Determination of the chemical structure of the capsular polysaccharide of strain B33, a fast-growing soya bean-nodulating bacterium isolated from an arid region of China

Miguel A. RODRÍGUEZ-CARVAJAL*, Pilar TEJERO-MATEO*, José L. ESPARTERO†, José. E. RUIZ-SAINZ‡, Ana M. BUENDÍA-CLAVERÍA‡, Francisco Javier OLLERO‡, Su S. YANG§ and Antonio M. GIL-SERRANO*[1]

*Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, 41071-Sevilla, Spain, †Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad de Sevilla, 41071-Sevilla, Spain, ‡Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, 41071-Sevilla, Spain, and §Department of Microbiology, China Agricultural University, 2 Yuan Ming Yuan West Road, Beijing 100094, China

We have determined the structure of a polysaccharide from strain B33, a fast-growing bacterium that forms nitrogen-fixing nodules with Asiatic and American soya bean cultivars. On the basis of monosaccharide analysis, methylation analysis, one-dimensional ^{1}H- and ^{13}C-NMR and two-dimensional NMR experiments, the structure was shown to consist of a polymer having the repeating unit →6)-4-*O*-methyl-α-D-Glc*p*-(1→4)-3-*O*-methyl-β-D-Glc*p*A-(1→ (where Glc*p*A is glucopyranuronic acid and Glc*p* is glucopyranose). Strain B33 produces a K-antigen polysaccharide repeating unit that does not have the structural motif sugar-Kdx [where Kdx is 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) or a Kdo-related acid] proposed for different *Sinorhizobium fredii* strains, all of them being effective with Asiatic soya bean cultivars but unable to form nitrogen-fixing nodules with American soya bean cultivars. Instead, it resembles the K-antigen of *S. fredii* strain HH303 (rhamnose, galacturonic acid)$_n$, which is also effective with both groups of soya bean cultivars. Only the capsular polysaccharide from strains B33 and HH303 have monosaccharide components that are also present in the surface polysaccharide of *Bradyrhizobium elkanii* strains, which consists of a 4-*O*-methyl-D-glucurono-L-rhamnan.

Key words: chemical structure, K-antigen polysaccharide, soya bean rhizobia.

INTRODUCTION

The family Rhizobiaceae is comprised of seven genera of plant-associating bacteria: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, *Agrobacterium* and *Phyllobacterium*. The first five genera are able to form root-nodule symbioses with members of the plant family Leguminosae [1]. Nodules are unique plant organs in which the bacteria are differentiated into bacteroids and fix atmospheric nitrogen. The *Rhizobium*–plant interaction is host-specific and determined by exchange of chemical signals between the partners [2]. While the development of nodules is induced by bacterial Nod factors (lipo-chito-oligosaccharides), invasion of the plant cells by rhizobia and formation of nitrogen-fixing nodules requires additional signals such as bacterial surface components [3,4]. The role of bacterial exopolysaccharides, lipopolysaccharides (LPS) and/or capsular polysaccharides (KPS; also called K-antigens) in the invasion process, protecting the bacterium from plant-host defences, or in the mechanisms of cell–cell communication, is largely unknown. In the genus *Sinorhizobium*, the outer membrane and the surrounding capsule are composed mainly of LPS and a 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)-containing polysaccharide that is structurally analogous to the group-II K-antigen polysaccharides of *Escherichia coli*.

The structures of K-antigen polysaccharides from different *Sinorhizobium fredii* and *S. meliloti* strains have recently been reported [3,5,6]. Most of the *Sinorhizobium* strains analysed produce a K-antigen polysaccharide that presents a structural motif of a disaccharide repeating unit composed of a variable glycosyl residue bonded to Kdo or a Kdo-related acid [(R^1)-glycosyl-(R^2)Kdx] (where glycosyl is any monosaccharide, Kdx is a 1-carboxy-2-keto-3-deoxysugar, and R^1 and R^2 are acetyl or 3-hydroxybutanoyl substituents). In *S. fredii* strains this conserved structural motif only appears in strains that form nitrogen-fixing (Fix$^+$) nodules with Asiatic soya bean cultivars. In contrast, the only two *S. fredii* wild-type strains (HH103 and HH303) that form Fix$^+$ nodules on both groups of soya bean cultivars are the only two strains that produce a K-antigen repeating unit that does not have the proposed consensus glycosyl-Kdx sequence.

Here we show that a fast-growing soya bean-nodulating rhizobium (strain B33) isolated from the Xinjiang Autonomous Region in Western China produces a K-antigen polysaccharide repeating unit consisting of the disaccharide →6)-4-*O*-methyl-α-D-Glc*p*-(1→4)-3-*O*-methyl-β-D-Glc*p*A-(1→ (where Glc*p*A is glucopyranuronic acid and Glc*p* is glucopyranose). This strain, which does not produce a Kdo-containing polysaccharide, is also able to form nitrogen-fixing nodules on both Asiatic and American soya bean cultivars. The fast-growing soya bean-

Abbreviations used: FSR, fast-growing soya bean rhizobium; LPS, lipopolysaccharide; KPS, capsular polysaccharide; HSQC, ^{1}H-detection mode via single-quantum coherence; HMBC, heteronuclear multiple-bond correlation; NOESY, nuclear Overhauser effect spectroscopy; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; 1D, one-dimensional; 2D, two-dimensional; Kdx, 1-carboxy-2-keto-3-deoxysugar; Glc*p*A, glucopyranuronic acid; Glc*p*, glucopyranose.

