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Unusual structure of the exopolysaccharide of *Rhizobium leguminosarum* bv. *viciae* strain 248

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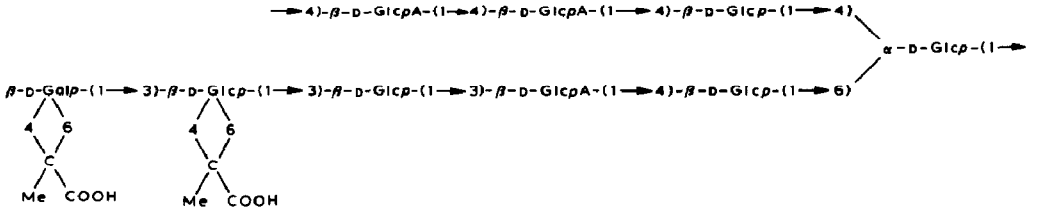
ABSTRACT

The exopolysaccharide from *R. leguminosarum* bv. *viciae* strain 248 differs from those of other *Rhizobium* strains with similar symbiotic behavior. ^{13}C-N.m.r. spectroscopy of fragments generated by partial hydrolysis, together with methylation analysis and ^{13}C-n.m.r. spectroscopy of the enzymically depolymerised exopolysaccharide, indicated the following nonasaccharide repeating-unit:



The locations of the acetyl and 3-hydroxybutanoyl substituents in the exopolysaccharide are assigned provisionally. *R. leguminosarum* bv. *viciae* strain 248, cured of its Sym plasmid pRL1JI, synthesised an exopolysaccharide in which the sites and degree of substitution were unchanged. A Tn5 mutant, derived from strain 248 and unable to induce nodules, synthesised small amounts of EPS that lacked galactose.

INTRODUCTION

The isolation of *Rhizobium* mutants that are defective in the synthesis of exopolysaccharide (EPS) and nodulation[1] indicates a role for the EPS in nodulation. The requirement of EPS, however, seems to be dependent on the cross-inoculation group. Borthakur *et al.*[2] described strains of *R. leguminosarum* bv. *phaseoli* which failed to synthesise EPS but still induced the formation of nitrogen-fixing nodules. On the other

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hand, the bv. *viciae* and bv. *trifolii* mutants in which the synthesis of EPS was affected were defective in nodulation on their respective host plants[3,4]. Djordjevic *et al.*[5] showed that an Exo$^-$ mutant of bv. *trifolii*, which induced non-nitrogen fixing nodules, was complemented by the addition of purified EPS.

The EPS from many *R. leguminosarum* biovar strains have octasaccharide repeating-units with pyruvate, 3-hydroxybutanoyl, and acetyl substituents[6–10]. Other types of EPS have been isolated from several bv. *phaseoli* strains[8,11–14] and one bv. *trifolii* strain[15].

Genetically, one of the best characterised strains is *R. leguminosarum* bv. *viciae* strain 248, which harbors the pRL1JI Sym plasmid[16] that is used commonly for the analysis of the function and localisation of *nod* genes. The structure of the EPS from strain 248 has not been characterised hitherto.

Many experiments have been performed in which *nod* genes were transferred between members of the *R. leguminosarum* family, including strain 248. In view of the probable involvement of the EPS in the nodulation assays, the structure of the EPS of strain 248 has been determined, and the influence of *nod* genes on its structure investigated.

EXPERIMENTAL

Growth of bacteria. — *R. leguminosarum* bv. *viciae* strain 248 (ref. 17) which harbours the Sym plasmid pRL1JI described by Johnston *et al.*[16], nodulates pea and vetch plants. Strain RBL1387 was obtained by curing strain 248 of its pRL1JI Sym plasmid[18]. Sym-plasmid-free strain RBL5515 is a derivative of *R. leguminosarum* bv. *trifolii* strain LPR5 described by McNeil *et al.*[8]. Unless otherwise indicated, the bacteria were grown in standard media, supplemented with antibiotics as described[19]. For the isolation of exopolysaccharides (EPS), bacteria were grown at 28° on a rotary shaker for 6 days in 2-L Erlenmeyer flasks each containing 500 mL of B$^-$ minimal medium[20]. The flasks were inoculated from a starter culture in B$^-$ minimal medium to an A_{620} value of 0.05.

Tn5 mutagenesis. — Tn5 mutagenesis and bacterial matings were performed on membrane filters (Sartorius) placed on a solid TY medium as described[21]. Only Exo$^-$ mutants that were not auxotrophic and had no apparent defects in their Sym plasmid were used.

Isolation of EPS. — After pelleting the bacterial cells, the EPS was isolated from the culture supernatant solution by using a hollow-fibre filtration procedure[22]. After freeze-drying, the crude EPS was dissolved in water and precipitated by adding 2 vol. of aqueous 96% ethanol[14]. The precipitate was collected by centrifugation for 10 min at 10 000 r.p.m., then dissolved in water, and the solution was freeze-dried.

Depolymerisation of the EPS. — The EPS (150 mg) was incubated for 6 days at 28° in phosphate buffer (pH 7.0) that contained mM $MgCl_2$ and $\sim 10^{10}$ plaque-forming units of phage RL38 (ref. 23) or phage 4S (ref. 24); the phages had the same enzymic activity. Repeating units from the EPS were isolated by precipitation with ethanol from 50% to 90%; the repeating units were isolated from the highest fraction. The oligosaccharides

were then purified by using[22] columns of Sephadex DEAE A25, Biogel P2, and Dowex 50W-X2.

The EPS was also hydrolysed partially by incubating several 100-mg batches in M trifluoroacetic acid at 95° for periods of up to 1.5 h. The time of hydrolysis was varied in order to optimise the yield of particular fragments. After freeze-drying, the batches were combined and eluted from Sephadex DEAE A25, followed by further purification as described above. The size of the fragments was estimated from their elution volumes from the column of Biogel P2 that was calibrated[22] with D-glucose, maltose, raffinose, and other model compounds.

