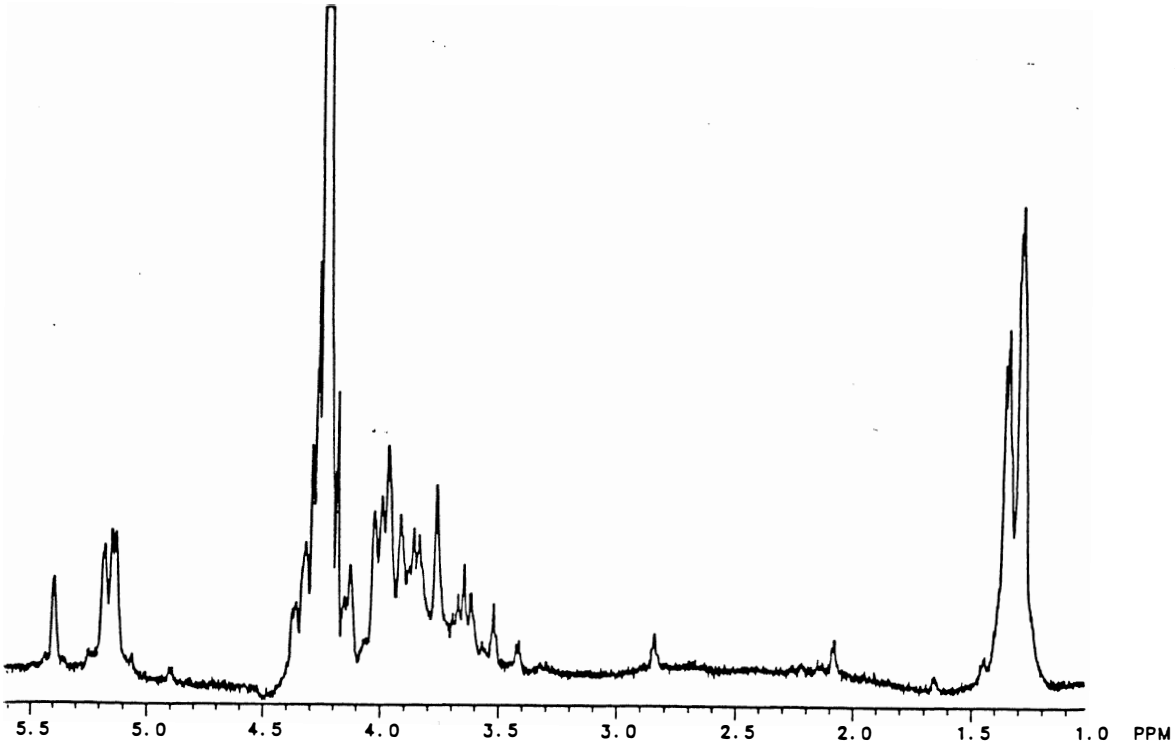


Figure 8. ^{1}H-NMR spectra of O-antigen from R. leguminosarum 128C53 in D_2O operating at 360 MHz.

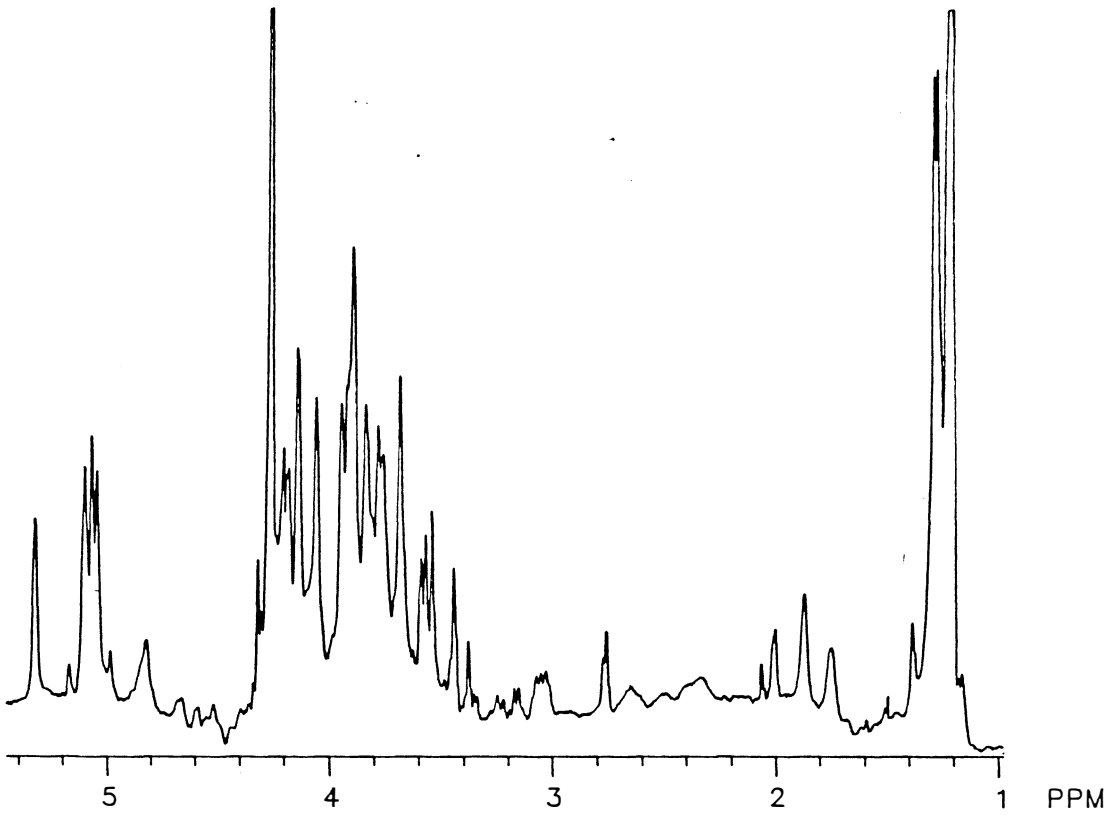


Figure 9. ^{1}H-NMR spectra of O-antigen from R. leguminosarum ANU54 mutant in D_2O operating at 360 MHz.

