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Note

Structure of the O-polysaccharide from the lipopolysaccharide of *Hafnia alvei* strain PCM 1546

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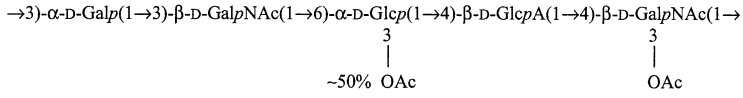
Abstract

An acidic O-polysaccharide isolated by mild acid hydrolysis from the lipopolysaccharide of *Hafnia alvei* PCM 1546 is composed of D-Gal, D-Glc, D-GlcA, D-GalNAc and *O*-acetyl groups in the ratios 1:1:1:2:1.6. On the basis of sugar and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, the following structure of the pentasaccharide repeating unit of the polysaccharide was established:



Keywords: *Hafnia alvei*; O-Antigen; Lipopolysaccharide; Bacterial polysaccharide structures

Hafnia alvei, a typical member of the family *Enterobacteriaceae*, is commonly distributed in natural environment and often associated with wounds and nosocomial infections, including enteric, urinary and respiratory tract disorders.[1]

Based on the specificity of the O-antigens (O-polysaccharide chains of the lipopolysaccharides), strains of *H. alvei* are divided into 39 O-serogroups,[2] and numerous cross-reactions between *Hafnia* and other genera of *Enterobacteriaceae* have been observed. Chemical structures of more than 25 O-antigens of *Hafnia* have been established [3–5] and the molecular basis of some cross-reactions has been elucidated.[5–7]

Now we report on the structure of the O-antigen of *H. alvei* PCM 1546, which, based on genetic studies, has been recently reclassified and moved from the genus *Citrobacter* to the genus *Hafnia*.[8]

Lipopolysaccharide (LPS) of *H. alvei* PCM 1546 was isolated from dry bacterial mass by phenol–water extraction and recovered from the water phase in a yield 2.1%. In SDS-PAGE analysis, the LPS showed a high molecular mass ladder-like pattern typical of smooth (*S*)-type LPS (Fig. 1).

Mild acid hydrolysis of the LPS released lipid sediment and a water-soluble carbohydrate portion, which was separated into four fractions by GPC on Sephadex

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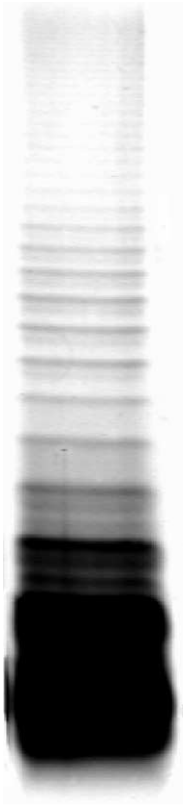


Fig. 1. Silver-stained SDS-PAGE of the LPS of *H. alvei* PCM 1546.

G-50. Fractions P_1 (long-chain O-polysaccharide), P_2 (a shorter-chain O-polysaccharide), P_3 (core oligosaccharides) and a Kdo-containing material were recovered in yields 25, 8, 34 and 33%, respectively, of the total material eluted from the column.

Enzymatic (D-glucose oxidase and D-galactose oxidase) and colorimetric assays showed that the O-polysaccharide contains D-Glc (13%; 0.72 μmol mg^{-1}), D-Gal (15%; 0.76 μmol mg^{-1}), D-GalN (29%; 1.6 μmol mg^{-1}), hexuronic acid (13.5%; 0.7 μmol mg^{-1}) and *O*-acetyl groups (1.6 μmol mg^{-1}). Glc and Gal were determined after hydrolysis of the polysaccharide with 2 M CF_3CO_2H (120 °C, 2 h) and GalN after hydrolysis with 4 M HCl (105 °C, 18 h). The double amount of D-Glc (26%; 1.44 μmol mg^{-1}) was determined in the carboxyl-reduced polysaccharide, thus showing the presence of D-GlcA. Sugar analysis by GLC of the alditol acetates obtained after hydrolysis of the polysaccharide with 10 M HCl (80 °C, 30 min) or 2 M CF_3CO_2H (120 °C, 2 h) revealed the presence of Glc, Gal and GalN in molar ratios 1.7:1.0:2.1 and 1.2:1.0:0.9, respectively. Similar analysis of the carboxyl-reduced polysaccharide after hydrolysis with 2 M CF_3CO_2H revealed Glc:Gal:GalN in molar ratios 2.1:1.0:1.5.

Methylation analysis of the polysaccharide (Table 1) revealed partially methylated alditol acetates derived from 3-substituted Gal, 6-substituted Glc, 4-substituited GalNAc and 3-substituted GalNAc. In methylation analysis of the carboxyl-reduced polysaccharide, a derivative of 2,3,6-tri-*O*-methylglucose was identified, which demonstrated 4-substituted GlcA.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 2 A) showed a structural heterogeneity, most likely owing to non-stoichiometric O-acetylation (there was signal for Me of *O*-acetyl groups at δ 21.4). The ^{13}C NMR spectrum of the O-deacetylated polysaccharide was typical of a regular polysaccharide having a pentasaccharide repeating unit (Table 2, Fig. 2 B). It contained signals for five anomeric carbons at δ 99.4–104.5, two nitrogen-bearing carbons (C-2 of GlcN and GalN) at δ 51.9 and 54.2, one carboxyl group (C-6 of GlcA) at δ 176.4, other sugar carbons in the region δ 61.7–80.7 and two *N*-acetyl groups at δ 23.4, 23.5 (both Me), 175.7 and 175.8 (both CO). The absence from the spectrum of signals at δ 82–88 ppm that are characteristic of furanosides[9] indicated that all monosaccharides are in the pyranose form. The ^1H NMR spectrum of the O-