[1] To whom correspondence should be addressed (e-mail agil@cica.es).

nodulating strain B33 most probably belongs to the species *S. fredii* or *Rhizobium xinjiangensis*. Because a detailed taxonomic study on strain B33 has not been carried out, we will use the acronym FSR (for fast-growing soya bean rhizobium) to designate strain B33.

EXPERIMENTAL

General methods

GLC-MS was performed with a Micromass AutoSpec-Q instrument fitted with an OV-1 column (25 m × 0.25 mm). The temperature programme for separating the trimethylsilylated methyl glycosides was isothermal at 150 °C for 2 min followed by a 10 °C/min gradient up to 250 °C, whereas that for the partially methylated alditol acetates was isothermal at 120 °C for 1 min followed by an 8 °C/min gradient up to 250 °C. The protocol for permethylated 2-butyl glycosides derivative was isothermal at 120 °C for 3 min followed by a gradient of 2 °C/min up to 150 °C and then 20 °C/min up to 250 °C. The ionization potential was 70 eV and the mass spectra were recorded using low-resolution electron impact.

Monosaccharides were identified on GLC-MS separation of their trimethylsilylated methyl glycosides obtained as described in [5]. The absolute configuration of monosaccharides was assigned following GLC-MS analysis of their permethylated (*S*)- and (*R*,*S*)-2-butyl glycosides, a modification of the method of Gerwig et al. [7]. The polysaccharide was hydrolysed with 2 M trifluoroacetic acid for 1 h at 120 °C and the released monosaccharides were methylated as described below. The permethylated monosaccharides were treated with HCl/(*S*)-2-butanol (0.625 M) and HCl/(*R*,*S*)-2-butanol (0.625 M).

Bacterial strains and isolation of the polysaccharide

FSR strain B33 was routinely grown at 28 °C in TY medium as described by Beringer [8]. Legume seeds were surface-sterilized and germinated as described by Pacios Bras et al. [9]. Nodulation tests were carried out on *Glycine soja*, *Cajanus cajan*, *Macroptilium atropurpureum*, *Macrotyloma axillare*, *Lotus japonicus*, *Indigofera tinctoria*, *Neonotonia wightii* and *Desmodium canadense* as described by Buendía-Clavería et al. [10]. Nitrogen fixation by nodules was assessed by acetylene-reduction assays [11].

TY liquid medium (10 l) was inoculated with 100 ml of early-stationary-phase cultures of B33 and incubated on an orbital shaker at 160 rev./min for 3 days at 28 °C. After incubation, the cells were harvested by low-speed centrifugation. The bacterial pellets were washed with 0.9 % (w/v) NaCl, freeze-dried and stored in sealed bottles at room temperature. The polysaccharide was extracted from the freeze-dried bacterial cells (5 g) with 1:1 hot phenol/water mixture (100 ml, 65 °C) [12] and the two phases were separated. The aqueous phase was dialysed against water, concentrated and passed through an IRA 400 Amberlite (AcO^-) anion-exchange resin column (30 cm × 1.6 cm) using water as eluent. The eluate was freeze-dried and redissolved in 10 mM $MgSO_4$ and 50 mM Tris/HCl solution (100 ml, pH 7.0); DNase (1 mg) and RNase A (1 mg) were added, and the solution was stirred overnight at 5 °C. Proteinase K (2 mg) was added, and the solution was shaken for 24 h at 37 °C, dialysed and then freeze-dried. The polysaccharide was chromatographed on Sephacryl S-500 (60 cm × 2.6 cm) using 0.05 M EDTA/triethylamine (pH 7.0) as eluent, and carbohydrates were detected using a refractive-index detector and the orcinol/sulphuric acid method on TLC plates. Fractions containing carbohydrates were dialysed and freeze-dried.

SDS/PAGE

Two different protocols were used to analyse the bacterial LPS and K-antigen polysaccharide profiles. SDS/PAGE of crude bacterial extracts was carried out as previously described [5,6]. Briefly, bacterial cultures of strain B33 were grown on solid TY medium. Bacterial cells were resuspended in 0.9 % NaCl and pelleted by centrifugation. The bacterial pellet was resuspended and lysed by heating at 100 °C in 125 μl of 60 mM Tris/HCl/2 % (w/v) SDS/1 mM EDTA (pH 6.8) for 5 min and then diluted to 1 ml with the same buffer without SDS. The crude bacterial extract was treated with RNase, DNase and proteinase K as described by Köplin et al. [13]. Electrophoresis of crude bacterial extracts or purified bacterial polysaccharide was performed on a 16.5 % (w/v) polyacrylamide gel with the tricine buffer system described by Lesse et al. [14]. For visualization of LPSs, gels were silver-stained as described by Kittelberger and Hilbink [15].

