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Characterization of structural defects in the lipopolysaccharides of symbiotically impaired *Rhizobium leguminosarum* biovar *viciae* VF-39 mutants

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ABSTRACT

The lipopolysaccharides (LPS) of a wild type strain of *Rhizobium leguminosarum* biovar *viciae* (strain VF-39) and two symbiotically defective Tn5 mutants (VF-39-32 and VF-39-86) have been studied. The LPS of the mutants reflected impaired synthesis of the O-antigen. In the LPS of one mutant, the core tetrasaccharide was lacking and in that of the other it was truncated to a disaccharide containing mannose and 3-deoxy-D-*manno*-oct-2-ulosonic acid (KdO). The latter mutant also synthesized an unusual carbohydrate component containing mannose, galactose, and an unidentified saccharide. The lipid A composition was similar to that found in other strains of *R. leguminosarum* biovar *viciae*. The O-antigen of the wild-type bacterium contained 2-*O*-methylfucose, fucose, 3,6-dideoxy-3-(methylamino)hexose, glucose, 2-amino-2,6-dideoxyhexose, and heptose. This study clearly defines a role for the bacterial LPS in the proper functioning of the *Rhizobium* legume symbiosis.

INTRODUCTION

The Rhizobiaceae are a family of Gram-negative bacteria which infect and form a symbiosis with legume plants, with consequent conversion or fixation of molecular nitrogen into ammonia. This process is marked by a high level of specificity between the bacteria and the plant, and cell-surface carbohydrates are thought to be involved in this process at the levels of initial recognition[1,2] and nodule development[3–6]. It has been demonstrated[3] that mutants of *R. japonicum*, which appeared to be defective in LPS biosynthesis, were incapable of forming nitrogen-fixing nodules in plants. This phenomenon was also demonstrated with *R. phaseoli* mutants[4] and for *R. leguminosarum* biovar *viciae* mutants[5]. Whereas these three studies lacked chemical analysis of the LPS structure, this aspect was provided in a study of symbiotically defective *R. phaseoli* mutants[6]. The work now reported

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extends the results of this study to the *R. leguminosarum* system and underscores the importance of the synthesis of an intact LPS by the bacterium for viable symbiosis.

The rhizobial LPS have unusual features which include transmembrane fatty acids[7,8], unusual sugars including uronic acids in the lipid A[9,10], and, for some LPS, a complete lack of phosphate in the lipid A[10]. There is a large degree of structural diversity from strain to strain among the different species[11] and complete structures are known only for two core oligosaccharide components[12–14], namely a trisaccharide containing KdO and two GalA residues and a tetrasaccharide containing KdO, Gal, Man, and GalA residues.

R. leguminosarum biovar *viciae* (strain VF-39), isolated from nodules of *Vicia faba*, can develop an efficient symbiosis with such host plants as *V. hirsuta* and *Pisum sativum*. Mutants of this strain, isolated by Tn5 mutagenesis, were impaired in their ability to elicit normal development of nodules. They were able to induce the formation of nodules and infection threads, but were affected[5] in the release and/or differentiation of the bacteria[5]. SDS-PAGE indicated that the symbiotic defect of these mutants was correlated with a failure to produce a normal LPS. This defect was also found for two mutants (including VF-39-32) by preliminary analysis of the LPS carbohydrate[15]. The present study was undertaken in order to determine the modified structures of the LPS of the genetically different mutants VF-39-32 and VF-39-86, so as to better understand the origin of the changes and to rationalize their effects on symbiosis events.

EXPERIMENTAL

Culture of bacteria and isolation of LPS and LPS fragments.—Bacteria were grown in a modified Bergensens medium as described[16] in shaken-broth cultures. Mutants were grown[5] in the presence of antibiotics. The LPS were isolated by the hot phenol method[17] and, after treatment with DNAse and RNAse, purified on Sepharose 4B, using an ammonium formate buffer as described[9,10]. Column eluates were monitored by the phenol–H_2SO_4 method[18]. Fragments were released from the LPS by hydrolysis for 2 h with aq 1% acetic acid at 100°. The hydrolysate was extracted with 5:1 $CHCl_3$–MeOH in order to remove lipid A, the aqueous layer was lyophilized, and the residue was chromatographed on a column of Biogel P2, using aq 0.1% formic acid. Fractions which appeared impure by ^{1}H-NMR spectroscopy and other analyses were subjected to ion-exchange chromatography on DEAE-Sephadex G25 in aq 0.01% formic acid adjusted to pH 6.5 with NH_3, using elution with a linear gradient of $0 \rightarrow 0.2$ M NH_4Cl.