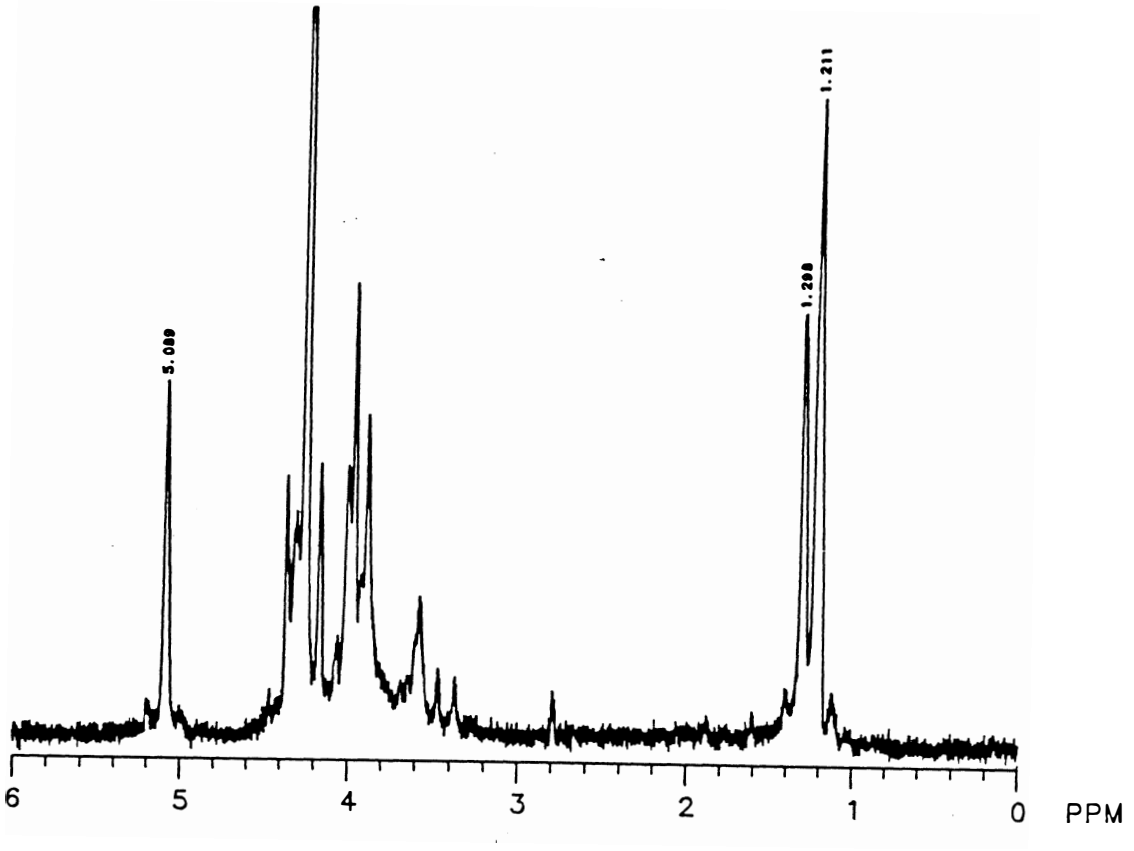


Figure 10. ^{1}H-NMR spectra of O-anti-SD from R. leguminosarum 128C53 in D_2O operating at 360 MHz.

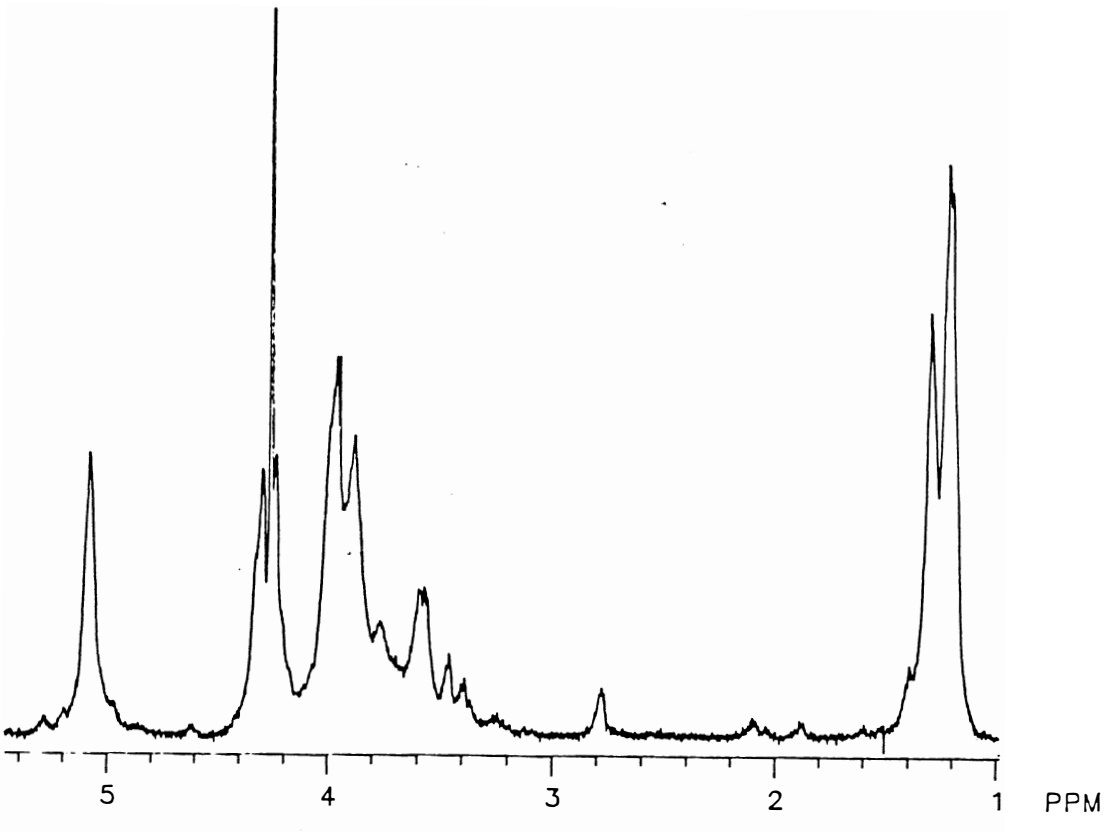


Figure 11. ^{1}H-NMR spectra of O-anti-SD from R. leguminosarum ANU54 mutant in D_2O operating at 360 MHz.

