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Structure of the O-specific polysaccharide from the lipopolysaccharide of *Citrobacter gillenii* O11, strain PCM 1540

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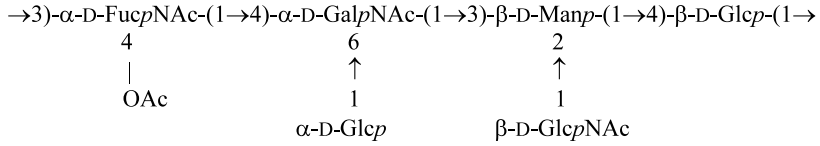
Abstract

The O-specific polysaccharide of the lipopolysaccharide of *Citrobacter gillenii* PCM 1540 (serogroup O11) consists of D-Glc, D-Man, D-GalNAc, D-GlcNAc, 2-acetamido-2,6-dideoxy-D-galactose (D-FucNAc) and *O*-acetyl groups in the ratios 2:1:1:1:1:1. On the basis of sugar and methylation analyses and Smith-degradation along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, the following structure of the branched hexasaccharide repeating unit was established:



Citrobacter werkmanii PCM 1541 belonging to the same serogroup O11 was found to have an R-form lipopolysaccharide devoid of the O-specific polysaccharide.

Keywords: *Citrobacter gillenii*; O-Antigen; Lipopolysaccharide; Bacterial polysaccharide structures; Serological classification

1. Introduction

Gram-negative bacteria of the genus *Citrobacter* are opportunistic pathogens that often cause serious human infections, especially in neonates, children and immunocompromised patients.[1–3] Since 1932, the classification of *Citrobacter* has undergone numerous changes, and these microorganisms were known under a variety of names. Serological studies on *Citrobacter* strains and chemical analysis of their lipopolysaccharides (LPS) enabled division of the genus into 43 O-serogroups[4] and 20 chemotypes.[5] Recently, on the basis of genetic studies, 11 *Citrobacter* species have been distinguished,[6–8] and all strains included formerly in the species *C. freundii* or to the Bethesda-Balleroup group reclassified by Miki et al.[9]

The chemical studies carried out on the lipopolysaccharides (LPS) of *Citrobacter* resulted in elucidation of about 30 structures of the O-specific polysaccharides (OPS, O-antigens) of strains belonging to different O-serogroups.[10] Now we report on the structure of the OPS of *Citrobacter gillenii* O11, strain PCM 1540. The LPS of another strain, *Citrobacter werkmanii* PCM 1541, belonging to the same serogroup O11 was also examined and found to be devoid of the OPS.

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2. Results and discussion

2.1. Isolation and characterisation of the LPS

LPS of *C. gillenii* PCM 1540 and *C. werkmanii* PCM 1541 were isolated by phenol–water extraction in a yield 1.1 and 5.3%, respectively. In SDS-PAGE, LPS of strain PCM 1540 showed a ladder-like banding pattern corresponding to S-form LPS with OPS chains of different length, whereas LPS of strain PCM 1541 gave only fast migrating bands characteristic for LPS of R-type (Fig. 1). In passive hemagglutination test, anti-*C. werkmanii* PCM 1541 serum reacted with the homologous LPS and did not cross-react with the LPS of *C. gillenii* PCM 1540.

On mild acid hydrolysis followed by fractionation of the carbohydrate material by GPC on Sephadex G-50, the LPS of strain PCM 1540 afforded five fractions: P_1 (a long-chain OPS), P_{1A} (a shorter-chain OPS), P_2 (a core oligosaccharide substituted with a very short-chain OPS), P_3 (an unsubstituted core oligosaccharide) and a low-molecular mass material containing 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in yields 38, 7, 14, 21 and 20%, respectively, of the total amount of the material eluted from the column. LPS of strain PCM 1541 gave only two fractions: P_3 (a core oligosaccharide) and a Kdo-containing material in yields 69 and 31%, respectively, with no high-molecular-mass OPS.

