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Structure of the O-polysaccharide of *Hafnia alvei* strain PCM 1189 that has hexa- to octasaccharide repeating units owing to incomplete glucosylation

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Abstract—The O-polysaccharide of *Hafnia alvei* PCM 1189 consists of D-glucose, D-galactose, D-GalNAc and D-GlcA and lacks the strict regularity. The intact and carboxyl-reduced polysaccharides as well as oligosaccharides obtained by partial acid hydrolysis were studied by chemical and enzymatic analyses, methylation and NMR spectroscopy. The following structure was established for the O-polysaccharide, which is built up of branched hexa- to octasaccharide repeating units differing in the number of lateral glucose residues:

$$\begin{array}{l} \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow \\ \quad\quad 4 \qquad\qquad\qquad\qquad\qquad\qquad 6 \\ \quad\quad\uparrow \qquad\qquad\qquad\qquad\qquad\qquad\uparrow \\ \quad\quad 1 \qquad\qquad\qquad\qquad\qquad\qquad 1 \\ \quad\alpha\text{-D-}Glcp \qquad\qquad \alpha\text{-D-}Glcp\text{-}(1\rightarrow 2)\text{-}\alpha\text{-D-Glc}p \end{array}$$

where the glucose residues shown in italics are nonstoichiometric substituents. The repeating units include also a minor *O*-acetyl group, whose position was not determined.

Keywords: *Hafnia alvei*; Enterobacteria; O-antigen; Lipopolysaccharide; Bacterial polysaccharide structure

1. Introduction

The genus *Hafnia* of the family Enterobacteriaceae contains only one species, *Hafnia alvei*, which is a rarely occurring but important opportunistic pathogen of humans found in many incidents of nosocomial infections.[1,2]

According to a serological classification, *H. alvei* strains are divided into 39 O-serotypes.[3] Aiming at elucidation of the chemical basis for the serological heterogeneity, structures of more than 25 O-polysaccharides from various *H. alvei* strains have been established (Refs. 4–6 and references cited therein). Most of the polysaccharides examined are neutral or acidic hexosaminoglycans, some of them containing rarely occurring monosaccharides and nonsugar components, such as 4-amino-4,6-dideoxyhexoses, 3-amino-3,6-dideoxyhexoses, 6-deoxy-D-talose, *N*-acetylneuraminic acid, D-allothreonine, glycerol phosphate, arabinitol phosphate,

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phosphoethanolamine, *N*-linked 3-hydroxybutanoyl and formyl groups.[6] Several polysaccharides with phosphodiester linkages resemble teichoic acids of Gram-positive bacteria.

The repeating units of the O-polysaccharides of *H. alvei* are linear or branched oligosaccharides, which range in size from two[7] to eight[8] monosaccharide residues, the most common being tetra- to hexasaccharides. Now we report on the structure of a new O-polysaccharide isolated from *H. alvei* PCM 1189, which lacks the strict regularity owing to nonstoichiometric glucosylation and is composed of hexa- to octasaccharide repeating units.

2. Results and discussion

The lipopolysaccharide (LPS) was extracted from dried bacterial mass of *H. alvei* 1189 by the phenol/water method in a yield 1.7%. SDS-PAGE of the LPS showed a ladder-like pattern of bands typical of smooth strains, which was observed also in immunoblotting with the LPS-specific rabbit serum (Fig. 1).

LPS was degraded by mild acid hydrolysis (1% HOAc, 100 °C, 1 h), and after the precipitation of lipid A (37% of LPS weight), the carbohydrate-containing supernatant (63% of LPS weight) was fractionated on Sephadex G-50 to give three main fractions: a polysaccharide (OPSI), a core oligosaccharide (OS) and a 3-deoxyoct-2-ulosonic acid-containing material (Kdo) in the yields 30%, 46% and 24%, respectively, of the total amount of the material eluted from the column. Alternatively, the LPS was hydrolysed with 0.1 M sodium acetate buffer pH 4.2 (100 °C, 4 h) to afford another polysaccharide (OPSII; 46%), a core oligosaccharide (OS; 42%) and a Kdo-containing fraction (~12%). The yields of OPSI and OPSII were 14% and 28% of the LPS weight, respectively.