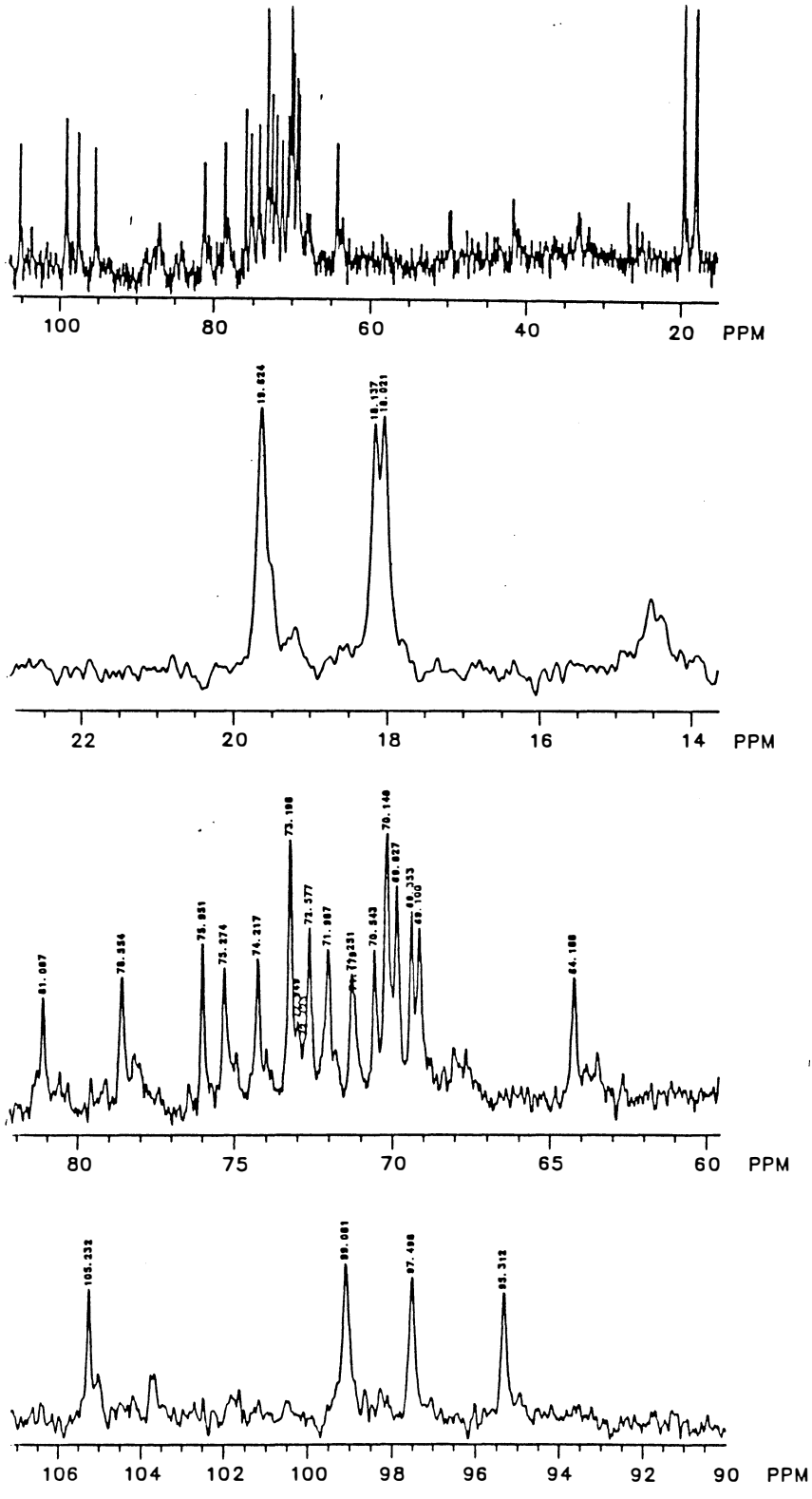


Figure 12. ^{13}C-NMR spectra of O-antigen from R. leguminosarum ANU54 in D_2O operating at 90 MHz for 17 hrs.