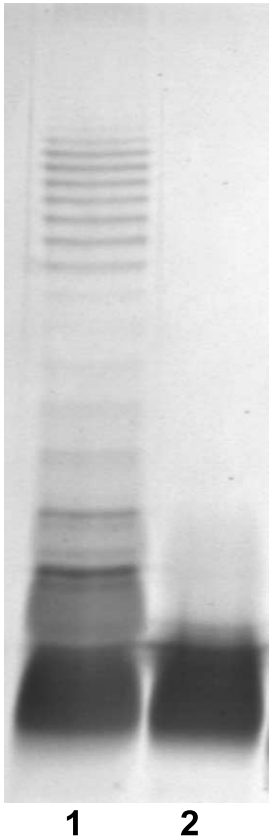


Fig. 1. Silver stained SDS-PAGE profiles of the LPS of *C. gillenii* PCM 1540 (lane 1) and *C. werkmanii* PCM 1541 (lane 2).

Therefore, *C. werkmanii* PCM 1541, which, together with *C. gillenii* PCM 1540, has been classified to serogroup O11, possesses an R-type LPS and should be reclassified as an O-antigen-lacking strain. Our preliminary chemical data (sugar and methylation analyses) of the core oligosaccharides (fractions P_3) showed that their structures may be identical in the two strains.

2.2. Chemical analyses of the O-specific polysaccharide

The OPS from *C. gillenii* PCM 1540 (fraction P_1) was used for detailed chemical studies. Sugar analysis of the OPS by GLC–MS of the alditol acetates revealed Glc, Man, GlcN, GalN and 2-amino-2,6-dideoxygalactose (fucosamine, FucN) in molar ratios 1.4:0.6:1.0:0.9:1.0 or 1.8:0.8:1.0:0.7:0.5, respectively, when 10 M HCl or 2 M CF_3CO_2H was used for hydrolysis. *O*-Acetyl groups were found in the amount of 0.6 μmol/mg. The presence of Man and FucN was confirmed by PC of the OPS hydrolysate (10 M HCl); the mobility of FucN was R_{Glc} 0.85.

The D configuration of Glc, Man and GlcN was demonstrated by treatment of the OPS hydrolysate (2 M CF_3CO_2H) with hexokinase in the presence of ATP, which resulted in full phosphorylation of these monosaccharides (data of GLC–MS of the alditol acetates). The D configuration of Glc was confirmed using D-glucose oxidase and that of GalN determined using D-galactose oxidase; the content of Glc and GalN in the OPS hydrolysates (2 M CF_3CO_2H, 120 °C, 2 h and 4 M HCl, 105 °C, 18 h) was 20 and 11.5%, respectively. FucN was proved to have the D configuration by GLC of the acetylated (*S*)-2-octyl glycosides.

Methylation analysis of the OPS (Table 1) revealed the terminal Glc, terminal GlcN, 4-substituted Glc, 3-substituted FucN, 2,3-disubstituted Man and 4,6-disubstituted GalN.

2.3. Smith degradation of the O-specific polysaccharide

The OPS (30 mg) was *O*-deacetylated with aq 12% ammonia (20 °C, overnight), and the *O*-deacetylated OPS was oxidised with 0.1 M $NaIO_4$ (20 °C, 72 h), reduced with $NaBH_4$ and desalted by GPC on Sephadex G-25. A portion of the oxidised OPS was methylated, the remaining material hydrolysed with aq 2% HOAc (100 °C, 4 h), and the products (Smith-degraded OPS) were fractionated by GPC on BioGel P-2 to give two oligosaccharides, OS-I and OS-II. The latter was repeatedly Smith-degraded to give OS-III.

