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Structures of two polysaccharides of *Campylobacter jejuni* 81116☆

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Dedicated to Professor Horton on the occasion of his 70th birthday

Abstract

Campylobacter jejuni 81116 has been extensively investigated in studies on genes associated with the synthesis of *Campylobacter* lipopoly/lipooligosaccharides (LPS/LOS). Despite these investigations, data on the chemical structure of polysaccharides from *C. jejuni* 81116 have been absent. The present study was undertaken to fill that void. Biomass was grown in large quantities on agar medium, harvested and extracted by hot phenol–water extraction. Subsequently, extracts were treated by DNase, RNase and proteinase K to remove contaminants. After mild acid treatment, followed by preparative gel-permeation and anion-exchange chromatography, fractions were isolated and studied by ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, ^{1}H,^{13}C HMQC and HMBC experiments. These advanced investigations revealed the occurrence of two different polysaccharides in the approximate ratio of 3:1, each having a tetrasaccharide repeating unit. Polysaccharide A contained glucose, glucuronic acid and mannose, and is *O*-acetylated. Polysaccharide B contained glucose, galactose and *N*-acetylglucosamine. Importantly, polysaccharide A is acidic, whereas polysaccharide B is neutral.

(A)

OAc (20%)
↓
6
→ 3)-β-D-Glc*p*-(1→2)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1 →
3
↑
OAc (30%)

(B)

→3)-β-D-Glc*p*NAc-(1→ 6)-α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→
3
↑
β-D-Glc*p*NAc-(1

Keywords: *Campylobacter jejuni*; Polysaccharide structure; NMR abstract

1. Introduction

Although *Campylobacter jejuni* is recognised as a leading cause of human enteritis,[1] antecedent infection with *C. jejuni* is the single most common predisposing factor for the development of the neurological disorder Guillain–Barré syndrome (GBS).[2,3] Molecular mimicry of gangliosides in the core oligosaccharides of *C. jejuni*

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lipopoly/lipooligosaccharides (LPS/LOS) has been postulated to play a role in the pathogenesis of GBS by inducing autoreactive antibodies to human gangliosides.[3,4] This has proven to be an impetus for the chemical characterisation of these *C. jejuni* glycolipids.[5] Previous structural studies have shown that the terminal regions of the core oligosaccharides from LPS/LOS of specific serotypes mimic the structures of human gangliosides, particularly those strains associated with GBS development.[3–5]

However, we have shown previously that phenol–water extraction, commonly used for isolation of LPS/LOS, can also extract independent polysaccharides of *C. jejuni*, e.g., those of serotypes HS:3 and HS:41.[6,7] Subsequently, a cluster of genes with significant sequence homology to the capsular polysaccharide (*kps*) genes of *Escherichia coli* were identified in *C. jejuni*[8] and, more recently, capsule-like structures have been observed in certain *C. jejuni* strains using electron microscopy and specialised staining.[9] Associated with these findings, genetic and mutational analysis of *kps* genes in a limited number of *C. jejuni* strains suggested that the heat-stable antigen serotyping scheme of *C. jejuni* is based on capsular polysaccharides alone.[8] On the other hand, more recent biochemical and serological evidence indicates the involvement of both polysaccharides and LPS/LOS in the serological reactions.[10]

C. jejuni 81116 was originally isolated from a human water-borne outbreak of gastroenteritis,[11] and has been extensively investigated in studies on genes associated with the synthesis of *C. jejuni* LPS/LOS.[12,13] Despite these investigations, data on the chemical structure of polysaccharides from *C. jejuni* 81116 have been absent and, thus, the present study was undertaken to fill that void.

2. Results and discussion

Mild-acid degradation of the phenol–water extract of *C. jejuni* 81116 with dilute acetic acid resulted in a polysaccharide mixture, which was isolated by GPC on Sephadex G-50 (fraction I) and further fractionated using anion-exchange chromatography on DEAE-Trisacryl M to yield neutral and acidic polysaccharides.

Sugar analysis using GLC of the alditol acetates derived after acid hydrolysis revealed glucose and mannose in the ratios 2.1:1 in the acidic polysaccharide and glucose, galactose and 2 amino-2-deoxyglucose in the ratios 1:0.8:1.7 in the neutral polysaccharide. GLC analysis of the acetylated glycosides with a chiral alcohol showed that all sugars have the D configuration.

The ^{13}C NMR spectrum of the polysaccharide (fraction I) showed a number of signals attributable to the methyl groups of *O*-acetyl groups. The spectrum was exceedingly complex, and, therefore, the de-O-acetylated polysaccharide was used for further investigations.