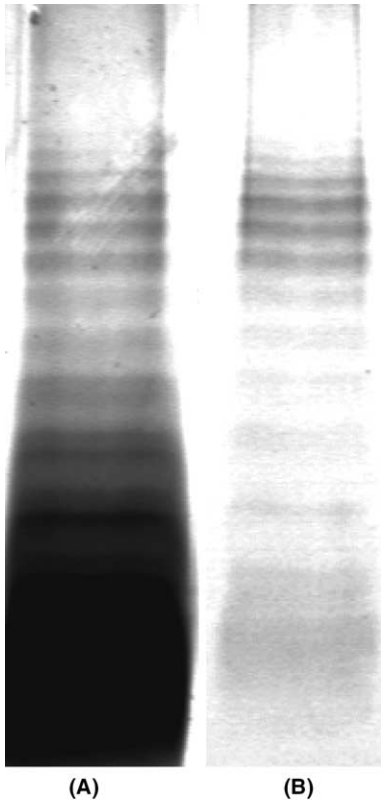

Figure 1. Silver-stained SDS-PAGE (A) and immunoblotting with anti-*H. alvei* PCM 1189 serum (B) of the LPS of *H. alvei* 1189.

Using enzymatic and colorimetric methods it was found that the OPSI contains D-glucose (42%), D-galactose (11.5%), hexuronic acid (12.5%) and *O*-acetyl groups (0.125 $\mu mol\,mg^{-1}$). The carboxyl-reduced polysaccharide (OPSIII) contained a higher amount of glucose (55%) as determined with D-glucose oxidase, which is an evidence for the D-*gluco* configuration of the hexuronic acid. Hydrolysis of the OPSI with 10 M HCl (80 °C, 30 min) followed by GLC–MS analysis of the derived alditol acetates revealed the presence of glucose, galactose and galactosamine in the molar ratio 4.5:1.0:1.8. The content of D-galactosamine (~30%) was determined also using D-galactose oxidase after hydrolysis with 4 M HCl (100 °C, 18 h). Therefore, it was concluded that the O-polysaccharide of *H. alvei* 1189 contains D-glucose, D-galactose, D-GlcA and D-GalN in the ratio ~4.5:1:1:2.

^{1}H and ^{13}C NMR (Fig. 2) spectra showed that the OPSI lacks the strict regularity, and further studies demonstrated that the reason of the irregularity is incomplete glucosylation (see below). The presence in the spectra of signals for *N*-acetyl groups [δ_H 2.04 and 2.09, δ_C 23.5, 23.8 (both CH_3), 175.1 and 176.3 (both CO)] indicated that the GalN residues are *N*-acetylated. The ^{13}C NMR spectrum of the OPSI contained also a minor signal for an *O*-acetyl group at δ 21.4. The OPSII showed essentially the same NMR spectra as the OPSI.

Methylation analysis of OPSI–OPSIII, including acid hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 30 min) (Table 1), showed the presence of eight major sugar residues, including terminal glucose (two residues), 2-substituted glucose, 3-substituted galactose, 4,6-disubstituted glucose, 4-substituted GlcA, 3-substituted GalNAc and 4,6-disubstituted GalNAc (one residue each) as well as a minor amount of 6-substituted glucose.

To obtain oligosaccharide fragments the OPSI (20 mg) was hydrolysed with 0.5 M HCl (80 °C, 30 or 45 min), and fractionation on Bio-Gel P2 resulted in a polysaccharide and two oligosaccharides, OSI and OSII, in different yields depending on the duration of the hydrolysis. Both oligosaccharides were found to contain GlcA (10% in OSI and 14.5% in OSII as determined using a colorimetric assay).