PAGE without detergent was also carried out to facilitate the visualization of K-antigens. Only the K-antigens (and not the LPS) of samples devoid of SDS can migrate into the gel. Samples of the purified K-antigen polysaccharide were analysed by vertical electrophoresis (Bio-Rad), as described for extracellular polysaccharides from *Bradyrhizobium* [16]. A continuous system was employed, using 0.75 mm-thick slab gels. The acrylamide concentration was 15 % (w/v), and the acrylamide/*N*,*N*′-methylenebisacrylamide ratio was 30:0.8. The electrophoresis buffer consisted of 50 mM Tris, 13 mM EDTA and 15 mM boric acid (pH 8.5). Samples were dissolved in the same buffer and diluted (1:2) in 1 M sucrose in deionized water. Gels were fixed using Alcian Blue in acetic acid [17] and stained by the silver method [15].

Methylation

The vacuum-desiccated sample of polysaccharide was methylated by the method of Ciucanu and Kerek [18], although using [^{2}H]iodomethane instead of iodomethane. The sample was purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge [19]. The permethylated polysaccharide was carboxyl-reduced by treatment with 2 mg of NaB^2H_4 dissolved in 500 μl of ethanol/water (75:25, v/v) at room temperature overnight [20]. Finally, the sample was hydrolysed, reduced and acetylated.

NMR spectroscopy

The samples were deuterium-exchanged several times by freeze-drying from ^2H_2O and then examined in solution (4 mg/ml) in 99.98 % ^2H_2O. Spectra were recorded at 303 K on a Bruker AMX500 spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). Chemical shifts are given in p.p.m., using the H^2HO signal (4.75 p.p.m.; ^{1}H) and external DMSO (39.5 p.p.m.; ^{13}C) as references. Selective-excitation one-dimensional (1D) experiments were carried out by application of the DANTE-Z pulse train ($n = 300$, $\tau = 100\ \mu$s, $\theta = 0.3°$) [21]. This train was also concatenated to a TOCSY sequence (isotropic mixing times of 141.1 and 56.4 ms, $\pi/2$ pulse width of 49 μs) [22] to yield the 1D-TOCSY subspectra. The number of accumulated scans was 512. The 2D homonuclear COSY was performed using the Bruker standard pulse sequence. The 2D heteronuclear one-bond proton-carbon correlation experiment was registered in the ^{1}H-detection mode via single-quantum coherence (HSQC). A data matrix of 256 × 1024 points was used to digitize a spectral width of 3000 and 26000 Hz in F_2 and F_1; 64 scans were used per increment with a delay between scans of 1 s and a delay corresponding to a J value of 150 Hz. ^{13}C decoupling was achieved by the GARP (globally optimized alternating-phase

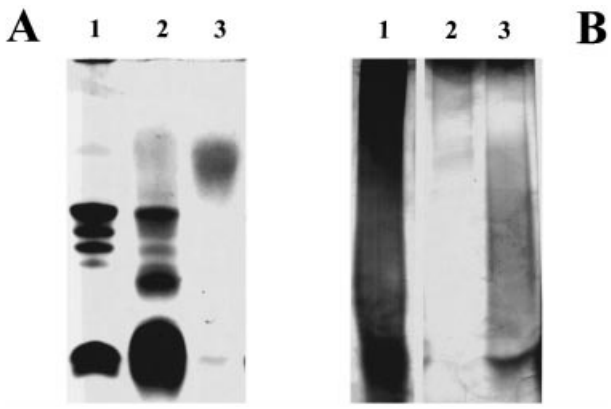

Figure 1 Silver-stained (A) and Alcian Blue/silver-stained (B) PAGE of whole-cell extracts of FSR strain B33 and fractions isolated by gel-permeation chromatography of the polysaccharide

(**A**) Lane 1, silver-stained SDS/PAGE of cell-wall extracts of FSR strain B33; lane 2, fraction F_1, high-molecular-mass fraction; lane 3, fraction F_2, low-molecular-mass fraction. (**B**) Lane 1, Alcian Blue/silver-stained PAGE of cell-wall extracts of FSR strain B33; lane 2, fraction F_1; lane 3, fraction F_2.

rectangular pulses) scheme. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 512×1024. This experiment was slightly modified by the implementation of an editing block in the sequence [23]. The heteronuclear multiple-bond correlation (HMBC) experiment was performed using the Bruker standard sequence with 256 increments of 2048 real points to digitize a spectral width of $3000 \times 28\,000$ Hz. 192 scans were acquired per increment with a delay of 50 ms for the evolution of long-range couplings. The pure absorption 2D nuclear Overhauser effect spectroscopy (NOESY) experiment was performed with mixing time of 200 ms. A data matrix of 256×1024 points was used to digitize a spectral width of 4000 Hz; 64 scans were used per increment with a delay between scans of 2 s.