Component and methylation analysis. — T.l.c. of the hydrolysate of the EPS was performed on Kieselgel 60 (Merck) with 1-butanol–pyridine–acetic acid–water (25:25:5:15) and detection by charring with H_2SO_4. Hexose was determined by the phenol–sulphuric acid method[25], and uronic acid by the carbazole assay[26]; pyruvate was estimated as described by Katsuki *et al.*[27]. The ratio of glucose to galactose was based on component analysis[28]. The molar ratios of sugars in hydrolysates were determined as described[14] and related to glucose (5 residues). Methylation analysis was performed as described[29], and uronic acid constituents were included by *O*-methylation and carboxyl reduction[14]. Partially methylated alditol acetates, obtained after g.l.c. on a Sil 43 CB column at 210°, were quantitated by making use of the effective carbon response factors[30], and identified by mass spectrometry using a VG Micromass 70-70-F system with a type DB-225 capillary column (30 m × 0.25 mm; J&W Scientific), resolution 1000, and an ionisation energy of 70eV at 200°.

N.m.r. spectroscopy. — Samples were dissolved in D_2O (99%) and spectra (^1H, 200 MHz; ^{13}C, 50.3 MHz) were recorded on a Varian XL-200 spectrometer at 21°. Chemical shifts were measured relative to that of internal trimethylpropanesulfonate for ^1H and to that of methanol (50.04 p.p.m.) for ^{13}C. For ^1H-n.m.r. spectroscopy, the samples were first exchanged against D_2O.

RESULTS

Isolation and nodulation abilities of R. leguminosarum *bv.* viciae *EPS mutants.* — In order to obtain mutants of *R. leguminosarum* bv. *viciae* strain 248 defective in the synthesis of EPS, mutagenesis was effected with transposon Tn5. The ~ 6000 independent mutants obtained were screened for differences in colony morphology after growth on YMB plates. The colony morphology of two mutants, namely, strains 248,*exo1*::Tn5 and 248,*exo9*::Tn5, indicated a significant decrease in the synthesis of EPS and they were characterised further.

When inoculated on *Vicia sativa*, *V. hirsuta*, and *Pisum sativum* plants, *R. leguminosarum* bv. *viciae* strain 248 induced nitrogen-fixing nodules. Mutant strains 248,*exo1*::Tn5 and 248,*exo9*::Tn5 were unable to induce nodules on these plants, but they induced root-hair deformations and abortive-infection threads in *V. sativa*.

Strain 248 and its Sym-plasmid-free derivative, strain RBL1387, each synthesised ~1.0 g of EPS per g of protein, strain 248,*exo1*::Tn5 produced only ~10% of this

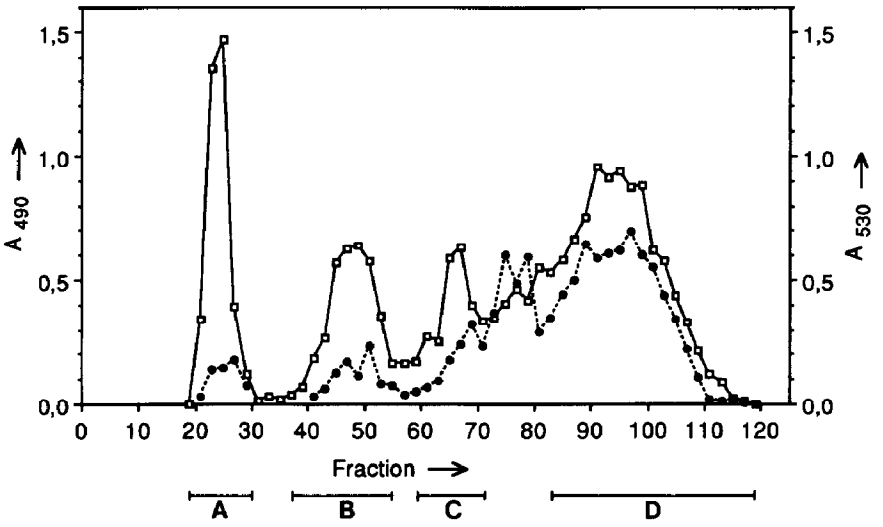

Fig. 1. Elution profile from a column of Sephadex DEAE A-25 of partially hydrolysed EPS isolated from *R. leguminosarum* bv. *viciae* strain 248: □, hexose content in 40-μL samples at A_{490}; ●, uronic acid content in 40-μL samples at A_{530}.

TABLE I

Fragments isolated by partial acid hydrolysis of EPS from *R. leguminosarum* bv. *viciae* strain 248

No.	*Fragment*
I	β-Glc*p*A-(1→4)-β-Glc*p*A-(1→4)-Glc*p*
II	β-Glc*p*A-(1→4)-β-Glc*p*A-(1→4)-β-Glc*p*-(1→4)-Glc*p*- 6 ↑ 1 β-Glc*p*A-(→4)-β-Glc*p*
III	β-Glc*p*A-(1→4)-β-Glc*p*-(1→6)-Glc*p*
IV	β-Glc*p*A-(1→4)-Glc*p*
V	β-Gal*p*-(1→3)-β-Glc*p*-(1→3)-Glc*p*
VI	R-Pyr⟨4,6⟩β-Gal*p*-(1→3)-β-Glc*p*-(1→3)-Glc*p*
VII	β-Glc*p*-(1→3)-Glc*p*

amount, and strain 248,*exo9*::Tn5 synthesised less than the detection limit of 0.003 g EPS per g of protein.