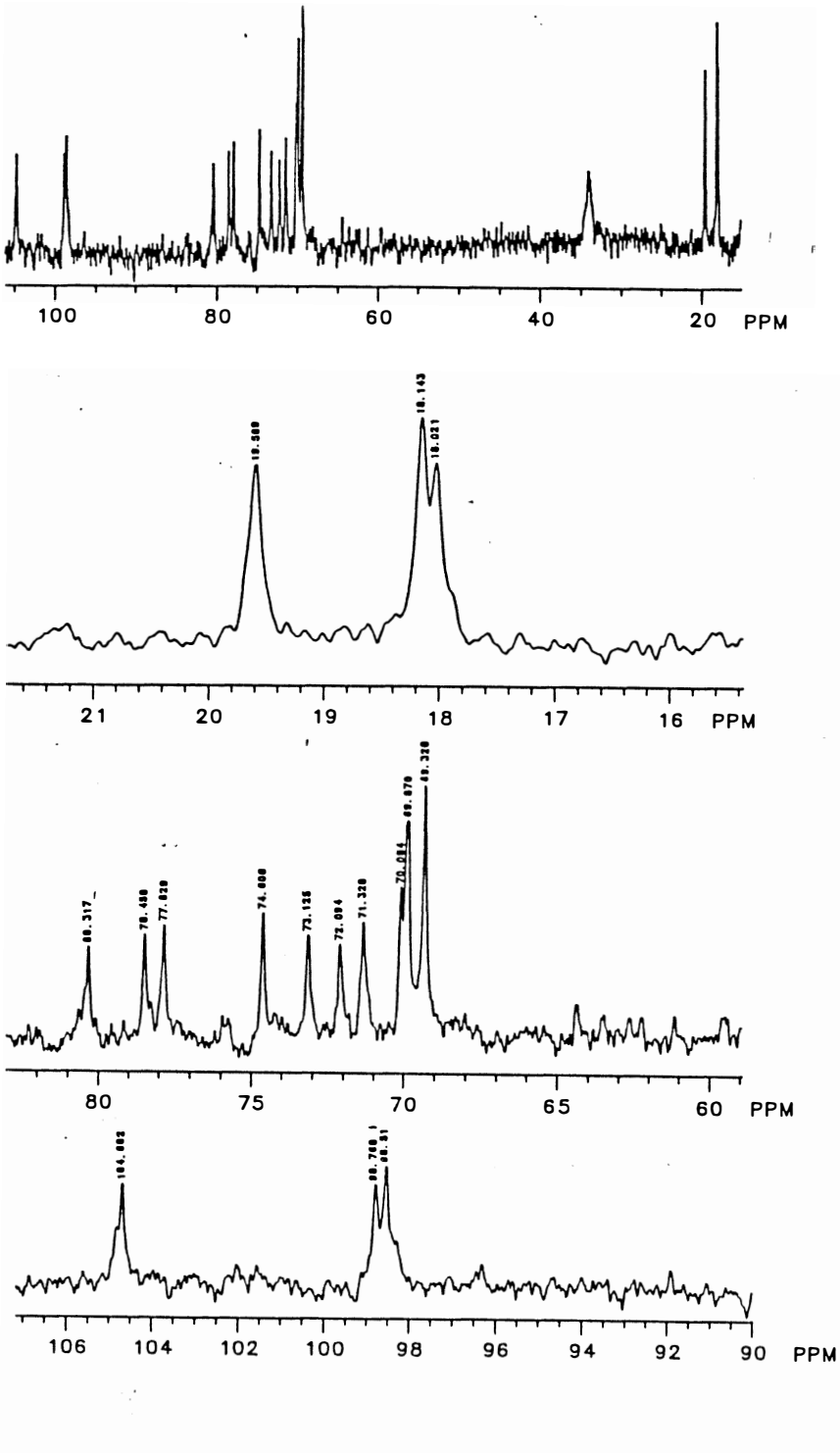


Figure 13. ^{13}C-NMR spectra of O-anti-SD from R. leguminosarum ANU54 mutant in D_2O operating at 90 MHz for 17 hrs.

DISCUSSION

R. leguminosarum parent strain 128C53 has the ability to form a symbiotic relationship with its host pea. It can nodulate pea (nod^+) and fix nitrogen (fix^+). However, its mutant ANU54 cannot carry out this role in the symbiotic process (nod^-, fix^-). It has been known that ANU54 is missing two plasmids, one of which carries the symbiotic genes (pSym). In order to understand the molecular basis for the interaction between *Rhizobium* and its legume hosts, numerous investigations on the surface polysaccharides of *Rhizobium* have been done. The LPS is thought to be important in symbiosis, therefore, it is necessary to determine its structure. In order to find out whether the gene or pSym are required for LPS synthesis, the comparison of structures between parent and mutant strains is important.

Structure analysis The resulting data show that the O-antigen from parent and mutant strains is a tetrasaccharide repeating unit. This is supported by the following experimental results:

1) Gas chromatography gives a composition ratio of Rha:Fuc:Man = 1:2:1.

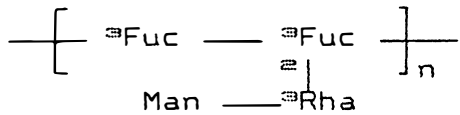
2) NMR analyses reveal that there are four anomeric proton and four carbon resonances. The integral ratio of Man to Rha, Fuc anomeric protons is 1:3. Also, all the anomeric proton chemical shifts are between 5 to 6 ppm indicating that all linkages are alpha.

3) Methylation analysis (GC) of O-antigen indicates that the area percentage ratio of 3-Rha:3-Fuc:2,3-Fuc:t-Man = 1:1:1:1.

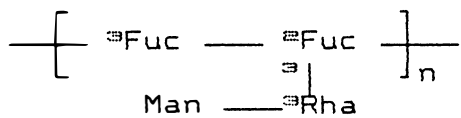
Although the composition data from GC show that the rhamnose value is slightly high (the data are not understood yet), the NMR and methylation data clearly confirm Rha:Fuc:Man = 1:2:1.

The methylation and GC/MS combination analysis indicates that these four sugar residue linkages are 1,3-linked-Rha, 1,3-linked-Fuc, 1,2,3-linked-Fuc and t-Man. Their arrangement may have four possibilities:

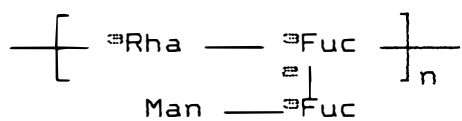
1)


3Fuc
3Fuc
2
Man
3Rha
n

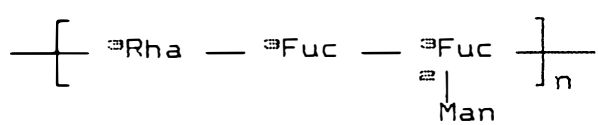
2)


3Fuc
2Fuc
3
Man
3Rha
n

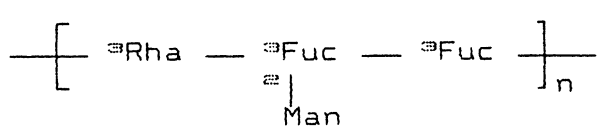
3)


3Rha
3Fuc
2
Man
3Fuc
n

4)


3Rha
3Fuc
3Fuc
2
Man
n

5)


3Rha
3Fuc
3Fuc
2
Man
n

The Smith degradation is used to determine the arrangment of sugar residues of O-antigen. The data indicate that the only glycosyl residue susceptible to removal by Smith degradation is the terminal mannose, because the 6-deoxyhexose residues do not contain any 1,2-diol (-CHOH-CHOH-) and will resist periodate oxidation. After Smith degradation, the data can be summarized as follows:

1) The GC shows that the composition of O-anti-SD is Rha:Fuc=1:2.