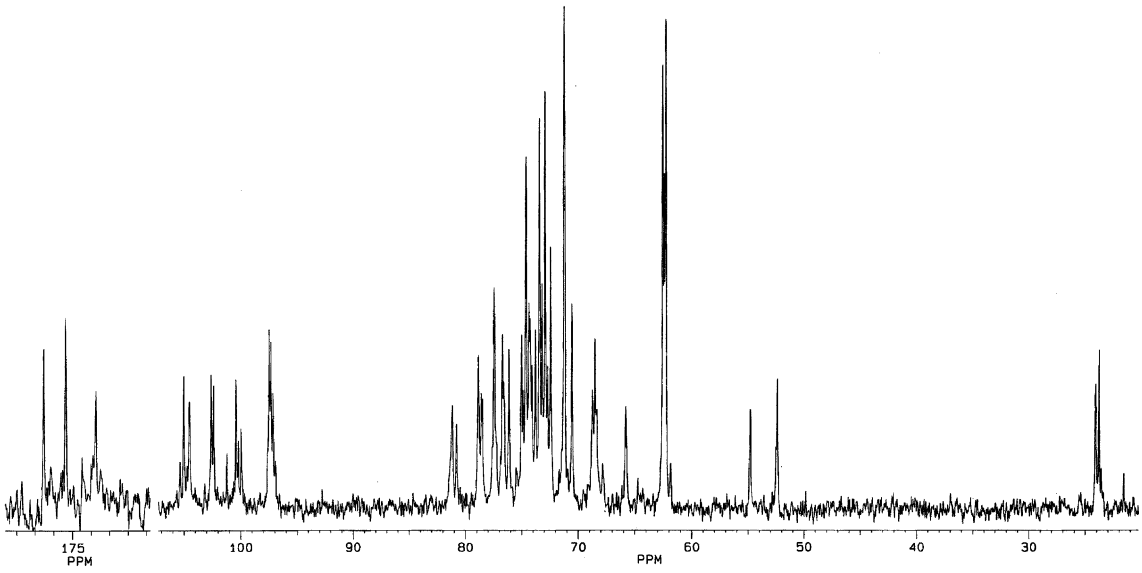

Figure 2. ^{13}C NMR spectrum of OPSI.

Table 1. Methylation analysis of the polysaccharides and oligosaccharides obtained from the LPS of *H. alvei* PCM 1189

Methylated sugar derivative	T_R	OPSI		OPSII		OPSIII	
		A	B	A	B	A	B
2,3,4,6-Me_4Glc	1.00	1.7	1.8	1.7	1.3	1.6	1.5
3,4,6-Me_3Glc	1.21	0.56	0.71	0.6	0.5	0.7	0.7
2,3,6-Me_3Glc	1.25					0.4	0.6
2,4,6-Me_3Gal	1.27	1.0	1.0	1.0	1.0	1.0	1.0
2,3,4-Me_3Glc	1.29	0.13	0.19	0.18	0.3	0.07	0.13
2,3-Me_2Glc	1.50	0.8	0.79	0.7	0.9	0.8	0.9
4,6-Me_2GalNMeAc	1.97	0.17	0.71	0.7	0.9	1.0	0.93
3-MeGalNMeAc	2.16	0.18	0.6	0.2	1.3	0.4	0.93
		OSI	OSI-$NaBD_4$	OSII	OSII–$NaBD_4$		
2,3,4,6-Me_4Glc	1.00	1.80	1.80	2.30	2.50		
3,4,6-Me_3Glc	1.20	1.00	1.00	1.00	1.00		
2,3,6-Me_3Glc	1.24	0.85	0.90	0.97	1.10		
2,4,6-Me_3Gal	1.26	0.94	0.84				
1,4,5,6-Me_4GalNMeAc[a]	1.43		0.47		tr		
1,3,5-Me_3GalNMeAc[a]	1.73				0.61		
4,6-Me_2GalNMeAc	1.96	0.94	0.07				
3-MeGalNMeAc	2.15	0.80	0.55	0.80	0.32		

Retention time in GLC–MS (T_R) for the alditol acetate is related to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol (2,3,4,6-Me_4Glc). Content of the methylated derivatives of monosaccharides is given as GLC–MS detector response. A and B refer to the conditions of hydrolysis of the methylated polysaccharides (2 M CF_3CO_2H, 120 °C, 2 h or 10 M HCl, 80 °C, 30 min, respectively). OPSI–OPSIII were obtained by hydrolysis of the LPS with aq 1% HOAc, with 0.1 M sodium acetate buffer pH 4.2, and by carboxyl reduction of OPSI, respectively. Hydrolysis of the methylated oligosaccharides was performed with 10 M HCl at 80 °C for 30 min. OSI and OSII were obtained by partial acid hydrolysis of the OPSI; OSI–$NaBD_4$ and OSII–$NaBD_4$ were prepared by reduction of OSI and OSII with $NaBD_4$; tr, trace amount.

[a] Alditol-1d derived by reduction of the reducing-end monosaccharide with $NaBD_4$.