Sugar analysis of the Smith-degraded OPS revealed FucN, Man, GalN and tetrose in the ratio

Table 1
Methylation analysis data. GLC retention time of the alditol acetates is related to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (2,3,4,6-Me_4Glc). OPS-D, the OPS degraded by periodate oxidation followed by borohydride reduction

Partially methylated sugar	Relative retention time	GLC detector response						
					Smith degradation products			
		OPS		OPS-D	OPS	OS-I	OS-II	OS-III
		A [a]	B	B	B	B	B	B
2,3,4,6-Me_4Glc	1.00	0.70	0.70					
2,3,6-Me_3Glc	1.25	0.50	1.00					
2,4,6-Me_3Man	1.26				0.90	0.24	0.40	0.95
4,6-Me_2Man	1.41	0.75	1.00	0.70	0.16	0.63		
3,4-Me_2FucNMeAc	1.52				1.00	1.00	1.00	
3,4,6-Me_3GlcNMeAc	1.70	1.00	1.00					
4-MeFucNMeAc	1.72	0.30	0.71	1.00				
3,4,6-Me_3GalNMeAc	1.79							1.00
3,6-Me_2GalNMeAc	1.91	0.07	0.08	0.10	0.80	0.75	0.40	
3,4-Me_2GalNMeAc	2.06	0.15						
3-MeGalNMeAc	2.17	0.40	0.60	0.55				

1.0:0.9:1.0:0.5. Methylation analysis data of the oxidised OPS, Smith-degraded OPS and oligosaccharides OS-I, OS-II and OS-III (Table 1) showed that the terminal Glc and GlcNAc and 4-substituted Glc were completely oxidised with periodate. OS-II that contains 3-substituted Man, 4-substituted GalN and the terminal FucN, is the expected product of Smith-degradation of the OPS. The terminal FucN resulted obviously from the 3-substituted FucN by the cleavage of the linkage of the oxidised 4-substituted Glc residue. Part of an oxidised terminal sugar (~25%; most likely, GlcNAc[11]) was not cleaved during mild acid hydrolysis of the oxidised OPS to give rise to OS-I containing a 2,3-disubstituted Man residue. OS-III that resulted from oxidation of the terminal FucN in OS-II, contained the terminal GalN and 3-substituted Man.

The methylation analysis and Smith degradation data showed the following sugar sequences in the Smith-degradation products and the OPS:

OS-III GalNAc-(1→3)-Man-(1→Gro

OS-II FucNAc-(1→4)-GalNAc-(1→3)-Man-(1→2)-erythritol

OS-I FucNAc-(1→4)-GalNAc-(1→3)-Man-(1→2)-erythritol
2
↑
1
GlcNAc-oxidised

OPS →3)-FucNAc-(1→4)-GalNAc-(1→3)-Man-(1→4)-Glc-(1→
6 2
↑ ↑
1 1
Glc GlcNAc

2.4. NMR spectroscopic studies and structure of the O-specific polysaccharide

The ^{13}C NMR spectrum of the OPS (Fig. 2) showed signals for six anomeric carbons at δ 99.6–104.8, three nitrogen-bearing carbons at δ 50.6–57.1 (C-2 of amino sugars), one CH_3–C group at δ 16.8 (C-6 of FucN), $HOCH_2$–C groups (C-6) at δ 61.9–62.9 and other sugar carbons in the region δ 67.3–81.5. The spectrum contained also signals for three *N*-acetyl groups and one *O*-acetyl group (Me at δ 23.5–24.0 at δ 21.9, respectively; CO at δ 175.7–176.0). The ^{1}H NMR spectrum of the OPS showed, inter alia, signals for six anomeric protons at δ 4.59–5.36, three *N*-acetyl groups at δ 2.05–2.10 and one *O*-acetyl group at δ 2.22. The NMR spectra of the *O*-deacetylated OPS were devoid of signals for the *O*-acetyl group.

