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THE DISCERNIBLE, STRUCTURAL FEATURES OF THE ACIDIC POLYSACCHARIDES SECRETED BY DIFFERENT *Rhizobium* SPECIES ARE THE SAME*,†

MICHAEL MCNEIL, JANET DARVILL, ALAN G. DARVILL**, PETER ALBERSHEIM**††,
Department of Chemistry, Campus Box 215, University of Colorado, Boulder, CO 80309 (U.S.A.)

RON VAN VEEN, PAUL HOOYKAAS, ROB SCHILPEROORT,
Department of Plant Molecular Biology, University of Leiden, Biochemistry Building, Wassenaarseweg 64, 2333 AL Leiden (The Netherlands)

AND ANNE DELL***
Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ (Great Britain)

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ABSTRACT

Octasaccharide repeating-units have been isolated from the acidic polysaccharides secreted by *Rhizobium trifolii* strain *NA30, R. trifolii* strain LPR5, *R. leguminosarum* strain LPR1, and *R. phaseoli* strain LPR49. (*R. trifolii* is the symbiont of clover, *R. leguminosarum,* of peas, and *R. phaseoli,* of beans). The repeating units were formed by treating the polysaccharides with an enzyme produced by a bacteriophage. The glycosyl sequence and the structures and locations of the non-glycosyl substituents were shown to be identical for repeating units derived from all of these polysaccharides, except for that derived from the polysaccharide produced by *R. trifolii* NA30. Therefore, the discernible structural features of the acidic polysaccharides secreted by *Rhizobium* species cannot be the determinant of host specificity. In support of this conclusion is the observation that *R. trifolii* LPR5045, produced by curing *R. trifolii* LPR5 of its Sym plasmid (the Sym plasmid is required for symbiosis and host specificity), secreted a polysaccharide having the same structure (including identities and locations of non-glycosyl substituents) as that of the polysaccharide secreted by its plasmid-containing parent. Thus, the structural genes that encode for synthesis of the acidic polysaccharide secreted by *R. trifolii* LPR5045 are not located on the Sym plasmid,

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**Present address: Complex Carbohydrate Research Center, P.O. Box 5677, Athens, GA 30613, U.S.A.

††To whom correspondence should be addressed.

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and neither are the genes that encode for synthesis and attachment of non-glycosyl substituents of the polysaccharide. The possibility remains that a quantitatively minor component of the acidic polysaccharide could be a host-specific determinant.

INTRODUCTION

Bacteria in the genus *Rhizobium* form nitrogen-fixing symbioses with legumes[2]. A given species of *Rhizobium* is usually capable of forming a symbiosis with only one, or a few, botanically related species of legume[3,4]. Although the molecular basis of the process by which *Rhizobia* induce symbiotic nodules in the roots of legumes has been studied at length, it remains unelucidated[2,3,5]. In fact, not one of the molecules that renders the symbiosis host-specific has been identified.

According to one hypothesis, the acidic polysaccharides secreted by *Rhizobium* are determinants of host specificity[3]; if this is correct, the acidic poly-

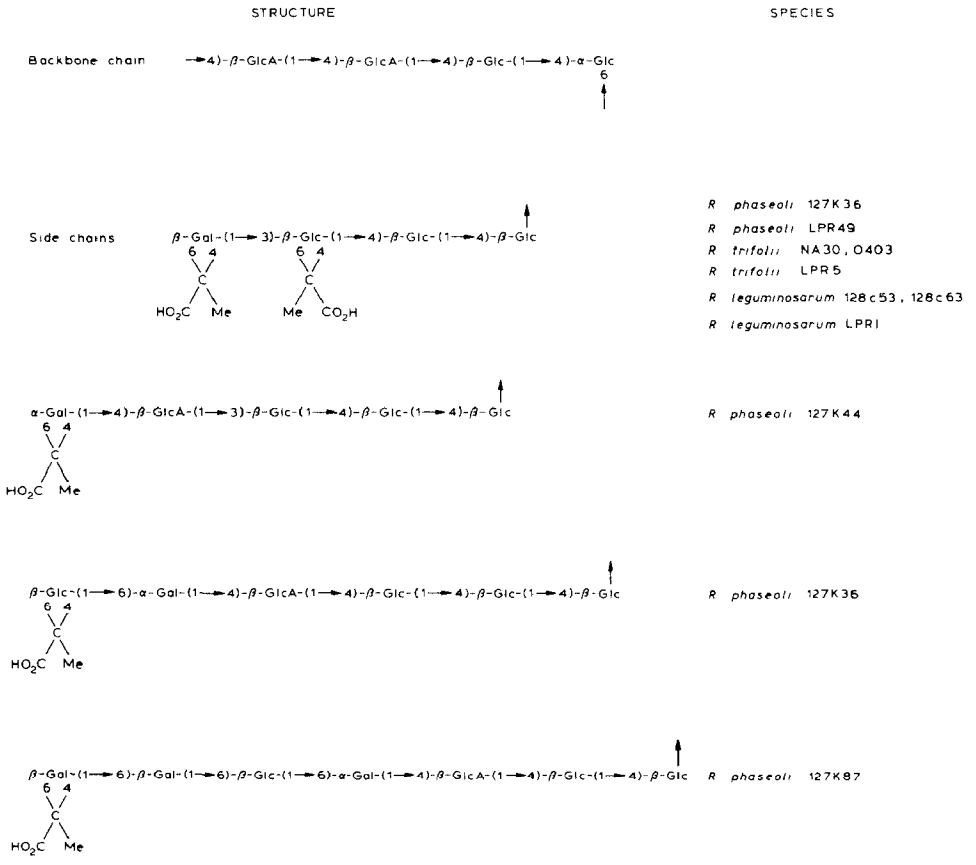

Fig. 1. Glycosyl sequences of the repeating units of polysaccharides secreted by 11 different strains of 3 species of *Rhizobium*. GlcA = glucosyluronic acid; Glc = glucosyl; Gal = galactosyl; Pyr = a pyruvic-acid ketal group.

saccharides secreted by the different species of *Rhizobium* would have different structures, as the *Rhizobium* species are defined by the legume species with which they can form a symbiosis. Structural studies in one of these laboratories[6-10] have elucidated the glycosyl sequences of the acidic polysaccharides secreted by eight different strains of the three most closely related *Rhizobium* species, namely, *R. leguminosarum, R. phaseoli,* and *R. trifolii,* the symbionts, respectively, of pea, bean, and clover (see Fig. 1). Upon examination of these structures, it was clear that different *Rhizobium* species do, in fact, secrete acidic polysaccharides having the same repeating unit (excluding possible differences in identity, and points of attachment, of base-labile, acyl substituents). Thus, *R. phaseoli* 127K36, *R. trifolii* NA30 and 0403, and *R. leguminosarum* 128c53 and 128c63 all secrete acidic polysaccharides having the same eight glycosyl (two pyruvic acetal) residue repeating-unit. These results established that the sequence of the glycosyl residues and the points of substitution of the pyruvic acetal groups cannot be the determinants of legume host-specificity.

A further notable phenomenon is that different strains of a single *Rhizobium* species do, at least in some instances, secrete acidic polysaccharides having different structures, for example, the polysaccharides secreted by the four *R. phaseoli* strains illustrated in Fig. 1. However, the polysaccharides secreted by the four *R. phaseoli* strains are structurally related by virtue of possessing identical backbones and the same, first two D-glucosyl residues of the side chains. Thus, it has been established that (*a*) different species of *Rhizobium* can secrete acidic polysaccharides having the same glycosyl-residue repeating-units and (*b*) a single species can secrete acidic polysaccharides having different glycosyl-residue repeating-units. These facts help define the possible biological functions of the polysaccharides.

The structures presented in Fig. 1 are not complete. The polysaccharides are esterified with acetyl groups[6-10] and with 3-hydroxybutanoyl groups[10a]. The 3-hydroxybutanoyl groups had been reported to be attached by ether linkages[11]; however, preliminary results of this laboratory, and those of Mort[10a], indicate that they are attached by ester, not ether, linkages. The sites of substitution of these *O*-acyl substituents have not been determined, and consequently, polysaccharides that are otherwise identical may prove to differ from each other in the sites of *O*-acyl substitution. Because they are known to be primary antigens of some capsular polysaccharides[12] and mammalian cells[13], it is possible that the *O*-acyl substituents endow the acidic polysaccharides secreted by *R. trifolii, R. leguminosarum,* and *R. phaseoli* with the ability to act as determinants of host selectivity. In the case of *R. phaseoli,* where different strains secrete acidic polysaccharides having different side-chains, it is possible that the *O*-acylated-backbone region of the polysaccharides is recognized by bean plants, and thereby functions in host selection.