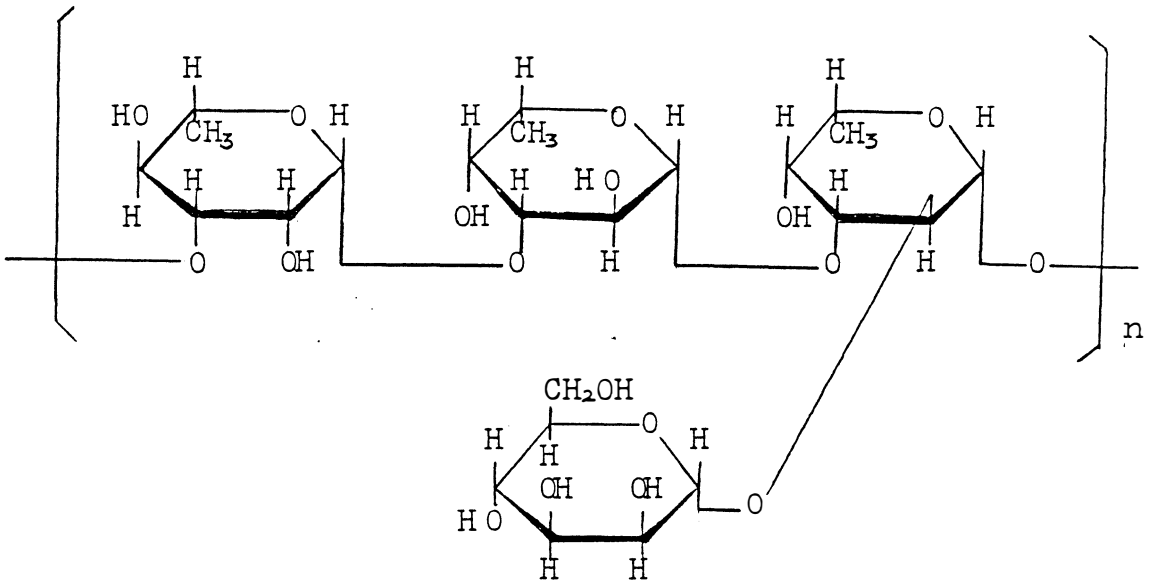
2) ^{1}H and ^{13}C-NMR show that the anomeric proton and carbon resonances of mannose have disappeared. Also, the C-6 resonance of mannose in ^{13}C-NMR is missing.

3) The GC/MS of methylation analysis shows that the composition and linkage are 1,3-linked-Rha:1,3-linked-Fuc = 1:2

According to these results, the only arrangements of O-antigen are the fourth and fifth posibilities above. Three 6-deoxyhexose residues are 1,3-linked in the backbone and the terminal mannose is attached to the 2-position of one of the two fucose residues since the original 1,2,3-linked-Fuc becomes 1,3-linked-Fuc after Smith degradation.

The results from various analytical methods and techniques confirm that the compositions and linkages of O-antigen from parent 128C53 and mutant ANU54 are identical to each other. The structural model of O-antigen can be given as Figure 14.

In comparison with the data from reference (6), some suppositions for the remaining resonances from ^{13}C-NMR spectra can be given, although these assignments were not listed in the Table 9. The resonances at 69.1 ppm, 70.5 ppm and 71.2ppm, which are missing in the O-anti-SD, can be attributable to the C-4, C-3 and C-2 of the terminal mannose residue respectively. Since one resonance of the O-antigen and two resonances of the O-anti-SD did not appear in the ^{13}C-NMR spectra, the C-5 resonance could be in 72.6 ppm or 75.3 ppm. The data for other three 6-deoxyhexose residues in reference are not complete, therefore, it is hard to identify them.



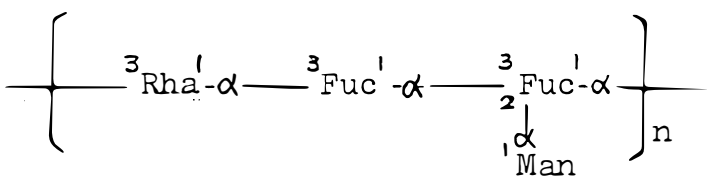
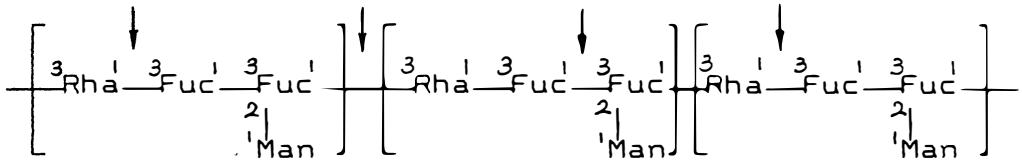


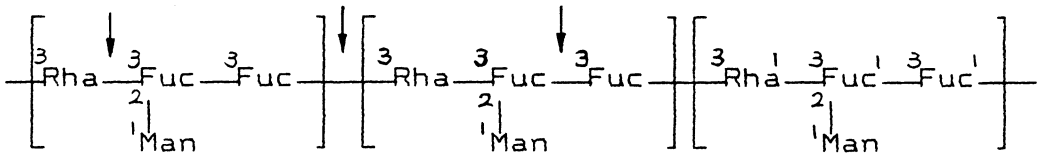
Figure 14. The structural model of O-antigen from R. leguminosarum 128C53 and ANU54 mutant.

Currently, we are working on the trisaccharide analysis of O-antigen using the partial acid hydrolysis method in order to determine to which fucose the terminal mannose is linked. In general, acid hydrolysis can partially break the main chain as the following two patterns:

1)



2)



In the first pattern, only two kinds of trisaccharides can possibly be obtained:

$$Fuc^{1} \text{ -- } {}^{3}Fuc^{2} \text{ -- } {}^{1}Man$$

$$Man^{1} \text{ -- } {}^{2}Fuc^{1} \text{ -- } {}^{3}Rha$$

In the second pattern, another two trisaccharides can possibly be obtained:

$$Rha^{1} \text{ -- } {}^{3}Fuc^{2} \text{ -- } {}^{1}Man$$

$$Man^{1} \text{ -- } {}^{2}Fuc^{1} \text{ -- } {}^{3}Fuc$$

These trisaccharides can be purified by Sephadex G-25 column and then by high performance liquid chromatography (HPLC). Their compositions and linkages can be deduced from the fast atom bombing mass spectrometry (f. a. b. m. s.).