The ^{1}H and ^{13}C NMR spectra of the *O*-deacetylated OPS were assigned using 2D COSY, TOCSY, NOESY, and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 2 and 3). The spin systems for α-Glc*p*, β-Glc*p*, β-Man*p*, β-Glc*p*NAc, α-Gal*p*NAc and α-Fuc*p*NAc were identified by ^3J coupling constant values; particularly, the $^3J_{1,2}$ values for α-linked sugars were <4 Hz and those for β-Glc*p* and β-Glc*p*NAc ~7 Hz. The configuration of the linkage of Man was determined based on H-1, H-3 and H-1, H-5 correlations in the NOESY spectrum, which are typical of a β-linked monosaccharide. The spin systems of amino sugars were distinguished by correlations of protons at nitrogen-bearing carbons (H-2) to the corresponding carbons (C-2) in the ^{1}H, ^{13}C HSQC spectrum.

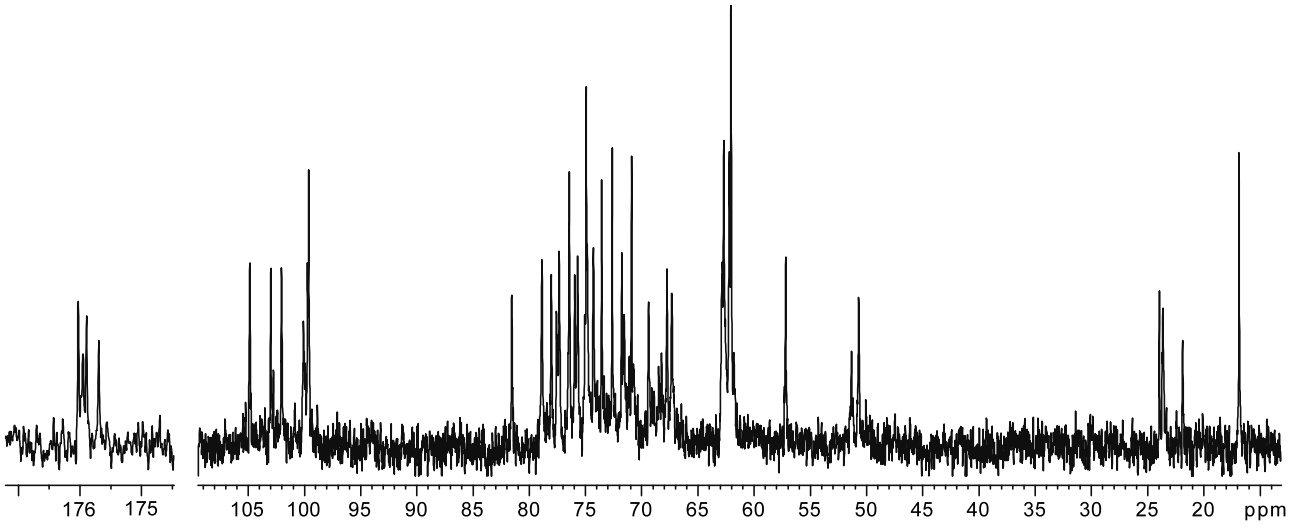


Fig. 2. ^{13}C NMR spectrum of the O-specific polysaccharide of *C. gillenii* PCM 1540.

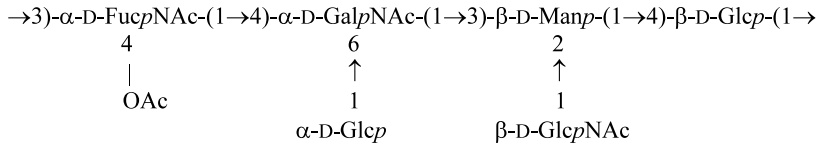
The glycosylation pattern of the monosaccharides was established by down-field displacements of the signals for linkage carbons to δ 76.0–81.4 as compared with their positions in the corresponding non-substituted monosaccharides.[12] The terminal position of α-Glc and β-GlcNAc followed from the ^{13}C NMR chemical shifts for C-2–C-5, which were close to those in the non-substituted monosaccharides.[12] The position of the *O*-acetyl group at FucNAc O-4 was determined by a significantly lower-field position of the signal for FucNAc H-4 at δ 5.43 in the ^{1}H NMR spectrum of the OPS as compared with its position at δ 4.08 in the spectrum of the *O*-deacetylated OPS. This conclusion was confirmed by characteristic displacements upon *O*-acetylation[13] of the ^{13}C NMR signals for C-3, C-4 and C-5 of FucNAc from δ 78.7, 72.4 and 68.3 to δ 76.4, 75.0 and 67.7, respectively.