Compositional analysis.—Analyses for fatty acids and carbohydrates in lipid A were carried out as described[7–10], after conversion into methyl esters and alditol acetates, respectively. Deuterium-labeled acetate groups were introduced by acylation with acetic anhydride-d_6. GLC of carbohydrates was carried out on a Hewlett–Packard 5890 gas chromatograph, using a capillary DB225 column and a

flame-ionization detector. Mass spectrometry detection was effected with a JEOL 505 mass spectrometer in the EI or CI (ammonia) mode.

Structural analyses.—Oligosaccharides were methylated after reduction of carboxyl and other carbonyl functions with $NaBD_4$. ^{1}H-NMR spectroscopy (500 MHz) was performed on solutions in D_2O with a Varian VXR500 spectrometer. FABMS was performed with a JEOL HX110-HF instrument in the negative- or positive-ion mode, using a Cs or Xe source.

RESULTS AND DISCUSSION

Chromatography on Sepharose 4B of the LPS from the wild-type strain *Rhizobium leguminosarum* biovar *viciae* VF-39 (Fig. 1) showed four major fractions, of which A–C corresponded to LPS and the late-eluting peak was composed almost entirely of a glucan. Hydrolysis of each of the fractions A–C with aqueous 1% acetic acid, followed by extraction of the lipid A with chloroform–methanol and chromatography of the components in the aqueous phase on Biogel P2, gave three sub-fractions and the profiles were similar (Fig. 1). GLC–MS indicated that peak 1 contained 2-*O*-methylfucose, fucose, a 3,6-dideoxy-3-(methylamino)hexose (probably the *galacto* isomer[19]), glucose, amino-2,6-dideoxyhexose, and heptose (Fig. 2). The presence of most of these components was also indicated by ^{1}H-NMR spectroscopy, which showed that the O-antigen was heavily acetylated (signals between 1.8 and 2.2 ppm; Fig. 3). There were signals at 0.8–1.4 (CHMe), 2.75 (NMe), and ~3.28 ppm (OMe). Peak 2 contained the deacetylated version of a tetrasaccharide made up of Man, Gal, GalA, and KdO, and first characterized in *R. trifolii* ANU843[12] (the linkage of mannose to KdO was later revised[13]). The composition and linkage positions were confirmed by methylation analysis, and the molecular weight by FABMS. The structure is shown in **1** and the ^{1}H-NMR spectrum, which is identical to that of the *R. trifolii* tetrasaccharide after deacetylation, is shown in Fig. 4. Peak 3 contained the trisaccharide (**2**) identified[6,13,14] in *R. trifolii* ANU843.

The gel-filtration profile on Sepharose 4B of the total LPS of the mutant VF-39-32 was radically different to that of the wild type (Fig. 5). Two major LPS fractions (A and B) which, after hydrolysis (with aqueous 1% acetic acid as described for the parent strain), gave profiles on Biogel P2 that were different to those obtained (Fig. 1) from the wild type. Thus, peak 1 from fraction A contained none of the amino sugars found in the wild type and appeared to contain only a small amount of a capsular antigen comprising mainly glucose. Peaks 2 and 3 from fractions A and B contained similar labeled major components. Peak 1 from fraction B contained small amounts of the O-antigen found in the parent strain. Peak 3 contained the trisaccharide **2**. The major peak contained a component, the ^{1}H-NMR spectrum of which is shown in Fig. 6. Two sets of upfield resonances (dd) at 2.02 (J 14.5 and 1 Hz) and 2.60 ppm. (J 14.5 and 7.6 Hz) indicated the presence of a deoxy sugar. GLC–(EI and CI)MS of the alditol acetate and trideuterioac-