The OSI and OSII were subjected to methylation analysis before and after reduction with $NaBD_4$ (Table 1). Identification of the corresponding methylated alditols-1d showed that the reducing end of these oligosaccharides is occupied by 3-substituted GalNAc and 4,6-disubstituted GalNAc residues, respectively, which were both present in the OPSI. The 4,6-disubstituted glucose residue (unit **B**) that was present in the OPSI was replaced with a 4-substituted glucose residue in both OSI and OSII, and, hence, a 1→6-linkage was cleaved selectively by partial acid hydrolysis. The absence from the OSII of the 3-substituted galactose (unit **G**) and

Table 2. ^{1}H NMR chemical shifts (δ) of OSI-2-red obtained by partial acid hydrolysis of the OPSI followed by borohydride reduction

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CO
Reduced octasaccharide **1**								
α-Glc*p*-(1→ (**A**)	5.38	3.56	3.73	3.41	3.76	3.76	3.87	
→4)-α-Glc*p*-(1→ (**B**)	5.44	3.56	3.96	3.64	3.88	3.79	3.85	
→4)-β-Glc*p*A-(1→ (**C**)	4.70	3.48	3.77	3.81	3.75			
α-Glc*p*-(1→ (**D**)	5.05	3.60	3.77	3.47	3.91	3.78	3.84	
→2)-α-Glc*p*-(1→ (**E**)	5.15	3.69	3.77	3.48	3.64	3.76	3.88	
→4,6)-β-Gal*p*NAc-(1→ (**F**)	4.73	4.01	3.87	4.27	3.87	3.84	3.86	2.06
→3)-α-Gal*p*-(1→ (**G**)	5.16	3.97	3.91	4.19	4.05	3.77	3.77	
→3)-GalNAc-ol (**H**)	3.72[a]	4.37	3.94	3.75	4.03	3.67	3.67	2.05
Reduced heptasaccharide **2**								
α-Glc*p*-(1→ (**A**)	5.38	3.56	3.73	3.41	3.76	3.76	3.87	
→4)-α-Glc*p*-(1→ (**B**)	5.43	3.56	3.95	3.64	3.86	3.79	3.85	
→4)-β-Glc*p*A-(1→ (**C**)	4.66	3.49	3.77	3.83	3.78			
α-Glc*p*-(1→ (**E**)	4.92	3.56	3.67	3.43	3.63	3.75	3.87	
→4,6)-β-Gal*p*NAc-(1→ (**F**)	4.73	4.00	3.87	4.23	3.89	3.75	3.88	2.06
→3)-α-Gal*p*-(1→ (**G**)	5.16	3.97	3.89	4.19	4.05	3.77	3.77	
→3)-GalNAc-ol (**H**)	3.72[a]	4.37	3.94	3.75	4.03	3.67	3.67	2.05

[a] H-1a; H-1b at δ 3.80.

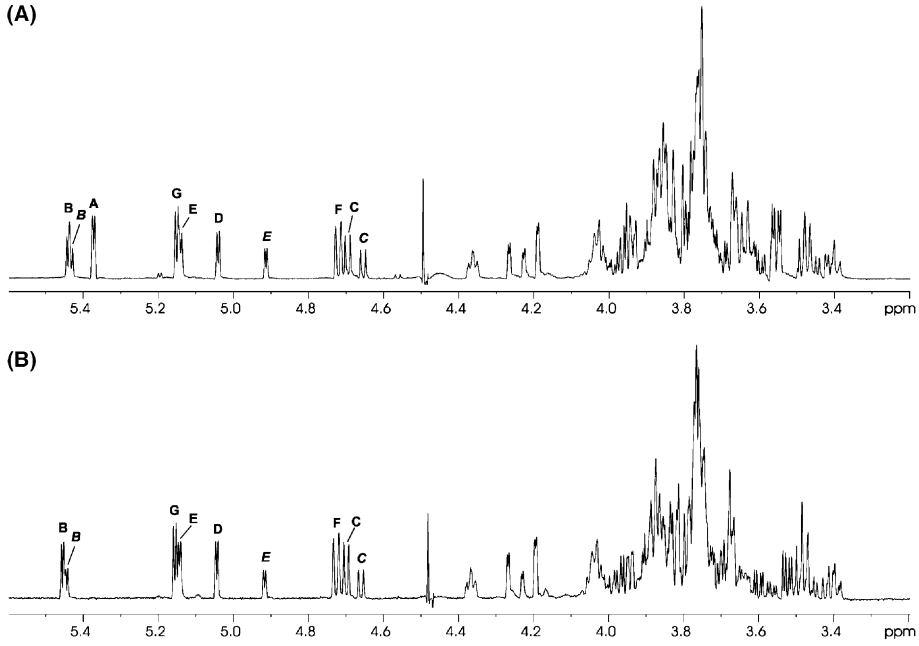

Figure 3. ^{1}H NMR spectra of OSI-2 (**1** and **2**) (A) and OSI-1 (**3** and **4**) (B). Designated are anomeric protons of monosaccharide residues denoted by letters as shown in Fig. 4. When different, designations for minor oligosaccharides (**2** and **4**) are italicised.