The ^{13}C NMR spectrum of the de-O-acetylated polysaccharide (fraction I) (Fig. 1, Table 1) contained signals for four anomeric carbons at δ 104.8, 101.0, 102.0 and 100.3 ppm, and signals of a lesser intensity for another four anomeric carbons at δ 100.0, 102.4 and 104.3 ppm (the peak at δ 100.0 ppm was of double intensity), indicating that a major and a minor series were present. Two signals at δ 23.4 and 23.3 ppm indicated the presence of two *N*-acetyl groups while two peaks at δ 55.2 and 56.8 indicated the presence of two N-substituted sugars. Five signals at δ 60.5, 61.4, 61.6, 62.0 and 62.1 ppm were indicative of C–CH_2OH hydroxymethyl groups (e.g., C-6 of hexoses). The presence of three signals at δ 175.4, 175.1 and 174.2 ppm,

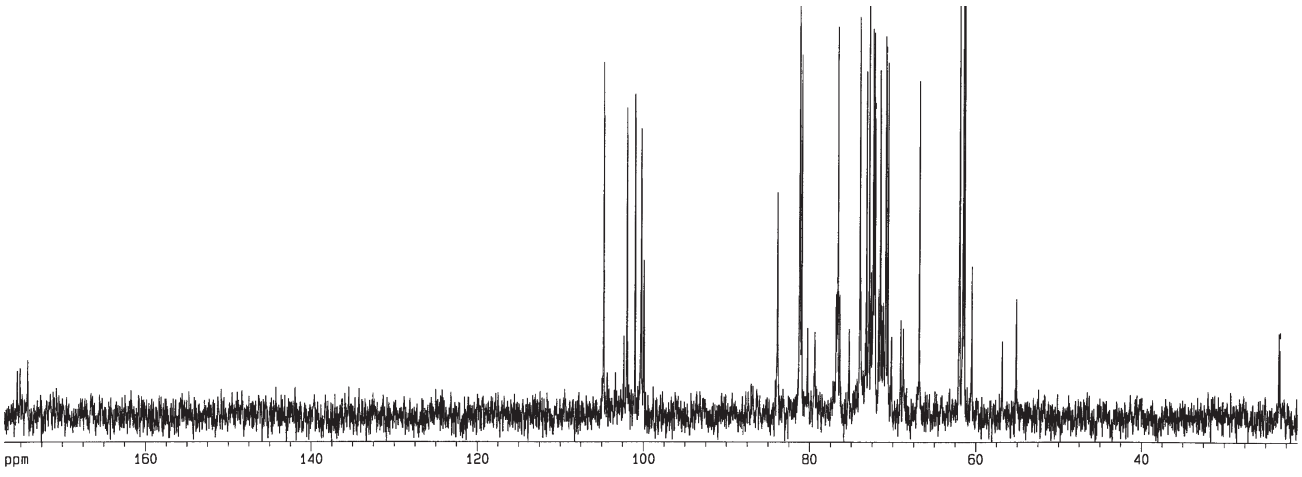


Fig. 1. 125 MHz ^{13}C NMR spectrum of the de-O-acetylated polysaccharide.

Table 1
^{13}C NMR data (δ, ppm) for the de-O-acetylated polysaccharide sample

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide A							
A	→3)-β-D-Glc*p*-(1→	104.8	73.2 (-1.2)	83.9	70.6 (-0.3)	76.6	61.6
B	→2)-α-D-Glc*p*A-(1→	101.0	81.2	72.4 (-1.6)	72.3	72.2	174.2
C	→3)-α-D-Man*p*-(1→	102.0	70.91 (-0.6)	81.0	66.9	74.0	62.0
D	→3)-α-D-Glc*p*-(1→	100.3	71.6 (-1.1)	81.2	70.8 (-0.1)	72.9	61.4
Polysaccharide B							
E	→3)-β-D-Glc*p*NAc-(1→	102.4	55.2 (-1.8)	80.3	71.8 (+0.6)	76.6	61.6
F	→6)-α-D-Glc*p*-(1→	100.0	73.2	73.9	70.2	71.2	68.8
G	→4)-α-D-Gal*p*-(1→ 3) ↑	100.0	69.1	79.4	76.9	72.7	60.5 (-1.9)
H	β-D-GlcpNAc-(1	104.3 (+8.1)	56.8	75.3	71.35	76.7	62.1