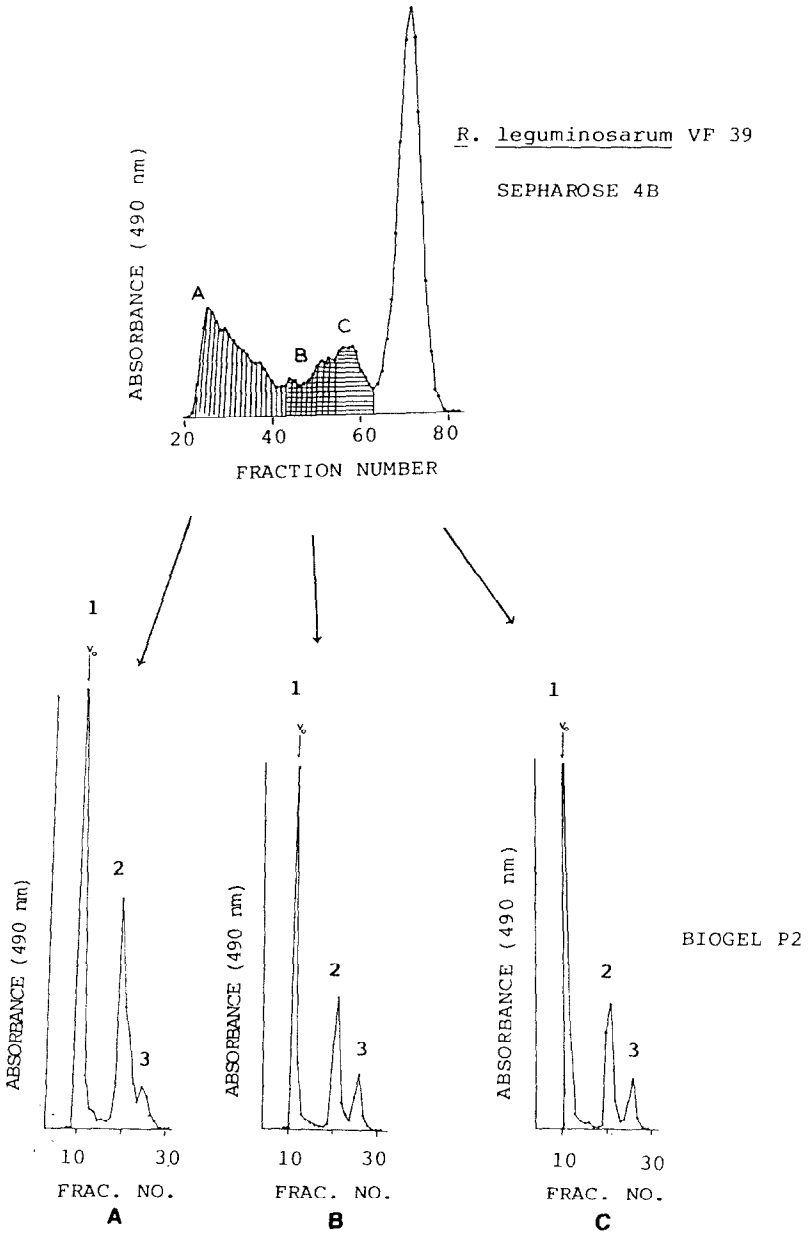


Fig. 1. Gel filtration on Sepharose 4B of the crude LPS isolated from *Rhizobium leguminosarum* biovar *viciae* VF-39. Fractions A–C were each subjected to mild hydrolysis with aqueous 1% acetic acid at 100° for 2 h and then extracted with 5:1 chloroform–methanol, and the aqueous layer was subjected to gel filtration chromatography on Biogel P2. Peaks with the same numbers had similar compositions and NMR spectra, and contained the O-antigen (1), a tetrasaccharide (2), and a trisaccharide (3). The vertical axes represent absorbance in the phenol–sulphuric acid assay.