The present study addressed the question of whether different *Rhizobium* species secrete acidic polysaccharides having distinctive *O*-acyl substitution-patterns by comparing the complete structures (including the identity and location

of *O*-acyl groups) of the acidic polysaccharides secreted by *R. leguminosarum*, *R. phaseoli*, and *R. trifolii*.

EXPERIMENTAL

Rhizobium *strains.* — *Rhizobium trifolii NA30* was obtained from Dr. William F. Dudman of the Division of Plant Industry of the Commonwealth Scientific and Industrial Research Organization (Canberra City, Australia); and *R. phaseoli* strain 127K36, from Dr. J. Burton, Nitragin Company, Milwaukee, WI; and *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49 were from the Rothamsted Culture Collection, Harpenden, U.K. (obtained under collection numbers 5, 1001, and 3622). Strain LPR5001 is a rifampicin-resistant derivative of LPR5, and LPR5045 is a derivative of LPR5001 cured of its Sym plasmid[14] and therefore nod$^-$.

Growth conditions. — The *Rhizobia* were grown on a modified, Bergersen medium (MBM). Each liter of medium contained the following: K_2HPO_4 (0.25 g), $MgSO_4 \cdot 7\ H_2O$ (0.10 g), sodium L-glutamate (1.1 g), D-mannitol (10 g), calcium pantothenate (4 mg), $CaCl_2 \cdot 2\ H_2O$ (6.62 mg), H_3BO_3 (0.14 mg), $FeSO_4 \cdot 7\ H_2O$ (125 μg), $CoCl_2 \cdot 6\ H_2O$ (59 μg), $CuSO_4 \cdot 5\ H_2O$ (5 μg), $MnCl_2 \cdot 4\ H_2O$ (4.3 μg), $ZnSO_4 \cdot 7\ H_2O$ (108 μg), Na_2MoO_4 (125 μg), riboflavin (0.02 mg), *p*-aminobenzoic acid (0.02 mg), nicotinic acid (0.02 mg), biotin (0.02 mg), thiamine · HCl (0.02 mg), pyridoxine HCl (0.02 mg), and inositol (0.12 mg). The pH of the medium was adjusted to 6.9. The *Rhizobia* were grown as described[6], but sometimes, as indicated later, they were grown on a salt-free medium, each liter of which contained sucrose (2.5 g), K_2HPO_4 (0.5 g), NaCl (0.1 g), and yeast extract[15] (0.5 g).

Determination of the optical absorbance of Rhizobium *cultures.* — The optical absorbance at 580 nm (A_{580}) of aliquots of *Rhizobium* suspensions was determined in a culture tube (1 cm diam.) in a Bausch and Lomb Spectronic 20 spectrometer.

Preparations of acidic polysaccharides from culture medium of Rhizobium *at different growth-phases.* — Two 1-L flasks of MBM medium were each inoculated with *R. trifolii* LPR5045 (7.5 mL; $A_{580} = 0.4$). Each flask was fitted with a side arm containing a stopcock to allow sampling during growth of the culture. The early-log and late-stationary phases of growth were defined by monitoring the A_{580} of cultures at ~6-h intervals. The flask contents harvested during the early-log phase of growth (25 h after inoculation) had $A_{580} = 0.4$; that harvested during the late-stationary phase (85 h after inoculation) had $A_{580} = 1.4$. The same experiment was repeated with *R. trifolii* LPR5001, a rifampicin-resistant strain of *R. trifolii* LPR5.

Purification of the acidic polysaccharides. — The acidic polysaccharides were purified from *Rhizobium* cultures as described[6].

Isolation and purification of a Rhizobium *phage.* — Soil (5 g) in which garden beans had been recently grown was added to MBM medium (10 mL), and shaken overnight at ~80 r.p.m. The suspension was centrifuged at 12,000*g* for 10 min, and the supernatant solution, which contained the phage, was decanted, and filtered

through a 0.2-μm Nalgene filter-unit, to remove bacteria. A culture of *R. phaseoli* 127K36 (1 mL; A_{580} 0.36) was added to the phage suspension, and the mixture was incubated for 24 h at room temperature. During this time, the phage used the *R. phaseoli* in order to multiply. The bacteria were removed by filtration through a 0.2-μm Nalgene filter-unit, and dilutions (10^{-2} to 10^{-12}) of the phage-enriched suspension were prepared. Aliquots (0.5 mL) of a liquid culture (A_{580} 0.315) of *R. phaseoli* were added to each of a series of test tubes containing 0.7% MBM-agar (2.5 mL) that had been melted and then maintained at 46°; a dilution (0.1 mL) of the phage suspension was added to each tube, and the contents of the tubes (phage, bacteria, and 0.7% MBM agar) were poured onto 1.5% MBM-agar plates[15]. The plates were swirled, and allowed to solidify. Plaques, due to phage clearing, were found in the bacteria lawn after two days.

A single plaque that contained a halo was selected for propagation and further purification. The presence of the halo suggested that the phage responsible for the plaque had a diffusible enzyme capable of digesting the acidic polysaccharide secreted by the *Rhizobium*[16]. A sterile transfer-needle was stabbed into the plaque, and the needle then rinsed into a flask containing *R. phaseoli* 127K36 (10 mL; A_{580} ~0.4) in MBM medium. The flask was shaken for 4 h, and the contents were then transferred to a flask containing low-salt medium (100 mL), the purpose of which was to inhibit the *Rhizobium* from secreting polysaccharides and thereby to allow more-efficient phage-infections. After incubation for 20 h, the solution was used to prepare phage plaques on a bacterial lawn. Phage from a single plaque were isolated as before. The procedure was repeated, so that the final phage-population was a result of three consecutive phage-isolations from infections due to a single phage.

Propagation of phage. — Large amounts of phage were prepared as follows. A *Rhizobium* suspension was made by transferring a suspension of *R. phaseoli* 127K36 (4 mL; A_{580} 0.4) into MBM medium (100 mL) in a 500-mL flask; this was incubated for 16 h at room temperature. The phage solution (containing a total of 10^{10} plaque-forming units of phage; 15 mL) was added to the 100 mL of culture. After incubation for 4 h at room temperature, this solution was added to low-salt solution (1 L), and shaken at ~80 r.p.m. for 24 h at room temperature; by then, the *Rhizobium* suspension had cleared, and it contained ~10^9 plaque-forming units of phage per mL.

Concentration of phage. — Phage solution (~500 mL) was dialyzed against de-ionized water (10 L) overnight at 4°, concentrated by rotary evaporation at room temperature to ~50 mL, and sterilized by filtration through a 0.2 μm Nalgene filter-unit.

Phage-enzyme-catalyzed depolymerization of the acidic polysaccharides. — To a solution of an acidic polysaccharide (15 mg) in distilled water (1.5 mL) a few drops of chloroform were added, to prevent bacterial growth. Purified phage (15 mL; containing 10^{10} phage plaque-forming units per mL) was added, and the mixture was incubated overnight at room temperature. The degree of depolymeri-

zation of the polysaccharides was monitored by the l.c. assay previously described[17].

