Structural determination of a 5-acetamido-3,5,7,9-tetradeoxy-7-(3-hydroxybutyramido)-L-*glycero*-L-*manno*-nonulosonic acid-containing homopolysaccharide isolated from *Sinorhizobium fredii* HH103

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The structure of a polysaccharide from *Sinorhizobium fredii* HH103 has been determined. This polysaccharide was isolated by following the protocol for lipopolysaccharide extraction. On the basis of monosaccharide analysis, methylation analysis, fast atom bombardment MS, matrix-assisted laser desorption ionization MS, electron-impact high-resolution MS, one-dimensional ^{1}H-NMR and ^{13}C-NMR and two-dimensional NMR experiments, the structure was shown to consist of a homopolymer of a 3:1 mixture of 5-acetamido-3,5,7,9-tetradeoxy-7-[(*R*)- and (*S*)-3-hydroxybutyramido]-L-*glycero*-L-*manno*-nonulosonic acid. The sugar residues are attached via a glycosidic linkage to the OH group of the 3-hydroxybutyramido substituent and thus the monomers are linked via both glycosidic and amidic linkages. In contrast with the *Sinorhizobium* K-antigens previously reported, which are composed of a disaccharide repeating unit, the K-antigen polysacharide of *S. fredii* HH103 is a homopolysaccharide.

Key words: capsular polysaccharide, K-antigen, pseudaminic acid.

INTRODUCTION

Sinorhizobium fredii is a Gram-negative soil bacterium that induces nodules on the roots of various legumes, such as *Glycine max* (soya bean). Within these nodules the bacteria, differentiated into bacteroids, fix atmospheric nitrogen. The development of legume nodules is controlled largely by reciprocal signal exchange between the symbiotic partners. Legume roots secrete specific flavonoids that induce the transcription of bacterial genes essential for nodule formation.

Many nodulation (*nod*) genes are involved in the synthesis and secretion of lipochitin oligosaccharides, known as LCOs or Nod factors. These bacterial signal molecules trigger the nodule development programme of the leguminous plant [1]. In addition to bacterial genes involved in nitrogen fixation (*nif* and *fix* genes), other bacterial determinants are required for the formation of a functional nodule. These determinants include certain polysaccharides that are present in the bacterial cell wall: β-glucans, acidic exopolysaccharides, capsular polysaccharides (CPSs) and lipopolysaccharides (LPSs) [2].

The cell surface of *Rhizobium*, as in other Gram-negative bacteria, consists of a cytoplasmic (inner) membrane and an outer membrane, separated by the periplasmic space containing the peptidoglycan. The outer membrane consists of proteins, LPSs and phospholipids. β-Glucans are located mainly in the periplasmic space. The inner membrane is composed of phospholipids and lipoproteins. The extracellular matrix of the bacterium (commonly termed the capsule) is composed of an acidic exopolysaccharide that is released into the cell's milieu. In the genus *Sinorhizobium*, the outer membrane and the surrounding capsule are composed in part of a complex array of LPS and a 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)-containing polysaccharide that is structurally analogous to the constituents of the group II K-antigen polysaccharides of *Escherichia coli* [3]. The structures of different K-antigens of several strains of *S. fredii* and *Sinorhizobium meliloti* have been reported previously [3–5]. Although possessing structural variability, it seems to be a conserved structural motif of a disaccharide repeating unit involving a variable glycosyl residue bonded to a 1-carboxy-2-oxo-3-deoxy sugar (Kdx). In all these structures studied, the Kdx sugar is either Kdo (in *S. fredii* strains) or a type of nonulosonic acid (in *S. meliloti* AK631, *Rhizobium* sp. NGR234 or *S. fredii* SVQ293) [3–5]. Kannenberg et al. [3] have proposed a consensus structure $[\rightarrow(R^1)\text{sugar}\rightarrow(R^2)\text{Kdx}\rightarrow]_n$ for most of the K-antigen CPSs of *Sinorhizobium* strains.

Here we show that *S. fredii* strain HH103 produces a K-antigen CPS that is not in accordance with the consensus structure. It consists of a homopolymer of a 3:1 mixture of 5-acetamido-3,5,7,9-tetradeoxy-7-[(*R*)-and (*S*)-3-hydroxybutyramido]-L-*glycero*-L-*manno*-nonulosonic acid. The sugar residues are attached via a glycosidic linkage to the OH group of the 3-hydroxybutyramido substituent; monomers are thus linked via both glycosidic and amidic linkages. Different pseudaminic acid derivatives have been described previously as components of the polysaccharides isolated from *Pseudomonas aeruginosa*, *Shigella boydii* and *Vibrio cholerae* [6–11] but this is the first report of the completely determined structure of a pseudaminic acid-containing homopolysaccharide in a rhizobial bacterium. Although different homopolysaccharides composed of a higher sugar of this class have been reported [12–15], and a polysaccharide with

Abbreviations used: CPS, capsular polysaccharide; FAB, fast atom bombardment; HSQC, ^{1}H-detection mode via single-quantum coherence; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; Kdx sugar, 1-carboxy-2-oxo-3-deoxy sugar; LPS, lipopolysaccharide; MALDI–TOF-MS, matrix-assisted laser desorption ionization–time-of-flight MS.

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this class of glycosidic linkage has been described [9], this is the first report of a homopolymer of pseudaminic acid derivative with this type of glycosidic linkage.

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 partly 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 the trimethylsilylated 2-octyl esters of 3-hydroxybutyric acid was isothermal at 70 °C for 3 min, followed by a gradient of 10 °C/min up to 170 °C and then 25 °C/min up to 280 °C. The ionization potential was 70 eV and the mass spectra were performed with both low-resolution and high-resolution electron-impact MS. Positive-ion fast atom bombardment MS (FAB-MS) spectra were obtained with a Kratos MS80RFA instrument with a xenon atom beam at an accelerating voltage of 4 kV. The probe was loaded with 1 μl of sample solution in water with a matrix of glycerol. Positive-ion matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS) was performed with a Micromass TofSpec mass spectrometer equipped with a 337 nm nitrogen laser, with 2,5-dihydroxybenzoic acid as matrix. A mixture of maltose oligosaccharides was used as an external calibration standard.

