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Structural studies of an exopolysaccharide produced by *Alteromonas macleodii* subsp. *fijiensis* originating from a deep-sea hydrothermal vent

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Abstract

The structure of the exopolysaccharide produced by *Alteromonas macleodii* subsp. *fijiensis* recovered from a deep-sea hydrothermal vent has been investigated. By means of chemical analysis and NMR studies, the repeating unit of the polymer was deduced to be a branched hexasaccharide with the structure shown.

→4)-β-D-Glc*p*-(1→4)-α-D-Gal*p*A-(1→4)-α-D-Gal*p*-(1→
3
↑
1
α-D-Glc*p*A
3
↑
1
β-D-Glc*p*A
4
↑
1
4,6-Pyr-β-D-Man*p*

Keywords: Bacterial polysaccharide; Hydrothermal vent; Pyruvic acetal residue; Uronic acid

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1. Introduction

Because of the extreme conditions (high pressures, high-temperature gradient and high concentrations of toxic elements) observed near deep-sea hydrothermal vents, it was expected that bacteria growing under these extreme conditions may produce unusual polysaccharides possessing properties of biotechnological interest. An initial screening of isolates from this environment showed that microbial polysaccharide producers exist under these conditions [1] and allowed the characterisation of several polysaccharides, obtained from laboratory-grown bacteria, for potential applications [2]. Following different oceanographic cruises, bacteria were selected, and their polysaccharides were partially analysed for their chemical and rheological properties [3]. It was shown that an aerobic, mesophilic, heterotrophic bacterium *Alteromonas macleodii* subsp. *fijiensis* [4] secreted significant amounts of a polysaccharide with interesting and potentially useful rheological properties. This report describes the structural elucidation of this polysaccharide, using monosaccharide analysis, methylation analysis, β-elimination studies, and NMR spectroscopy.

2. Results and discussion

Isolation, purification and composition of the polysaccharide.—A crude preparation of the polysaccharide was obtained by precipitation of the culture supernatant of *A. macleodii* subsp. *fijiensis* with EtOH at 40% (v/v). The polymer was purified by anion-exchange chromatography, and fractions corresponding to the main peak, which were eluted with 450 mM NaCl, were combined and lyophilised.

The neutral glycosyl residue composition of the polysaccharide, determined by acid hydrolysis and the preparation and analysis of alditol acetates, indicated that it was composed of glucose, galactose, and mannose in the molar ratio of 1:1:1. GLC analysis of the per-*O*-(trimethylsilyl) (TMS) methyl glycoside derivatives showed glucose, galactose, mannose, glucuronic acid, and galacturonic acid in the relative proportions of 1:1:0.6:2:1, along with an additional peak [t_R 0.67 relative to per-*O*-(trimethylsilyl)-*myo*-inositol]. Each glycosyl residue was determined to be in the D-configuration [5]. The lower amount of mannose observed from the analysis of trimethylsilylated methyl glycosides compared to that observed from alditol acetate analysis indicated that the mannosyl residue was linked to a substituent released by acid hydrolysis and partially released by methanolysis. 1D ^{1}H NMR spectroscopy of the native polysaccharide showed an upfield signal at 1.5 ppm attributed to the protons of a methyl group and suggested the presence of an *O*-(1-carboxyethylidene) group. This was confirmed by the determination of a pyruvic acid content of 7% (w/w) by chemical analysis [6]. GLC–MS analysis of the per-*O*-(trimethylsilyl) methyl mannoside (t_R 0.67) gave a unambiguous characterisation of the mannosyl pyruvic substituent. The mass spectrum (Fig. 1) showed a characteristic fragment ion at m/z 363 $(M-COOMe)^+$, formed by an α-cleavage from the carboxyethylidene ring and consistent with a molecular mass of 422, the predicted value for methyl *O*-(1-carboxyethylidene)hexopyranoside methyl ester di-*O*-TMS ethers [7]. The prominent ion at m/z 204, requires