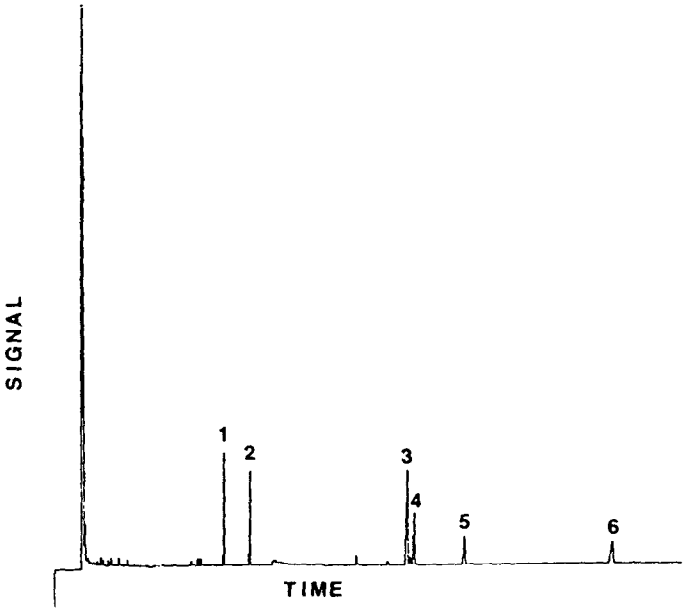


Fig. 2. Gas chromatogram (see Experimental) of the O-antigen carbohydrate components of *Rhizobium leguminosarum* biovar *viciae* VF-39 as the alditol acetates derived from 2-*O*-methylfucose, fucose, glucose, 2-amino-2,6-dideoxyglucose, 2-amino-2,6-dideoxyhexose, and heptose in the order 1–6, respectively.

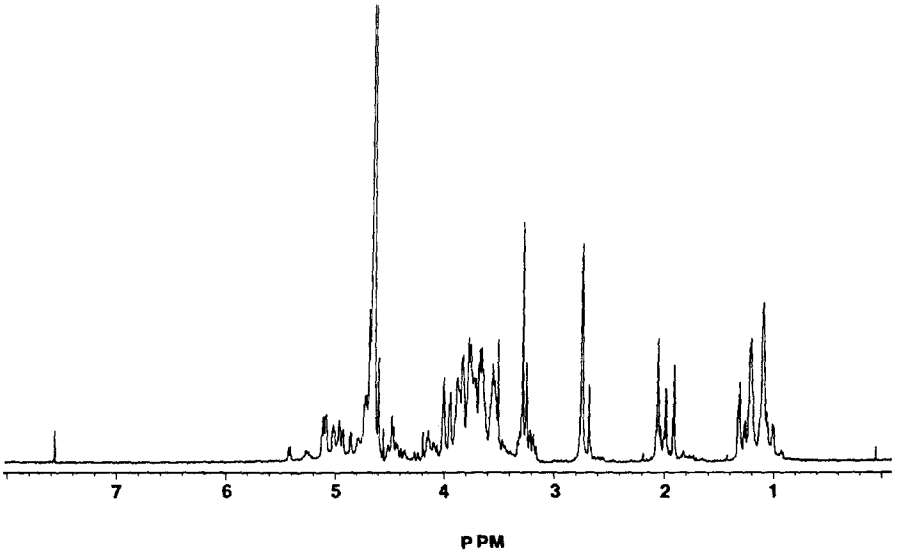


Fig. 3. 500-MHz ^{1}H-NMR spectrum of the O-antigen (peak 1 in Fig. 1).

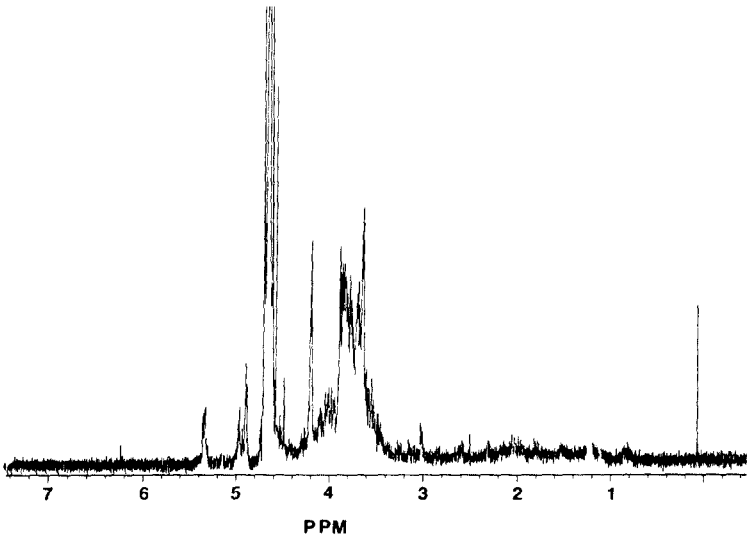
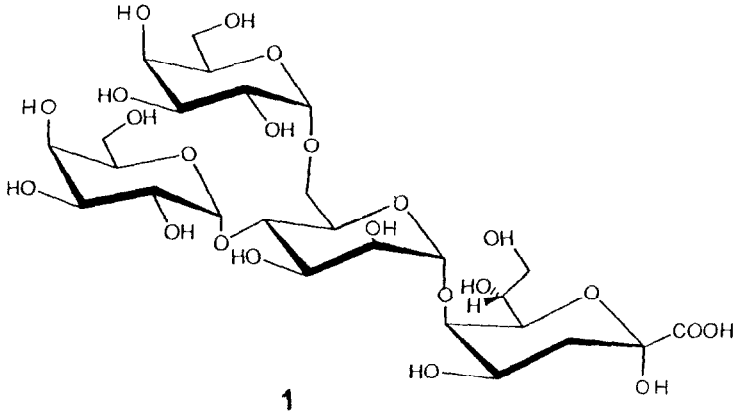