Since the exact position of terminal mannose residue has not been determined, the only possible difference between the parent and mutant strains in the O-antigen structure is in the position of t-man. However, immunochemical data show that the antiserum to ANU54 react as well with parent 128C53 as with mutant ANU54 LPS (14). This supports the idea which the structure of O-antigen from parent and mutant strains should be identical.

Role of LPS in nodulation According to present data, the O-antigen chain of LPS from the mutant ANU54 is identical to the parent 128C53. However, the mutant ANU54

does not have the ability to attach to root hair, cause root hair curling, form infection threads and produce nodules in the host pea (34). This implies that LPS may not be directly involved in the early stages of attachment, hair curling or infection thread formation; or it is not the only molecule required for these stages. Also, the genes dictating the synthesis of the LPS do not reside in the symbiotic plasmid since pSym is missing in the mutant ANU54. But, it is still possible that the LPS has a role in later symbiotic steps such as bacteroid formation. As a result, more studies are needed to be done before a decisive outcome can be reached. Presently, the core structure of LPS from mutant ANU54 is still under study. In addition, we are working on a collaborative project with Dr. Dale Noel at Marquette University. Dr. Noel has a mutant from R. phaseoli in which a symbiotic defect is linked to a defect in LPS synthesis.

APPENDIX I

Hexose Assay

Preparation:

1. Prepare or obtain from the freezer a 1 mg/ml solution of glucose standard.
2. Prepare 1 mg/ml solutions of samples to be assayed.

Procedure:

1. Prepare a series of test tubes containing increasing amounts of the standard from 0 to 200 ul. Add sufficient deionized water for a final volume of 500 ul.
2. Prepare a series of tubes for the samples but use larger volumes of the samples (50-300 ul). Add deionized water to a final volume of 500 ul.
3. Add 1 ml of 0.2% anthrone in conc. H_2SO_4. Vortex.
4. Wait 5 minutes and read absorbance at 620 nm.

Prepare a graph with the absorbance reading of the standard as the vertical axis and the number of micrograms of glucose as the horizontal axis. Read the number of micrograms in the poly-saccharide samples from their absorbance readings. Calculate the percent hexose in the polysaccharide.

APPENDIX II

KDO Assay: 2-Keto-3-deoxyoctonic acid

Reagents:

1. Standard of 0.1 mg/ml KDO
2. 0.4 N H_2SO_4
3. 0.04 N HIO_4 in 0.4 N H_2SO_4
4. 2% $NaAsO_2$ (Sodium Arsenite) in 0.5 N HCl
5. 0.3% Thiobarbituric Acid

Procedure:

1. Prepare a set of standards of 0-100 ul of KDO and samples of at least 200 ul. Total volume must be 200 ul.
2. Add 20 ul of H_2SO_4 to standards and samples. Vortex. Heat 30 mins in a hot water bath.
3. Add 250 ul of HIO_4. Vortex. Let stand at room tmperature at least 40 mins.
4. Add 500 ul of $NaAsO_4$. Vortex. Allow to stand 5 mins.
5. Add 2ml of thiobarbituric acid. Vortex. Place in a hot water bath for 20 mins.
6. Read absorbance immediately at 548 nm. Centrifuge any cloudy samples 1 min. in tabletop centrifuge.

APPENDIX III

Uronic Acid Assay

Reagents:

1. 0.0125 M sodium tetraborate ($Na_2B_4O_7.10\ H_2O$) in conc. H_2SO_4. Stored in cold room.
2. 0.15% m-hydroxybiphenyl in 0.5% NaOH. Stored in cold room.
3. Standard of 0.1 mg per 1.0 ml glucuronic or galacturonic acid.

Procedure:

1. Prepare a set of standards with a total volume of 200 ul using 0-200 ul of standard.
2. Prepare samples with a total volume of 200ul.
3. Add 1.2 ml of cold tetraborate and vortex all tubes.
4. Heat 5 min. in boiling water bath.
5. Cool 1-2 min. in cold water in cold room.
6. Add 20 ul of m-hydroxy-biphenyl to standrads and samples. Vortex.
7. Allow to stand 5 mins. at room temperature.
8. Read absorbance at 520 nm.

APPENDIX IV

Acetylation Procedure

Procedure

1. Prepare a standard sugar solution containing 1 mg/ml of each standard sugar. This solution may be stored in the freezer after each use.
2. Prepare a 1 mg/ml solution of inositol to use as the internal standard. This also may be stored in the freezer and re-used. (Note: Dry your standard sugars and your samples in the vacuum oven before preparing the solutions.)
3. Prepare a 1 mg/ml solution of your sample polysaccharide.
4. Determine the % hexose of your sample using the anthrone assay.
5. Place a volume of your sample equivalent to not more than 250 micrograms of hexose in a screw-cap test tube. In another tube place 100 microliters of your standard sugar solution. Add 20 microliters of inositol solution to each tube. Dry your samples and standards using filtered air or by freeze-drying.
6. Add 500 microliters of a 2 M TFA(trifluoroacetic acid)

solution to each tube. Seal with the teflon-lined screw cap and heat at 121^{O}C for 2 hours.

7. Remove tubes and blow-dry with filteres air. This may be done at 40 to 50^{O}C unless your sample is a methylated polysaccharide. For a methylated polysaccaride blow dry at no more than 35^{O}C.
8. Prepare a 10 mg/ml solution of sodium borohydride(or sodium borodeuteride) in 1 M ammonium hydroxide. For methylated polysaccharides always use sodium borodeuteride. Add 250 microliters of this solution to each sample, mix and allow to stand for 1 hour at room temperature. Allow to stand for 2 hours for methylated samples.
9. Add 50 microliters of glacial acetic acid. Vigorous bubbling should take place! Repeat two more times.
10. Add 500 microliters of a 9/1 = MeOH/HAc solution to each tube. Blow dry using filtered air. (For methylated samples keep temp at 35^{O}C or below.) Repeat 4 times. Be sure each sample is dry after each addition.
11. Add 500 microliters of MeOH to each tube and blow dry as in step 10. Repeat 4 times.
12. Add 50 microliters of pyridine and 50 microliters of acetic anhydride to each tube. Mix, seal with teflon cap and heat at 121^{O}C for 30 minutes.