GalNAc (unit **H**) residues and the presence of the other sugar constituents of the OSI defined the structure of OSII as a partial structure of OSI (see below).

For the NMR spectroscopic studies, the OSI was reduced with $NaBH_4$ and fractionated by high-performance anion-exchange chromatography (HPAEC) on CarboPac PA1 to give two fractions, OSI-1-red and OSI-2-red. ESI MS showed that the latter, major product is a mixture of hepta- and octasaccharides, and the former, minor product a mixture of hexa- and heptasaccharides, all oligosaccharides differing in the number of hexose (glucose) residues (the experimental molecular masses 1412.475, 1250.420 and 1088.366 Da differed from the calculated masses by <0.01 Da).

OSI-2-red was studied by 2D NMR spectroscopy according to the published methodology,[9] including assignment of the ^{1}H and ^{13}C NMR spectra (Fig. 3A, Tables 2 and 3) with the aid of ^{1}H,^{1}H COSY, TOCSY and ^{1}H,^{13}C HSQC experiments. The anomeric configurations of the monosaccharides (units **A**–**G**) were determined by the $^{3}J_{H1,H2}$ coupling constant values, and the glycosylation pattern by significant low-field displacements of the ^{13}C NMR signals for the linkage carbons in the substituted units **B**, **C**, **E**–**H** (Table 3). A ROESY experi-

Table 3. ^{13}C NMR chemical shifts (δ) of OSI-2-red obtained by partial acid hydrolysis of OPSI followed by borohydride reduction

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
Reduced octasaccharide **1**						
α-Glc*p*-(1→ (**A**)	100.8	73.0	74.4	71.0	74.0	62.1[a]
→4)-α-Glc*p*-(1→ (**B**)	99.6	73.3	74.6	78.1	71.8	61.6
→4)-β-Glc*p*A-(1→ (**C**)	104.8	75.1	77.7	78.4	78.3	
α-Glc*p*-(1→ (**D**)	97.3	72.6	74.4	70.9	73.2	61.9
→2)-α-Glc*p*-(1→ (**E**)	97.0	76.4	73.1	70.9	73.2	62.0[a]
→4,6)-β-Gal*p*NAc-(1→ (**F**)	104.3	54.7	72.8	78.2	74.2	68.3
→3)-α-Gal*p*-(1→(**G**)	101.0	68.6	80.5	70.5	72.8	62.4
→3)-GalNAc-ol (**H***)	62.5	53.4	78.7	71.4	71.5	64.4
Reduced heptasaccharide **2**						
α-Glc*p*-(1→(**A**)	100.8	73.0	74.4	71.0	74.0	62.1[a]
→4)-α-Glc*p*-(1→ (**B**)	99.6	73.3	74.6	78.1	71.8	61.6
→4)-β-Glc*p*A-(1→ (**C**)	105.2	75.1	77.7	78.5	78.3	
α-Glc*p*-(1→ (**E**)	99.7	72.7	74.6	71.1	73.3	62.0[a]
→4,6)-β-Gal*p* NAc-(1→ (**F**)	104.3	54.7	72.7	78.6	73.7	67.8
→3)-α-Gal*p*-(1→ (**G**)	101.0	68.6	80.4	70.4	72.8	62.4
→3)-Gal*p*NAc-ol (**H***)	62.5	53.4	78.7	71.4	71.6	64.4

Signals for CH_3 of NAc are at δ 23.5 and 23.7, for CO of NAc and COOH of GlcA at 175.7–176.5.
[a] Assignment could be interchanged.

ment showed cross-peaks between the anomeric protons of units **A**–**G** and protons at the linkage carbons of the substituted units (Table 2) and thus confirmed the linkage positions and defined the monosaccharide sequence.