Fig. 4. ^{1}H-NMR spectrum of the tetrasaccharide **1**. The three H-1 signals in the range 4.8–5.4 ppm are due to α-Gal, Man, and GalA, respectively, in decreasing order of chemical shift. Signals for the poorly resolved deoxy function appear at ~ 2 ppm.

etate from this residue confirmed the presence of Gal and Man, and indicated the presence of a 2-deoxyaldose [the signal at 4.53 ppm (dd) was coupled to those for the methylene protons and the chemical shift, consistent with its being due to an anomeric proton, is far downfield of that for H-4 of KdO]. The structure of this molecule is still under investigation, but it does not correspond[6] to a truncated form of the tetrasaccharide **1**.

The gel-filtration profile on Sepharose 4B of the LPS of the mutant VF-39-86 was also different from that of the wild type (Fig. 7). The major fractions A–C,



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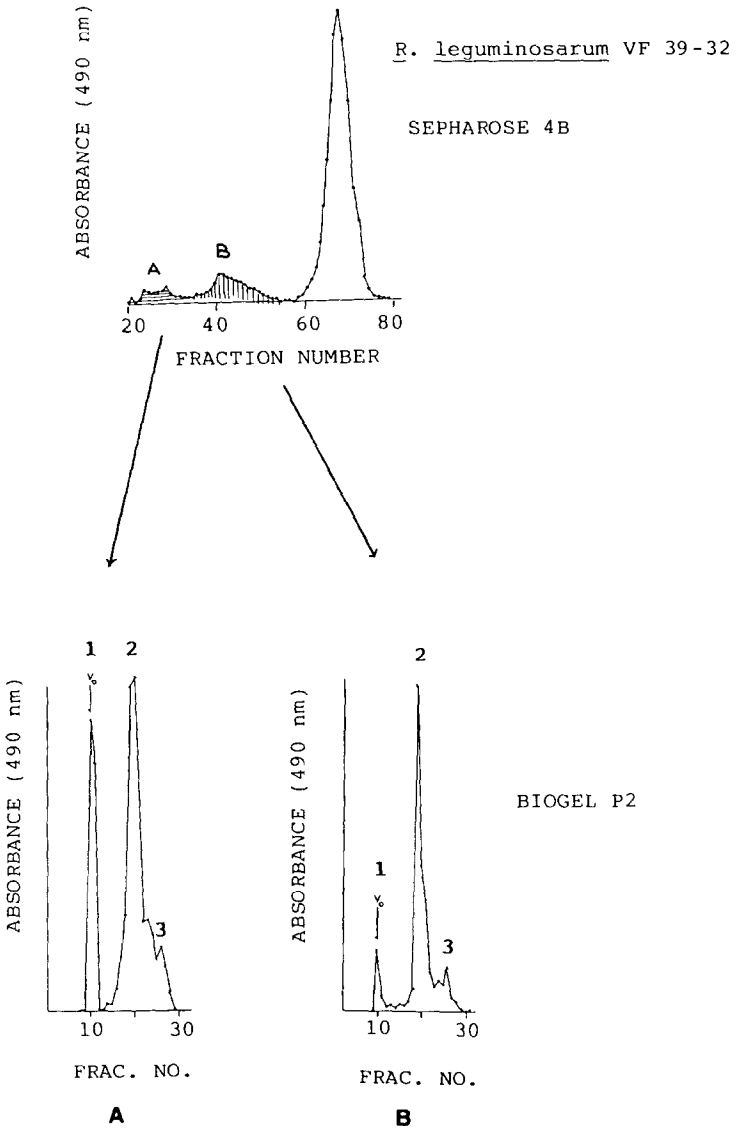