Sugars were identified on GLC–MS separation of their trimethylsilylated methyl glycosides obtained as described [5]. The absolute configuration of 3-hydroxybutyric acid was assigned after the GLC–MS analysis of their trimethylsilylated (*S*)- and (*R*,*S*)-2-octyl esters. The polysaccharide was hydrolysed with 2 M trifluoroacetic acid for 1 h at 120 °C; 3-hydroxybutyric acid was extracted with ethyl acetate and esterified by heating at 140 °C overnight in the presence of a drop of trifluoroacetic acid [16]. The octyl ester was trimethylsilylated conventionally. Optical rotation was measured with a Perkin-Elmer model 241 MC polarimeter at 25 °C. Monosaccharides and oligosaccharides were obtained by mild acid hydrolysis of the polysaccharide with 1 % (v/v) acetic acid at 100 °C for 1 h, followed by gel-permeation chromatography on Sephadex G-50 and G-15, with water as eluent. Autohydrolysis of fraction F3 was performed by heating a 1 % (v/v) aqueous solution at 60 °C for 2 h.

Bacterial strains and isolation of the polysaccharide

Wild-type *S. fredii* HH103 was grown routinely at 28 °C in TY medium as described by Beringer [17]. When required, media were supplemented with streptomycin (400 μg/ml). Early-stationary-phase cultures (300 ml, TY medium) of *S. fredii* HH103 were used to inoculate 30 litres of TY liquid medium supplemented with the antibiotic streptomycin. After inoculation, cultures were incubated for 3 days at 28 °C. Bacterial cultures were aerated by pumping sterile air through at a flow of 50 l/min. After incubation, the cells were harvested by low-speed centrifugation. The bacterial pellets were washed with 0.9 % NaCl, freeze-dried and stored in sealed bottles at room temperature. The polysaccharide was extracted from the freeze-dried bacterial cells (5 g) with 100 ml of hot phenol/water (1:1, v/v) [18] 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), with water as eluent. The eluate was freeze-dried and redissolved in 10 mM $MgSO_4$/50 mM Tris/HCl (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 (10 mg) was added; 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) with 0.2 M NaCl as eluent; carbohydrates were detected with a refractive-index detector and the orcinol/sulphuric acid method on TLC plates. Fractions containing carbohydrates were dialysed and freeze-dried.

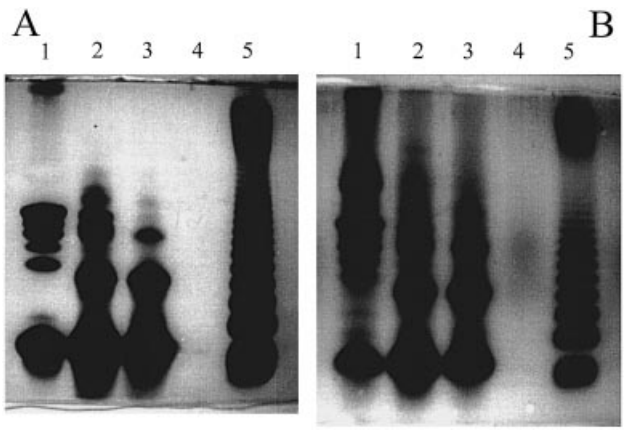

Figure 1 Silver-stained (A) and Alcian Blue/silver-stained (B) SDS/PAGE of extracts of *S. fredii* HH103 and comparison with LPS from *Salmonella typhimurium*

Lanes 1, whole-cell extracts; lanes 2, F1 (very-high-molecular-mass fraction); lanes 3, F2 (high-molecular-mass fraction); lanes 4, F3 (lower-molecular-weight fraction obtained by GPC of the polysaccharide); lanes 5, LPS from *Salmonella typhimurium*.

SDS/PAGE

Three different protocols have been used to analyse the bacterial LPS and K-antigen polysaccharide profiles. SDS/PAGE electrophoresis of crude bacterial extracts (Figures 1A and 1B, lanes 1) was performed as described previously [5]. In brief, bacterial cultures of *S. fredii* HH103 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 (pH 6.8)/2 % (w/v) SDS/1 mM EDTA for 5 min and then diluted to 1 ml with the same buffer without SDS. The bacterial crude extract was treated with RNase, DNase and proteinase K as described by Köplin et al. [19]. The electrophoresis of crude bacterial extracts (Figures 1A and 1B, lanes 1) or purified bacterial polysaccharide (Figures 1A and 1B, lanes 2–4) was performed on a 16.5 % (w/v) polyacrylamide gel with the Tricine buffer system described by Lesse et al. [20]. For the detection of LPSs, gels (Figure 1A) were stained with silver as described by Kittelberger and Hilbink [21]. For the detection of LPSs and K-antigens, gels (Figure 1B) were fixed with Alcian Blue as described by Corzo et al. [22] and then stained with silver by the method described by Tsai and Frasch [23].

PAGE without detergent was also performed to facilitate the detection of K-antigens (Figure 2). Under the same conditions, only the K-antigens (and not the LPS) can migrate into the gel. Samples of the purified K-antigen polysaccharide were analysed by vertical electrophoresis (Hoefer-Pharmacia Biotech), as described for extracellular polysaccharides from *Bradyrhizobium*

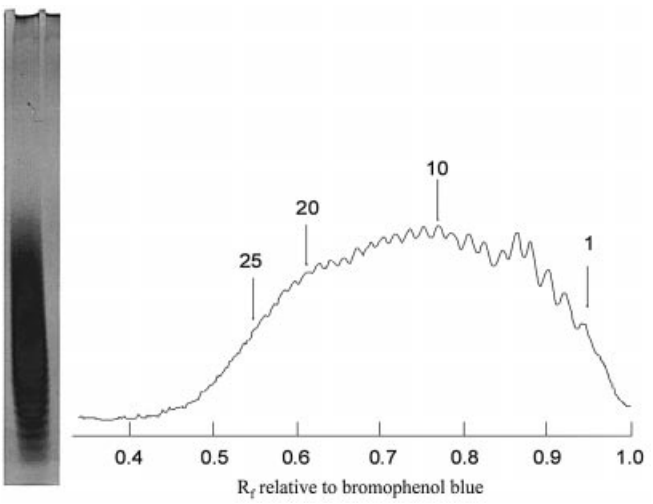

Figure 2 Alcian Blue/silver-stained PAGE, densitometric tracing and band numbering of fraction F3 (lower molecular mass) performed in the absence of SDS

The absence of SDS meant that only K-antigen non-hydrophobic polysaccharides could migrate into the gel. The electrophoresis was stopped when the Bromophenol Blue reached 10 mm from the bottom of the gel. No bands were detected below band 1.