The NOESY spectrum of the *O*-deacetylated OPS showed interresidue correlations between the following anomeric protons and protons at the linkage carbons: α-FucNAc H-1,α-GalNAc H-4; α-GalNAc H-1,β-Man H-3; β-Man H-1,β-Glc H-4; β-Glc H-1,α-FucNAc H-3; α-Glc H-1,α-GalNAc H-6a; β-GlcNAc H-1,β-Man H-2. These data were in agreement with the ^{13}C NMR chemical shift data and defined the monosaccharide sequence in the repeating unit. Similar NMR spectroscopic analysis of OS-II obtained by Smith degradation of the OPS (Tables 2 and 3) confirmed its structure that was suggested based on the chemical analyses data (see above). Therefore, the OPS of *C. gillenii* PCM 1540 from serogroup O11 has the following structure:

Table 2
^{1}H NMR chemical shifts (δ, ppm) of the *O*-deacetylated OPS and OS-II

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CON [a]
O-Deacetylated OPS								
→3)-α-D-Fuc*p*NAc-(1→	4.91	4.33	4.13	4.08	4.47	1.17		2.05
→4,6)-α-D-Gal*p*NAc-(1→	5.36	4.27	4.08	4.06	4.03	3.64	3.89	2.08
→2,3)-β-D-Man*p*-(1→	4.73	4.26	3.77	3.66	3.40	3.71	3.93	
→3)-β-D-Glc*p*-(1→	4.61	3.36	3.70	3.63	3.63	3.77	3.87	
α-D-Glc*p*-(1→	4.82	3.56	3.57	3.46	3.70	3.60	3.75	
β-D-Glc*p*NAc-(1→	4.65	3.74	3.59	3.42	3.42	3.79	3.85	2.10
OS-II								
α-D-Fuc*p*NAc-(1→	4.87	4.15	4.02	3.83	4.43	1.17		2.02
→4)-α-D-Gal*p*NAc-(1→	5.19	4.22	4.08	4.01	4.16	3.73	3.73	2.08
→3)-β-D-Man*p*-(1→	4.77	4.23	3.69	3.71	3.39	3.73	3.91	
→2)-erythritol	3.74 [b]	3.84	3.82	3.61 [c]				

[a] Assignment could be interchanged.
[b] H-1a; H-1b at δ 3.82.
[c] H-4a; H-4b at δ 3.73.

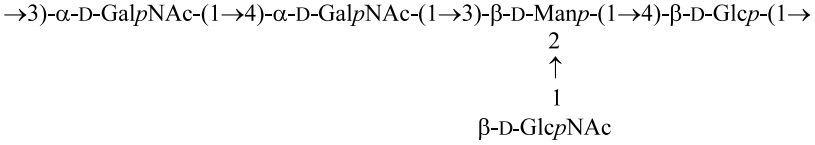


Similarly, the ^{1}H and ^{13}C NMR spectra of OS-II were assigned (Tables 2 and 3) and the structure of this compound was determined, which is in full agreement with the OPS structure.