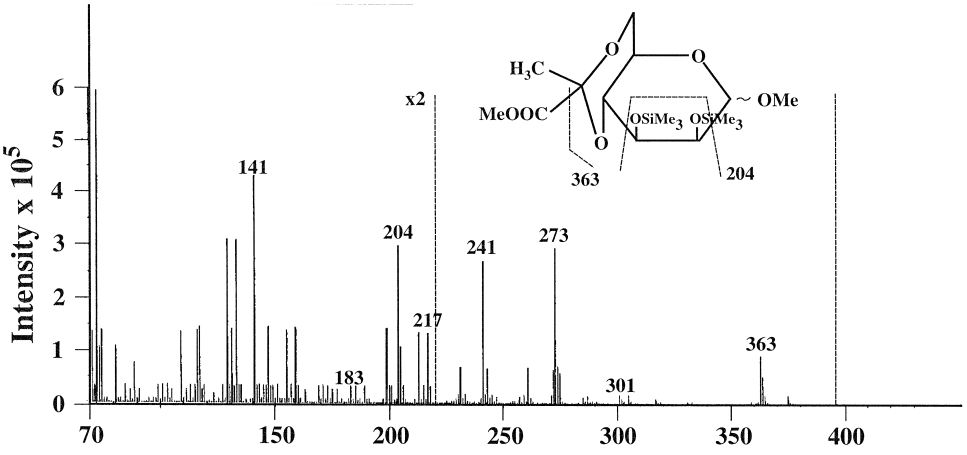


Fig. 1. Electron-impact mass spectrum of methyl 4,6-*O*-(1-methoxycarbonylethylidene)-2,3-di-*O*-trimethylsilylmannoside released by methanolysis of the *A. macleodii* subsp. *fijiensis* exopolysaccharide

adjacent trimethylsilyl groups, and, thus, establishes the 4,6-linkage position of the carboxyethylidene ring of the mannosyl residue [8].

Glycosyl-linkage analysis.—Methylation analysis of the polysaccharide (Table 1) showed the presence of 4-substituted Glc*p*, 4-substituted Gal*p* and 4,6-di-substituted Man*p*. When the polysaccharide was methylated and then reduced with 'Superdeuteride' [$LiB(C_2H_5)_3D$], subsequent analysis revealed three additional components, namely, 3,6-disubstituted Glc*p*, 4,6-disubstituted Glc*p* and 3,4,6-trisubstituted Gal*p*, all of which contained two deuterium atoms on C-6. Thus, these three residues exist in the polysaccharide as 3-substituted Glc*p*A, 4-substituted Glc*p*A, and 3,4-disubstituted Gal*p*A, respectively. These results are consistent with a hexasaccharide repeating unit containing a branch point at a Gal*p*A residue and a side-chain terminated by a 4,6-*O*-(1-carboxyethylidene)-Man*p*.

Position of the uronic acid residues.—β-Elimination of the per-*O*-methylated polysaccharide, followed by ethylation and GLC–MS analysis of the

Table 1
Analysis of the alditol acetates derived from alkylation of *A. macleodii* subsp. *fijiensis* exopolysaccharide before and after β-elimination

Alkylated sugars (as alditol acetates)	t_R[a]	Detector response (%)	
		I[b]	II[b]
4-Et-2,3,6-Me_3Gal[c]	0.51	–	38
2,3,6-Me_3Gal	0.66	26	14[d]
2,3,6-Me_3Glc	0.67	20	43
2,3-Me_2Man	0.75	13	5
2,4-Me_2Glc-6-d_2[e]	0.77	22	–
2,3-Me_2Glc-6-d_2	0.79	11	–
2-MeGal-6-d_2	0.85	8	–

[a] t_R = Retention time for the corresponding alditol acetate relative to that of *myo*-inositol hexaacetate (t_R = 1.00).
[b] I, methylated and carboxyl-reduced polysaccharide; II, methylated, uronic acid-degraded and ethylated polysaccharide.
[c] 4-Et-2,3,6-Me_3Gal = 1,5-di-*O*-acetyl-4-*O*-ethyl-2,3,6-tri-*O*-methyl-galactitol, etc.
[d] Due to the incomplete degradation of the uronic acids.
[e] 2,4-Me_2Glc-6-d_2 = 1,3,5,6-tetra-*O*-acetyl-6,6-dideutero-2,4-di-*O*-methyl-glucitol, etc.