The chemical shifts for NAc are δ 23.3 and 23.4 (Me) and 175.1 and 175.4 (CO). The glycosylation effects important for the determination of relative absolute configuration[16] are given in parentheses.

attributable to carbonyl groups, indicated that either *N*-acetyl substituents and/or uronic acid(s) were present. The absence, from the ^{13}C NMR spectrum, of any signals for non-anomeric sugar carbons at a lower field than δ 81 ppm demonstrated the pyranoid form of all sugar residues.[14]

The low-field region of the ^{1}H NMR spectrum of the de-O-acetylated polysaccharide sample (Fig. 2, Table 2) contained signals for eight anomeric protons, four of which were of greater magnitude than the other four and were in the approximate ratio 3:1, a further indication that a more abundant series (**A**) and less abundant

Table 2
^{1}H NMR data (δ, ppm) for the de-O-acetylated polysaccharide sample

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Polysaccharide A								
A	→3)-β-D-Glc*p*-(1→	4.70	3.48	3.67	3.67	3.50	3.93	3.76
B	→2)-α-D-Glc*p*A-(1→	5.48	3.78	3.98	3.68	4.37		
C	→3)-α-D-Man*p*-(1→	5.22	4.21	4.02	3.92	4.01	3.87	3.78
D	→3)-α-D-Glc*p*-(1→	5.33	3.65	3.88	3.57	4.03	3.84	3.77
Polysaccharide B								
E	→3)-β-D-Glc*p*NAc-(1→	4.59	3.86	3.77	3.77	3.50	3.92*	3.80*
F	→6)-α-D-Glc*p*-(1→	4.95	3.48	3.70	3.46	4.26	4.16	3.95
G	→4)-α-D-Gal*p*-(1→ 3) ↑	5.46	3.96	3.85	4.28	3.94	3.81-3.76	
H	β-D-GlcpNAc-(1	4.65	3.62	3.58	3.44	3.44	3.94	3.77

The chemical shifts for NAc are δ 2.03 and 2.08. * Assignment could be interchanged.

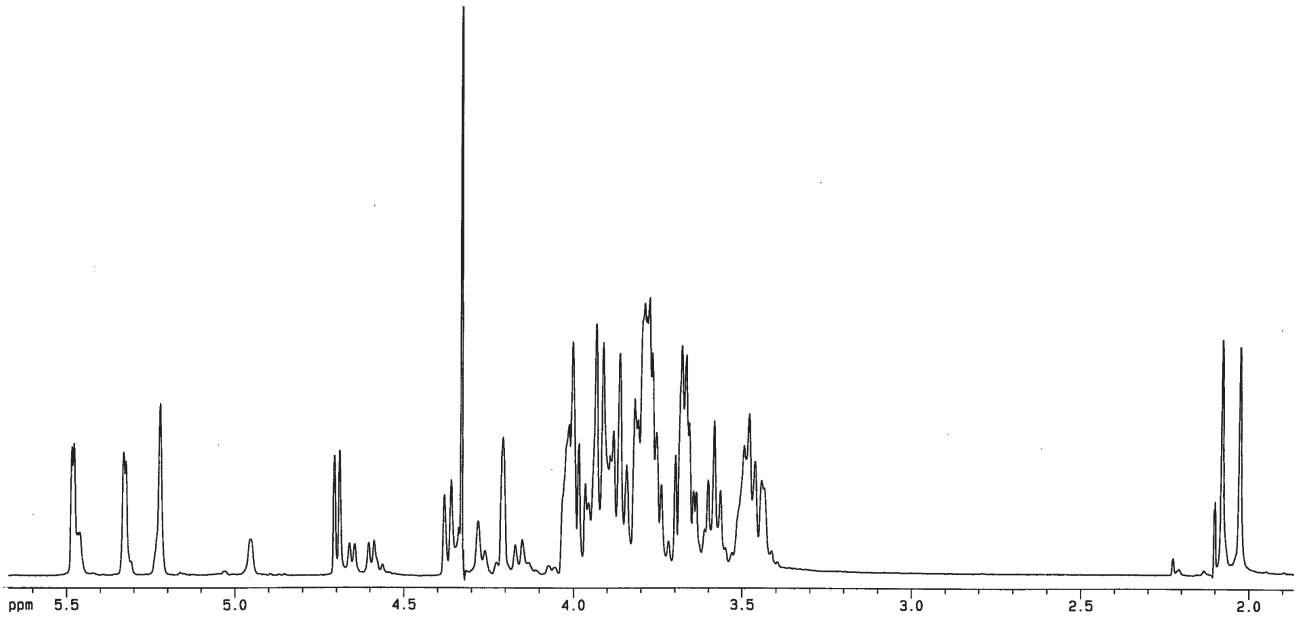


Fig. 2. 500 MHz ^{1}H NMR spectrum of the de-O-acetylated polysaccharide.

series (**B**) were present. The high-field region of the spectrum contained signals for two *N*-acetyl methyl groups at δ 2.03 and 2.08 ppm.