Fig. 5. Gel filtration on Sepharose 4B of the crude LPS of *Rhizobium leguminosarum* biovar *viciae* VF-39-32. Fractions A and B were each subjected to mild hydrolysis with aqueous 1% acetic acid at 100° for 2 h and then extracted with 5:1 chloroform–methanol, and the aqueous layer was subjected to gel-filtration chromatography on Biogel P2. Peak 1 from fraction **A**, which contained mainly glucose indicating that it might be due to small amounts of capsular material, was devoid of amino sugars. Peak 1 from fraction B contained a small amount of the O-antigen. Peak 3 from fractions **A** and **B** contained the trisaccharide **2** and peak 2 contained an unidentified product.

when subjected to mild acid hydrolysis followed by gel filtration on Biogel P2, yielded profiles (Fig. 7) that were different from those obtained from the parent strain. Thus, peak A yielded three peaks, of which peak 1 corresponded to traces

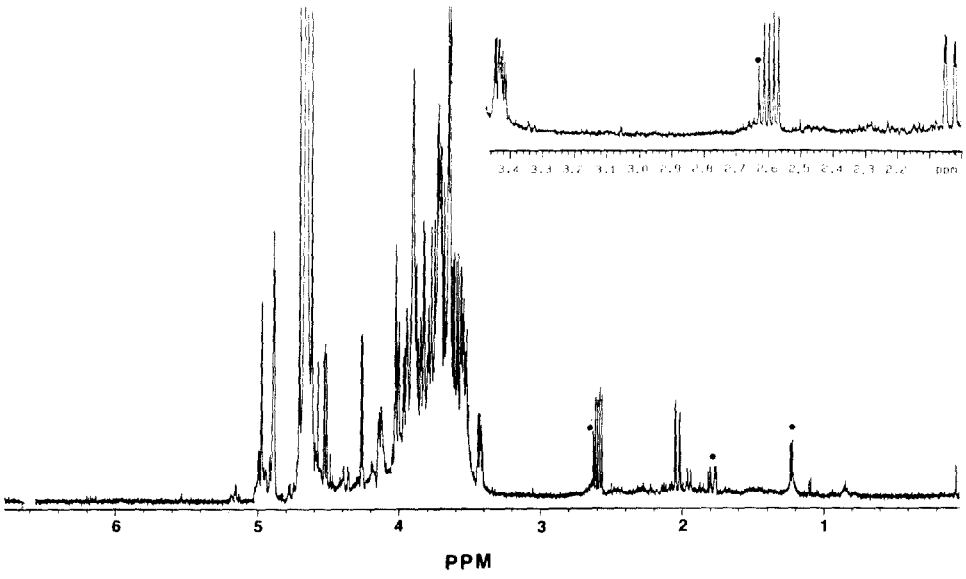
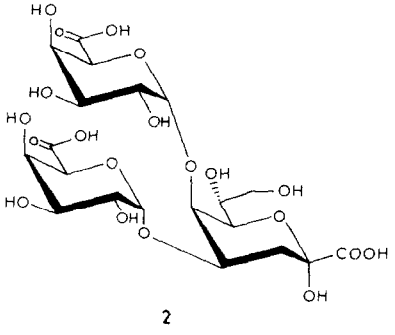


Fig. 6. ^{1}H-NMR spectrum of an unidentified component from strain VF-39-32 (peak 2 in Fig. 5). The peaks labeled with asterisks are due to contaminants.

of capsular material but none of the components present in the O-antigen of the parent strain (Fig. 2). The quantity of material isolated was too low for ^{1}H-NMR spectroscopy. Peaks 2 and 3 also contained very small amounts of material and their ^{1}H-NMR spectra contained peaks downfield of 6 ppm, indicating that they may contain nucleic acid. Fraction B gave several small peaks, each of which contained carbohydrate in quantities too small for study. However, the major peak (4) from fractions B and C contained material whose ^{1}H-NMR spectrum (Fig. 8) indicated the presence of three aldopyranoside components because of three H-1 resonances between 4.8 and 5.2 ppm. However, ion-exchange chromatography of this material on DEAE-Sephadex yielded two components, one of which had a



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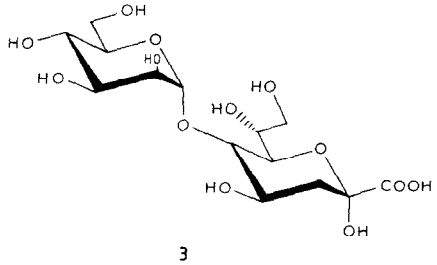