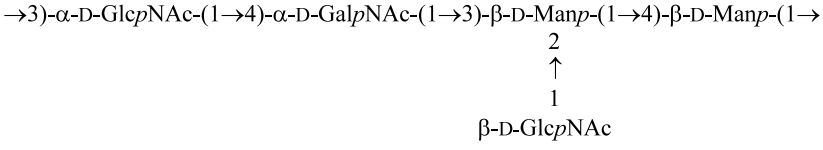
As most *Citrobacter* O-antigens, the OPS studied is neutral. Its sugar composition is consistent with chemotyping of *Citrobacter* serogroup O11 to chemotype CC-B, which is characterised by the presence of D-FucN, GalN, D-Man and the so-called common sugars, which may originate from either the OPS or the LPS core and lipid A.[5]

Keleti et al.[5] and Lányi[14] reported on the serological relatedness of *Citrobacter* O11 to *Salmonella riogrande* O40 and *Escherichia coli* O6, and later the structures of the O-antigens of these bacteria have been established (see below).[15,16] Comparison of the O-antigen structures showed that *S. riogrande* O40 and *C. gillenii* O11 share a tetrasaccharide fragment that includes the →4)-α-D-Gal*p*NAc-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→ trisaccharide with the terminal β-D-Glc*p*NAc residue attached at C-2 of Man. In *E. coli* O6:K54, the common structure is limited to a branched →4)-α-D-GalNAc-(1→3)-[β-D-Glc*p*NAc-(1→2)]-β-D-Man trisaccharide, and in *E. coli* O6:K2 and O6:K13 the terminal β-D-GlcNAc is replaced with the terminal β-D-Glc. However, the glycosidic linkages between the corresponding monosaccharides and the size of the backbone repeating unit are the same in all polysaccharides in question, which implies their similar conformation. This feature and sharing of the oligosaccharide fragments seem to be responsible for the observed serological cross-reactivity.[5,14]

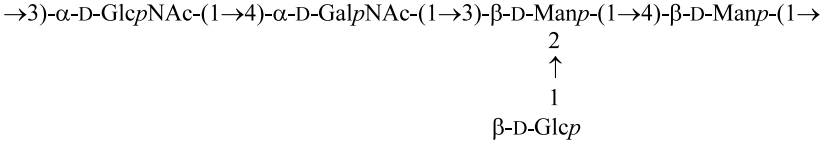
Salmonella riogrande O40[15]



Escherichia coli O6:K54[16]



Escherichia coli O6:K2 and O6:K13[16]



3. Experimental

3.1. General methods

The passive hemagglutination test[17] was carried out with anti-*Citrobacter* PCM 1541 serum. SDS-PAGE was performed according to Laemmli[18] and silver staining of the gels as described.[19] GPC was carried out on

Table 3
^{13}C NMR chemical shifts (δ, ppm) of the *O*-deacetylated OPS and OS-II

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CON[a]	CH_3CON[b]
O-Deacetylated OPS								
→3)-α-D-Fuc*p*NAc-(1→	99.9	49.9	78.7	72.4	68.3	16.6	23.3	175.8
→4,6)-α-D-Gal*p*NAc-(1→	99.5	51.2	70.4	78.9	70.1	67.2	23.4	175.8
→2,3)-β-D-Man*p*-(1→	101.9	77.4	76.0	69.2	77.1	62.4		
→3)-β-D-Glc*p*-(1→	105.0	74.0	75.4	81.4	75.4	61.6		
α-D-Glc*p*-(1→	99.3	72.4	74.6	70.6	73.3	61.5		
β-D-Glc*p*NAc-(1→	102.9	56.9	74.5	71.5	77.8	62.5	23.6	175.5
OS-II								
α-D-Fuc*p*NAc-(1→	99.9	51.3	68.7	72.6	68.8	16.7	23.3	175.8
→4)-α-D-Gal*p*NAc-(1→	101.2	51.5	68.5	78.8	73.8	62.3	23.3	175.9
→3)-β-D-Man*p*-(1→	101.3	72.1	82.5	67.4	77.6	62.4		
→2)-erythritol	62.8	82.6	72.3	63.7				

[a,b]Assignment could be interchanged.