[24]. A continuous system was employed, with slab gels 0.75 mm thick. The acrylamide concentration was 17% (w/v), the acrylamide-to-*N*,*N*′-methylenebisacrylamide ratio being 30:0.8. The electrophoresis buffer was 50 mM Tris/HCl (pH 8.5)/13 mM EDTA/15 mM boric acid. Samples were dissolved in the same buffer and diluted (1:2) in 1 M sucrose in deionized water. Gels were fixed by Alcian Blue in acetic acid [22] and stained with silver [23]. After being dried, the stained gels were scanned with a Pharmacia-LKB Ultroscan XL laser densitometer.

Methylation

The vacuum-desiccated sample of monosaccharide was carbonyl-reduced by dissolving in 1 M NH_4OH (400 μl) containing 10 μg/μl NaB^2H_4 for 2 h at room temperature. The borate was eliminated with acetic acid and by co-evaporation with methanol. The resulting polyol was esterified with 0.625 M HCl in methanol at 20 °C for 2.5 h and then carboxy-reduced by treatment with 2 mg of $NaBH_4$ dissolved in 500 μl of ethanol/water (3:1, v/v) at room temperature overnight. Finally, the carboxy-reduced polyol was methylated by the method of Ciucanu and Kerec [25]. For the oligosaccharide, the sample was carbonyl-reduced, methylated and carboxy-reduced as above, then hydrolysed with 1% (v/v) trifluoroacetic acid for 1 h at 100 °C and finally 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 (^1H) and 125.75 MHz (^{13}C). Chemical shifts are given in p.p.m., with the H^2HO signal (4.75 p.p.m.) (^1H) and external DMSO (39.5 p.p.m.) (^{13}C) as references. In one experiment, ^{13}C data were recorded with inverse gated decoupling to measure the integral of the ^{13}C signals correctly. The two-dimensional homonuclear proton double-quantum-filtered COSY experiments were performed in the phase-sensitive mode with the Bruker standard pulse sequence. A data matrix of 512×1024 points was used to digitize a spectral width of 4500 Hz; 32 scans were used per increment with a delay between scans of 1 s. Before Fourier transformation, zero-filling was used in F_1 to expand the data to 1024×1024 points. The two-dimensional heteronuclear one-bond proton–carbon COSY experiment was registered in the ^1H-detection mode via single-quantum coherence (HSQC). A data matrix of 256×1024 points was used to digitize a spectral width of 4000 and 20000 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 globally optimized alternating-phase rectangular pulses ('GARP') scheme. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 512×1024 points. This experiment was slightly modified by the implementation of an editing block in the sequence [26]. The two-dimensional HSQC-TOCSY spectra were recorded with the conventional phase-cycle HSQC pulse sequence concatenated to a WALTZ 16-pulse train with a spin-locking π pulse of 98 μs. Two spectra were recorded with 20 and 140 ms of isotropic mixing time respectively.

RESULTS

Isolation and purification of the polysaccharide

The polysaccharide was extracted from bacterial pellets of the parental strain *S. fredii* HH103 with hot phenol/water, treated with different enzymes and finally purified and fractionated by gel-permeation chromatography. Three fractions were isolated on the basis of their molecular masses: a very high-molecular-mass fraction, F1, which was excluded from Sephacryl S-500, a high-molecular-mass fraction, F2, which was partly excluded from S-500, and a lower-molecular-mass fraction, F3, which was further chromatographed on Sephacryl S-400 with the same eluent and whose molecular mass was estimated to be 26 kDa. 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 and the silver-staining method as described by Kittelberger and Hilbink [21] were used to stain and detect the LPS component of the samples. The second method included a fixation treatment with Alcian Blue before LPS and K-antigens were stained with silver as described [23]. The treatment with Alcian Blue was developed by Corzo et al. [22] to detect K-antigen polysaccharides. Apparently, the fixation step with Alcian Blue prevents the escape of K-antigens from the gel so that they can be detected by silver staining.

Figure 1 shows that fractions F1 (lanes 2) and F2 (lanes 3) still contained LPS, which gives a ladder pattern resembling the characteristic LPS profile of the crude cell-wall extract of strain HH103 (lanes 1). The higher mobility of the bands in samples subjected to purification procedures (Figures 1A and 1B, lanes 2 and 3) was probably due to partial hydrolysis of the LPS. The upper part of those lanes containing *S. fredii* samples (Figure 1B, lanes 1–3) in the gel treated with Alcian Blue were more heavily stained than the corresponding pairwise lanes (Figure 1A, lanes 1–3) in the gel without Alcian Blue. This difference in staining intensity was probably due to the differences in the silver-staining protocols used and/or the presence of high-molecular-mass material (probably K-antigens) that did not escape from the gel because of the fixation with Alcian Blue.