13. Cool on ice, blow dry at room temperature and extract by adding 500 microliters of water and 500 microliters of chloroform. Mix, centrifuge on table top centrifuge for 5 minutes, remove chloroform layer (bottom) with a Pasteur pipette and transfer to another test tube. Extract water layer with another 500 microliters of chloroform and combine chloroform layers from first and second extraction. Blow chloroform layers dry with filtered air.
14. Analyze by dissolving sample in 20 to 100 microliters of dichloromethane and injecting 1 to 5 microliters onto the GC. (Note: To insure purity of all solvents they should be spectrometric grade, HPLC grade or distilled prior to use.)

APPENDIX V

Sep-Pak C_{18} Column

Precondition of the Sep-Pak column

1. Wash with 5 ml CH_3OH.
2. Wash with 5 ml ethylacetate
3. Wash with 5 ml CH_3OH.
4. Wash with 10 ml H_2O.

Purification

1. Dilute samples with 5 ml of H_2O and add to the Sep-Pak column.
2. Rinse samples with 10 ml H_2O, 4 ml of 15%CH_3CN and then 4 ml of 20% CH_3CN.
3. Elute samples with 2 ml of 100% CH_3CN and save.
4. Rinse column with 2 ml of 95% ETOH and save.
5. Combine both of above and blow-dry.

LITERATURE CITED

1. Albersheim, P., Nevens, D. J., English, P. D. and Karr, A., "A Method for the Analysis of Sugars in Plant Cell-wall Polysaccharides by Gas-liquid Chromatography." Carbohydr. Res., 5, 340-345 (1967).

2. Bauer, W. D., "Infection of Legumes by Rhizobia." Annu. Rev. Plant Physiol, 32, 407-449 (1981).

3. Bhagwat, A. A. and Thomas, J., "Dual Binding Sites for Peanut Lectin on Rhizibia." J. Gen. Microbiol, 117, 119-125 (1980).

4. Bjorndal, H., Hellerquist, C. G., Lindberg, B. and Svensson, S., "Gas-liquid Chromatograph and Mass Spectrometry in Methylation Analysis of Polysaccharides." Angew. Chem. Internat. Edit., 9, 610 (1970).

5. Blumenkrantz, N. and Asboe-Hamsen, G., "A New Method for the Quantitative Determination of Uronic Acid." Anal. Biochem., 54, 484 (1973).

6. Bock, K. et al, "Carbon-13 NMR Data for Oligosaccharides." Advances in Carbohydrate Chemistry and Biochemistry, R. Stuart Tipson (ed.), Academic Press, Inc., New York, 42, 193-224 (1984).

7. Carlson, R. W., "Host-Symbiont Interactions. III. The Purification and Partial Characterization of Rhizobium Lipopolysaccharides." Plant Physiol., 62, 912-917 (1978).

8. Carlson, R. W., "Surface Chemistry." Nitrogen Fixation-Rhizobium, W. J. Broughton (ed.), Clarendon Press, Oxford, 2, 199-234 (1982).

9. Carlson, R. W. and Lee, R. P., "A Comparison of the Surface Polysaccharides from R. Leguminosarum 128C53 $sm^r rif^r$ with the Surface Polysaccharides from Its exo^{-1} Mutant." Plant Physiol., 71, 223-228 (1983).

10. Carlson, R. W., "Heterogeneity of Rhizobium Lipopolysaccharides." J. Bacteriol, 158, 1012-1017 (1984).

11. Carlson, R. W., Hollingsworth, R. and Dazzo, F. B., "A Novel Core Oligosaccharide Component of the Lipopolisaccharide from Rhizobium trifolii ANU843." submitted to Carbohydrate Res. (1987).

12. Carlson, R. W. and Kalembasa, S., "Characterization of the Lipopolisaccharide from a Mutant of Rhizobium phaseli which is Defective in Infection Thread Development." submitted to J. Bacteriol. (1987).

13. Carlson, R. W. and Shatters, R. et al, "The Isolation and Partial Charcterization of the Lipopolysaccharides from Several Rhizobium trifolii Mutants Affected in Root Hair Infection." Plant Physiol.,in press (1987).

14. Cummins, L. L., "Partial Characterization of the Extracellular Polysaccharides Produced by Mutants of R. leguminosarum and R. trifolli." MS. Thesis, Eastern Illinois University, (1985).

15. Dazzo, F. B., Napoli, C. and Hubbell, D. H. "Adsorption of Bacteria to Roots as Related to Host Specificity in the Rhizobium-Clover Symbiosis." Appl. Environ. Microbiol. 32, 166-171 (1976).

16. Dazzo, F. B., "Adsorption of Microorganisms to Roots and Other Plant Surfaces." Adsorption of Microorganisms to Surfaces, New York: Wiley, 253-316 (1979).

17. Dazzo, F. B. and Brill, W. J., "Bacterial Polysaccharide Which Binds Rhizobium Trifolii to Clover Root Hair." J. Bacteriol, 137, 1362-1373 (1979).

18. Dazzo, F. B. and Hubbel, D. H., "Control of Root Hair Infection." Nitrogen Fixation:Rhizobium, Clarendon Press, Oxford, 2, 274-310 (1982)

19. Dilworth, M. J. and Parker, C. A., "Development of the Nitrogen Fixing System in Legumes." J. Theor. Biol., 25, 208-218 (1969).

20. Dische, Z., "Color Reaction of Carbohydrates." Methods in Carbohydrate Chemistry, Academic Press, New York, 1, 478-481 (1962).

21. Droge, W., "Structural Investigations on the 2-Keto-3-Deoxyoctonate Region of lipopolysaccharides.", Eur. J. Biochem., 14, 175-184 (1970).

22. Gerhardt, P. (ed.), Manual of Methods of General Bacteriology, American Society for Microbiology, Washington D.C., 26-27 (1981).