The sugar composition of the EPS of the *R. leguminosarum* bv. *viciae* strains was verified after isolation and purification. Composition analysis by t.l.c. revealed glucose, galactose, and glucuronic acid in the EPS of strains 248 and RBL1387, whereas, in the

EPS of strain 248,*exo1*::Tn5, galactose was absent. Colorimetric assays and g.l.c. of the alditol acetates of the components of the EPS indicated molar ratios for glucose, galactose, glucuronic acid, and pyruvate of 5.0:1.2:3.0:2.1 for the EPS from strain 248, and 5.0:0.0:2.8:1.1 for the EPS from strain 248,*exo1*::Tn5. The molar ratios for the parent strain were not the same as those (5:1:2:2) reported for the EPS of other *R. leguminosarum* strains[6-8,10]. For a representative of these *R. leguminosarum* strains, strain RBL5515, molar ratios of 5.0:1.0:2.2:2.1 were found. Because of this difference in sugar composition and because strain 248,*exo1*::Tn5 synthesised EPS that lacked galactose, the structures of the EPS of *R. leguminosarum* bv. *viciae* strain 248 and its derivatives were investigated.

Structure of the EPS synthesised by strains 248, RBL1387, and 248,exo1::Tn5. — Fragments obtained by partial hydrolysis of EPS of strain 248 were fractionated by ion-exchange chromatography into neutral, and singly and doubly charged species (Fig. 1, peaks A–C, respectively), plus an unresolved mixture of species with more than two acidic residues (peak D). Size-exclusion chromatography resolved these fractions according to the number of sugar residues. Most of the fractions, which ranged in size from mono-to hexa-saccharide, were shown by ^{13}C-n.m.r. spectroscopy to each contain a single molecular species. The molecular sizes deduced from retention volumes and by n.m.r. spectroscopy (the relative areas of the H-1 signals for the reducing and non-reducing residues) agreed. The structures of seven fragments were determined by n.m.r. spectroscopy (Table I). The assignments of the ^{13}C resonances are listed in Table II.

The spectra of fragments V and VII were assigned by comparison with the chemical shift data reported for similar oligosaccharides[32]. Each fragment contained a (1→3) linkage between glucose residues, which was not present in the EPS from strain LPR5[8]. The C-3 resonances at ~85 p.p.m. that are characteristic of this linkage were absent from the spectrum of the octasaccharides obtained from strain RBL5515 (Fig. 2), which accords with the expectation that the EPS of strain RBL5515 is similar to that of the strain (LPR5[8]) from which it was derived.

The spectrum of the acidic fragment I was assigned by combining reasonable substitution shifts[32] with published chemical shifts for glucuronic acid[33]. A fragment identical to I was obtained by hydrolysis of the EPS from strain ANU843, which has the same linkages as the EPS from strain LPR5[7,8,10].

The ^{13}C-n.m.r. spectra of fragments III and IV were assigned by comparison with that of fragment I and the previously reported[32] spectrum of β-Glc-(1→6)-Glc, which permitted assignment of the spectrum for fragment II by comparison with the data for fragments I and III.

These results support the structure shown in Fig. 3 for the repeating unit of the EPS of *R. leguminosarum* bv. *viciae* strain 248.

Enzymic depolymerisation of the EPS. — The EPS of strains 248, RBL1387, and 248,*exo1*::Tn5 were depolymerised enzymically. After purification of the products by column chromatography and de-esterification in the n.m.r. tube by the addition of 100mM NaOD in D_2O, the ^{13}C-n.m.r. spectra of the oligosaccharides of strains 248 and RBL1387 (Fig. 2A) accorded with the structure in Fig. 3, as summarised in Table III,

TABLE II

^{13}C-Chemical shifts[a] (p.p.m.) for oligosaccharides I–VII (see Table I) produced by partial acid hydrolysis of the EPS from *R. leguminosarum* strain 248

Residue[b]	*Carbon*	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*
a	1	103.5*	103.4*					
	2	73.7*	73.6*					
	3	76.4	76.1					
	4	72.7	72.3					
	5	76.9[d]	-					
	6	175.8	173.5*					
b	1	103.2*	103.3*					
	2	74.2*	74.0*					
	3	75.3	75.4					
	4	82.0	80.9					
	5	76.9[d]	-					
	6	176.4	173.1*					
c	1	92.9, 96.9	103.4*					
	2	72.3*, 75.0	73.8					
	3	72.4*, 75.3	74.8					
	4	79.9, 79.8	79.6					
	5	71.2, 75.9	75.7					
	6	61.0, 61.2	61.0					
d	1		93.0, 97.0	93.2, 97.1				
	2		72.3, 74.6*	72.5, 75.1				
	3		72.3, 75.0*	73.7, 76.7				
	4		79.1, 79.1	70.5, 70.5				
	5		70.0, -	71.5, 75.4				
	6		68.6, 68.6	69.8, 69.9				
e	1		103.4*	103.6	92.8, 96.7			
	2		73.8	73.9	72.3*, 74.9			
	3		74.8	75.2	72.4*, 75.4			
	4		79.9	79.9	79.9, 79.9			

	5	75.7	75.8	71.2, 75.8			
	6	61.0	61.0	61.0, 61.1			
f	1	103.3*	103.5	103.3			
	2	73.8	73.8	74.0			
	3	76.1	76.1	76.4			
	4	72.3	72.3	72.8			
	5	-	-	76.9			
	6	173.5*	173.6	?			
g	1				93.2, 96.8	93.1, 96.8	93.2, 96.9
	2				72.0, 74.9	71.9, 74.9	72.2, 75.0
	3				83.6, 85.8	83.3, 85.5	83.4, 85.7
	4				69.2, 69.2	69.2, 69.2	69.3, 69.3
	5				72.3, 76.7	72.2, 76.7	72.4, 76.7
	6				61.7, 61.9	61.7, 61.8	61.7, 61.9
h	1				104.1,104.0	103.7,103.6	103.9,103.8
	2				74.6	74.3	74.5
	3				85.3	85.4	77.2
	4				69.3	69.3	70.7
	5				76.7	76.7	76.7
	6				61.9	61.8	61.9
	pyr[c] CH_3						
	pyr C						
	pyr CO						
i	1				104.4	104.1	
	2				72.4	72.6	
	3				73.7	67.3	
	4				69.7	72.1	
	5				76.5	71.9	
	6				62.2	66.2	
	pyr[c] CH_3					26.3	
	pyr C					102.0	
	pyr CO					177.1	

[a] The resonances marked * are also given by other fragments or residues. [b] See Fig. 3. [c] Pyruvate group. [d] These resonances were broader than the other resonances.