The ^{1}H NMR spectrum of the de-O-acetylated polysaccharide (Fig. 2) was assigned using 2D COSY and ^{1}H,^{1}H relayed COSY experiments (Table 2). The

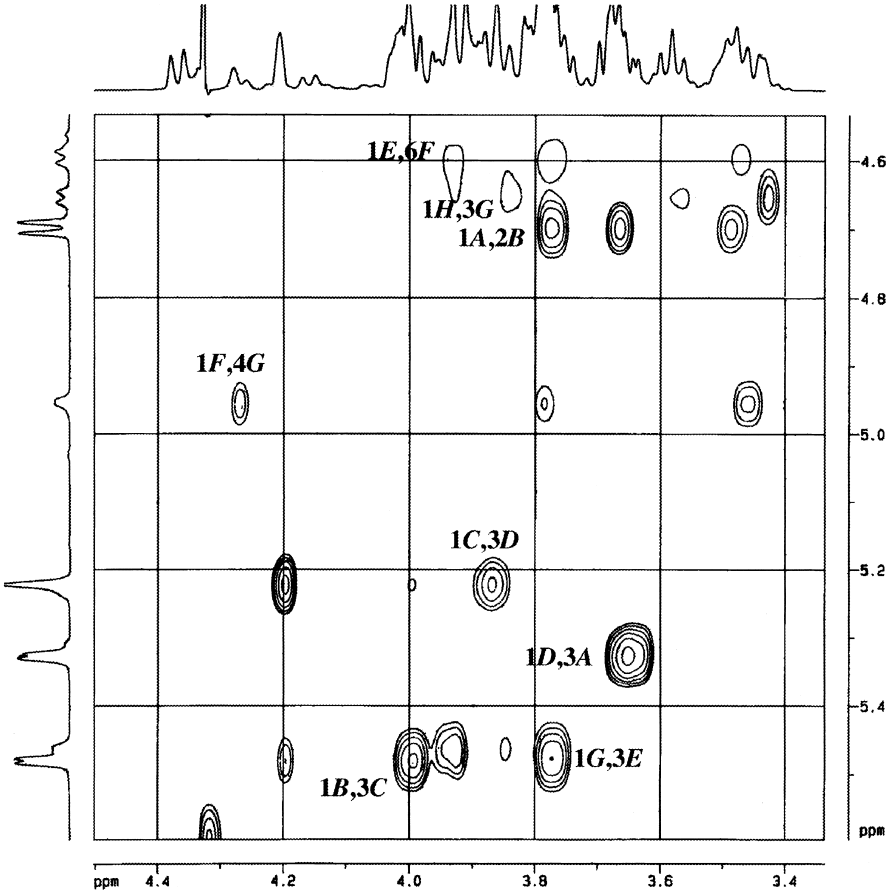


Fig. 3. Part of a 2D NOESY spectrum of the de-O-acetylated polysaccharide. The corresponding parts of the ^{1}H spectrum are displayed along the vertical and horizontal axes.

Table 3
HMBC inter-residue correlations for the de-O-acetylated polysaccharide sample

	Sugar Residue	Connectivities	
A	→3)-β-D-Glc*p*-(1→	H1-C2***B***	H3-C1***D***
B	→2)-α-D-Glc*p*A-(1→	H1-C3***C***	H2-C1***A***
C	→3)-α-D-Man*p*-(1→	H1-C3***D***	H3-C1***B***
D	→3)-α-D-Glc*p*-(1→	H1-C3***A***	H3-C1***A***
E	→3)-β-D-Glc*p*NAc-(1→	H1-C6***F***	
F	→6)-α-D-Glc*p*-(1→		
G	→4)-α-D-Gal*p*-(1→ 3) ↑	H1-C3***E***	H4-C1***F***
H	β-D-GlcpNAc-(1	H1-C3***G***	

spin-systems for all residues were assigned on the basis of their $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constant values, thus identifying three residues with *gluco*- and one with *manno*-configuration in series **A**, and three residues with *gluco*- and one with *galacto*-configuration in series **B**.