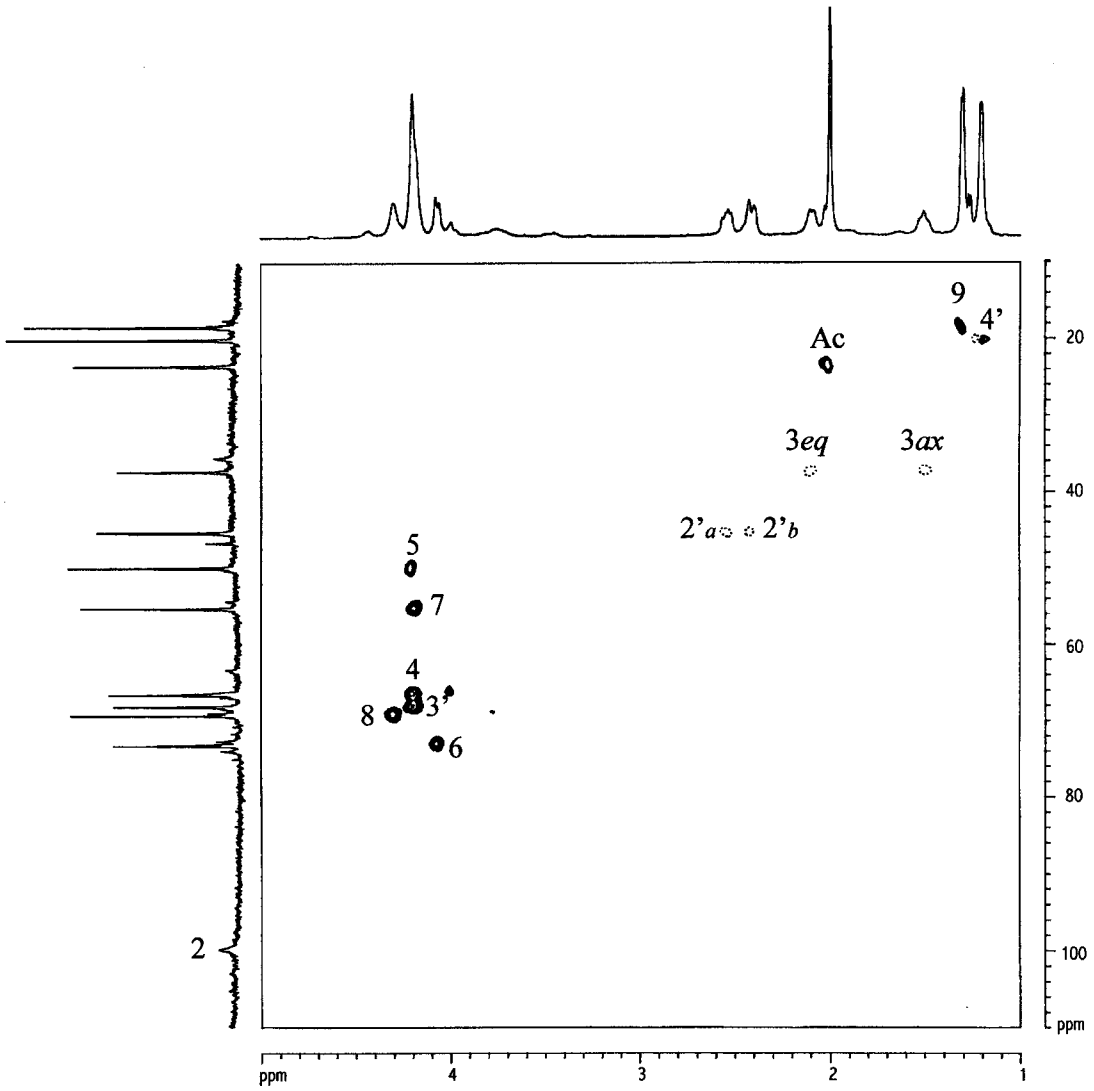

Figure 3 ^{1}H-NMR, ^{13}C-NMR and multiplicity-edited two-dimensional HSQC experiment at 500 MHz obtained for fraction F3

Positive peaks (solid lines) correspond to methine and methyl groups; negative peaks (broken lines) correspond to methylenic groups. The numbers followed with an apostrophe correspond to the 3-hydroxybutyryl moiety.

The lower-molecular-mass fraction (F3) apparently did not contain significant amounts of LPS, because the LPS ladder was absent. No material was silver-stained in the gel that was not treated with Alcian Blue (Figure 1A, lane 4) and only a weakly stained smear was visible in the gel treated with Alcian Blue (Figure 1B, lane 4). NMR analyses of fraction F3 did not detect the presence of LPS; the major component was another polysaccharide. Because of its apparent homogeneity, fraction F3 was chosen for NMR studies. Fractions F2 and F3 were used for sugar and methylation analyses.

NMR analysis of the polysaccharide

The ^{1}H-NMR and ^{13}C-NMR spectra (edited HSQC in Figure 3) of the homogeneous lower-molecular-mass fraction F3 showed two signals (at δ_C 18.3 and 19.9 p.p.m.) that revealed the presence of two methyl groups, and another (at δ_C 23.4 p.p.m., correlated with the singlet at δ_H 2.01 p.p.m.) that was assigned to the methyl group of an acetyl group. The methylenic nature of the carbons at δ_C 37.2 and 45.2 p.p.m. is demonstrated unequivocally by the negative sign (broken lines) of their one-bond proton–carbon correlation cross peaks in this spectrum. The signals at δ_C 49.8 and 55.1 p.p.m. were tentatively assigned to methine carbons carrying nitrogen. In the region of δ_C 65–75 p.p.m. four carbon signals appeared; these were correlated with ^{1}H signals in the region of δ_H 4–4.5 p.p.m. and were tentatively assigned to methine carbons linked to oxygen. The broad signal at δ_C 99.7 p.p.m. (which showed no proton correlation cross-peak) was assigned to a ketalic anomeric carbon. In addition, the low-field region of the ^{13}C spectrum (results not shown) contained signals at δ_C 173.7, 175.7 and 178.0 p.p.m., which corresponded to three carbonyl carbons.

On the basis of the COSY and HSQC-TOCSY experiments, the methyl group at δ_C 18.3 and δ_H 1.3 p.p.m. was correlated with at least three ^{1}H signals in the δ_H 4–4.5 p.p.m. region and was therefore identified as the terminal methyl group of a deoxy

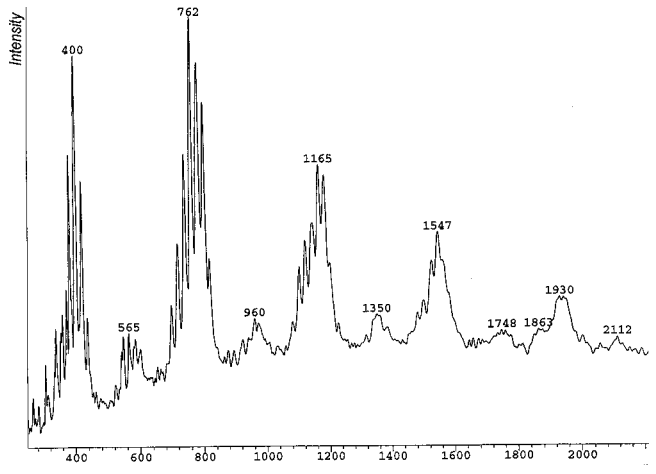

Figure 4 Positive-ion MALDI–TOF mass spectrum of the autohydrolysate of fraction F3

The indicated mass value over each peak corresponds to an average for all the ions in the cluster. The abscissa scale is in units of *m/z*.

sugar. The methylenic group at δ_C 37.2, δ_H 1.52 and 2.11 p.p.m. was correlated with two ^{1}H signals in the same region as before. These two connectivity pathways are representative of the C-3 to C-9 carbon framework of a 3,9-dideoxynonulosonic acid. In addition, the methyl group at δ_C 19.9 and δ_H 1.21 p.p.m. was correlated with the methylenic signal at δ_C 45.2, δ_H 2.43 and 2.57 p.p.m. and with a methine group at δ_C 68.0 and δ_H 4.2 p.p.m. This set of signals was identified as corresponding to a 3-hydroxybutyryl moiety.