Purification of the repeating units. — A solution containing phage and depolymerized polysaccharide was concentrated by rotary evaporation at 40° to ~2 mL, and the concentrate loaded onto a column (2 × 55 cm) of Bio-Gel P-4 which was then eluted with 50mM sodium acetate, pH 5.2. Fractions (1.8 mL) were collected, and assayed for neutral sugars by the anthrone method[18]. The principal carbohydrate-containing peak was shown to be the octasaccharide repeating-unit by fast-atom-bombardment mass spectrometry (f.a.b.-m.s.). The fractions containing the repeating unit were combined, and concentrated by rotary evaporation at 40° to ~4 mL. The concentrate was desalted by gel filtration on a column (50 × 2 cm) of Sephadex G-50 that was eluted with distilled water. Fractions (3 mL) were collected, and assayed by the anthrone method[18]. The purified repeating-units (the anthrone-positive fractions), which were eluted ahead of the salts (detected by conductivity), were combined. The repeating units from the *R. trifolii* LPR5 and *R. trifolii* LPR5045 polysaccharides, whose ^{1}H-n.m.r. spectra are shown in Fig. 9, were desalted on a column (0.5 mL) of Dowex 50 (H^+) from Aldrich; this resulted in repeating units having their carboxyl groups in the hydrogen form, in contrast to those purified by means of a column of Sephadex G-50, which had the carboxyl groups in the sodium form. Because the ^{1}H-n.m.r. spectrum was dependent on the salt form of the repeating unit, it was necessary to convert the carboxyl groups of the Dowex 50-desalted repeating-units into the sodium salt form by the addition of an excess of sodium acetate.

Per-O-methylation of the repeating units. — The octasaccharide repeating-unit was reduced with sodium borodeuteride, to afford the heptaglycosyl-alditol, which was per-*O*-methylated as previously described[19].

Reduction of methyl-esterified carboxyl groups. — Per-*O*-methylation of the heptaglycosyl-alditols resulted in methyl-esterified carboxyl groups, which were reduced to dideuterated primary alcohol groups by treatment with M lithium triethylborodeuteride in oxolane (0.25 mL; Aldrich) for 1 h at room temperature. The excess of this reagent was then decomposed with a few drops of glacial acetic acid, the sample evaporated to dryness with nitrogen gas, and a solution of the residue in 50% ethanol passed through a column (0.5 mL) of Dowex 50 (H^+) to remove the lithium ions. The product was a partially *O*-methylated heptahexosyl-alditol.

Formation and analysis of partially O-acetylated partially O-methylated alditols. — The partially *O*-methylated heptaglycosyl-alditol derived from the repeating unit of the acidic polysaccharides was converted into its constituent, partially *O*-acetylated, partially *O*-methylated alditols as previously described[19].

Preparation of samples for ^{1}H-n.m.r. spectroscopy. — Samples were prepared for ^{1}H-n.m.r. spectroscopy by dissolution in deuterium oxide (99.8% D; Aldrich), and the deuterium oxide was evaporated by lyophilization or with a stream of dry nitrogen gas, the procedure being performed at least twice. The samples were then

dissolved in deuterium oxide (99.96% D; 0.5 mL; Stoller Isotope Chemicals), and the 1-dimensional, 250-MHz, ^{1}H-n.m.r. analysis was performed as described[20], except that it was conducted at 80°. A Bruker WM 250 spectrometer with an Aspect 2000A computer was used for all such analyses. Chemical shifts were referenced to internal acetone (δ 2.25). For the *J*-resolved, two-dimensional, ^{1}H-n.m.r. spectroscopy, a Bruker *J*-resolved microprogram in the DISN84 software was used. The experimental parameters included SW1, ±31.25 Hz; SW2, 2 kHz; TD1, 64W; TD2, 2048W; F2, zero-filled to 4 k; and F1, zero-filled to 128W. A sine-bell window was used in the F2 dimension, and a sine-bell window shifted $\pi/4$ was used in the F1 dimension. The *J*-correlated spectra were obtained by using the Bruker microprogram for COSY45 in the DISNMR82 software. The parameters included TD2, 1 k; TD1, 512W; SW2, 1.5 kHz; SW1, ±750 Hz; and F1, zero-filled to 1 k. A sine-bell window was used in both dimensions.

Fast-atom-bombardment mass spectrometry. — Fast-atom-bombardment mass spectrometry (f.a.b.-m.s.) was performed as described[21].

RESULTS AND DISCUSSION

^{1}H-N.m.r. analyses of the acidic polysaccharides secreted by the three Rhizobium *species suggested that the polysaccharides have the same* O*-acyl substitution-pattern.* — The *O*-acyl substitution-patterns of polysaccharides that have the same sequence of glycosyl residues were compared by ^{1}H-n.m.r. spectroscopy. Specifically, the chemical shifts of the hydrogen atoms of the *O*-acyl substituents, namely, methyl hydrogen atoms of the *O*-acetyl groups and the methyl and methylene hydrogen atoms of the *O*-(3-hydroxybutanoyl) groups were compared. In the region where the hydrogen atoms in the methyl and methylene groups of the acyl substituents resonate (δ 2.9–1.3), the ^{1}H-n.m.r. spectra of the acidic polysaccharides secreted by *R. leguminosarum* LPR1, *R. trifolii* LPR5, and *R. phaseoli* LPR49 appeared to be identical. This result suggested that the *O*-acyl substitution-pattern of these polysaccharides was the same. However, the result was not conclusive, because the viscosity of the polysaccharides caused the line width of the ^{1}H-n.m.r. signals to broaden. Therefore, it was decided that, in order to obtain definitive, ^{1}H-n.m.r. spectra, the polysaccharides would have to be depolymerized (converted into their repeating units) without removing or changing the location of the *O*-acyl substituents. A feasible way in which to accomplish this goal was by means of a phage enzyme[22–26].

A phage enzyme depolymerizes the acidic polysaccharides secreted by the Rhizobium *species and strains thereof studied herein.* — In garden soil in which beans had been grown, a phage was found that could lyse *R. phaseoli* 127K36 and that formed plaques that had halos, a phenomenon indicating a diffusible enzyme capable of depolymerizing the acidic polysaccharide secreted by the *Rhizobium*[16]. The phage was purified by three successive, single-colony isolations (see Experimental section). The purified, intact phage was used to depolymerize the

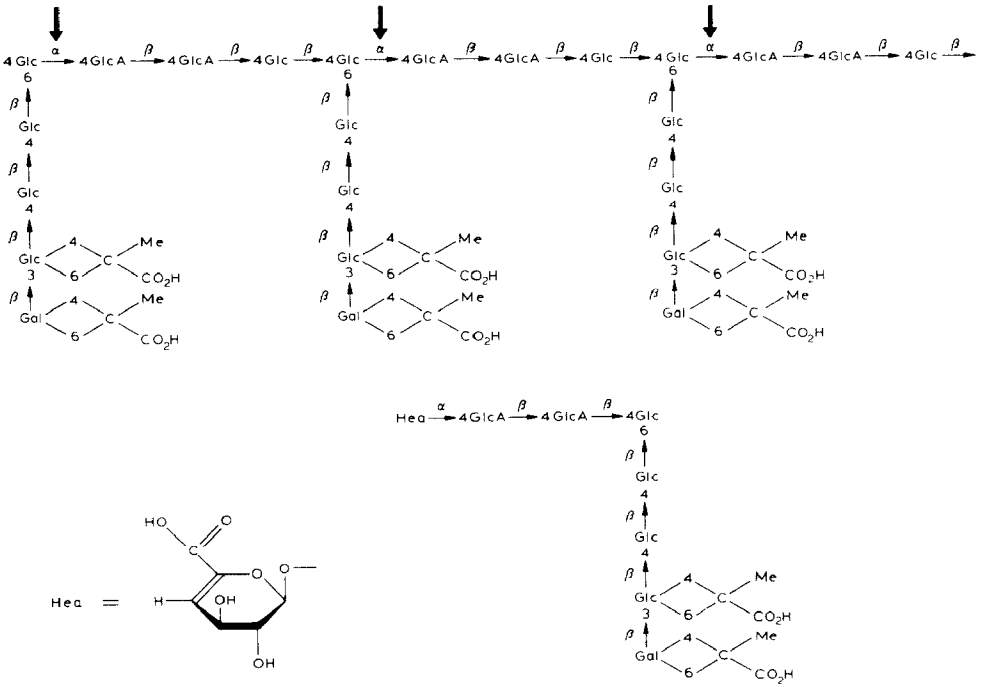

Fig. 2. Point of phage-enzyme cleavage (bold arrows) of the polysaccharides secreted by *R. trifolii* NA30, *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49. The lyase action of the enzyme results in a double bond between C-4 and C-5 of a D-glucosyluronic acid residue, which thus becomes a terminal 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid (HeA) group.

polysaccharide[23] (see Fig. 2). The rate at which the repeating units were formed was determined by liquid chromatography (l.c.) using a Waters I-125 column as previously described[17]. L.c. profiles obtained by incubating phage with the acidic polysaccharide secreted by *R. trifolii* NA30 are shown in Fig. 3. All of the acidic polysaccharides shown in Fig. 1 could be depolymerized by the same phage.