Relatively large coupling constant values of ~8 Hz showed that one *gluco* residue in series **A** and two *gluco* residues in series **B** were β-linked, while two *gluco* residues from series **A** and the *galacto* residue and the remaining *gluco* residues from series **B** were α-linked ($J_{1,2} < 3$ Hz). The anomeric configuration of the *manno* residue was determined using a HMQC experiment (see below). *N*-Acetylation of the two sugars with β-*gluco* configuration in series **B** was confirmed upon assignment from a HMQC experiment of their C-2 peaks at δ 55.2 and 56.8 which are characteristic of N-substitution. The peak at δ 174.2 was assigned as C-6 of α-Glc*p*A in the main series (**A**) due to its lower-field position relative to the peaks at 175.4 and 175.1 (spectrum was recorded at pH 3.5). These latter peaks were assigned to the *N*-acetyl carbonyl groups of the two β-Glc*p*NAc residues in series **B**.

Sequence and linkage analyses of the de-O-acetylated polysaccharide sample were performed using a NOESY experiment (Fig. 3) which revealed all the linkages for the two polysaccharides. For the main series (**A**), the following correlations were observed: Glc*p*-β(1→2)-Glc*p*A (δ 4.70/3.78 ppm); Glc*p*A-α(1→3)-Man*p* (δ 5.48/4.02 ppm); Man*p*-α(1→3)-Glc*p* (δ 5.22/3.88 ppm) and Glc*p*-α(1→3)-Glc*p* (δ 5.33/3.67 ppm). For the minor series (**B**), the following correlations were observed: Glc*p*-α(1→4)-Gal*p* (δ 4.95/4.28 ppm); Gal*p*-α(1→3)-Glc*p*NAc (δ 5.46/3.77 ppm); Glc*p*NAc-β(1→6')-Glc*p* (δ 4.59/3.95 ppm) and Glc*p*NAc-β(1→3)-Gal*p* (4.65/3.85 ppm). These results were confirmed by a HMBC experiment (Table 3). No evidence of linkage between the two series of signals was present.

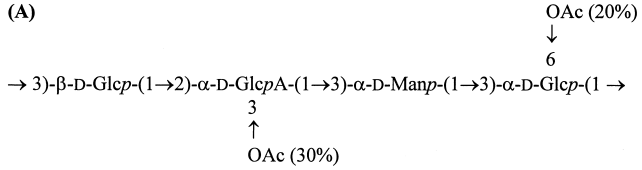
The ^{13}C NMR spectrum (Fig. 1) of the polysaccharide sample was assigned using a 2D ^{1}H, ^{13}C HMQC experiment (Table 1). This revealed the position of C-5 of α-Man to be δ 74.0, thus indicating an α configuration for this residue.[15] Analysis of the glycosylation effects in the spectrum confirmed the positions of substitution of the monosaccharide residues[15,16] and allowed determination of the absolute configurations of the residues; all were found to be D sugars (Table 1).

Therefore, the de-O-acetylated sample contains two polysaccharides present in the ratio 3:1. The more abundant polysaccharide (**A**) consists of an unbranched tetrasaccharide repeating unit containing one residue each of β-D-Glc*p*, α-D-Glc*p*A, α-D-Man*p* and α-D-Glc*p*. The second polysaccharide (**B**) consists of a branched tetrasaccharide repeating unit, containing one residue each of α-D-Glc*p*, α-D-Gal*p*, β-D-Glc*p*NAc and a terminal residue of β-D-Glc*p*NAc.

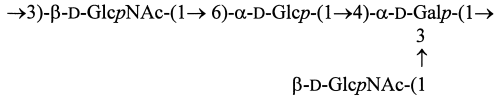
The position of O-acetylation was investigated by recording proton spectra of the native polysaccharide sample. COSY and TOCSY experiments showed that the more abundant polysaccharide was 30% O-acetylated at position 3 of α-D-Glc*p*A and 20% at position 6 of α-D-Glc*p*.