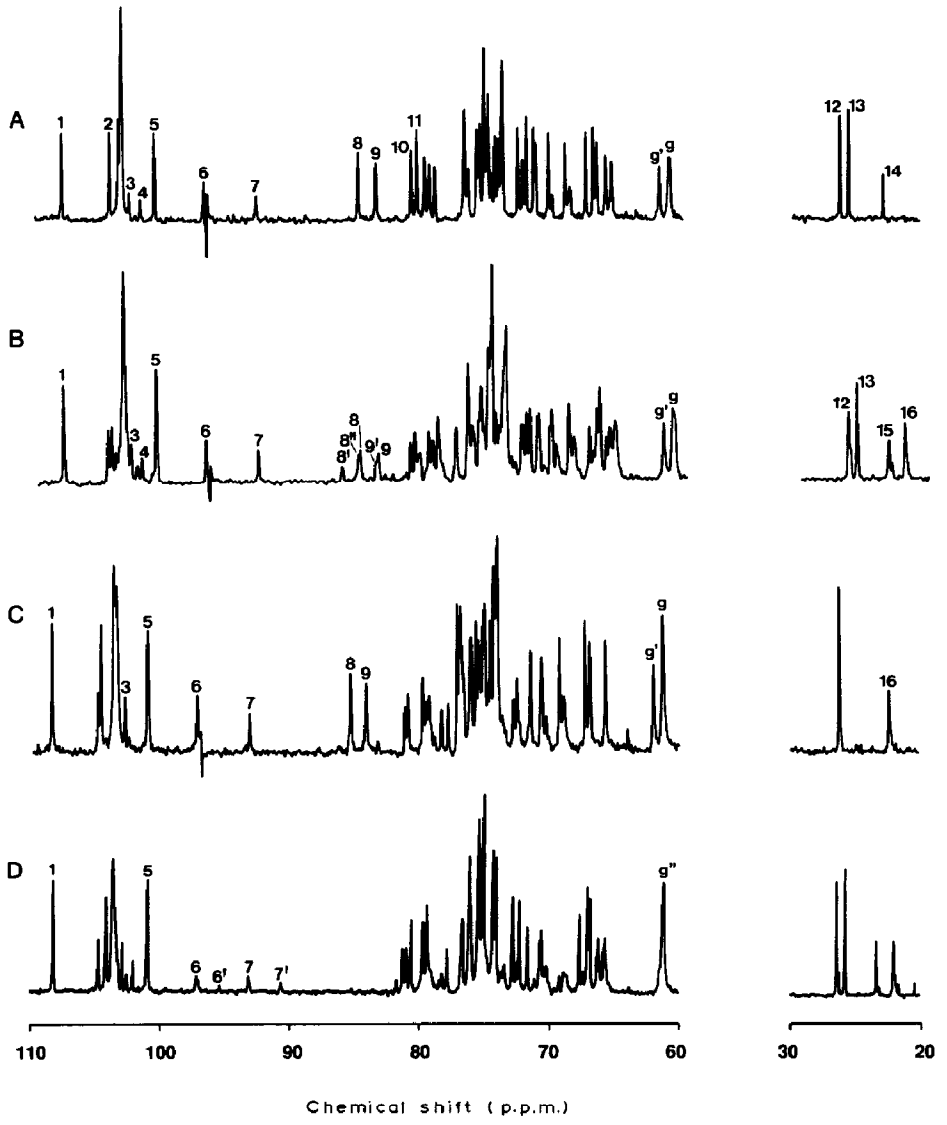

Fig. 2. ^{13}C-N.m.r. spectra of the enzymically depolymerised EPS from *R. leguminosarum* bv. *viciae* strains: A, strain 248, de-esterified; B, strain 248 (identical to the spectrum of the EPS of strain RBL1387); C, strain 248,*exo1*::Tn5; D, strain RBL5515; 1, C-4 of 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid (residue *a*); 2, C-1 of carboxyethylidene-glucose residue *h*; 3, C-1 of the carboxyethylidene group on residue *h*; 4, C-1 of the carboxyethylidene group on galactose residue *i*; 5, C-1 of residue *a*; 6, C-1 of β-glucose residue *d* in spectra A–C; spectrum D: 6, C-1 of residue *d* and 3-*O*-acetyl-β-glucose; 6′, C-1 of 2-*O*-acetyl-β-glucose residue *d*; 7, C-1 of α-glucose residue *d* in spectra A–C; spectrum D: 7, C-1 of α-glucose residue *d* and 3-*O*-acetyl-α-glucose; 7′, C-1 of 2-*O*-acetyl-α-glucose residue *a*; 8, C-3 of glucose residue *g*; 8′ and 8″, C-3 of glucose residue *g* shifted as influenced by the 3-hydroxybutanoyl and acetyl substituents; 9, C-3 of glucuronic acid residue *f*; 9′, C-3 of glucuronic acid residue *f* as influenced by the 3-hydroxybutanoyl and acetyl substituents; 10, C-4 of glucuronic acid residue *b*; 11, C-3 of glucose residue *h*; 12, methyl resonance of the carboxyethylidene group on galactose residue *i*; 13, methyl resonance of the carboxyethylidene group on glucose residue *h*; 14, de-esterified 3-hydroxybutanoyl methyl resonance; 15, methyl resonance of esterified 3-hydroxybutanoyl; 16, acetyl methyl resonance; g, C-6 of glucose residue *c* and *e*; g′, C-6 of glucose residue *g*; g″ in spectrum D, C-6 of glucose residues *c*, *e*, and *f*.