RESULTS

Isolation and purification of the polysaccharide

FSR strain B33 was isolated from a soil sample from the arid Xinjiang Autonomous Region of Western China. Strain B33 nodulates both American and Asiatic soya bean cultivars, such as Williams and Jing Dou 19, respectively [24]. This strain also formed nitrogen-fixing nodules with other legumes such as *G. soja*, *C. cajan*, *M. atropurpureum* and *I. tinctoria*, although in this latest legume the scored nitrogen-fixation activity was low. Only a fraction of *N. wightii* and *D. canadense* plants inoculated with strain B33 formed nodules and they showed very weak nitrogen-fixation activity (results not shown). Strain B33 did not nodulate *L. japonicus*, but it formed swellings on the legume roots. Hence, the host range for nodulation of strain B33 is similar to that of other strains belonging to the species *S. fredii*.

The polysaccharide was extracted from bacterial pellets of strain B33 with hot phenol/water, treated with different enzymes, and finally purified and fractionated by gel-permeation chromatography. Two fractions were isolated on the basis of their molecular masses; a high-molecular-mass fraction F_1, and a

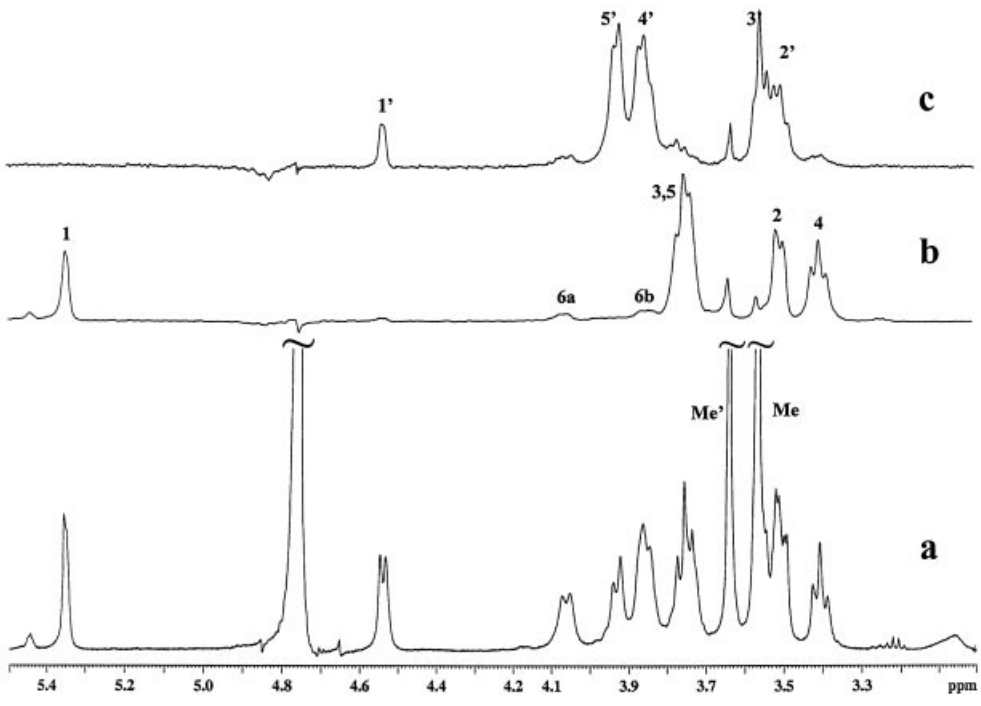

Figure 2 ^{1}H-NMR spectrum (500 MHz; a) and 1D-TOCSY subspectra obtained by selective excitation of the signals at δ_H 3.85 p.p.m., t_m 141.1 ms (b) and δ_H 3.91 p.p.m., t_m 56.4 ms (c) for the polysaccharide

The signal assignments are indicated in each subspectrum. The numbers with ' correspond to the glucuronic acid residue. The signals at 3.63 and 3.56 p.p.m. arise from the intense signals corresponding to the methoxyl groups.