These data allowed us to conclude that the analysed sample consists of a homopolymer of a 3,9-tetradeoxydiaminononulosonic acid bearing an acetyl group and a 3-hydroxybutyryl group as substituents.

Sugar analysis

GLC–MS analysis of the trimethylsilylated methyl glycosides obtained after methanolysis of the heterogeneous high-molecular-mass fraction F2 revealed that the fraction contained glucose, Kdo and traces of galacturonic acid, galactose, mannose, glucuronic acid and rhamnose (results not shown). However, no major chromatographic peak was found arising from the main component of the polysaccharide identified by NMR, probably owing to the known lability of this kind of compound under acidic conditions [16,27].

FAB-MS and MALDI–TOF-MS analyses

To determine fully the structure of the monosaccharidic repeating unit, a mild hydrolysis [1 % (v/v) acetic acid] was performed and the monosaccharide was isolated by gel-permeation chromatography. On positive-ion FAB-MS analysis of the monosaccharide, $[M+H]^+$, $[M+Na]^+$ and $[M+2Na-H]^+$ pseudomolecular ions were observed at m/z 379, 401 and 423 respectively. The observed mass was thus consistent with an acetyl, 3-hydroxybutyryl derivative of a tetradeoxydiaminononulosonic acid.

In addition, positive-ion MALDI–TOF mass spectra of the fraction F3 and its autohydrolysate were performed. The two spectra were very similar. Figure 4 shows the spectrum of the hydrolysate. Clusters of ions arising from different sodium salts and adduct ions were detected at the following mass values: m/z 401, assigned as before to the sodium adduct of the monomer (reported m/z 400); m/z 761, sodium adduct of the dimer (reported m/z 762); m/z 1165, sodium adduct of the disodium salt of the trimer (reported m/z 1165); m/z 1547, sodium adduct of the trisodium salt of the tetramer (reported m/z 1547); m/z 1929, sodium adduct of the tetrasodium salt of the pentamer (reported m/z 1930). The presence of the sodium adduct of the

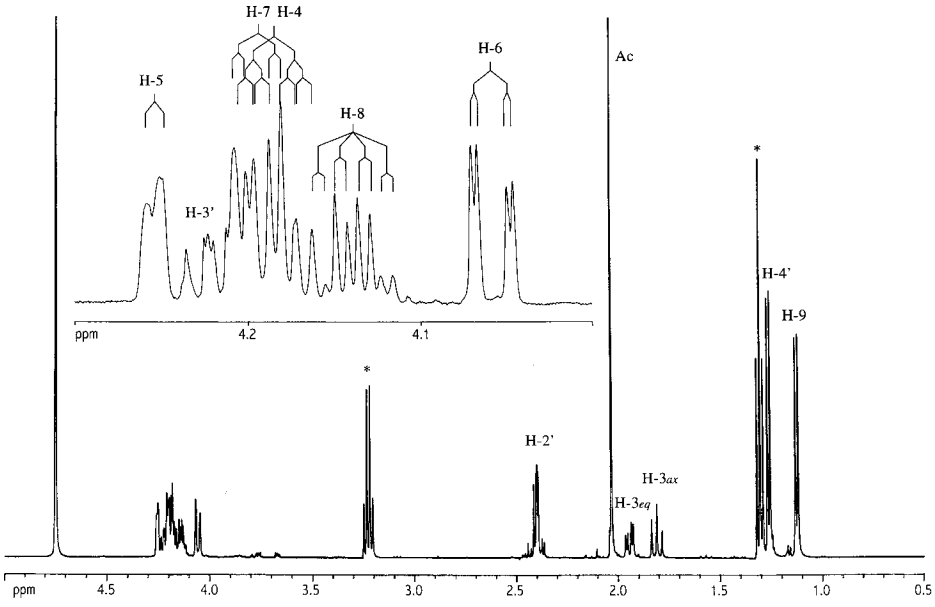

Figure 5 ^{1}H-NMR spectrum obtained at 500 MHz for the monosaccharide

The inset shows the δ = 4.0–4.3 p.p.m. zone. Signals marked with an asterisk correspond to redisual ethanol.

Table 1 ^{1}H[NMR and ^{13}C-NMR chemical shifts (δ) and coupling constants (J) for the CPS from *S. fredii* and the derived monosaccharide

Compound	Parameter	Position ...1	2	3	4	5	6	7	8	9
Polysaccharide	δ_H (p.p.m.)	–	–	1.52 (ax) 2.11 (eq)	4.20	4.21	4.07	4.19	4.30	1.30
	δ_C (p.p.m.)	173.7	99.7	37.2	66.4	49.8	73.1	55.1	69.2	18.3
3-Hydroxybutyryl residue	δ_H (p.p.m.)	–	2.57 (a) 2.43 (b)	4.20	1.21					
	δ_C (p.p.m.)	178.0*	45.2	68.0	19.9					
Acetyl residue	δ_H (p.p.m.)	–	2.01							
	δ_C (p.p.m.)	175.7*	23.4							
Monosaccharide	δ_H (p.p.m.)	–	–	1.81 (ax) 1.94 (eq)	4.18	4.25	4.06	4.19	4.14	1.13
	J (Hz)	–	–	−12.7 (3ax,3eq) 12.7 (3ax,4)	5.0 (3eq,4) 4.2 (4,5)	1.7 (5,6)	10.5 (6,7)	3.3 (7,8)	6.5 (8,9)	
	δ_C (p.p.m.)	175.0†	97.7	36.0	66.6	50.1	71.3	54.2	68.4	16.8
3-Hydroxybutyryl residue	δ_H (p.p.m.)	–	2.41 (a) 2.39 (b)	4.23	1.26					
	J (Hz)	–	−14.5 (2′a,2′b) 8.1 (2′a,3′)	4.9 (2′b,3′) 6.3 (3′,4′)						
	δ_C (p.p.m.)	177.4†	46.4	66.2	23.37					
Acetyl residue	δ_H (p.p.m.)	–	2.03							
	δ_C (p.p.m.)	175.9†	23.2							

*,† These assignments could be interchanged.

sodium salt has been reported for sialylated oligosaccharides [28]. These experiments confirmed the homopolymeric character of the polysaccharide and the molecular mass of the repeating unit.