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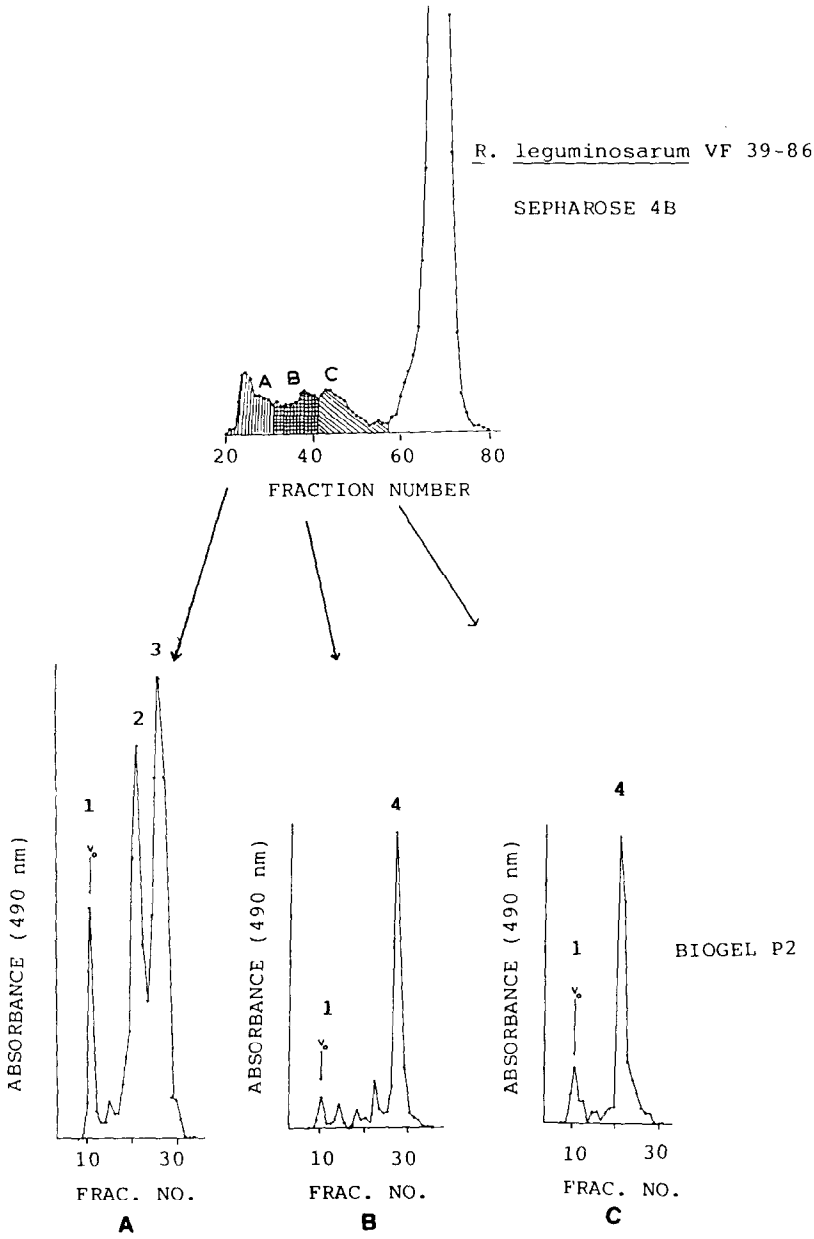

Fig. 7. Gel filtration on Sepharose 4B of the crude LPS isolated from *Rhizobium leguminosarum* biovar *viciae* VF-39-86. Fractions **A**–**C** were each submitted to mild hydrolysis with acid and then to gel filtration on Biogel P2 as described for the parent strain and strain VF-39-32. Fraction **A** yielded small amounts of capsular material (peak 1) and possibly nucleic acid material (peaks 2 and 3). Fractions **B** and **C** gave one major component (peak 4).