As a result, it was established that the major and minor oligosaccharides present in the OSI-2 have structures **1** and **2** differing in the presence or absence of the lateral glucose residue (unit **D**), respectively (Fig. 4). The lack of unit **D** influences significantly the ^1H NMR chemical shifts of not only the neighbouring glucose residue (unit **E**) but also those of the GalNAc residue (unit **F**) at the branching point of the main chain and the neighbouring GlcA residue (unit **C**) (Table 2). Similar studies of the OSI-1-red (for the ^1H NMR spectrum see Fig. 3B) indicated that OSI-1 contains the oligosaccharides that have structures **3** and **4** (Fig. 4), which differ from **1** and **2** only in the absence of the terminal 4-linked glucose residue (unit **A**). Finally, based on NMR spectroscopic and methylation analysis data (Table 1), structure **5** was established for the major oligosaccharide present in the OSII, which differs from structure **1** in the absence of the Gal→GalNAc disaccharide fragment from the reducing end (Fig. 4).

Comparison of the NMR and methylation data of the oligosaccharides and the *O*-deacetylated OPSI (OPSIV) showed that structures **1**–**4** correspond to four types of the repeating units that are present in the O-polysaccharide. ^1H and ^{13}C NMR spectroscopic studies of the OPSIV as described above for the OSI-2 showed that the main chain of the O-polysaccharide consists of pentasaccharide repeating units that are linked to each other by the β-(1→6)-linkage between the 3-substituted GalNAc residue (**H**) and the 4-substituted glucose residues (**B**). Particularly, this conclusion followed from the position of the H-1 signal at δ 4.61 and $^3J_{H1,H2}$ 7 Hz of unit **H** and from a correlation between H-1 of unit **H** and H-6a,6b of unit **B** at δ 4.61/3.90 and 4.61/4.09 shown by the NOESY spectrum of the OPSIV. The ratio of integral intensities of the H-1 signals showed that the content of the repeating units containing the terminal glucose residue **D** is 65–70% of the total. The content of the repeating units containing the terminal glucose residue **A** was estimated as 80–85% based on the ratio of 4-substituted and 4,6-disubstituted glucose residues (unit **B**) revealed by methylation analysis (Table 1). The *O*-acetyl group was present in a minor amount (~15% as judged by the ratio of the integral intensities of the *O*–Ac and *N*–Ac signals in the ^{13}C NMR spectrum of the OPSI) and its location was not determined.

Therefore, based on the data obtained, it was concluded that the O-polysaccharide of *H. alvei* PCM 1189 has structure **6** shown in Fig. 4. It has hexa- to octasaccharide repeating units owing to incomplete glucosylation at two sites. Such large repeating units are uncommon in bacterial LPSs. For the first time an octasaccharide repeating unit was found in the O-polysaccharide of *H. alvei* strain 2 (PCM 2386),[8] which, in common with the polysaccharide studied in this work, is structurally heterogenous owing to a partial substitution with a lateral glucose residue.[10] In contrast, side-chain glucosylation that usually occurs as a post-polymerisation modification of bacterial polysaccharides,[11] is often nonstoichiometric. Remarkably, the main chain of the branched O-polysaccharide of *H. alvei* PCM 1189 has the same structure as the linear O-polysaccharide of *H. alvei* PCM 1546 reported earlier,[4] thus indicating a close similarity of the O-antigen gene clusters in the two bacterial strains.