In a typical, "large-scale", depolymerization experiment, an acidic polysaccharide (15 mg) was treated with 1.5×10^{11} plaque-forming units of the phage. It was not practical to purify that amount of carbohydrate on an analytical, l.c. column, and so the products of the reaction were fractionated by gel-filtration chromatography on a column of Bio-Gel P-4 in 50M sodium acetate, pH 5.2. The purified, octasaccharide repeating-unit was desalted by gel filtration on a column of Sephadex G-50 eluted with water.

The purified octasaccharide from *R. trifolii* NA30 was analyzed by f.a.b.-m.s. (see Table I), which showed that the polysaccharide repeating-unit contained zero, one, or two *O*-acetyl groups, and that some of the repeating units were substituted with a single *O*-(3-hydroxybutanoyl) group. The f.a.b.-mass spectral analysis indicated that, in all probability, the phage enzyme was an endo-lyase, not a hydrolase, because the mass of the repeating unit was 18 amu lower (due to loss of water) than that expected for the intact octasaccharide (see Fig. 2, and later).

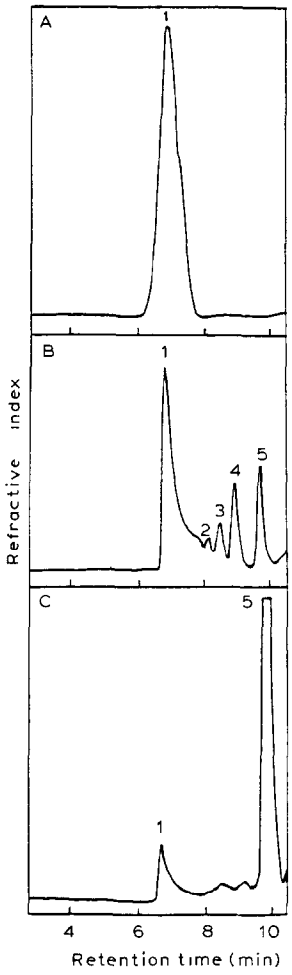

Fig. 3. The elution profile, as detected by a refractive-index monitor, of the products from ~60 μg of the acidic polysaccharide secreted by *R. trifolii* NA30 after the following treatments: A, no treatment; B, partial digestion with a purified phage that contained an endolyase; C, complete digestion with the phage endolyase. The numbered peaks correspond to (1) in A and B, incompletely digested polysaccharide and material in the phage preparation, and, in C, material in the phage preparation; (2) an oligosaccharide composed of four of the octasaccharide repeating-units; (3) an oligosaccharide composed of three of the octasaccharide repeating-units; (4) an oligosaccharide composed of two of the octasaccharide repeating-units;and (5) the octasaccharide repeating-unit.

The phage enzyme cleaves the polysaccharide in front of the branched D-*glucosyl residue.* — The site in the polysaccharide backbone at which the phage enzyme attacks was ascertained by sodium borodeuteride reduction at C-1 of the glycosyl residue released by the enzyme. The octasaccharide repeating-unit was thus converted into a heptaglycosyl-alditol, which was then per-*O*-methylated. The carboxyl groups of the glycosyluronic acids, which were methyl-esterified by the methylation procedure, were reduced with lithium triethylborodeuteride to primary

TABLE I

THE *m/z* VALUES AND NORMALIZED INTENSITIES OF THE (M − 1) IONS DETECTED DURING NEGATIVE-ION FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY OF THE REPEATING UNITS PREPARED FROM THE POLYSACCHARIDES SECRETED BY THREE STRAINS OF *Rhizobium*

m/z *value* (*M* − *1*)	*Number of O-acetyl groups*	*Number of O-(3-hydroxybutanoyl) groups*	Rhizobium *strain from which the polysaccharide was isolated*		
			R. trifolii *NA30*	R. trifolii *LPR5*	R. trifolii *LPR5045*
			Normalized relative intensity		
1463	0	0	0	3	0
1505	1	0	66	14	45
1547	2	0	0	17	36
1591	1	1	33	33	10
1633	2	1	0	34	9

alcohol groups. The partially *O*-methylated heptahexosyl-alditol resulting was then hydrolyzed to a mixture of partially *O*-methylated aldoses and, from its reducing end, a single, partially *O*-methylated alditol. This mixture was reduced with sodium borodeuteride, to afford a mixture of partially *O*-methylated alditols which was *O*-acetylated, to give a mixture of partially *O*-acetylated, partially *O*-methylated alditols that was identified by g.l.c.–m.s.[19].

The sole, partially *O*-acetylated, partially *O*-methylated alditol with *O*-methyl groups at O-1 and O-5 was derived from the glycose residue originally present at the reducing end of the precursor of the heptaglycosyl-alditol. The other, partially *O*-acetylated, partially *O*-methylated alditols were acetylated on O-1 and O-5, because O-5 of each residue was protected from *O*-methylation by the glycosyl ring, and O-1 by the presence of another glycosyl substituent. The preponderant, partially *O*-acetylated, partially *O*-methylated alditol that possessed methyl, not acetyl, groups on O-1 and O-5 was 4,6-di-*O*-acetyl-1,2,3,5-tetra-*O*-methylglucitol. (A significant proportion of 2,3,4,6-tetra-*O*-acetyl-1,5-di-*O*-methylglucitol was also detected. This compound resulted from undermethylation at O-2 and O-3, a phenomenon that has previously been observed[27] in glucitol residues substituted with a β-glucosyl group at O-6. The presence of the acetyl groups on O-4 and O-6 in the preponderant alditol (with methyl groups on O-1 and O-5) showed that, in the repeating units, O-4 and O-6 were protected from methylation, and that this residue was 4,6-linked in the repeating unit. Thus, the glycosidic linkage of the branched residue is the cleavage point of the phage enzyme (see Fig. 2).

The ^{1}*H-n.m.r. spectrum of the repeating unit of the* R. trifolii *NA30 polysaccharide corresponds to the proposed structure of the repeating unit, and confirms that the phage enzyme is a lyase.* — F.a.b.-m.s. analysis of the repeating unit formed by the phage enzyme had suggested that the phage enzyme is a lyase; analysis of the repeating-unit octasaccharide by ^{1}H-n.m.r. spectroscopy (Fig. 4) confirmed this

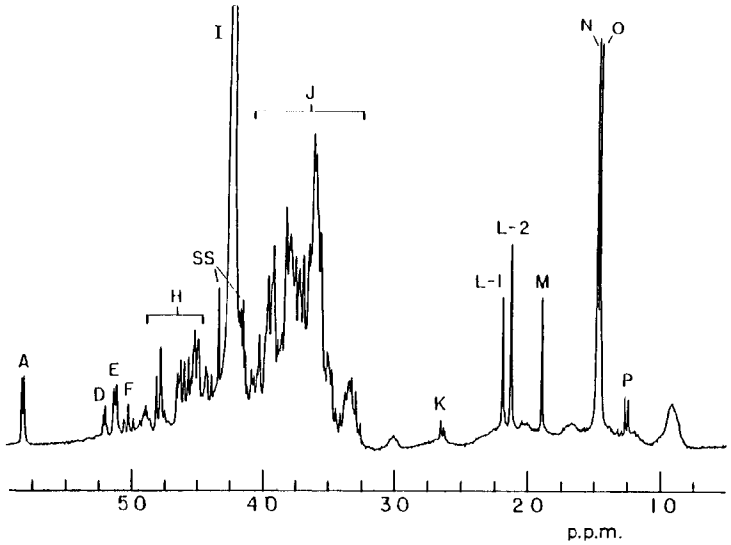

Fig. 4. ^{1}H-N.m.r. spectrum of the octasaccharide repeating-unit prepared from the acidic polysaccharide secreted by *R. trifolii* NA30. The labeled signals are identified in the legend to Fig. 7, and are discussed in the text. The broad signals at δ 3.0 and 0.9 are of unknown origin.

concept. The doublet at δ 5.83 ("A" in Fig. 4) was the signal of a proton on the double-bonded carbon atom formed by action of the lyase. Comparing the coupling constant and chemical shift of the doublet to those of similar types of known compounds[28,29] and to the known structure[6] of the polysaccharide secreted by *R. trifolii* NA30 confirmed this doublet is caused by H-4 of a 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue (see Fig. 2). There is no signal at, or near, δ 5.83 in the ^{1}H-n.m.r. spectrum of the intact polysaccharide, and this signal would not have been present in the ^{1}H-n.m.r. spectrum of the repeating unit had the phage enzyme been a hydrolyase. Thus, the phage endo-lyase catalyzed the elimination, in the polysaccharide, of the branched D-glucosyl residue from O-4 of a D-glucosyluronic acid residue, resulting in the formation of the 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue (see Fig. 2).