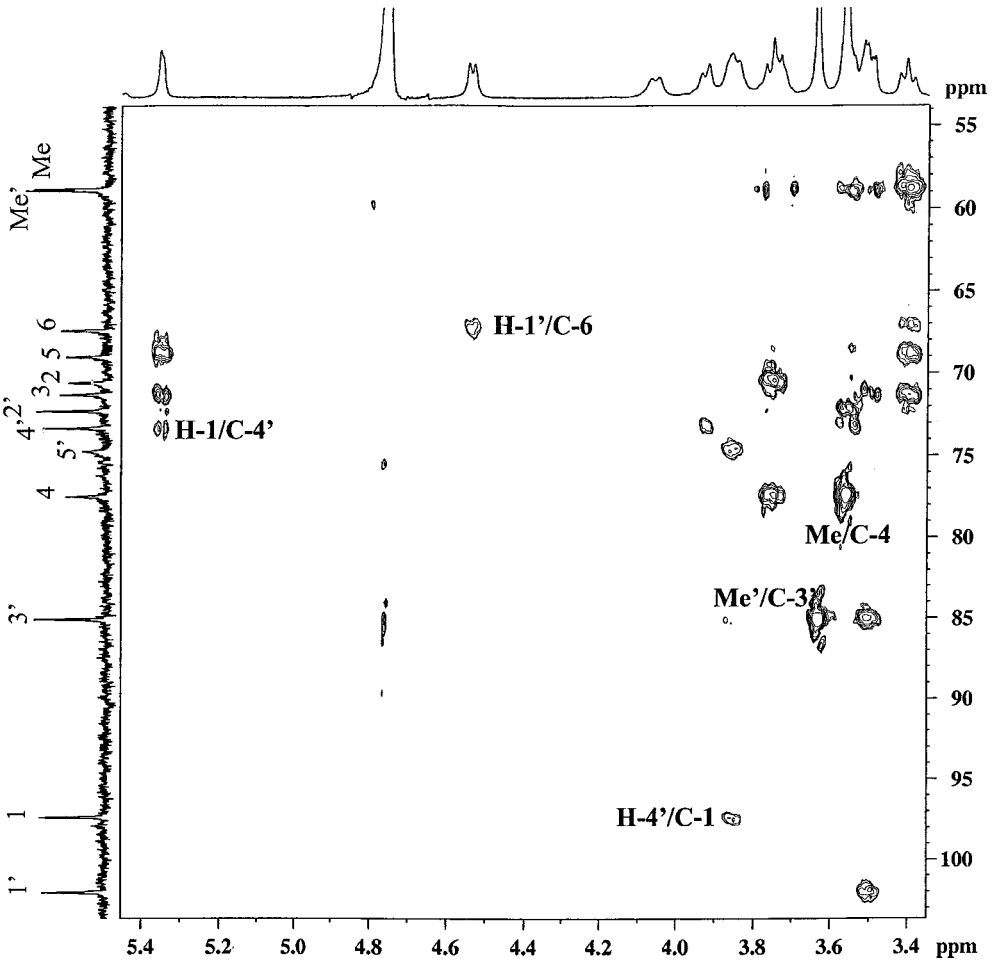

Figure 3 500 MHz ^{1}H-NMR, 125 MHz ^{13}C-NMR and 2D-HMBC registered for the polysaccharide

Inter-residue cross signals are indicated.

lower-molecular-mass fraction F_2. These fractions were analysed for the presence of LPS and/or K-antigens by using two different protocols for PAGE. SDS/PAGE with the tricine buffer system followed by silver staining was carried out to visualize the LPS component of the samples (Figure 1A). In this method, K-antigen polysaccharides escape from the gel during the washing steps before silver staining. The second method allows the visualization of KPS antigens and it consists of PAGE without detergent, followed by a fixation step of KPS antigens with Alcian Blue before silver staining (Figure 1B). Using this method, the LPS molecules do not migrate into the gel devoid of SDS.

Silver staining of crude B33 cell-wall extracts subjected to SDS/PAGE shows an LPS profile (Figure 1A, lane 1) that is very similar to that previously reported for *S. fredii* HH103 [5,6]. It consists of a ladder with a variable number of bands which probably correspond to the complete LPS molecules (called the LPS-I region) and one or two faster-migrating bands that could correspond to the lipid-A plus core and one, or no, O-antigen subunits (LPS-II region). Fraction F_1 (Figure 1A, lane 2) gives an LPS pattern that is similar to that given by the crude cell-wall extracts. The higher mobility of some bands of fraction F_1, as well as the high intensity of new faster-migrating bands was probably due to partial hydrolysis of complete LPS molecules. Fraction F_2 (Figure 1A, lane 3) only showed a new heavily stained slow-migrating band of unknown origin (possibly a bacterial polysaccharide chemically altered in the purification process). This fraction did not give bands in the LPS-I region and only a faint band in the LPS-II region (Figure 1A, lane 3), indicating that this sample does not contain significant amounts of LPS-I and LPS-II observed in the crude B33 cell-wall extracts or fraction F_1 (Figure 1A, lanes 1 and 2, respectively).

PAGE without SDS of crude B33 cell-wall extracts fixed with Alcian Blue before silver staining showed a very dark smearing that covered the whole lane (Figure 1B, lane 1). The silver-stained material is assumed to correspond to a polysaccharide that is lost in the gel if the fixation with Alcian Blue is omitted (as in Figure 1A, lane 1) and thus it is most probably the KPS. This stained smearing, although less dark and ending in a fast-migrating band, is also observable in fraction F_2 (Figure 1B, lane 3). Fraction F_1 yields a clear LPS pattern in SDS/PAGE (Figure 1A, lane 2) but it does not give a stained region in the gel devoid of detergent (Figure 1B, lane 2). All these results indicate that fraction F_1 mainly contains LPS whereas F_2 probably contains the bacterial K-antigen.