Therefore, the two polysaccharides have the following structures:

(A)



(B)



Thus, the results collectively show that an acidic polysaccharide (structure **A**) and a neutral polysaccharide (structure **B**) were present in the approx ratio 3:1 in the acid-degraded phenol–water extracts of *C. jejuni* 81116. Similar to serotypes HS:3 and HS:41 in which we have found polysaccharides independent of core and lipid A of LPS/LOS,[6,7] core sugars (e.g., heptoses and Kdo) were not detected in the two polysaccharides of *C. jejuni* 81116. Although this may indicate that the polysaccharides are independent of LPS, the proportion of core sugars on a weight basis would be expected to be low and may be below the detection limits of the analysis. Nevertheless, this contrasts with other bacterial species, e.g. *E. coli*, where simultaneous production of neutral and acidic polysaccharides occur as an O-chain polysaccharide and as an independent polysaccharide.[17] On the other hand, phase variation in capsular polysaccharide expression has been observed in *C. jejuni* 81-176 and two polysaccharides with differing physical properties have been observed after mutational analysis of the *kpsM* gene.[18] However, no structural data is available, to date, on these polysaccharides. Importantly, capsular polysaccharide of *C. jejuni* 81116 has been visualised in electrophoretic analysis using selective staining with Alcian blue.[19] The cloning and characterisation of a 16 kb region of DNA involved in LPS/LOS biosynthesis of *C. jejuni* 81116 has been described.[12] Moreover, it was suggested that this 16 kb region was sufficient to produce high-molecular-weight LPS in heterologous expression experiments in *E. coli*, but no genes similar to *kpsM*, *kpsC* and *kpsS* genes, required for polysaccharide synthesis, were present in that region.

At present, we are attempting to chemically separate the two polysaccharides from phenol–water extracts of *C. jejuni* 81116 before acid hydrolysis and hence verify their origin. Preliminary NMR analyses of gel-chromatography fractions enriched for structure A-containing molecules are consistent with this polysaccharide being derived from capsular polysaccharide, whereas the neutral polysaccharide B appears LPS-related (Kilcoyne et al., unpublished results). However, further and more detailed analyses are required to resolve this identity issue. Nevertheless, the occurrence of an LPS-independent, capsular-like polysaccharide in *C. jejuni* 81116 is consistent with the recent findings that mutation of the *waaF* gene, encoding a heptosyltransferase, an enzyme important for LPS core synthesis in this strain, did not inhibit polysaccharide production.[20]

3. Experimental

Bacterial strain, growth conditions, and isolation and degradation of polysaccharides.—*C. jejuni* 81116 was grown in large quantities on blood agar under microaerobic conditions and harvested as described previously. The LPS and associated polysaccharides produced by the bacterium were extracted from freeze-dried biomass by hot phenol–water treatment into the water phase.[21] Subsequently, this phase was enzymatically treated with RNase A, DNase II and proteinase K to ensure purity from contaminating nucleic acids and proteins.[22] The yield of extract was 18% (bacterial dry weight). The extract (82 mg) was dispersed in water and degraded with 1% HOAc at 100 °C for 2 h. A lipid precipitate was removed by centrifugation, and the supernatant solution was subjected to GPC on a Sephadex G-50 column (80×2.5 sm) with 0.05 M pyridinium acetate buffer pH 4.5. The effluent was monitored using a Knauer differential refractometer and yielded three fractions, one of which was a polysaccharide (fraction I) and was studied directly using NMR spectroscopy. Also, this fraction was further fractionated by anion-exchange chromatography on a DEAE-Trisacryl M column (18×1.5 cm) to yield neutral (0.005 M sodium phosphate buffer, pH 6.3.) and acidic (0.1 M sodium phosphate buffer, pH 6.3) fractions, both of which were examined by NMR and shown to be the major components of the polysaccharide mixture (fraction I). O-Deacetylation of the polysaccharide (fraction I, 32 mg) was performed with aq 12.5% ammonia (16 h, 37 °C) followed by GPC on a TSK-40 column (70×2.0 cm) in aq 1% HOAc.

Sugar analysis.—LPS was hydrolysed with 2 M trifluoroacetic acid (120 °C, 2 h), the hydrolysate was evaporated, and monosaccharides identified by GLC of the derived alditol acetates[23] using a Hewlett–Packard 5880 instrument equipped with a DB-5 fused-silica capillary column (25 m $\times$ 0.25 mm) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The absolute configurations of the monosaccharides were determined essentially as described.[24]

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined as solutions in 99.97% D_2O. All spectra were recorded, using internal acetone (δ_H 2.225, δ_C 31.45) as a reference, at a temperature of 50 °C on a Bruker DRX-500 MHz spectrometer equipped with an SGI INDY workstation, where data were acquired and performed using XWINNMR 1.1 version software. The parameters used for 2D NMR experiments were the same as described previously.[7]

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