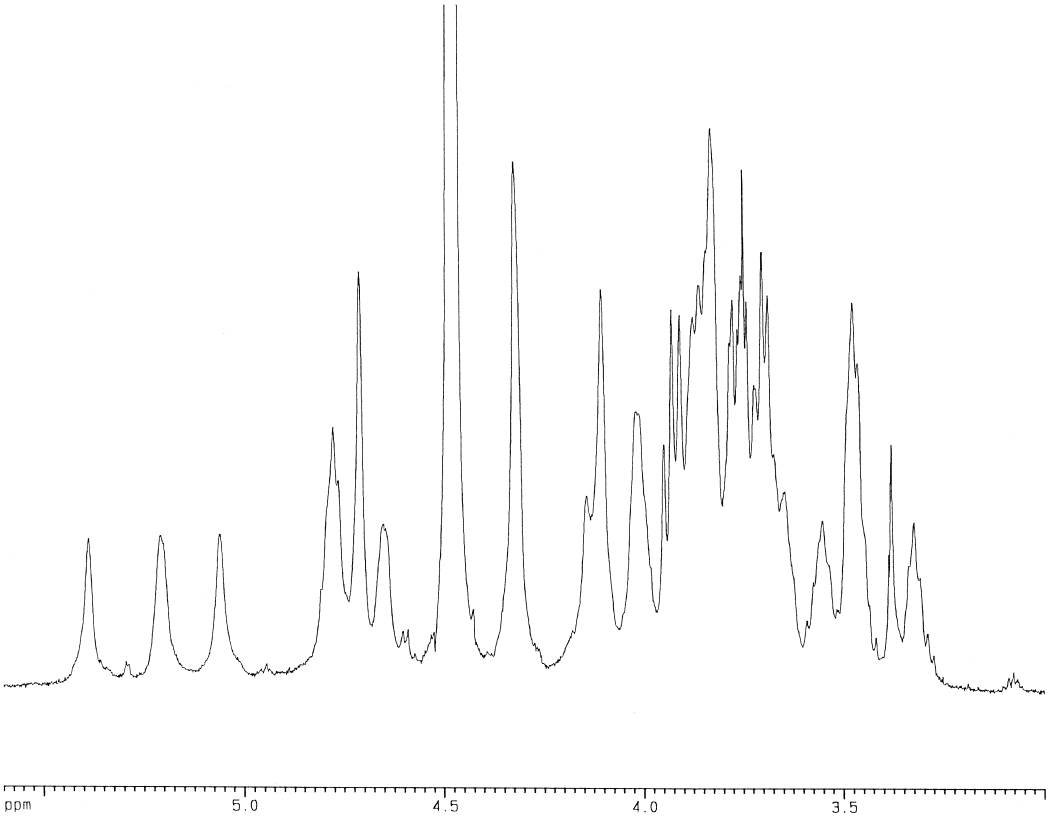


Fig. 2. 500 MHz ^{1}H NMR spectrum recorded at 320 K in deuterium oxide of the depyruvylated *A. macleodii* subsp. *fijiensis* exopolysaccharide

partially methylated and ethylated alditol acetates, showed 4-*O*-ethyl-2,3,6-*O*-trimethyl-Gal*p* as the only ethylated derivative; no ethylated Glc*p* derivative was observed. This ethylated Gal*p* derivative was derived from the 4-substituted Gal*p* (Table 1) whose O-4 was converted to a hydroxyl group on degradation of the uronic acids. This result suggested that the three uronic acid residues constituted a trisaccharide linked to position-4 of the galactopyranosyl residue.

NMR studies.—NMR spectroscopy was performed on the polysaccharide after removal of the pyruvyl group in order to reduce the high viscosity of the solution. The 1D ^{1}H NMR spectrum (Fig. 2) showed a complex anomeric region, but the analysis of the HSQC spectrum (Fig. 3) indicated six signals for H-1/C-1, three α-linked at δ 5.39/101.6, 5.21/97.3 and 5.06/101.4 and three β-linked at δ 4.77/104.1, 4.71/104.1 and 4.70/101.1, defining a hexasaccharide repeating unit, in agreement with the constituent analysis. The residues were labelled A–F in decreasing order of chemical shift of the H-1 resonance.