GLC-MS analysis of the trimethylsilylated methyl glycosides obtained after methanolysis of fraction F_2 yielded three peaks that could not be identified as arising from any common monosaccharide (results not shown). The corresponding mass

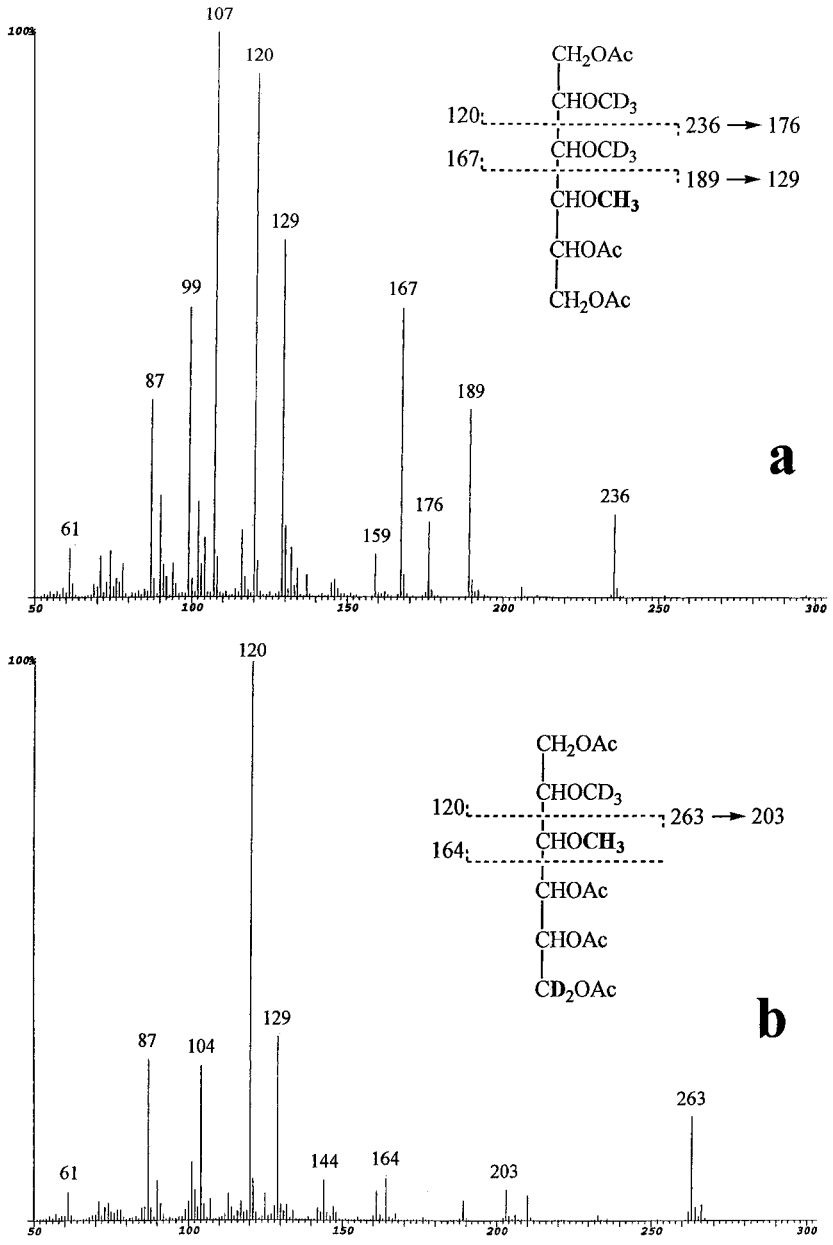

Figure 4 Electron-impact-MS and fragmentation scheme for partially methylated alditol acetates obtained on methylation analysis of the polysaccharide

(**a**) 1,2,6-Tri-*O*-acetyl-4,5-di-*O*-trideuteromethyl-3-*O*-methylhexitol. (**b**) 1,2,3,6-Tetra-*O*-acetyl-1,1-dideutero-5-*O*-trideuteromethyl-4-*O*-methylhexitol.

spectra in all cases contain a relatively intense fragment at m/z 146, which is characteristic of methylated trimethylsilylated methyl glycosides. The ^{1}H-NMR spectrum (Figure 2) of this fraction has two intense signals (at δ_H 3.64 and 3.56 p.p.m.) that are assigned to two methoxyl groups. The ^{1}H-NMR spectrum also has signals for two anomeric protons at δ_H 5.35 p.p.m. (corresponding to an α-anomer) and δ_H 4.53 p.p.m. (corresponding to a β-anomer) in a 1:1 ratio. These data suggest that the sample consists of a polysaccharide composed of a disaccharidic repeating unit whose components are both O-methylated, as all the peaks arising from the monosaccharide analysis yielded the fragment at m/z 146.

The ^{13}C-NMR spectrum (in the HMBC in Figure 3) has two signals at δ_C 59.0 and 58.9 p.p.m., assigned to the methyl groups, nine signals in the region between δ_C 67 and 85 p.p.m., two signals at δ_C 102.0 and 97.4 p.p.m., assigned to anomeric carbons, and a signal at 173.0 p.p.m., which was assigned to a carbonyl group.