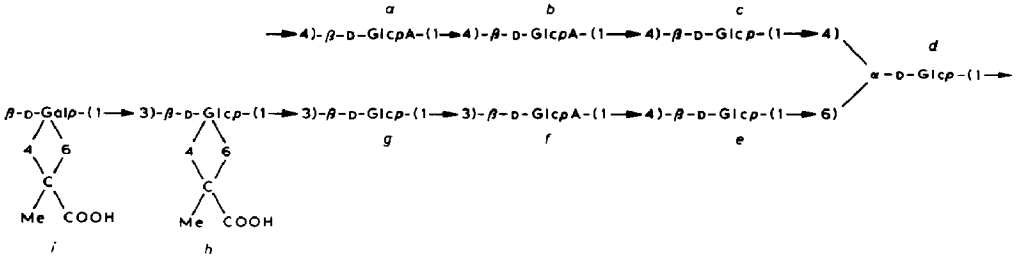

Fig. 3. Structure of the repeating unit of the EPS synthesised by *R. leguminosarum* bv. *viciae* strain 248.

TABLE III

Chemical shifts (p.p.m.) for ^{13}C resonances in the n.m.r. spectrum of the de-esterified nonasaccharide repeating-unit from EPS produced by *R. leguminosarum* bv. *viciae* strain 248

Carbon	*Sugar residue*[a]									
	a	b	c	d(α)	d(β)	e	f	g	h	i
1	100.8	103.3	103.3	93.0	97.0	103.3	103.3	103.3	104.3	103.6
2	70.3	74.9	73.9	72.3	74.6	73.9	74.2	74.4	74.1	72.7
3	66.6	75.2	75.2	72.3	75.0	74.9	83.7	85.1	80.5	66.8
4	107.9	81.0	79.5	79.1	79.1	79.9	71.3	69.0	75.6	72.0
5	145.6	76.5	75.8	70.0	—	75.8	76.8	76.8	67.4	71.5
6	170.4	176.2	61.0	68.7	68.7	61.0	176.4	61.7	65.5	65.9
pyr[b] CH_3									25.5	26.2
pyr C									102.8	102.1
pyr CO									176.4	177.3

[a] See Fig. 3. [b] Pyruvate group.

except that residue *a* had been converted into 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid.

The same spectrum was obtained for the de-esterified repeating unit isolated from the EPS of strain RBL1387. The chemical shifts of the ^{13}C resonances in the 4,6-*O*-(1-carboxyethylidene)galactose residue of the nonasaccharide and fragment VI were similar to those for 4,6-*O*-(1-carboxyethylidene)galactose[22], except that the presence of the glycosidic bond produced a larger than expected upfield shift (1.4 p.p.m.) of the C-5 resonance. The C-5 and C-3 resonances were affected[22] by the 1-carboxyethylidene group (Table IV), presumably because of changes in conformation. Verification that the assigned peaks were correctly assigned as galactose resonances was provided by their absence from the ^{13}C-n.m.r. spectrum of the repeating unit from the EPS of strain 248,*exo1*::Tn5 (Fig. 2C).

The presence of 4,6-*O*-(1-carboxyethylidene)-β-D-glucose (residue *h*, Fig. 3) was verified by comparing the spectra of the oligosaccharide with that for the octasaccharide isolated from the EPS of strain RBL5515 (Fig. 2D). Strain RBL5515 is closely related to *R. leguminosarum* bv. *trifolii* strain LPR5, which contains 4,6-*O*-(1-carboxyethylidene)-

TABLE IV

^{13}C-Chemical shifts (p.p.m.) for the 4,6-*O*-(1-carboxyethylidene)galactose residue *i* in the nonasaccharide (*A*) produced by de-esterification of the depolymerised EPS from *R. leguminosarum* bv. *viciae* strain 248 compared with those for 4,6-*O*-(1-carboxyethylidene)galactose (*B*)

Position	A	B[a]
1	103.6	97.2 [−0.1]
2	72.5	72.6 [−0.3]
3	66.7	67.2 [−6.6]
4	71.9	72.2 [+2.5]
5	71.4	72.8 [−3.2]
6	65.8	66.2 [+4.2]

[a] The figures in parentheses are the differences in chemical shift (p.p.m.) compared to the corresponding resonances for β-galactose[32].

β-D-glucose[8]. The linkage between galactose and the residue *h* was determined from the spectrum of fragment VI.

Substitution shifts produced by the carboxyethylidene group were deduced by comparing the spectra of the oligosaccharide and fragment VI. The shifts for the resonances of C-2, C-3, C-4, C-5, and C-6 were 0.2, −4.9, +6.3, −9.3, and +3.6 p.p.m., respectively. Although there is a broad similarity with the pattern of shifts observed for the β-D-galactose residue, the magnitudes of the shifts of the resonances for C-3, C-4, and C-5 are different. The cause of the large substitution shifts is likely to be due to a change in conformation. Interchange of any of the proposed assignments would lead to a less acceptable pattern of substitution shifts.

The resonances for H-1/4 in residue *a* were assigned by proton homonuclear decoupling and those of C-1/4 were then located by heteronuclear correlation spectroscopy. The chemical shifts of the resonances for C-5 and C-6 were distinctive. Resonances for all other residues were assigned by comparison with data for the hydrolysis fragments.