The signal at δ 5.20 ("D") resulted from H-1 of the α anomer of the branched (reducing) D-glucose residue, and was present at about half of the intensity of signal "A", as expected, because the branched, D-glucose residue was also present in the β anomeric form. The signal resulting from H-1 of the β form of the residue was obscured by other signals in the spectrum.

The signal at δ 5.11 ("E") (see Fig. 4) was shown, by *J* connectivity (COSY 45), ^{1}H-n.m.r. spectroscopy (see Fig. 5), to result from H-1 of the 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue. The arrows in Fig. 5 indicate the connectivity between H-4 and the other ring protons of this residue. This connectivity allowed the chemical shifts of H-3 (δ 4.13), H-2 (δ 3.85), and H-1 ("E", δ 5.11) to be determined, and this information confirmed that the signal at δ 5.83 was, in fact, that of H-4 of the unsaturated uronic acid residue.

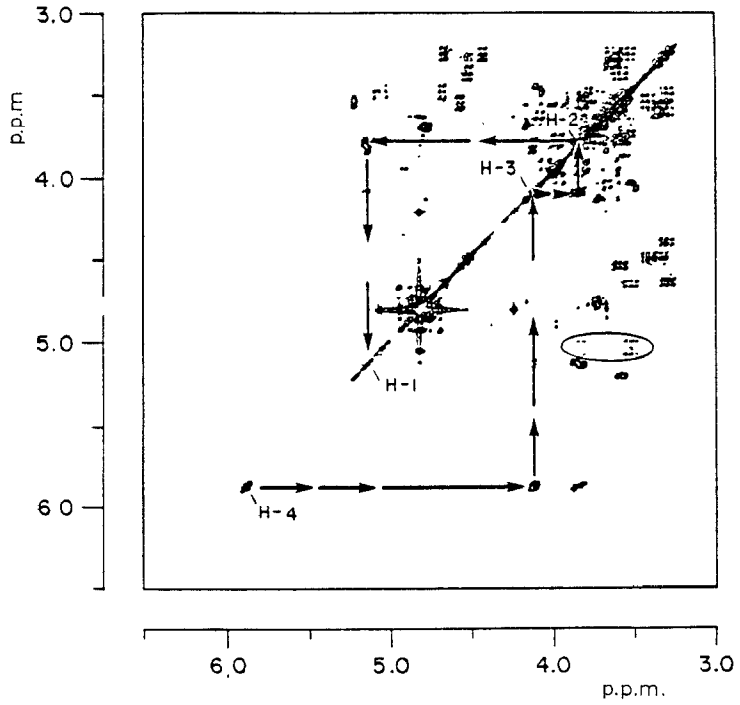

Fig. 5. The contour plot of the *J*-correlated (COSY 45), ^{1}H-n.m.r. spectrum of the acidic polysaccharide secreted by *R. trifolii* NA30. The arrows indicate connectivity between H-4, H-3, H-2, and H-1 of the α-L-*threo*-hex-4-enopyranosyluronic acid residue. The circle indicates the connectivity of signal "G" (see text and Fig. 7) to an H-4 and an H-2 atom.

It is known that hydrogen atoms attached to *O*-acyl-bearing carbon atoms resonate at δ values greater than 4.3, as do hydrogen atoms attached[30,31] to C-1. Because all ^{1}H-n.m.r. signals from H-1 atoms are singlets or doublets, the triplet at δ 5.03 ("F") must result from a hydrogen atom attached to an acyloxy-bearing carbon atom. The chemical shifts of the off-diagonal signals in the *J* connectivity, two-dimensional spectrum (see Fig. 5) showed that this atom was H-3 of a glycosyl residue that had an acyl group attached to O-3.

The signals between δ 4.3 and 4.9, labeled "H" in Fig. 4, resulted from the H-1 atoms of all of the remaining β-glycosyl residues. Integration showed that these signals contained approximately one hydrogen atom more than could be expected if only H-1 atoms were present (7.3 hydrogen atoms, not 6.5). The presence of the additional hydrogen atom can be explained by the signals of hydrogen atoms on carbon atoms bearing acyloxy groups.

The ^{1}H-n.m.r. signals at δ 2.20 and 2.14 ("L-1" and "L-2" in Fig. 4) resulted from methyl hydrogen atoms of *O*-acetyl substituents. The signals at δ 2.64 and 1.34 ("K" and "P") resulted from methylene and methyl hydrogen atoms, respectively, of *O*-(3-hydroxybutanoyl) substituents. The signals at δ 1.44 and 1.50 ("N" and "O") resulted from methyl hydrogen atoms of two pyruvic acetals. The

positions of the pyruvic acetals on the repeating unit of the polysaccharide had previously been determined[6] (see Fig. 1). Thus, the ^{1}H-n.m.r. spectrum of the repeating unit is in complete agreement with that expected from the structure of the acidic polysaccharide secreted by *R. trifolii* NA30.

The average number of *O*-acetyl and *O*-(3-hydroxybutanoyl) groups per octasaccharide repeating-unit derived from the *R. trifolii* NA30 polysaccharide was calculated by integration of the appropriate peaks in the ^{1}H-n.m.r. spectrum shown in Fig. 4; the result was 0.75 *O*-acetyl group and 0.14 *O*-(3-hydroxybutanoyl) group per repeating unit. The f.a.b.-m.s. data (see Table I) suggested ~1.0 *O*-acetyl group and 0.33 *O*-(3-hydroxybutanoyl) group per repeating unit. Because integration of ^{1}H-n.m.r. peaks is known to give reliable quantitation, it seems likely that the *O*-acetyl- and *O*-(3-hydroxybutanoyl)-containing repeating-units ionize more readily during f.a.b.-m.s. than those repeating units not *O*-acetylated; this agrees with similar observations made on other acetylated carbohydrates[32].

The glycosyl sequences of the four Rhizobium *acidic polysaccharides are identical.* — The glycosyl sequences of the acidic polysaccharides secreted by *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49 had not previously been determined. Methylation analysis of these polysaccharides showed[33] that they are composed of the same glycosyl residues, in the same proportions, as the acidic polysaccharide secreted by *R. trifolii* NA30. Furthermore, these polysaccharides were depolymerized by the same phage-enzyme that depolymerized the *R. trifolii* NA30 polysaccharide, and this further established their structural similarity. F.a.b.-m.s. analysis (see Table I) of the phage-enzyme-produced repeating-unit of the *R. trifolii* LPR5 polysaccharide (see Table I) established that it has the same molecular weight as that of the polysaccharide secreted by *R. trifolii* NA30, except that the former possesses greater variation in the number of *O*-acetyl groups per repeating unit. The ^{1}H-n.m.r. spectra of the repeating units prepared from the polysaccharides secreted by *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49 were the same as the spectrum of the repeating unit prepared from the polysaccharides secreted by *R. trifolii* NA30, except for differences explicable by variations in the *O*-acetyl substitutions. That the differences were due entirely to differences in *O*-acyl substitution was established by comparison of the ^{1}H-n.m.r. spectra of the de-esterified repeating units of the polysaccharides of *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. trifolii* NA30 (see Fig. 6). The three ^{1}H-n.m.r. spectra were virtually superposable, and this established that the structure of the glycosyl portion of the repeating units of the polysaccharides of *R. trifolii* LPR5 and *R. leguminosarum* LPR1 is identical to that of *R. trifolii* NA30. The repeating unit of the polysaccharide secreted by *R. phaseoli* LPR49 will now be shown to be identical to those of the polysaccharides secreted by *R. trifolii* LPR5 and *R. leguminosarum* LPR1.