Due to the poor resolution of the spectra and severe overlaps, it was difficult to extract, without any ambiguities, all the proton assignments. However, values of some chemical shifts in combination with the data of the methylation analysis allowed us to identify the different residues (Table 2). Residues D and E showed upfield chemical shifts of the H-2 signals as expected for sugars with the β-D-*gluco* configuration. Residue E was confirmed as the 4-substituted-β-D-Glc*p* residue due to the presence of the signals for H-6 and C-6, and the downfield C-4 signal at δ 79.3. Residue D was identified as the 4-substituted-β-D-Glc*p*A residue due to the downfield location (δ 82.0) of its C-4 signal. The α-D-*galacto* configuration attributed to residues A and C was in complete agreement with the downfield chemical shift of the H-1 and H-2 resonances. Residue C was attributed to the 3,4-disubstituted-α-D-Gal*p*A residue due to the downfield locations (δ 76.7 and 77.5, respectively) of its C-3 and C-4 signals, along with the downfield position (δ 4.65) of its H-4 resonance.

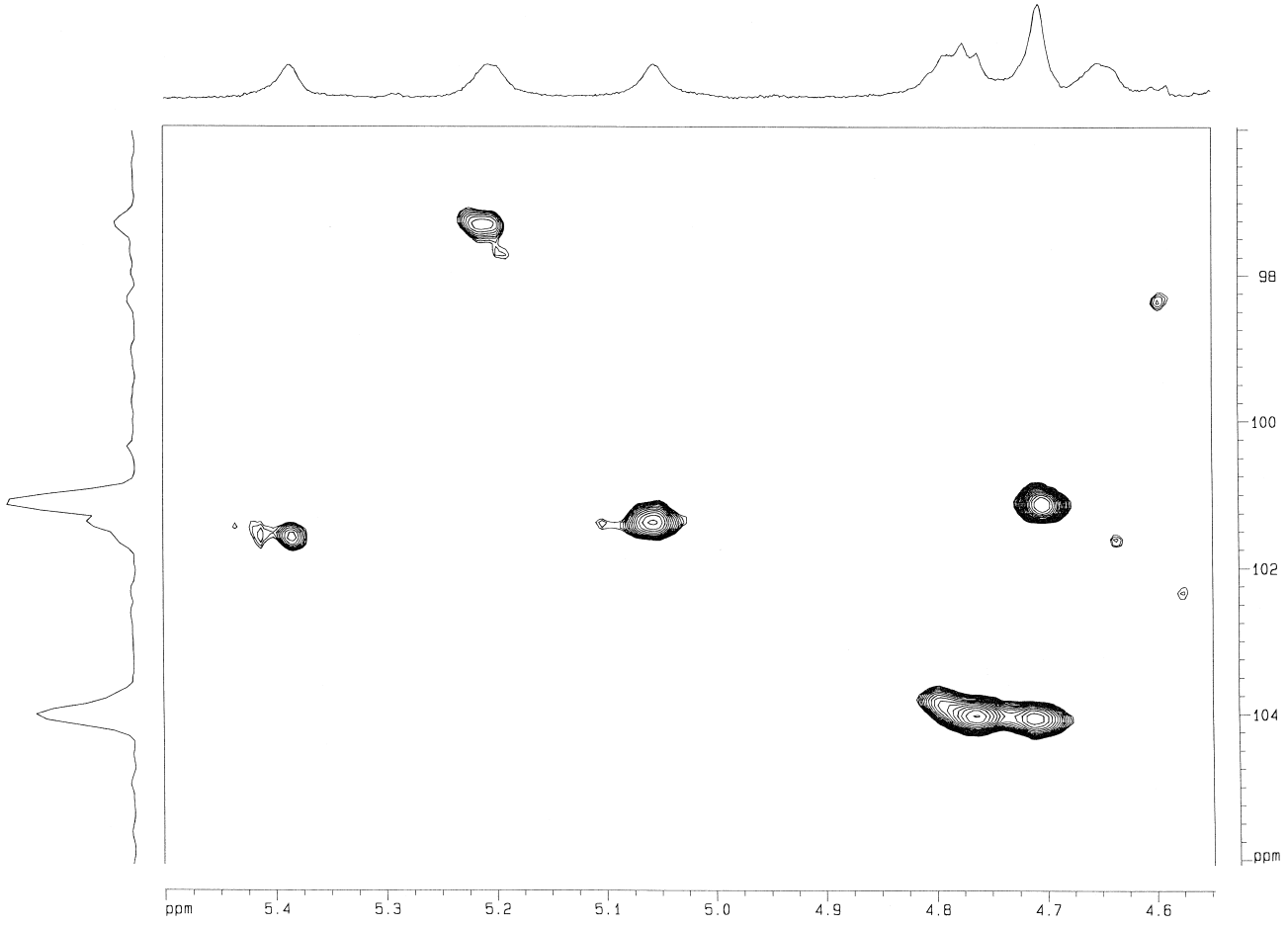


Fig. 3. Anomeric region of the 600 MHz HSQC spectrum recorded at 320 K in deuterium oxide of the depyruvylated *A. macleodii* subsp. *fijiensis* exopolysaccharide

Table 2
Chemical shift (δ, ppm) of the signals in the ^{1}H and ^{13}C NMR spectra of the depyruvylated *A. macleodii* subsp. *fijiensis* exopolysaccharide

Residue	$^{1}H/^{13}C$[a]					
	1	2	3	4	5	6
→4)-α-D-Gal*p*-(1→	5.39	3.89	3.92	4.14	–	
A	101.6	70.3	70.3	78.8	–	–
→3)-α-D-Glc*p*A-(1→	5.21	3.83	4.01	–	–	–
B	97.3	72.1	83.1	–	–	–
→3,4)-α-D-Gal*p*A-(1→	5.06	4.10	4.11	4.65	–	–
C	101.4	68.8	76.7	77.5	–	–
→4)-β-D-Glc*p*A-(1→	4.77	3.46	3.70	3.77	3.86	
D	104.1	74.8	75.7	82.0	77.3	–
→4)-β-D-Glc*p*-(1→	4.71	3.32	3.68	3.55	3.47	3.77–3.94
E	104.1	75.1	77.9	79.3	77.6	62.6
β-D-Man*p*-(1→	4.70	4.32	–	–	–	–
F	101.1	70.7	–	–	–	–

[a]Carbons involved in interglycosidic linkages are underlined.