The position of the glycosidic linkages in the polysaccharide, as well as the position of the methyl groups, were determined by methylation analysis. The polysaccharide was trideuteromethylated, to identify the position of the endogenous methyl groups, and carboxyl-reduced using NaB^2H_4, to identify the position of the carboxyl group. Finally, it was hydrolysed, reduced and acetylated. GLC-MS analysis of the resulting partially trideuteromethylated alditol acetates showed the presence of a 1,2,6-tri-*O*-acetyl-4,5-di-*O*-trideuteromethyl-3-*O*-methylhexitol (Figure 4a), derived from a 6-linked 4-*O*-methyl-

Table 1 ^{1}H- and ^{13}C-NMR chemical shifts (δ, p.p.m.) and coupling constants (J, Hz) for the KPS from FSR strain B33

ND, not determined.

	Position						
Residue	1	2	3	4	5	6	Methyl
→ 6)-4-*O*-Me-α-D-Glc*p*-(1 →							
H	5.35	3.50	3.75	3.41	3.75	4.06a, 3.84b	3.56
J	(1,2) 3.9	(2,3) 10.0	(3,4) 9.7	(4,5) 9.6	(5,6a) < 3; (5,6b) 2.5	(6a,6b) −11.1	–
C	97.4	70.6	71.3	77.5	69.0	67.4	58.9
→ 4)-3-*O*-Me-β-D-Glc*p*A-(1 →							
H	4.53	3.50	3.56	3.85	3.93	–	3.64
J	(1,2) 7.3	(2,3) ND	(3,4) ND	(4,5) 8.8	–	–	–
C	102.0	72.3	85.1	74.7	77.5	173.0	59.0

hexopyranose, and a 1,2,3,6-tetra-*O*-acetyl-1,1-dideutero-5-*O*-trideuteromethyl-4-*O*-methylhexitol (Figure 4b). This latter could arise from a 4-linked 3-*O*-methylhexopyranosyluronic acid as well as a 5-linked 3-*O*-methylhexofuranosyluronic acid.

The NMR analysis allowed us to determine the configuration of carbohydrates and to confirm the linkage position in the polysaccharide. Thus, the chemical shifts for the ^{1}H and ^{13}C resonances of the polysaccharide chain were assigned (Table 1) from the ^{1}H and 1D-TOCSY (Figure 2), and ^{13}C, COSY, HSQC and HMBC spectra (Figure 3). The subspectrum obtained from the 1D-TOCSY experiment by selecting the signal at δ_{H} 3.75 p.p.m. (Figure 2b) contains the signals corresponding to the residue with α-anomericity and this corresponds to the hexose residue. In the same way, the subspectrum obtained by selecting the signal at δ_{H} 3.93 p.p.m. (Figure 2c) contains the signals corresponding to the residue with β-anomericity and this corresponds to the hexuronic acid residue.

The position of the two methyl groups and the linkages in the polysaccharide were confirmed from the results of the HMBC experiment (Figure 3). Thus, the inter-residue cross signals H-1/C-4′ and H-4′/C-1 indicated the linkage α-Hex*p*-(1-4)-β-Hex*p*A (where Hex*p* is hexopyranose and Hex*p*A is hexopyranuronic acid), which in effect demonstrates the pyranosyl ring of the hexuronic acid. The cross signal H-1′/C-6 indicates the linkage β-Hex*p*A-(1-6)-α-Hex*p*, and in the same way the cross signals Me/C-4 and Me′/C-3′ allowed us to confirm the position of the two *O*-methyl groups.

The relative configuration was assigned from the ^{1}H-NMR data. From the ^{1}H and 1D-TOCSY experiments (Table 1 and Figure 2), the ^{3}J 9.6 Hz for H-4 and the two coupling constants ^{3}J 10.0 and 3.9 Hz for H-2 indicate the trans-diaxial relative disposition of each pair of hydrogens from H-2 to H-5 [25] for the hexopyranose residue. This residue, therefore, has the *gluco* configuration. For the β-hexopyranosyl uronic acid, the value of $^{3}J_{1',2'}$ 7.3 Hz indicates the trans-diaxial disposition of H-1′ and H-2′ and the doublet for H-5′, with ^{3}J 8.8 Hz, indicates the trans-diaxial dispositon of H-5′ and H-4′. The relative disposition of H-3′ was not clearly determined from these data and therefore we propose the *gluco* or *alo* configurations. Finally, in the NOESY spectrum, the anomeric proton of the glucopyranose residue H-1 showed intra-residue NOE contact with H-2 and inter-residue NOE contacts with H-4′ and Me′, which confirm the position of the linkage. The anomeric proton of the hexopyranosyluronic residue H-1′ showed inter-residue NOE contacts with H-6a and H-6b, which confirm the position of the linkage, and intra-residue NOE contacts with H-3′ and H-5′, which demonstrate the axial disposition of H-3′ and, thereafter, the *gluco* relative configuration of the uronic acid.