NMR analysis of the monosaccharide

The ^{1}H and ^{13}C chemical shift resonances from the monosaccharide, obtained as described above, were assigned from ^{1}H (Figure 5) and ^{13}C-NMR spectra, COSY and multiplicity-edited HSQC spectra (Table 1). Data for C-5 and C-7 indicated that these positions were aminated. The difference between chemicals shifts of the signals for H-3_{ax} and H-3_{eq} was only 0.13 p.p.m., indicating the equatorial disposition of the vicinal carboxy group [13]. The $J_{H,H}$ pattern found for all the protons in the monosaccharide residue, and the δ_C value for C-9 (16.8 p.p.m.) [7,8,13] suggested that the sugar was the L-*glycero*-L-*manno* isomer or its enantiomer. The large negative value for the optical rotation, $[\alpha]_D$ −45° (*c* 1.1 in water), allowed us to conclude that the monosaccharide residue was a diacyl derivative of 5,7-diamino-3,5,7,9-tetradeoxy-L-*glycero*-α-L-*manno*-nonulosonic (pseudaminic) acid. This result is in accordance with previous results reported for pseudaminic acid derivatives [7,8,10,11].

Electron-impact high-resolution MS analysis of the monosaccharide

The positions of the acyl substituents were determined by an electron-impact high-resolution mass spectrum of a derivative of the monosaccharide repeating unit. The sugar was carbonyl-reduced, carboxy-reduced and methylated. GLC–MS analysis of the resulting monosaccharide derivative showed the presence of (2*R* or 2*S*)-3,5,7,9-tetradeoxy-2-deutero-1,2,4,6,8-penta-*O*-methyl-5-(*N*-methylacetamido)-7-[*N*-methyl-(3-methoxybutyramido)]-L-*glycero*-L-*manno*-nonitol (Figure 6). The fragmentation scheme was confirmed by accurate mass measurements in a high-resolution electron-impact mass spectrum. The results for the diagnostic fragments were as follows: M^+, m/z 479.331282, $C_{23}H_{45}{}^{2}HN_2O_8$; m/z 464.307513, $C_{22}H_{42}{}^{2}HN_2O_8$; m/z 448.313575, $C_{22}H_{42}{}^{2}HN_2O_7$; m/z 434.295509, $C_{21}H_{40}{}^{2}HN_2O_7$; m/z 420.281651, $C_{20}H_{38}{}^{2}HN_2O_7$; m/z 331.223404, $C_{16}H_{31}N_2O_5$; m/z 277.187385, $C_{13}H_{25}{}^{2}HNO_5$; m/z 246.170080, $C_{12}H_{24}NO_4$; m/z 233.161089, $C_{11}H_{21}{}^{2}HNO_4$; and m/z 202.143719, $C_{10}H_{20}NO_3$. The base peak at m/z 102.091674, $C_5H_{12}NO$, was assigned to a secondary fragment arising from the loss of $CH_3CH(OMe)CH=C=O$ from the primary fragment at m/z 202.143719.

Linkage analysis

The position of the glycosidic linkage of the pseudaminic acid derivative in the homopolysaccharide was determined by methylation analysis of a disaccharide isolated from the mild hydrolysis of the polysaccharide. The oligosaccharide was carbonyl-reduced, methylated, carboxy-reduced, hydrolysed, reduced and acetylated. Only the derivative arising from the reduced reducing-end unit, (2*R* or 2*S*)-1-*O*-acetyl-3,5,7,9-tetradeoxy-2-deutero-2,4,6,8-tetra-*O*-methyl-5-(*N*-methylacetamido)-7-[*N*-methyl-(3-*O*-acetylbutyramido)]-L-*glycero*-L-*manno*-nonitol, was detected and identified by GLC–MS (Figure 7). The fragmentation scheme was confirmed by accurate mass measurements. The results for the diagnostic fragments were as follows: M^+, m/z 535.320986, $C_{25}H_{45}{}^{2}HN_2O_{10}$; m/z 520.301017, $C_{24}H_{42}{}^{2}HN_2O_{10}$; m/z 504.303188, $C_{24}H_{42}{}^{2}HN_2O_9$; m/z 476.271663, $C_{22}H_{38}{}^{2}HN_2O_9$; m/z 359.216887, $C_{17}H_{31}N_2O_6$; m/z 305.182540, $C_{14}H_{25}{}^{2}HN_2O_6$; m/z 274.165515, $C_{13}H_{24}NO_5$; m/z 261.157209, $C_{12}H_{21}{}^{2}HNO_5$ and m/z 230.139220, $C_{11}H_{20}NO_4$. The base peak m/z 102 had the same origin as before. These results showed clearly that the glycosidic linkage was established between the hemiketalic OH and the hydroxyl group of the 3-hydroxybutyramido residue. In consequence, the sugar repeating units were attached via two linkages: one ketalic and the other amidic. Additionally, the small difference between H-3_{ax} and H-3_{eq} δ values (0.59 p.p.m.) in the ^{1}H-NMR spectrum (Table 1) indicated that the orientation of the carboxy group of the pseudaminic diacyl derivative in the polysaccharide is equatorial

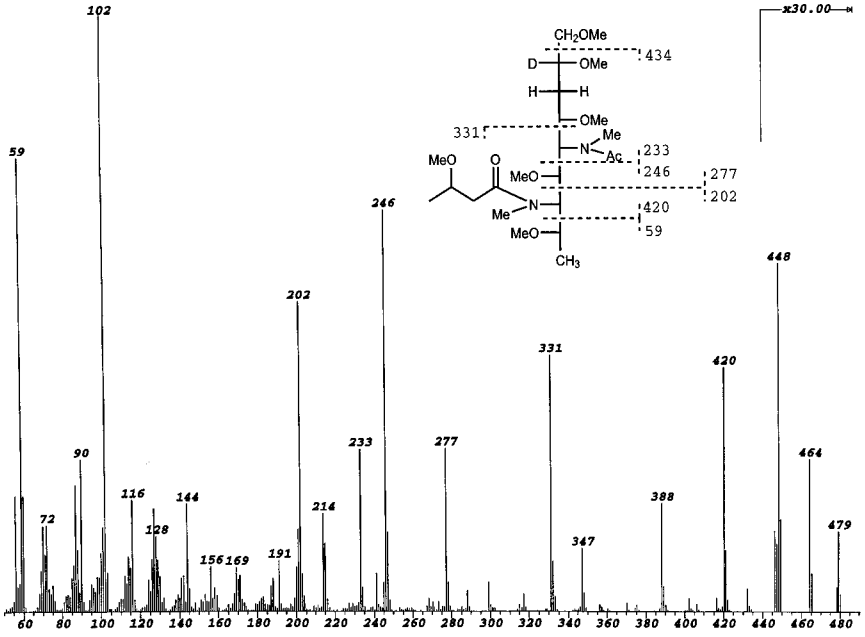

Figure 6 Electron-impact MS and fragmentation scheme for (2*R* or 2*S*)-3,5,7,9-tetradeoxy-2-deutero-1,2,4,6,8-penta-*O*-methyl-5-(*N*-methylacetamido)-7-[*N*-methyl-(3-methoxybutyramido)]-L-*glycero*-L-*manno*-nonitol