The acidic polysaccharides secreted by R. phaseoli *LPR49,* R. trifolii *LPR5, and* R. leguminosarum *LPR1 have identical structures.* — The ^{1}H-n.m.r. spectra of

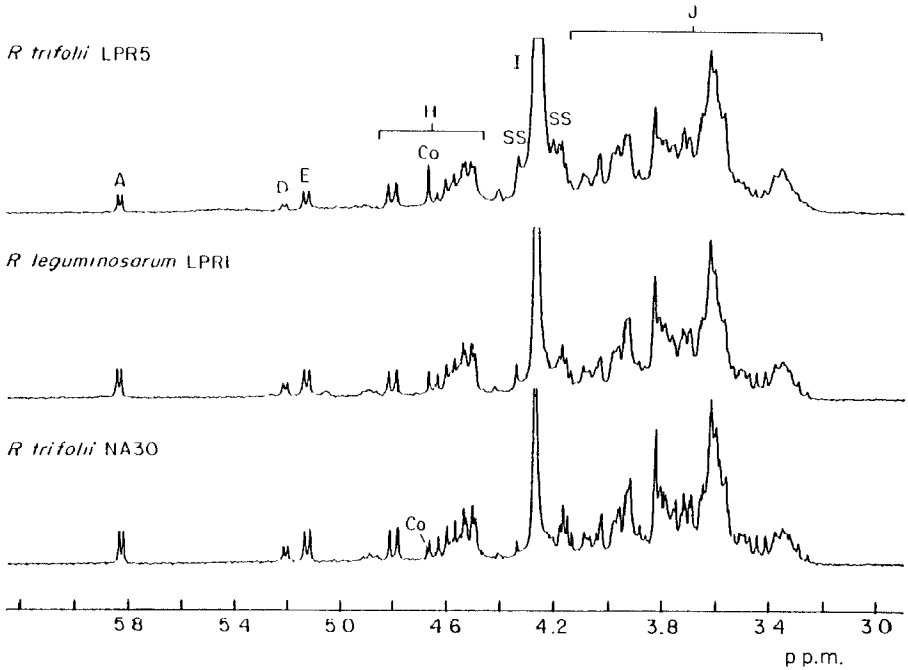

Fig. 6. A comparison of the ^{1}H-n.m.r. spectra of the de-esterified, repeating-unit octasaccharides prepared from the polysaccharides secreted by *R. trifolii* LPR5 (top), *R. leguminosarum* LPR1 (middle), and *R. trifolii* NA30 (bottom) The labeled signals are identified in the legend to Fig. 7, and are discussed in the text.

the repeating units of the polysaccharides secreted by *R. trifolii* NA30, *R. trifolii* LPR5, *R. phaseoli* LPR49, and *R. leguminosarum* LPR1 are compared in Fig. 7. This comparison shows clearly that the repeating units derived from the *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49 polysaccharides are the same, whereas the repeating unit from that secreted by *R. trifolii* NA30 has a different *O*-acyl substitution pattern; the proportion of 3-hydroxybutanoate in the polysaccharides varies, and this variation is discussed later. The signals at δ 5.33, 5.26, 5.03, and 4.90 ("B", "C", "F", and "G" in Fig. 7) arise from hydrogen atoms on glycosyl residues that possess *O*-acyl substituents. These signals can result either from hydrogen atoms on C-1 of glycosyl residues (the chemical shift of the hydrogen atom on C-1 is different, depending on whether or not the glycosyl residue is substituted with an *O*-acyl group[30,31]) or from those attached directly to the *O*-acyl-bearing carbon atoms[30,31]. These signals are present in the same locations, and in very similar proportions for the repeating units of the polysaccharides of *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49. Furthermore, in the spectra of these polysaccharides, there are at least four signals that arise from the methyl groups of the *O*-acetyl groups (labeled "L" in Fig. 7); these signals have the same chemical shifts, and are present in similar proportions, for each of the repeating units of each of the three polysaccharides.

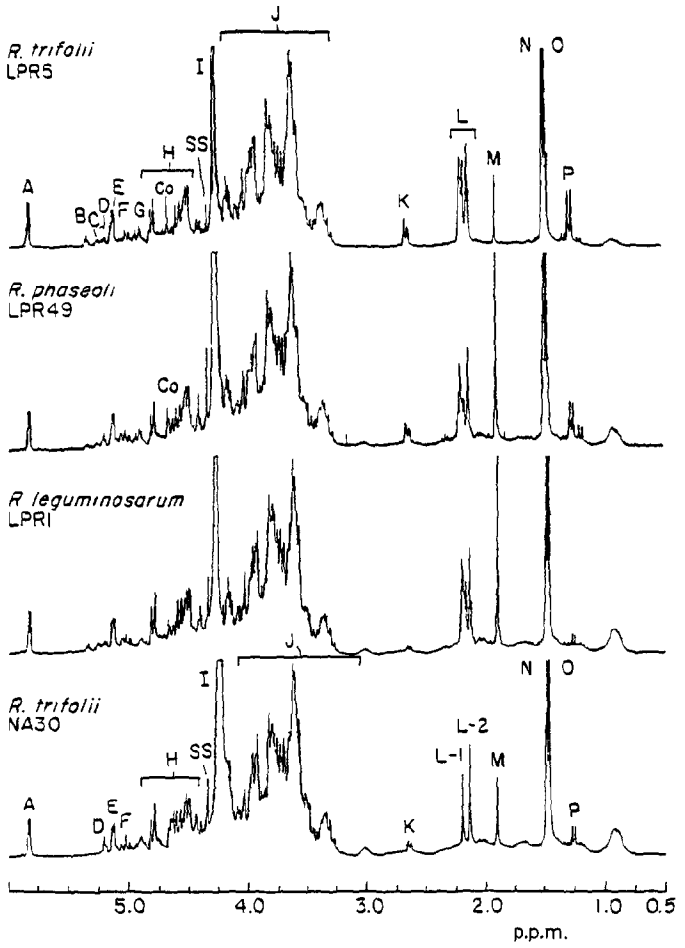

Fig. 7. A comparison of the ^{1}H-n.m.r. spectra of the repeating units prepared from the polysaccharide secreted by (from top to bottom) *R. trifolii* LPR5, *R. phaseoli* LPR49, *R. leguminosarum* LPR1, and *R. trifolii* NA30. The signal labeled "A" results from H-4 of the 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue; the signals labeled "B", "C", "F", and "G" result from hydrogen atoms attached to *O*-acyl-substituted glycosyl residues (see text); the signal labeled "D" results from H-1 of the α anomer of the (reducing) D-glucose residue; the signal labeled "E" results from H-1 of the 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid residue; the signals labeled "H" result from the H-1 atoms of the remaining glycosyl residue (see text); the signal labeled "I" results from water (present as DOH); the signals labeled "J" result from the ring hydrogen atoms (except H-1) of the glycosyl residues; the signal labeled "K" results from the methylene hydrogen atoms of the 3-hydroxybutanoic ester; the signals labeled "L" result from the methyl hydrogen atoms of acetic esters; the signal labeled "M" results from the methyl hydrogen atoms of sodium acetate (a contaminant of the repeating units); the signals labeled "N" and "O" result from the methyl hydrogen atoms of pyruvic acetals; the signal labeled "P" results from the methyl hydrogen atoms of the 3-hydroxybutanoic ester; the signals labeled "SS" result from spinning side-bands; and the signals labeled "Co" are caused by a volatile contaminant.