23. Gorin, P. A. J., "Carbon-13 Nuclear Magnetic Resonance Spectroscopy of polysaccharides." In: Advances in Carbohydrate Chemistry and Biochemistry, 38, R. Stuart Tipson (ed.), Academic Press, Inc., New York, 13-97 (1984).

24. Gram, T. L., Sequeira, L. and Huang, T. R., "Bacterial Lipopolysaccharides As Inducers of Disease Resistance in Tabacco." Appl. Environ. Microbiol., 34, 424-432 (1977).

25. Hakimori, S., J. Biochem.(Tokyo), 55, 205-208 (1964).

26. Hrabak, E. M. Urbano, M. R. and Dazzo F. B., "Growth-phase-dependent Immunodeterminants of Rhizobium trifolii Lipopolysaccharides which Bind Trifoliin A, a White Clover Lectin." J. Bacteriol., 148,697-711 (1981).

27. Jann, B., Reske, K. and Jann, K., "Hetrogeneity of Lipopolysaccharide. Analysis of Polysaccharide Chain Lengths by Sodium Dodecylsulfate-polyacrylamide gel electrophoresis." Eur. J. Biochem., 60, 239-246 (1975).

28. Jansson, P. E., "Structural Studies on the Hexose Region of The Core in Lipopolysaccharides from Enterobacteriaceae." Eur. J. Biochem., 115,571-577 (1981).

29. Kamberger, W.,"An Ouchterlony Double Diffusion Study on the Interaction between Legume Lectins and Rhizobial Cell Suface Antigens." Arch. Microbiol., 121, 83-90 (1979).

30. Kamberger, W., " Role of the Surface Polysaccharides in Rhizobium-Pea Symbiosis." FEMS Microbiol Left., 6, 361-365, (1979).

31. Kato, G., Maruyama, Y. and Nakamura, M., "Role of Lectins and Lipopolysaccharides in the Recognition Process of Specific Legume-Rhizobium symbiosis." Agric. Biol. Chem., 43, 1085-1092 (1979).

32. Kato, G., Maruyaman, Y. and Nakamura, M., "Role of Bacterial Polysaccharides in the Adsorption Process of the Rhizibium-pea symbiosos." Agric. Biol. Chem., 44, 2843-2855 (1980).

33. Luderitz, O., Freudenberg, M. A., Galanos, C., Lemann, V., Rietschel, E. and Shaw, D. H., "Lipoposaccharides of Gram-Negative Bacteria." Current Topics in Membranes and Transport, 17, Academic Press, Inc., New York, 79-134 (1982).

34. Napoli, C. and Albersheim, P., "Rhizobium leguminosarum Mutants Incapable of Normal Polysaccharides Production." J. Bacteriol., 141, 1454-1456 (1980).

35. Noel, K. D., VandenBosch, K.A. and Kulpaca, B.,"Mutations in Rhizobium phaseoli that Lead to Arrested Infection Thread Development." J. Bacteriol.,168, 1392-1401 (1986).

36. Penlin, A. S. and Casu, B., "Spectroscopic Methods." Polysaccharides, Gerald O. Aspinall (ed.), Academic Press, Inc., New York, 1, 135-169 (1982).

37. Planque, K. and Kijne, J. W., "Binding of Pea Lectins to A Glucan-type Polysaccharide in the Cell Walls of Rhizobium leguminosarum." FEBS Lett., 73, 64-66 (1977).

38. Sanders, R. E., Carlson, R. W. and Albersheim, P., "Host- Symbiont Interaction. II. A Mutant of Rhizobium leguminosarum Has Simultaneously Lost the Ability to Nodulate Pea Seedlings and to Produce Extracellular Polysaccharides." Nature, 217, 240 (1978).

39. Sanders, R. E., Raleigh, E. and Signer, E., "Lack of Correlation between Extracellular Polysacchrides and Nodulation Ability in Rhizobium." Nature, 292, 148-149 (1981).

40. Sharon, N., Complex Carbohydrates, Addison-Wesley Publishing Co., London, 320-331 (1975).

41. Stryer, L., Biochemistry, W. H. Freeman and Company (2nd ed.), San Francisco, 782-785 (1981).

42. Sweet, D. P., Shapiro, R. H., and Albersheim, P., "Quantitative Analysis by Various GLC Response-factor Theories for Partially Ethylated Alditol Acetates." Carbohydr. Res., 40, 217-225 (1975).

43. Vincent, J. M., "Root-Nodule Symbioses with Rhizobium." The Biology of Nitrogen Fixation, A. Quispel (ed.), North-holland Publishing Co., Amsterdam, Oxford, 33, 226-332 (1974).

44. Weissbach, A. and Hurwitz, J., "The Formation of 2-Keto-3-deoxheptonic Acid in Extracts of E. Coli." J. Biol. Chem., 234, 705 (1958).

45. Weslphal, O. and Jann, K., "Bacterial Lipopolysaccharides." Methods in Carbohydr. Chem., 5, 83-91 (1965).

46. Whatley, M. H., Bodwin, T. S., Lippincott, B. B. and Lippincott, J. A., "Role for Agrobacterium Cell Envelope Lipopolyssacharide in Infection Site Attachment." Infect. Immun., 13, 1080-1083 (1976).

47. Whatley, M. H. and Spiess, L. D., "Role of Bacterial Lipopolysaccharide in Attachment of Agrobacterium to Moss." Plant Physiol., 60, 765-766 (1977).

48. Wilkinson, S. G., "Composition and Structure of Bacterial Lipopolysaccharides." Surface Carbohydrates of the Procaryotic Cell, Academic Press, Inc., London, 97-175 (1977).

49. Wolpert, J. S. and Albersheim, P., "Host-Symbiont Interactions. I. The Lectins of Legumes interact with the O-antigen Containing Lipopolysaccharides of Their Symbiont Rhizobia." Biochem. Biophys. Res. Commun., 70, 729-737 (1976).

50. Zevenhuizen, L. P. T. M., Scholten-Koerselman, I. and Postthumus, M. A., "Lipopolysaccharides of Rhizobium." Arch. Microbiol., 125, 1-8 (1980).