The linkages in the EPS of strain 248 were identified by methylation analysis (g.l.c.–m.s. of the partially methylated alditols; Table V). These results were in agreement with the structure proposed in Fig. 3.

Location of the ester substituents. — The nonasaccharide repeating-units, purified from the enzymically depolymerised EPS from strains 248 and RBL1387, contained both acetyl and 3-hydroxybutanoyl substituents. There were four major resonances for OAc in the ^{1}H-n.m.r. spectrum of the esterified nonasaccharide (Fig. 4A). Two had chemical shifts similar to those assigned to AcO-2 and AcO-3 on residue *b* in octasaccharides from strain ANU843[7,10], whereas those of the others differed from reported values[7,10]. The presence of AcO-3 on residue *b* was confirmed by the presence[7] of the characteristic triplet at 5.15 p.p.m. for H-3 of residue *b* and the shift of the resonance for C-4 (Table VIA). The two new sites for acetyl groups cannot be assigned with certainty. Because the C-3 resonances for residues *f* and *g* are affected, the substituents must be

TABLE V

Methylation analysis data for the EPS from *R. leguminosarum* bv. *viciae* strain 248 (See Experimental)

Alditol	T[a]	*Strain*			
		RBL5515[b]	*248*[b]	*248*[c]	*248*,exo1::*Tn5*[b]
2,4,6-Me_3-Glc[d]	1.38	0	20	10	25
3,4,6-Me_3-Glc[d,e]	1.38	0	4	3	1
2,3,6-Me_3-Glc	1.56	50	22	19	28
2,4-Me_2-Glc	2.36	0	0	11	0
2,3-Me_2-Glc	2.42	15	18	33	46
2,3-Me_2-Gal	2.52	16	15	11	0
2-Me-Glc	3.22	19	21	13	0

[a] Retention time relative to that of 2,3,4,6-Me_4-Glc (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol). [b] Samples were methylated, then hydrolysed, and the partially methylated sugars were converted into alditol acetates. [c] The methylated EPS was carboxyl-reduced ($NaBH_4$). Increased proportions of 2,3-Me_2-Glc and 2,4-Me_2-Glc are derived from (1→4)- and (1→3)-linked glucosyluronic acid residues, respectively. [d] These compounds could not be separated by g.l.c. at 210°, but were resolved by applying a temperature program (140–240°, 2°/min). [e] Traces of 3,4,6-Me_3-Glc stem from a (1→2)-β-D-glucan impurity in the EPS.

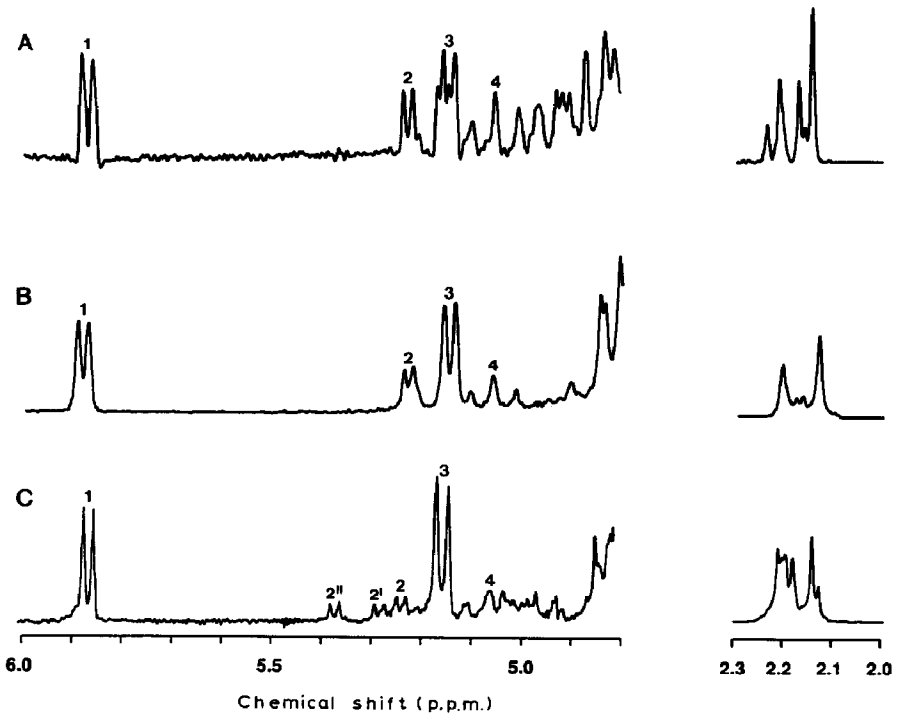

Fig. 4. ^{1}H-N.m.r. spectra of the enzymically depolymerised EPS from *R. leguminosarum* bv. *viciae* strains: A, strain 248 (the spectrum of the EPS from strain RBL1387 was identical); B, strain 248,*exo1*::Tn5; C, strain RBL5515; 1, H-4 of 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; 2, H-1 of α-glucose residue *d*; 2′ and 2″, H-1 of 2- and 3-*O*-acetyl-α-glucose (residue *d*); 3, H-1 of 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; 4, H-3 of 3-*O*-acetyl-β-glucuronic acid residue *b*.