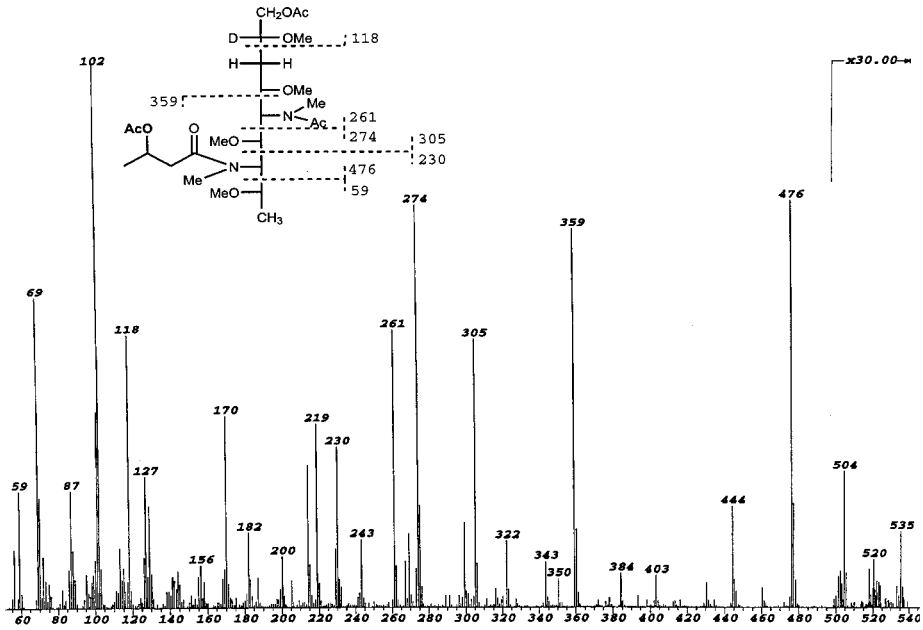

Figure 7 Electron-impact MS and fragmentation scheme for (2*R* or 2*S*)-1-*O*-acetyl-3,5,7,9-tetradeoxy-2-deutero-2,4,6,8-tetra-*O*-methyl-5-(*N*-methylacetamido)-7-[*N*-methyl-(3-*O*-acetylbutyramido)]-L-*glycero*-L-*manno*-nonitol

(reported $\Delta\delta = 0.6$ p.p.m. for the equatorial carboxy group and $\Delta\delta = 0.9$ p.p.m. for the axial carboxy group) [8,13]. Moreover, the chemical shift for C-2 is reported to be in the range $\delta =$ 99–100 p.p.m. when the carboxy group is equatorial and in the range $\delta = 101$–104 p.p.m. when it is axial [10]. Our δ value (99.7 p.p.m.) indicates that the carboxy group was equatorial. Because the configuration of the sugar residue is L-*glycero*-L-*manno*, and the carboxy group was equatorial, the anomerism should be defined as α, in accordance with the recommendation for the nomenclature of carbohydrates.

Determination of the absolute configuration of the 3-hydroxybutyramido group

Hydrolysis of the polysaccharide followed by extraction of the hydrolysate with ethyl acetate gave free 3-hydroxybutyric acid,

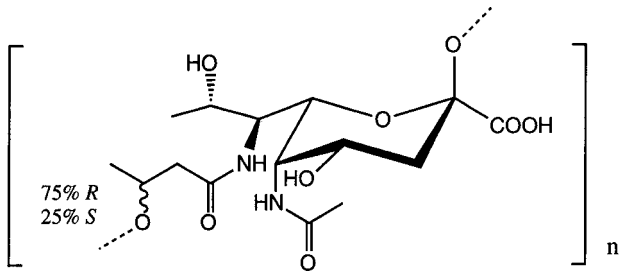

Figure 8 Proposed structure of the CPS from *S. fredii* HH103

which was converted into the (*R*,*S*)-oct-2-yl or the (*S*)-oct-2-yl ester, trimethylsilylated and analysed by GLC–MS. The chromatogram showed a 3:1 diasteroisomeric mixture obtained in the esterification with (*S*)-octan-2-ol. These results allowed us to propose that 75% of the 3-hydroxybutyric acid in the polysaccharide had the configuration *R* and 25% the configuration *S*.

On the basis of the above results, we propose that the CPS produced by *S. fredii* HH103 has the structure shown in Figure 8.

Figure 1 indicates that fraction F3 does not contain LPS, or that its presence is negligible. To improve the detection of the K-antigens, we performed PAGE in the absence of SDS to prevent the migration of LPS molecules into the gel. Figure 2 shows the electrophoretic profile of the K-antigen polysaccharides of strain HH103 when the gel was fixed with Alcian Blue and silver-stained. Under these conditions, at least 26 discrete bands and a trailing smear were present. The CPS in the sample was composed of small molecules with a polymerization degree ranging from 1 to more than 26 monosaccharide units. There was no predominant size because the quantities of molecules composed of 3–20 units were similar (Figure 2). In the absence of Alcian Blue fixation, the K-antigen polysaccharide was hardly visible, even with a large amount of sample loaded on the gel. These results are in accordance with those described for *Sinorhizobium* K-antigens [4]. However, the sensitivity of the silver-staining method after fixation with Alcian Blue was rather low because it was necessary to apply 5–10-fold the amount of sample normally used to reveal polysaccharides. The structure of the K-antigen of strain HH103 does not contain *cis*-diol groups that could reduce the silver ion during the silver-staining procedure. This explains why fraction F3 (which contained only the K-antigen polysaccharide) was so poorly silver-stained when standard protocols were used for the detection of K-antigen polysaccharides. It is important to determine the size of oligosaccharides because this can be an important factor in their biological activity [29]. The sizes of small molecules can be very accurately determined by different techniques such as FAB or MALDI–TOF. However, these techniques are not useful for the quantification of each component in a polydisperse sample, because their sensitivity decreases sharply as polymer size rises and because oligosaccharides that are larger than a few carbohydrate units are too big to be resolved adequately by these techniques. Nevertheless, the range of polymerization and the relative abundance of each molecule in the whole sample can easily be obtained from the electrophoretic profile (Figure 2). The absolute sizes of the molecules cannot be directly obtained by electrophoresis but in this case it was possible to ascribe the fastest band to the monomer because we knew from MALDI–TOF that in the CPS sample the smallest molecule was the monomer. Electrophoresis of whole-cell extract in Tris/borate buffer showed a CPS profile similar to that of the purified F3 sample (results not shown), so the small size of the purified CPS of *S. fredii* HH103 was not due to its degradation during purification.