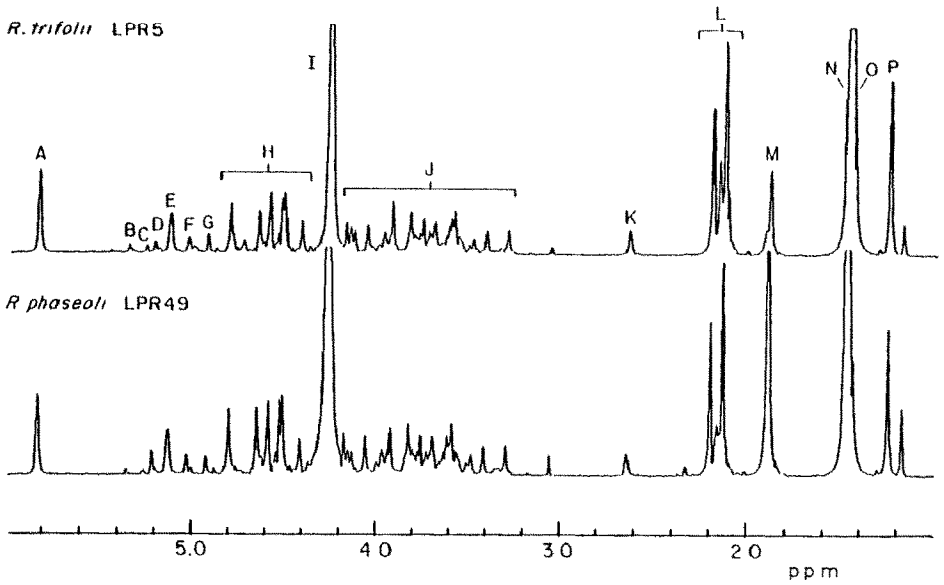

Fig. 8. Comparison of the F2 projection of the *J*-resolved, two-dimensional, ^{1}H-n.m.r. spectra of the repeating units prepared from the polysaccharides secreted by *R. trifolii* LPR5 (top) and *R. phaseoli* LPR49 (bottom). The labeled signals are identified in the legend to Fig. 7.

The structures of the repeating units of the polysaccharides secreted by *R. trifolii* LPR5 and *R. phaseoli* LPR49 were more accurately compared by means of *J*-resolved, two-dimensional, ^{1}H-n.m.r. spectroscopy. In this technique, the F2 projection is a fully proton-decoupled spectrum; thus, each proton in the repeating unit yields a single, unsplit peak in the F2 projection. The F2 projections resulting from these analyses are compared in Fig. 8. The only substantial difference between the two spectra is the signal at δ 4.75, which is present in the spectrum of the octasaccharide derived from the polysaccharide secreted by *R. trifolii* LPR5, but not in that of the octasaccharide, derived from the polysaccharide secreted by *R. leguminosarum* LPR49. This signal comes from a volatile contaminant, of unknown origin, that can be removed by evaporating the sample; it is also present in some of the spectra shown in Fig. 7. In all other respects, the two *J*-resolved spectra are indistinguishable.

The structure of the polysaccharide secreted by R. trifolii *LPR5045 is the same as that secreted by* R. trifolii *LPR5.* — *R. trifolii* LPR5045 was prepared by curing *R. trifolii* LPR5 of its Sym plasmid[14]. As it was known that the Sym plasmid is required for symbiosis, and controls the specificity of the *Rhizobium*–legume infection-process[14], it was not surprising that *R. trifolii* LPR5045 was unable to nodulate (form a symbiosis with) clover (the host plant of *R. trifolii*) or any other legume. However, when the Sym plasmid from *R. leguminosarum* LPR1 is introduced into *R. trifolii* LPR5045, the resultant *Rhizobium* could, as expected, form a symbiosis with peas (the host plant of *R. leguminosarum*), but not with clover[14].

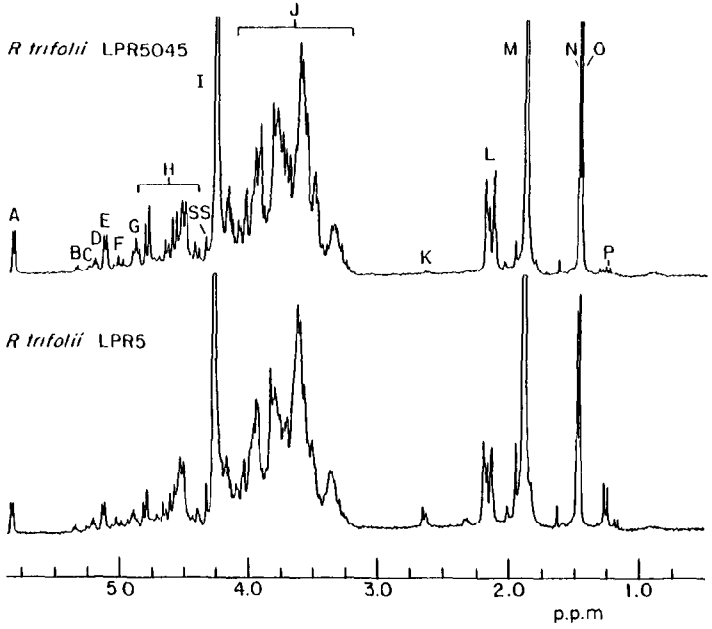

Fig. 9. A comparison of the ^{1}H-n.m.r. spectra of the repeating-unit octasaccharides prepared from the polysaccharides secreted by *R. trifolii* LPR5045 (top) and *R. trifolii* LPR5 (bottom). The labeled signals are identified in the legend to Fig. 7.

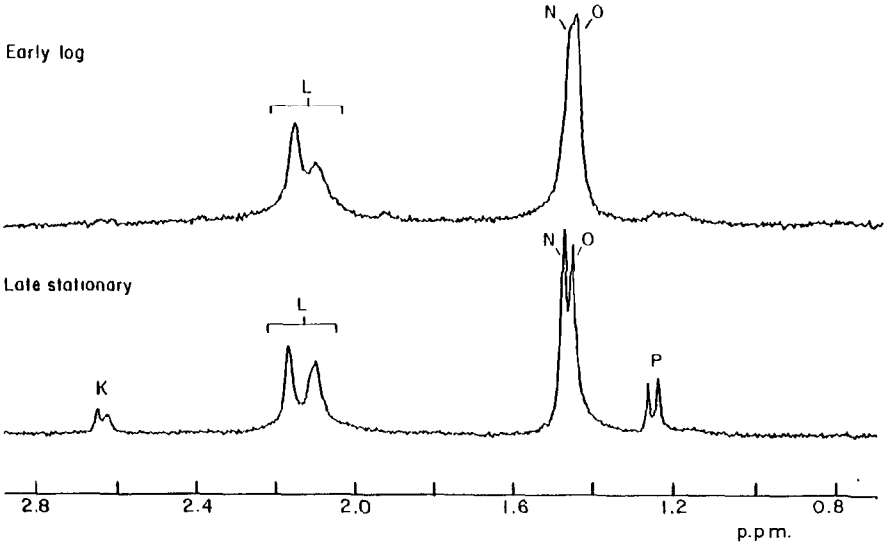

Fig. 10. A comparison of the region between δ 0.8 and 2.8 in the ^{1}H-n.m.r. spectra of the polysaccharide secreted by *R. trifolii* LPR5045 during different stages of culture growth. The polysaccharide whose ^{1}H-n.m.r. spectrum is shown in the top trace was harvested when the *Rhizobia* were in the early-log phase of growth. The polysaccharide whose ^{1}H-n.m.r. spectrum is shown in the bottom trace was harvested when the *Rhizobia* were in the late-stationary phase of growth. The labeled signals are identified in the legend to Fig. 7.

The phage enzyme was used to prepare the repeating unit from the polysaccharide secreted by *R. trifolii* LPR5045. F.a.b.-m.s. analysis of this repeating unit (see Table I) showed that it had the same molecular weight as the repeating unit derived from the polysaccharide secreted by *R. trifolii* LPR5. Furthermore, *O*-acetyl groups were present in proportions equivalent to those observed in the repeating unit derived from the polysaccharide secreted by the parent strain. The particular preparation of the polysaccharide secreted by *R. trifolii* LPR5045 (from which the octasaccharide was prepared) was substituted only rarely with 3-hydroxybutanoyl groups [see the subsequent discussion of *O*-(3-hydroxybutanoyl) groups]. The ^{1}H-n.m.r. spectra of the repeating units of *R. trifolii* LPR5045 and *R. trifolii* LPR5 are compared in Fig. 9. This analysis established that the two repeating units differ only in the degree of *O*-(3-hydroxybutanoyl) substitution. Thus, the genes that encode for the synthesis of the acidic polysaccharides are not on the Sym plasmid.