TABLE VI

Esterification effects[a] in the ^{1}H- and ^{13}C-n.m.r. spectra of nonasaccharides obtained from enzymically cleaved EPS isolated from *R. leguminosarum* bv. *viciae* strains 248 and RBL1387

A. Effects of AcO-3 on residue b

Residue	*Position*	^{13}*C Chemical shift*	^{13}*C Substitution shift*	^{1}*H Chemical shift*
a	2	70.5	+0.2	3.92
b	1	104.6	+1.3	4.54
	2	72.8[b]	−2.1	3.60
	3	76.5	+1.3	5.15
	4	81.4	+0.4	3.88
	5	78.1	+1.6	3.91
	6	175.9	−0.3	—
c	4	77.7	−1.8	3.78

B. Effects of AcO-2 on residue b

Residue	*Position*	^{13}*C Chemical shift*	^{13}*C Substitution shift*	^{1}*H Chemical shift*
b	1	100.8[c]	−2.5	4.90
b	2	73.5	−1.4	
b	3	73.2	−2.0	

C. Effects of the 3-hydroxybutanoyl group on residue i

Residue	*Position*	*3-O-Hydroxybutanoyl*		*2-O-Hydroxybutanoyl*	
		^{13}*C Chemical shift*	*Shift on de-esterification*	^{13}*C Chemical shift*	*Shift on de-esterification*
i	1	102.4	−1.20	—	—
i	3			67.2	+0.4
i	5	70.9	−0.6	—	—
pyr C		101.9	−0.2		
pyr CH_3		26.2	−0.2		

D. Effects of the 2-O-(3-hydroxybutanoyl) group on residue h

Residue	*Position*	^{13}*C Chemical shift*	*Shift on de-esterification*
g	3	86.4	+1.3
h	1	103.9	−0.4
h	3	80.7	+0.2
pyr C		102.9	+0.1

[a] Chemical shifts (p.p.m.) are recorded for ^{13}C resonances that disappeared on de-esterification, together with suggested assignments. [b] Observed only in material from the strain 248,*exo1*::Tn5, which has no galactose in the side chain. [c] Obscured by the A-1 peak in the normal spectrum. The different ^{1}H resonance frequency allows it to be seen in the ^{1}H–^{13}C heteronuclear correlation spectrum.

located on the side chain in the vicinity of these residues. There were five ^{13}C resonances in the region from 82–88 p.p.m. for the nonasaccharide, which disappeared on deacylation, but it was not possible to deduce which were due to substitution by acetyl or to 3-hydroxybutanoyl. Provisionally, the two new species are assigned as AcO-2 and AcO-4 on residue *f*, each of which should affect the C-3 resonance for residue *f*, and the peak at 85.1 p.p.m. does decrease in intensity when the acetyl groups are present. A new peak at 83.3 p.p.m. was ascribed to the C-3 resonance of residue *f* in the presence of AcO-4. The ^{1}H–^{13}C correlation spectrum of the acetylated oligosaccharide contained a ^{1}H peak at 4.99 p.p.m., which clearly represented a site of esterification. The corresponding ^{13}C resonance was 73.8 p.p.m. and the peaks were assigned to C-2/H-2 on residue *f*.

From the intensities of the AcO resonances, it was estimated that there were between 2–3 times more of one of its acetyl groups. Because de-esterification produced little change in the intensity of the peak assigned to C-4 of residue *f*, but significant change in the C-2 region, the more abundant species is believed to be AcO-2. Even if these assignments are reassessed, the shifts in the resonance for C-3 of glucuronic acid residue *f* indicate that acetylation occurs on or near that residue.

There were also four types of 3-hydroxybutanoyl substituents as indicated by their C-2 resonances at 44.0, 44.3, 44.9, and 45.0 p.p.m. Two are assigned to positions 2 and 3 on the galactose ring. The effects of 3-hydroxybutanoylation of galactose on the ^{1}H-n.m.r. spectrum of the EPS from strain LPR5035 has been reported[10]. The ^{1}H spectrum for the depolymerised EPS from the closely related strain RBL5515 was identical to that reported for LPR5035, and it is concluded that this strain also has a 3-hydroxybutanoyl group on the galactose residue. The ^{13}C-n.m.r. spectrum of the depolymerised EPS from strain RBL5515 showed all the shifts ascribed to substitution of the galactose residue (Table VIC). In particular, the resonance (102.1 p.p.m.) for pyruvate on residue *i* was split when about half the molecules had 3-hydroxybutanoyl substituents.

The corresponding resonance for the pyruvate on the glucose residue *h* at 102.8 p.p.m. (Table III) is not split in spectra from RBL5515 (Fig. 2D), but is split in the spectrum of the nonasaccharides from strain 248 (Fig. 2B). The most probable site for one of the remaining 3-hydroxybutanoyl groups, therefore, is position 2 of residue *h*. Consistent with this suggestion are the observations that the presence of ester causes (*a*) a decrease in the intensities of the C-1,3,5 resonances of residue *h* and the appearance of new peaks nearby (Table VID), (*b*) a shift of the C-3 resonance for residue *g* (Table VID), and (*c*) increased complexity (eight separate frequencies) of the pyruvate methyl proton resonance (Fig. 2B). The fourth resonance must be due to multiply subsituted residues with shifts caused by interaction, direct or indirect, of the substituents. Thus, the EPS of strain 248 has acetyl substituents on residues *b* and *f*, and 3-hydroxybutanoyl substituents on residues *h* and *i*.

For each of the proposed sites of substitution, there is at least one ^{13}C resonance that can be assigned unambiguously and the intensity of which is reduced markedly by esterification: C-4 of residue *b*, C-3 of residues *f* and *g*, and C-1 of both carboxyethyli-

dene groups. The locations of the acetyl groups on residue *b* and the 3-hydroxybutanoyl groups on residue *i* are strongly supported by similarities between the spectra of the EPS from strains 248 and RBL5515. Although the remaining substituents are probably located in the general regions indicated, the exact positions are less certain because of the difficulty in assigning the resonances in crowded regions of the n.m.r. spectra.

The ^{1}H- and ^{13}C-n.m.r. spectra of the EPS of strain 248 and its Sym-plasmid-free derivative, namely, strain RBL1387, were indistinguishable, which indicated that the genes of the Sym plasmid have no influence on the substituents present in the EPS.