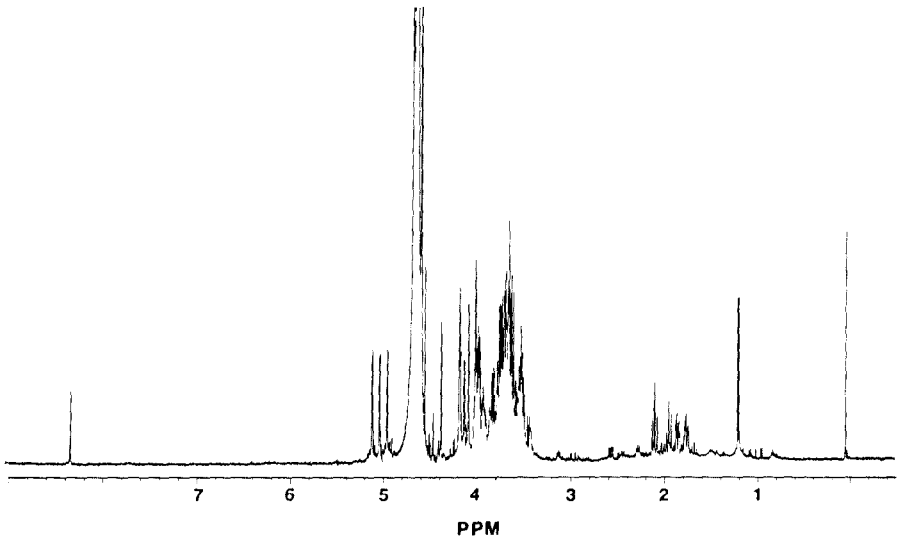

Fig. 8. ^{1}H-NMR spectrum of the material in peak 4 of Fig. 7.

^{1}H-NMR spectrum (Fig. 9) that contained only one H-1 resonance and which corresponded to a disaccharide containing Man and KdO. The same molecule was obtained[6] from the LPS of a *R. phaseoli* mutant and shown by NMR spectroscopy and methylation analysis to be α-Man-(1 → 5)-KdO (**3**). The molecular weight of this component was confirmed by FABMS (m/z 445 for $[M + Na]^+$). The fatty

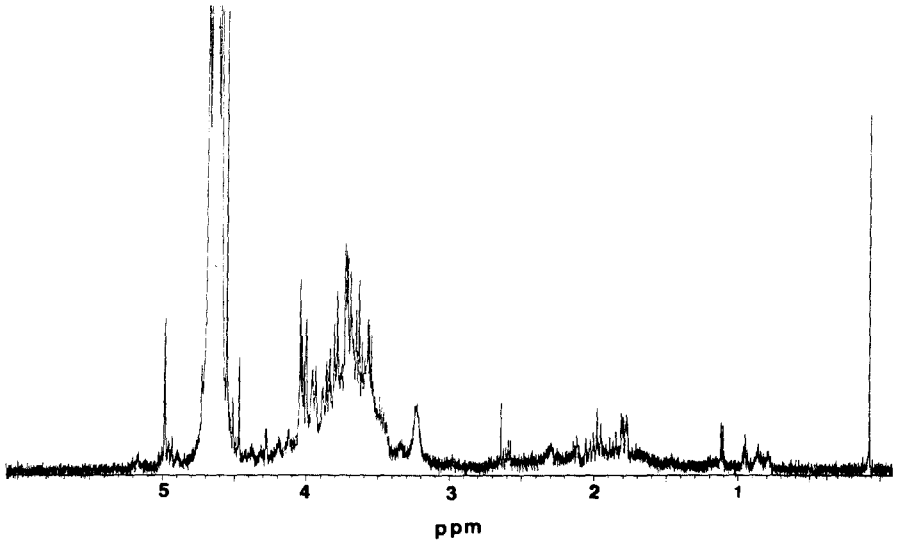

Fig. 9. ^{1}H-NMR spectrum of the disaccharide from peak 4 of Fig. 7 purified by ion-exchange chromatography.

acids found in the lipid A of the parent strain and both mutants were the same and in the same proportion as those found in *Rhizobium leguminosarum* biovar *trifolii* ANU843[7,8], namely C_{14}, C_{15}, C_{16} and C_{18} 3-hydroxy acids and 27-hydroxyoctacosanoic acid.

The results obtained for the symbiotically deficient mutants VF-39-32 and VF-39-86A of *Rhizobium leguminosarum* indicate that (*a*) the ability of the bacterium to synthesize an intact LPS containing the O-antigen is necessary for proper symbiosis, (*b*) the synthesis of the core trisaccharide **2** component is independent of the synthesis of the tetrasaccharide **1**, and (*c*) the bacterium can synthesize more than one type of LPS and impairment or suppression of the synthesis of the usual LPS can lead to the synthesis of an alternative LPS which is not normally expressed. This work underlines the importance of bacterial cell surface polysaccharides in infection.

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