The 3-hydroxybutanoyl content of the acidic polysaccharides secreted by R. trifolii *LPR5 and* R. trifolii *LPR5045 depends on the growth phase of the bacteria at the time harvest.* — During the course of this investigation, we observed that the extent of *O*-(3-hydroxybutanoyl) substitution differed in different preparations of an acidic polysaccharide secreted by the same strain of a *Rhizobium* species. Therefore, we determined, by ^{1}H-n.m.r. analysis, the *O*-(3-hydroxybutanoyl) content of the acidic polysaccharides harvested at various times during culture of *R. trifolii* LPR5045. Portions of the ^{1}H-n.m.r. spectra (between δ 0.5 and 2.9) of the intact polysaccharide isolated during the early-log phase (top) and late-stationary phase (bottom) are compared in Fig. 10. The result was unequivocal; the content of *O*-(3-hydroxybutanoyl) groups in the polysaccharide harvested from Rhizobia in the late-stationary phase of growth is much greater than that of that from Rhizobia in the early-log phase of growth. The same result was obtained with the polysaccharide secreted by a rifampicin-resistant strain of *R. trifolii* LPR5. The biological significance of this observation is not yet known, but the observation explains the variable 3-hydroxybutanoate content of the repeating units examined herein.

CONCLUSION

This study has shown that the repeating units of the acidic polysaccharides secreted by *R. trifolii* LPR5, *R. leguminosarum* LPR1, and *R. phaseoli* LPR49 have the same sequence of glycosyl residues as that secreted by *R. trifolii* NA30. The structure of this repeating unit is illustrated in Figs. 1 and 2. Furthermore, the polysaccharides secreted by *R. trifolii* LPR5, *R. phaseoli* LPR49, and *R. leguminosarum* LPR1 have now been shown to have the same structure, including the identities and locations of *O*-acyl groups. This finding is incompatible with the hypothesis that the acidic polysaccharides secreted by *Rhizobium* species are determinants of host specificity. In keeping with this conclusion, *R. trifolii* LPR5045, a non-nodulating *Rhizobium* strain prepared by removal of the Sym

plasmid from *R. trifolii* LPR5, secretes a polysaccharide whose structure is identical to that of the parent. Because the Sym plasmid has been shown to control the synthesis of the molecules necessary for host specificity[14], the failure of the acidic polysaccharide secreted by LPR5045 to lack any of the structural features of that secreted by the parent *Rhizobium* is strong confirmation that the acidic polysaccharide is not a determinant of host specificity.

The possibility remains that a quantitatively minor component of the acidic polysaccharides could be a host-specific determinant. For example, if a single repeating unit in 50 had an altered sequence of glycosyl residues, and if this specific sequence were to be recognized by host plants, the analytical procedures used in these studies would not detect the critical sequence. The analytical methods used were also insufficiently sensitive to detect a single *O*-acyl substituent occurring only once in every ten repeating units. Thus, the presence on the Sym plasmid of a gene encoding for an occasional, but precise, modification of the acidic polysaccharides, a modification that results in host-specific recognition of the polysaccharide, cannot be excluded by our experiments. If this were the case, the development of a sensitive bioassay to detect molecules that determine host specificity would be necessary. A bioassay that is at least semi-quantitative (*e.g.*, root-hair curling) could be used to detect, and purify, bioactive repeating-units of the acidic polysaccharides secreted by *Rhizobium* species were such repeating units the determinants of host specificity.

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REFERENCES

1 A. DELL, W. S. YORK, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 185–200.
2 D. P. S. VERMA AND S. LONG, *Int. Rev. Cytol., Suppl.*, 14 (1983) 211–245.
3 W. D. BAUER, *Annu. Rev. Plant Physiol.*, 32 (1981) 407–409.
4 J. M. VINCENT, in R. W. F. HARDY AND W. S. SILVER (Eds.), *A Treatise on Dinitrogen Fixation, Section III: Biology*, Wiley, New York, 1977, pp. 277–366.
5 T. L. GRAHAM, in A. ATHERLY AND K. GILES (Eds.), *Biology of the Rhizobiaceae*, Academic Press, New York, 1980, pp. 127–248.
6 B. ROBERTSEN, P. ÅMAN, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Plant Physiol.*, 67 (1981) 389–400.
7 P. ÅMAN, L.-E. FRANZÉN, J. E. DARVILL, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 103 (1982) 77–100.
8 W. F. DUDMAN, L.-E. FRANZÉN, J. E. DARVILL, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 141–156.
9 L.-E. FRANZÉN, W. F. DUDMAN, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 157–167.
10 W. F. DUDMAN, L.-E. FRANZÉN, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 168–184.
10a M.-S. KUO AND A. J. MORT, *Carbohydr. Res.*, 145 (1986) 247–265..

11 R. I. HOLLINGSWORTH, M. ABE, F. B. DAZZO, AND K. HALLENGA, *Carbohydr. Res.*, 134 (1984) c7–c11.
12 K. JAHN AND B. JANN, in W. SUTHERLAND (Ed.), *Surface Carbohydrates of the Prokaryotic Cell*, Academic Press, London, 1977, pp. 247–287.
13 D. A. CHERESH, A. P. VARKI, N. M. VARKI, W. B. STALLCUP, J. LEVINE, AND R. A. REISFELD, *J Biol. Chem.*, 259 (1984) 7453–7459.
14 P. J. HOOYKAAS, F. G. M. SNIJDEWINT, AND R. A. SCHILPEROORT, *Plasmid*, 8 (1982) 73–82.
15 J. M. VINCENT, *Nitrogen Fixation in Legumes*, Academic Press, New York, 1982
16 A. H. LINDBERG, in ref. 12, pp. 289–356.
17 A. R. WHITE, A. G DARVILL, W S YORK, AND P. ALBERSHEIM, *J. Chromatogr.*, 298 (1984) 525–530.
18 Z. DISCHE, *Methods Carbohydr. Chem.*, 1 (1962) 477–512.
19 B. S. VALENT, A. G. DARVILL, M MCNEIL, B. K. ROBERTSEN, AND P. ALBERSHEIM, *Carbohydr. Res.*, 78 (1980) 165–192.
20 M. W. SPELLMAN, M. MCNEIL, A. G DARVILL, P. ALBERSHEIM, AND A DELL, *Carbohydr. Res.*, 122 (1983) 131–153.
21 M. W. SPELLMAN, M. MCNEIL, A. G. DARVILL, P. ALBERSHEIM, AND K. HENRICK, *Carbohydr. Res.*, 122 (1983) 115–129.
22 A. AMEMURA, T. HARADA, M. ABE, AND S. HIGASHI, *Carbohydr. Res.*, 115 (1983) 165–174.
23 G. G. S. DUTTON, J. L. DI FABIO, D M. LEEK, E. H. MERRIFIELD, J. R. NUNN, AND A. M. STEPHEN, *Carbohydr. Res.*, 97 (1981) 127–138.
24 R. I. HOLLINGSWORTH, M. ABE, J. E. SHERWOOD, AND F. B. DAZZO, *J. Bacteriol.*, 160 (1984) 510–516.
25 Y. M. BARNET AND B. HUMPHREY, *Can. J. Microbiol.*, 21 (1975) 1647–1650.
26 S. HIGASHI AND M. ABE, *J. Gen. Appl. Microbiol.*, 24 (1978) 143–153.
27 J. K. SHARP, M. MCNEIL, AND P. ALBERSHEIM, *J. Biol. Chem.*, 259 (1984) 11,321–11,336
28 A. S. PERLIN, D. M. MACKIE, AND C. P. DIETRICH, *Carbohydr. Res.*, 18 (1971) 185–194.
29 N. OTOTANI AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 76 (1974) 545–551.
30 A. VAN DER KAADEN, J. I. M. VAN DOORN-VAN WAKEREN, J. P. KAMERLING, J. F. G. VLIEGENTHART, AND R. H. TIESJEMA, *Eur. J. Biochem.*, 141 (1984) 513–519.
31 J. HAVERKAMP, H. VAN HALBEEK, L. DORLAND, J. F. G. VLIEGENTHART, R PFEIL, AND R. SCHAUER, *Eur. J. Biochem.*, 122 (1982) 305–311.
32 A. DELL, unpublished results.
33 M. MCNEIL, M. QUIST, AND J. DARVILL, unpublished results.