DISCUSSION

The structures of K-antigen polysaccharides of different *S. fredii* and *S. meliloti* strains have recently been reported [3–5]. There are structural variations between the K-antigen polysaccharide of *S. meliloti* and those of *S. fredii*, and also from strain to strain within a single species. Despite these variations, the structure seems to be a conserved structural motif of a disaccharide repeating unit composed of a variable glycosyl residue bonded to a Kdx sugar [3]. This Kdx sugar is Kdo in *S. fredii* strains and derivatives of the nonulosonic acid in *S. meliloti* and *Rhizobium* sp. NGR234 [3,4]. The repeating unit of the K-antigen polysaccharide of *S. fredii* strain HH103 is pseudaminic acid, an analogue of Kdo. Therefore it is most probable that the Kdo detected by GLC–MS analyses was due to the presence of trace amounts of LPS in the purified samples.

In contrast with the *Sinorhizobium* K-antigens reported previously [3], which are composed of a disaccharide repeating unit, the K-antigen polysaccharide of strain HH103 is a homopolysaccharide in which the glycosidic linkage is established between the hemiketalic OH and the hydroxy group of the 3-hydroxybutyramido residue. In consequence, the sugar repeating units are attached via two linkages: one hemiketalic and the other amidic. The monomer of the K-antigen polysaccharide of strain HH103 is structurally related to the Kdx sugar of the disaccharide repeating unit of the CPSs of *S. meliloti* AK631 and *Rhizobium* sp. NGR234. These two bacteria seem to be related to *S. fredii* strains. The species *S. fredii* and *S. meliloti* are very closely related [30] and some *S. fredii* strains are capable of establishing an effective nodulation with alfalfa cultivars [31]. Although *Rhizobium* sp. NGR234 does not nodulate on soya beans, this bacterium carrying the symbiosis plasmid of *S. fredii* USDA191 can form nitrogen-fixing nodules with soya beans [32]. HH103 and NGR234 show a very broad host range of nodulation, the legumes nodulated by HH103 being a subset of those nodulated by NGR234. The DNA sequence of genes located in the symbiotic plasmid of NGR234 is strikingly similar to that in HH103 [33]. Strain NGR234 is also closely related to *S. fredii* on the basis of serology, partial SSU (**s**mall **su**b**u**nit) rRNA nucleotide sequences, and FAME (**f**atty **a**cid **m**ethyl **e**ster) analysis [34].

All the reported K-antigens of *S. meliloti* strains contain N-acetyl groups. In contrast, this characteristic is not present in the *S. fredii* K-antigens, with the sole exception of strain HH303 [3]. Here we show that the K-antigen of *S. fredii* strain HH103 also contains *N*-acetyl groups. Interestingly, both strain HH103 and HH303 also have a host specificity different from those of other *S. fredii* strains in that they are able to nodulate, and fix nitrogen, with American soya-bean cultivars. The conserved structural motif of the K-antigen polysaccharides of *Sinorhizobium* strains [(R^1)sugar-(R^2)Kdx] has been proposed as a result of a comparative study of the K-antigens of *S. meliloti* (unable to nodulate soya beans), *Rhizobium* sp. NGR234 (unable to nodulate soya beans), and *S. fredii* strains USDA201, USDA205, USDA208 and USDA257. All these *S. fredii* strains form effective symbioses with Asiatic soya-bean cultivars but they fail to nodulate with most American soya-bean cultivars [35]. In contrast, *S. fredii* strains HH103 and HH303 are able to nodulate with American soya-bean cultivars and they are the only two strains that do not

produce a K-antigen polysaccharide repeating unit involving a variable glycosyl residue bonded to Kdo or a derivative of Kdo. Although the number of *S. fredii* strains investigated is still too small to obtain a consistent conclusion, there is other evidence supporting the idea that K-antigen polysaccharides could have a role in the specificity of soya-bean cultivars/bacterial strains. For instance, mutations in the *nolWXBTUV* genes of *S. fredii* USDA257 result in extending the host range of this strain to include different American soya-bean cultivars that are not normally infected by it. These host-range mutants show clear differences in their LPSs and K-antigens [3].

Although no clear-cut functions have been assigned to rhizobial K-antigen polysaccharides, it has been shown recently that they could be involved in the onset of the alfalfa defence response [36]. The structural diversity of *Sinorhizobium* K-antigen polysaccharides makes it difficult to establish a relationship between their structure and their possible biological activity. In addition, the expression of rhizobial K-antigens has been shown to be affected by legume-derived compounds [3]. This report shows that the repeating unit of the K-antigen polysaccharide can also be a monosaccharide instead of a disaccharide. In addition, strain SVQ293 (a mutant derivative of HH103 that shows a pleiotropic phenotype) produces a K-antigen polysaccharide in which the repeating unit is a trisaccharide composed of galactose, ribose and 5-*O*-methyl-3-deoxynonulosonic acid [5].

S. fredii strain USDA257 can produce two different K-antigen polysaccharides, both containing Kdo in the disaccharide repeating unit. Our results show that, although strain HH103 apparently produces only one K-antigen polysaccharide, it has the intrinsic capacity to produce another CPS (in mutant SVQ293) that is very different in structure [5]. This situation resembles that described for *S. meliloti* Rm 1021 (also called Rm2011), in which two completely different types of exopolysaccharide can be produced. Exopolysaccharide I, or succinoglycan, is produced by the wild-type strain, whereas exopolysaccharide II, or galactoglucan, is produced by some specific mutants [37].

It seems that the presence of an acidic CPS could be a general feature of a broad range of plant symbiont and pathogenic bacteria [3]. Here we have shown that the K-antigen polysaccharide of strain HH103 is not easily detected by using the standard Alcian Blue method unless a very high concentration of the polysaccharide sample is used in the electrophoresis. This fact is a warning of the possibility that the presence of certain types of rhizobial CPS might not be detected in rhizobial strains.

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