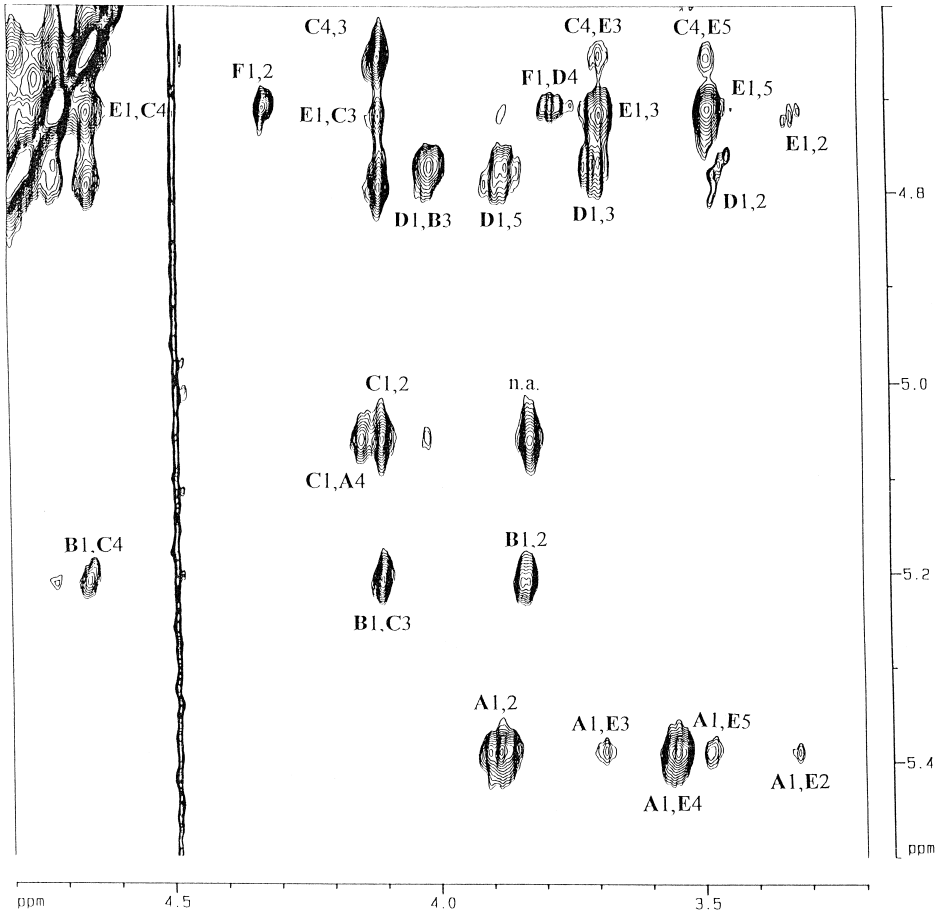
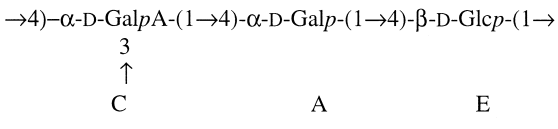


Fig. 4. Anomeric region of the 600 MHz NOESY spectrum recorded at 320 K in deuterium oxide of the depyruvylated *A. macleodii* subsp. *fijiensis* exopolysaccharide

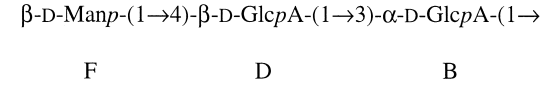
Residue A then could be attributed to the 4-substituted-α-D-Gal*p* residue, as supported by the downfield location of its C-4 and H-4 signals at δ 78.8 and 4.14, respectively. Residue B was attributed to the 3-substituted-α-D-Glc*p*A residue due to the presence of a downfield chemical shift of the C-3, δ 83.1. The only remaining glycosyl component, F, could, therefore, be assigned to the β-D-Man*p* residue, as supported by the downfield position of its H-2 signal at δ 4.32.

Glycosyl sequence information was obtained from a NOESY experiment (Fig. 4). The spectrum showed a clear interresidue contact between H-1 of C and H-4 of A, a result consistent with the data obtained by β-elimination. The H-1 of residue A gave a NOE contact with H-4 of E, as well as contacts of lower intensity with H-2, H-3 and H-5 of E. Therefore, the repeating unit of the polysaccharide contained the trisaccharide unit **1**,



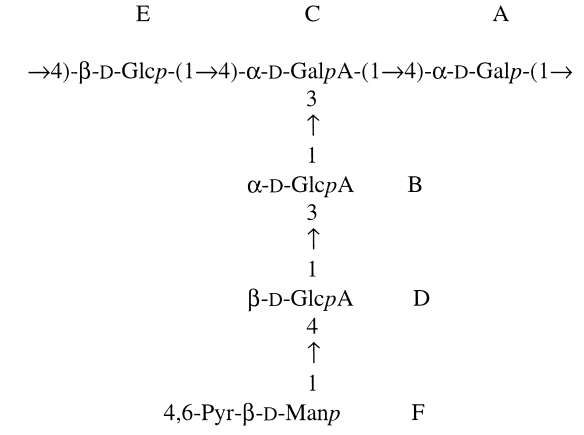
1

H-1 of **F** showed a long-range NOE correlation to H-4 of D, and the H-1 of residue D gave a NOE contact with H-3 of B. On the basis of these data, it was concluded that the repeating unit of the polysaccharide also contained the trisaccharide unit **2**,