Ester groups present in the EPS of mutant strain 248,exo1::Tn5. — The ^{1}H- and ^{13}C-n.m.r. spectra from the repeating unit isolated from the EPS of strain 248,*exo1*::Tn5 contained resonances ascribed to acetylation on residue *b*, but only traces of the peaks that indicated acetylation of residue *f* in the EPS of strain 248 (Figs. 2B,C and 4). Resonances for 3-hydroxybutanoyl groups on residues *h* and *i* (Fig. 3) were absent, as were the ^{13}C resonances for residue *i* and its carboxyethylidene group. Therefore, it is concluded that the repeating unit of the EPS synthesised by strain 248,*exo1*::Tn5 lacks the terminal galactose residue *i* and its substituents, lacks the 3-hydroxybutanoyl group attached to residue *h*, and also has many fewer acetyl groups on the side chain.

DISCUSSION

Structures of the EPS of R. leguminosarum bv. viciae *strains 248 and RBL1387.* — The structure proposed for the EPS of strains 248 and RBL1387 differs from that of other *R. leguminosarum* strains like strain LPR5[8]. Thus, the EPS has ratios of glucose:glucuronic acid:galactose:pyruvate of ~5:3:1:2. Also, the fragments III and IV, obtained by partial hydrolysis of the EPS, contain a single glucuronic acid residue (Table I). Such fragments were not obtained from the EPS similar to that of strain LPR5, because the glucuronosidic linkages are more resistant to acid hydrolysis than those of glucose or galactose. The C-3 resonances of residues *g–i* of fragments V and VI (Tables I and II) were much further downfield than the corresponding resonances for (1→2) or (1→4) linkages. Methylation analysis demonstrated the presence of a (1→3)-linked glucose residue, which is not present in the EPS from strain LPR5[8].

The sugar sequence determined for the repeating units of the EPS isolated from strains 248 and RBL1387 has some features in common with those of *R. leguminosarum* bv. *viciae* strains 128C53 and 300 (refs. 9 and 10) and *R. leguminosarum* bv. *trifolii* strains LPR5[8], ANU843[7], 0403, and TA1[10]. These common features are the sugar backbone (residues *a–d*), the terminal galactose residue of the side chain together with its 3-hydroxybutanoyl and carboxyethylidene substituents, a glucose residue next to the terminal galactose residue that carries a carboxyethylidene group, and acetyl substituents at positions 2 and 3 of residue *b*. The structure differs from that of the other strains in that the repeating unit has an additional glucuronic acid residue in the side chain (Fig. 3, residue *f*), acetyl substituents are found in the side chain, presumably on residue *f*, no acetyl groups are found on residue *c* or *d*, and 3-hydroxybutanoyl substituents are present on, or near, residue *h*.

In comparison with the EPS of other *R. leguminosarum* bv. *viciae* strains, the EPS of strain 248 seems to carry more ester groups. It is not certain whether this feature is under direct genetic control or is due to variation in the metabolic activity of the bacterial cell, which could influence the amount of acetylcoenzyme A and hence the acetylation of EPS[34]. Similarly, 3-hydroxybutanoic acid is used as an electron donor when bacteria are grown under anaerobic conditions. Therefore, the level of 3-hydroxybutanoyl substitution of the EPS might vary according to growth conditions, as described[8] for other *R. leguminosarum* strains. The sugar sequence and substituents of the EPS of strain 248 seem to be determined by the chromosomal DNA, since they are not affected by the absence of the Sym plasmid.

*Synthesis and structure of the EPS from the Exo⁻ mutant strains 248,*exo1*::Tn5 and 248,*exo9*::Tn5.* — Strain 248,*exo9*::Tn5 synthesised no EPS, whereas strain 248,*exo1*::Tn5 synthesised small amounts of EPS that lacked the galactose residue *i*, the carboxyethylidene group attached to galactose residue *i*, and had no 3-hydroxybutanoyl substituents.

Influence of the structure of the EPS on nodulation. — Previous analyses of *Rhizobium* mutants affected in the synthesis of EPS[2,3,4,35] have indicated that the EPS is involved in nodulation, which can explain the behaviour of strain 248,*exo9*::Tn5 which produces no EPS and fails to nodulate. On the other hand, strain 248,*exo1*::Tn5 also fails to nodulate, which seems to indicate that either the EPS has to meet certain structural requirements or not enough EPS is synthesised.

If the structure of the EPS is a determinant in the nodulation process, it is possible that the inability of strain 248,*exo1*::Tn5 to nodulate is due to the absence of galactose and 3-hydroxybutanoyl groups from its EPS. *R. leguminosarum* bv. *trifolii* strain 4S, however, also synthesises EPS without a terminal galactose residue[15]. When the Sym plasmid pRL1JI was introduced into strain 4S, it acquired the ability to nodulate pea and vetch plants (data not shown), just as most other *R. leguminosarum* bv. *trifolii* strains do when plasmid pRL1JI is introduced. Hence, neither the terminal galactose residue, nor the carboxyethylidene group attached there, nor the hydroxybutanoyl substituent, is required for nodulation of pea or vetch plants.

Moreover, *R. leguminosarum* bv. *viciae* strain 248 and 300 (ref. 9) both nodulate pea and vetch plants, even though their EPS differ in the sites of acetylation and in the structures of the side chains. These differences apparently have no influence on nodulation.

Thus, for the nodulation strains 248, 300, and Rt4s, and for the non-nodulating mutant strain 248,*exo1*::Tn5, only the amount of EPS synthesised is different. Therefore, the possibility remains that the amount of EPS synthesised by mutant strain 248,*exo1*::Tn5, which is only $\sim 10\%$ of that produced by the wild type strain, is insufficient to induce nodules.

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