columns (2 × 100 cm) of Sephadex G-50, Sephadex G-25 and BioGel P-2 in pyridinium acetate buffer pH 5.6; sugar content in the fractions was determined by the phenol–sulfuric acid reaction. GPC on a column (1.6 × 100 cm) of TSK-HW 40 (S) in aq 1% HOAc was monitored with a Knauer differential refractometer. GLC–MS was performed with a Hewlett-Packard 5971 instrument equipped with HP-1 glass capillary column (12 m × 0.2 mm) using a temperature program of 150→270 °C at 8 °C/min. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 spectrometer for solutions in D_2O at 40 °C for the polysaccharides and 33 °C for OS-II. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45 ppm) as reference. Mixing time of 150 and 200 ms were used in TOCSY and NOESY experiments, respectively.

3.2. Bacterial strains, isolation and degradation of the lipopolysaccharide

C. gillenii O11:77, 78 (PCM 1540, IHE Be 69/57, Ind 1401[5,9,14]) and *C. werkmanii* O11:83 (PCM 1541, IHE Be 70/57, SR 724[5,9,14]) were from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were harvested from a liquid medium,[17] and the LPS obtained from acetone-dried bacterial mass by phenol–water extraction.[20,21] The LPS were hydrolysed with aq 1% HOAc (100 °C, 1.5 h), and, after removal of a lipid sediment, the carbohydrate-containing material (50 and 60% of the LPS weight of strains PCM 1540 and 1541, respectively) was fractionated by GPC on a column of Sephadex G-50.

3.3. Sugar and methylation analysis

The polysaccharides and oligosaccharides were hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 0.5 h), and monosaccharides were converted conventionally into the alditol acetates and analysed by GLC–MS. Methylation was performed according to Gunnarsson;[22] the methylated polysaccharides and oligosaccharides were hydrolysed as in sugar analysis, converted into the partially methylated alditol acetates and analysed by GLC–MS.

The absolute configuration of Glc, Man and GlcNAc was determined by treatment of the OPS hydrolysate (2 M CF_3CO_2H, 120 °C, 2 h) with hexokinase[23] followed by GLC–MS analysis of the phosphorylated with hexokinase products after inactivation of the enzyme by heating at 100 °C for 5 min. The absolute configuration of FucNAc was established by GLC analysis of the acetylated (*S*)-2-octyl glycosides as described.[24] The absolute configuration of GalNAc was established with D-galactose oxidase.[25] Glucose and *O*-acetyl groups were determined with D-glucose oxidase[26] and by the Hestrin procedure,[27] respectively. PC was performed on Whatmann 1 paper using a system of butanol–pyridine–water (v/v/v, 6:4:3) and staining with the Trevelyan reagent.

3.4. Smith degradation

OPS was *O*-deacetylated with aq 12% ammonia (ambient temperature, overnight) and evaporated. The *O*-deacetylated OPS (30 mg) was oxidised with 0.1 M $NaIO_4$ (3 mL) for 72 h at 20 °C, then ethylene glycol (0.15 mL) was added and in 1 h the sample was reduced with $NaBH_4$ (150 mg, ambient temperature, overnight),

neutralised with aq 50% HOAc, evaporated and co-distilled four times with MeOH. The degraded OPS was desalted by GPC on Sephadex G-25 and hydrolysed with aq 2% HOAc (100 °C, 4 h). The resultant products were fractionated on BioGel P-2 to give two oligosaccharides: OS-I (3.8 mg) and OS-II (8.6 mg). The latter (2.2 mg) was again oxidised with 0.1 M $NaIO_4$ (0.2 mL, 20 °C, 48 h), reduced with $NaBH_4$ (10 mg), the reaction mixture was neutralised with aq 50% HOAc, co-distilled with methanol, desalted on TSK HW-40(S) and the product was hydrolysed with aq 2% HOAc (100 °C, 2 h) to yield OS-III.

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