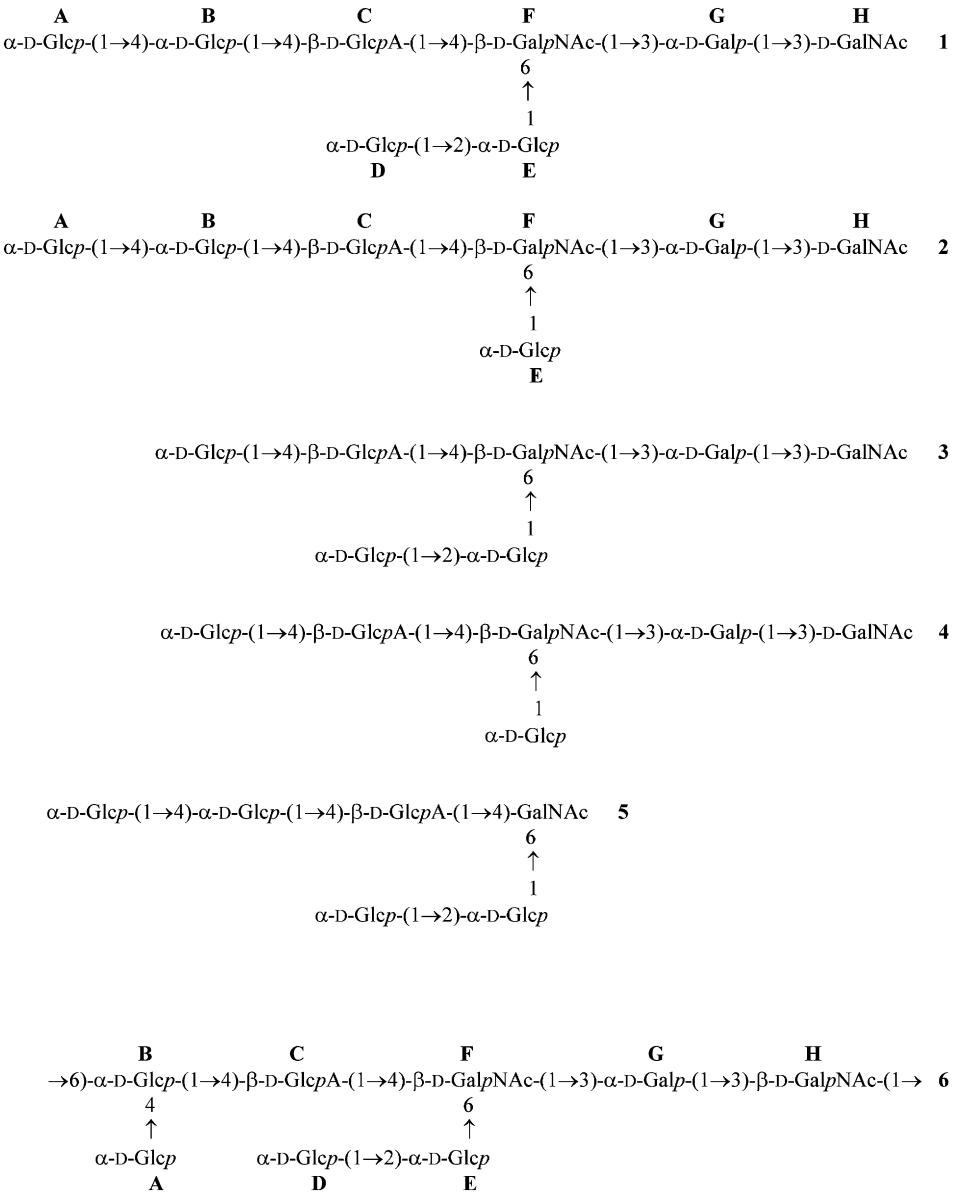

Figure 4. The structures of the oligosaccharides contained in OSI-2 (**1** and **2**), OSI-1 (**3** and **4**) and OSII (**5**) and the fully glucosylated repeating unit of the O-polysaccharide of *H. alvei* PCM 1189 (**6**). In the O-polysaccharide, the degree of substitution with glucose residues **A** and **D** is 80–85% and 65–70%, respectively; a minor O-acetyl group (15%) is present at unknown position.

3. Experimental

3.1. Bacterial strain, isolation of the lipopolysaccharide and preparation of the polysaccharides

H. alvei strain PCM 1189 from the collection of the Institute of Immunology and Experimental Therapy (Wrocław, Poland) was cultivated in a liquid medium with aeration at 37 °C for 24 h as described.[12] The LPS was isolated from dry bacterial mass by the phenol–water extraction[13] and purified by GPC on Sepharose 2B as described.[14] The LPS was degraded with aq 1% HOAc at 100 °C for 1 h or with 0.1 M sodium acetate buffer pH 4.2 at 100 °C for 4 h, a lipid precipitate was removed by centrifugation and the carbohydrate portion was fractionated by GPC on Sephadex G-50 to give the OPSI and OPSII, respectively. OPSIII was prepared by carboxyl reduction of the OPSI according to the procedure of Taylor et al.[15] OPSI was *O*-deacetylated by treatment with aq 12% ammonia (20 °C, 16 h) followed by lyophilisation to give the OPSIV.