2

The H-1 protons of residues E and B did not provide conclusive structural information because both gave a clear interresidue contact with H-3 and H-4 of C. However, H-4 of **C** (δ 4.65) gave NOE contacts with H-3 and H-5 of E. These results demonstrate that **E** is linked to the 4-position of C. Therefore, structure **2** must be attached to structure **1** at the 3-position of the galacturonosyl residue. Thus, the structure of the repeating unit of the *A. macleodii* subsp. *fijiensis* polysaccharide can be written as:



3. Experimental

Bacterial strain.—*A. macleodii* subsp. *fijiensis* was discovered near an active hydrothermal vent located in the rift system of the North Fiji Basin during the 'Starmer' oceanographic cruise in 1989.

Production and isolation of the polysaccharide.—The polysaccharide was produced as previously described [1,9] in a 2-L fermenter containing 1 L of 2216E medium [10] supplemented with glucose, at atmospheric pressure, a temperature of 25 °C and at pH 7.2. After 30 h, cells were removed from the medium by high-speed centrifugation at 20,000 *g* for 2 h. The polysaccharide was precipitated from the supernatant with EtOH at 40% (v/v) and washed with EtOH–water (70% to 100%, v/v). The yield was 5 g of polysaccharide, which was stored at room temperature.

Purification of the polysaccharide.—The crude polysaccharide was dissolved in 50 mM Tris solution (pH 7.5) and applied to an anion-exchange chromatographic column (35×2.6 cm) of Sepharose CL-6B. Elution was performed using the same buffer and a 0.1 M–1 M NaCl gradient. Fractions were collected, assayed for uronic acid [11] and for neutral sugars [12], dialysed and lyophilised.

General methods.—GLC–MS was carried out on alditol acetates and partially methylated alditol acetates on an HP-5890 system using a SP2330 fused silica column (0.25 mm×30 m) and a temperature program of 170 °C to 240 °C at 4 °C/min. GLC–MS on the per-*O*-trimethylsilyl derivatives was performed on a HP-5890 system using a DB-5 column (0.25 mm×15 m) and a temperature programme of 160 °C to 260 °C at 3 °C/min.

Constituent analysis.—Methanolysis was performed in methanolic 1 M HCl, and the methyl

glycosides were converted to the corresponding trimethylsilyl derivatives as described by Montreuil et al. [13]. Hydrolysis was performed in 2 M TFA, and the monosaccharides were reduced and converted in the corresponding alditol acetates as described by Blakeney et al. [15]. The absolute configuration of the sugars was determined as devised by Gerwig et al. [5]. Pyruvic acid content was estimated colorimetrically by the 2,4-dinitrophenylhydrazone procedure [6].

Methylation analysis.—Glycosyl-linkage positions were determined using a modification of the Hakomori procedure [14] using lithium methanide-methylsulfinyl [16] and MeI in Me_2SO. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore) [17]. Reduction of ester groups with 'Superdeuteride' [$LiB(C_2H_5)_3D$] was carried out according to York et al. [18]. The methylated product was then hydrolysed in 2 M TFA (2 h, 120 °C), reduced, and acetylated.

β-*Elimination*.—The sample was β-eliminated according to the procedure of Aspinall and Rosell [19]. Briefly, the methylated polysaccharide was dissolved in Me_2SO, BuLi was added to form the lithium methylfinylmethanide anion, and the sample was stirred overnight at room temperature. The sample was then ethylated as described [19], hydrolysed, and converted to its alditol acetate by conventional methods.

NMR spectroscopy.—After removal of the pyruvic acid (in aqueous 2% AcOH for 2 h at 100 °C), NMR spectra of solutions in deuterium oxide containing 0.1 M NaCl (in order to reduce the viscosity) were recorded at 320 K using acetone as the internal standard (δ_H 2.225 ppm, δ_{CH_3} 31.45 ppm) on Bruker AMX 500 and AMX 600 spectrometers using UXNMR software. ^1H–^1H-correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), and heteronuclear single quantum coherence (HSQC) were used to assign signals and were performed according to standard pulse sequences. For interresidue correlations, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment was used with a mixing time of 160 ms.

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