The absolute configuration and the confirmation of the presence of 4-*O*-methylglucose and 3-*O*-methylglucuronic acid in the polysaccharide were achieved by comparing the GLC results for the permethylated *S*- and (*R*,*S*)-2-butyl glycosides of standard D-glucose and D-glucuronic acid with those obtained for the residues in the polysaccharide. On the basis of these results we propose that the KPS isolated from FSR strain B33 is composed of disaccharide repeating units with the following structure:

→ 6)-4-*O*-methyl-α-D-Glc*p*-(1 → 4)-3-*O*-methyl-β-D-Glc*p*A-(1 →

DISCUSSION

The structures of the K-antigens of different strains belonging to *S. meliloti* (a microsymbiont of alfalfa), *S. fredii* (a microsymbiont of soya bean) and *Rhizobium* sp. NGR234 (a broad-host-range rhizobial strain) have been reported recently [3,5,6,26]. These studies demonstrated that the KPS from these bacteria show clear structural variations, even between strains within a single species. The repeating unit can be a monosaccharide (as in *S. fredii* HH103), a disaccharide (as in *S. meliloti* strains NGR185, NGR247 and AK631, *S. fredii* USDA205, USDA257, USDA208 and HH303, and *Rhizobium* sp. NGR234), a trisaccharide (as in *S. fredii* SVQ293) or a tetrasaccharide (as in *S. fredii* USDA201). In most of the structures studied, there appears to be a conserved structural motif of a disaccharide repeating unit involving a variable glycosyl residue bonded to a Kdx. This Kdx residue is either Kdo or a type of nonulosonic acid [3,22]. Because of this, Kannenberg et al. [3] have proposed a consensus structure [-(R^1)-glycosyl-(R^2)-Kdx-]$_n$ for most of the K-antigen KPSs of *Sinorhizobium* strains.

Here we show that the FSR strain B33 produces a K-antigen that it is not in accordance with the consensus structure since it is composed of a disaccharide repeating unit composed of → 6)-4-*O*-methyl-α-D-Glc*p*-(1 → 4)-3-*O*-methyl-β-D-Glc*p*A-(1 → . This fact aligns FSR strain B33 with two other *S. fredii* strains (HH103 and HH303) that also lack the glycosyl-Kdx structural motif in their K-antigens [3,6]. Although no clear functions have been assigned to rhizobial K-antigen polysaccharides, their presence appears to be a common characteristic of Gram-negative bacteria (pathogens and symbionts) that interact with plants [3]. Thus it is reasonable to expect that they play a role in plant–microbe interactions. One possible role of KPSs is that they could be somehow involved in the marked soya bean cultivar-specificity for nodulation exhibited by *S. fredii* strains. All the *S. fredii* strains (USDA201, USDA205, USDA208 and USDA257) that produce a KPS showing the structural motif glycosyl-Kdx

form effective symbioses (Fix^+) with Asiatic soya bean cultivars but fail to nodulate (Nod^-) most of the advanced American soya bean cultivars [26,27].

In contrast, the only three wild-type strains (HH103, HH303 and B33) that do not have the structural motif glycosyl-Kdx are also the only *S. fredii* strains, of those for which the structure of the KPS has been determined, that form nitrogen-fixing nodules on American soya bean cultivars. Although the number of *S. fredii* strains in which the KPS structure has been determined is still too small, the emerging picture indicates a possible association between the absence of the glycosyl-Kdx motif in the bacterial KPS and the bacterial ability to effectively nodulate American soya bean cultivars.

More intriguing coincidences at the level of monosaccharide composition between *S. fredii* K-antigens (with and without the sugar-Kdx motif) and the exopolysaccharides of the slow-growing soya bean microsymbionts *Bradyrhizobium japonicum* and *B. elkanii* can be found. *B. japonicum* strains produce exopolysaccharides containing mannose, galactose, galacturonic acid, glucose and 4-*O*-methylglucose. These bacterial strains interact with the classical *N*-acetylgalactosamine/galactose-specific soya bean lectin. Similarly, mannose and/or galactose is also present in all *S. fredii* KPSs that have the glycosyl-Kdx structural motif. On the other hand, *B. elkanii* strains produce exopolysaccharides composed of rhamnose and 4-*O*-methyl-glucuronic acid [4]; they bind soya bean lectin very poorly but interact with a 4-*O*-methylglucuronic acid-specific soya bean lectin [28]. Rhamnose and 4-*O*-methylglucuronic acid are absent from all the K-antigens analysed that have the glycosyl-Kdx structural motif but they are present in some of KPSs lacking the consensus (rhamnose in HH303 and methylglucuronic acid in FSR B33). Because some varieties of soya bean are preferentially nodulated by one of the *Bradyrhizobium* species [29], it would be interesting to investigate any possible difference in lectin-binding ability between *S. fredii* strains that produce KPS having the glycosyl-Kdx consensus and those strains producing a KPS without the structural motif.

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