3.2. Chromatography

HPAEC was performed on a semi-preparative CarboPac PA1 column (250 × 9 mm, Dionex) using a linear gradient of 0.05 → 0.15 M NaOAc in 0.1 M NaOH at flow rate 2 mL min^{-1} for 70 min. Fractions (2 mL) were

collected and analysed by HPAEC with pulsed amperometric detection (Dionex) on an analytical CarboPac PA1 column (250 × 4.6 mm) using a linear gradient of 0.05 → 0.25 M NaOAc in 0.1 M NaOH at a flow rate of 1 mL min^{-1} for 30 min.

GPC was performed on a column (2 × 100 cm) of Sephadex G-50 or Bio-Gel P2 equilibrated with 0.05 M pyridine–HOAc buffer pH 5.6; eluates were monitored by the phenol–sulfuric acid reaction. Fractions obtained by HPAEC were desalted by GPC on a column (40 × 2.6 cm) of Sephadex G-50 in 0.1 M NH_4HCO_3.

GLC–MS was carried out with a Hewlett–Packard 5971A chromatograph using an HP-1 glass capillary column (12 m × 0.2 mm) and a temperature program of 150 → 270 °C at 8 °C min^{-1}.

3.3. NMR spectroscopy

Prior to the measurements, the samples were lyophilised twice from ^2H_2O. The ^1H and ^{13}C NMR spectra of the polysaccharides were run on Bruker DRX-500 or Bruker DRX-600 spectrometers (Germany) in 99.96% ^2H_2O at 50 °C. Chemical shifts were referenced to internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0) and external acetone (δ_C 31.45). A mixing time of 100 ms was used in 2D TOCSY experiments and 200 ms in ROESY and NOESY experiments, respectively.

3.4. Mass spectrometry

ESI MS was performed on a Fourier transform ion cyclotron resonance mass analyser (ApexII, Bruker Daltonics, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source. Samples were dissolved in a 30:30:0.01 (v/v/v) mixture of 2-propanol, water and Et_3N at a concentration of ~20 ng μL^{-1} and sprayed with a flow rate of 2 $\mu L\,min^{-1}$.

3.5. Sugar and methylation analyses

The polysaccharides and oligosaccharides were hydrolysed with 2 M CF_3CO_2H at 120 °C for 2 h or 10 M HCl at 80 °C for 30 min and the monosaccharides were determined using colorimetric and enzymatic assays or by GLC–MS as the alditol acetates. Hexuronic acid was determined as described[16] and *O*-acetyl groups were estimated by the method of Hestrin.[17] D-Glucose (both present originally and derived from GlcA) and D-galactose were identified using D-glucose oxidase[18] and D-galactose oxidase,[19] respectively, after hydrolysis of the OPSI and OPSIII with 2 M CF_3CO_2H at 120 °C for 2 h. D-GalN was identified after hydrolysis of the OPSI with 4 M HCl at 105 °C for 18 h. GLC–MS analysis of the alditol acetates was performed as described.[20]

Methylation was performed according to the procedure of Gunnarsson,[21] the methylated products were purified by extraction with chloroform/water (1:1, v/v), hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 30 min), reduced with $NaBH_4$, acetylated with acetanhydride in pyridine and analysed by GLC–MS. Prior to methylation, a portion of OSI and OSII (~1 mg) was reduced with $NaBD_4$.

3.6. Partial acid hydrolysis

The OPSI (20 mg) was hydrolysed with 0.5 M HCl (2 mL) at 80 °C for 30 min. After neutralisation with 5 M NaOH, the products were fractionated on a column of Bio-Gel P2 to give a polysaccharide (6.2 mg) and two oligosaccharides OSI (8.4 mg) and OSII (2 mg). When OPSI was hydrolysed for a longer time (45 min), the yield of the polysaccharide decreased (2.4 mg) and that of the OSI and OSII increased (9.6 and 3.8 mg, respectively). OSI (16 mg) was reduced with $NaBH_4$ in water and fractionated by HPAEC on CarboPac PA1. After desalting, two major fractions, OSI-1-red and OS1-2-red, having retention times 13.5 and 15.7 min in analytical HPAEC, were isolated in yields 2.6 and 9.7 mg, respectively.

3.7. SDS-PAGE and immunoblotting

Preparation of rabbit serum against whole cells of *H. alvei* PCM 1189, SDS-PAGE, staining of the gels and immunoblotting were performed as described.[22–24]

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