Table 1
Methylation analysis data

Partially methylated monosaccharide	Relative retention time	Relative GLC detector response		
		Polysaccharide		Carboxyl-reduced polysaccharide
		A	**B**	**B**
2,3,6-Me_3Glc	1.25			1.3
2,4,6-Me_3Gal	1.27	1	1	1
2,3,4-Me_3Glc	1.30	1.0	0.8	1.2
3,6-Me_2GalNMeAc	1.91	0.3	0.7	0.9
4,6-Me_2GalNMeAc	1.98	0.8	0.9	0.9

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). Hydrolysis conditions: (A) 2 M CF_3CO_2H, 120 °C, 2 h; (B) 10 M HCl, 80 °C, 0.5 h.

Table 2
One hundred and twenty-five megahertz ^{13}C NMR data of the O-deacetylated polysaccharide (δ, ppm)

	C-1	C-2	C-3	C-4	C-5	C-6
→3)-α-D-Gal*p*-(1→ (**A**)	99.4	68.2	80.7	70.2	72.1	61.7
→3)-β-D-Gal*p*NAc-(1→ (**B**)	102.9	51.9	76.5	65.0	76.0	62.2
→6)-α-D-Glc*p*-(1→ (**C**)	99.6	72.7	73.8	70.2	71.8	68.8
→4)-β-D-Glc*p*A-(1→ (**D**)	104.5	74.7	77.5	77.5	77.4	176.4
→4)-β-D-Gal*p*NAc-(1→ (**E**)	104.5	54.2	72.4	77.4	75.4	61.8

Signals for NAc are at δ 23.4, 23.5 (Me), 175.7 and 175.7 (both CO).

deacetylated polysaccharide (Table 3) showed, inter alia, signals for five anomeric protons at δ 4.60–5.44 and two *N*-acetyl groups at δ 2.02 and 2.07.

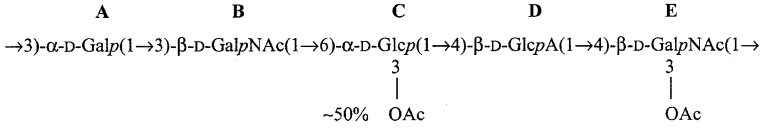
The ^{1}H and ^{13}C NMR spectra of the O-deacetylated polysaccharide were assigned using 2D COSY, TOCSY (Fig. 3), NOESY and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 2 and 3), and spin systems of one residue each of Glc, Gal and GlcA and two residues of GalNAc. The monosaccharide residues were designated as units A–E according to their sequence in the repeating unit (see below). Intense intraresidue H-1, H-2 cross-peaks in the NOESY spectrum showed that Glc and Gal are α-linked, whereas H-1, H-3 and H-1, H-5 cross-peaks indicated that the three other residues are β-linked.

The modes of glycosylation of the monosaccharides were determined by significant downfield displacements of the signals for C-3 of Gal (**A**) and GalNAc (**B**), C-4 of GlcA (**D**) and GalNAc (**E**) and C-6 of Glc (**C**) by 4–10 ppm, as compared with their positions in the corresponding non-substituted monosaccharides.[9,10]

The NOESY spectrum showed strong interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: Gal (**A**) H-1,GalNAc (**B**) H-3 at δ 5.08/3.82; GalNAc (**B**) H-1,Glc (**C**) H-6a and H-6b at δ 4.52/3.84 and 4.52/4.07; Glc (**C**) H-1,GlcA (**D**) H-4 at δ 5.44/3.77; GlcA (**D**) H-1,GalNAc (**E**) H-4 at δ 4.65/4.14; and GalNAc (**E**) H-1,Gal (**A**) H-3 at δ 4.60/3.77. These data are in accordance with the glycosylation pattern determined by the ^{13}C chemical shift data and defined the monosaccharides sequence in the repeating unit.

Comparison of the ^{1}H, ^{13}C HSQC spectra of the initial and O-deacetylated polysaccharides showed a significantly lower-field position of the H-3 signal of GalNAc (**E**) (δ 5.09 in the former vs. 3.81 in the latter). This difference is due to a deshielding effect of the *O*-acetyl group and indicated O-acetylation of GalNAc (**E**) at position 3. A similar down-field displacement of part of the H-3 signal of Glc (**C**) (δ 5.07 vs. 3.65) showed a partial O-acetylation of this monosaccharide at position 3. The ratio of the signals for O-acetylated and non-acetylated Glc (**C**) showed that the *O*-acetyl groups are present on about half of the residues.

On the basis of the data obtained, the following structure of the linear pentasaccharide repeating unit of the O-polysaccharide of *H. alvei* PCM 1546 LPS was established:



1. Experimental

1.1. General methods

SDS-PAGE analysis according to Laemmli[11] and silver staining of the gels[12] were performed as described. GPC was carried out on columns (2 × 100 cm) of Sephadex G-50 and BioGel P-2 in pyridinium acetate buffer pH 5.6; sugar content in the fractions was determined by the phenol–sulfuric acid reaction. GLC–MS was performed with a Hewlett-Packard 5971 instrument equipped with a HP-1 glass capillary column (12 m × 0.2 mm) using a temperature program of 150→270 °C at 8 °C min^{-1}.

1.2. Bacterial strain, isolation and degradation of the LPS

H. alvei strain PCM 1546 (strain 6109-50, Ref. 8, previously Citrobacter O13:66; IHE Be 76/57) derived

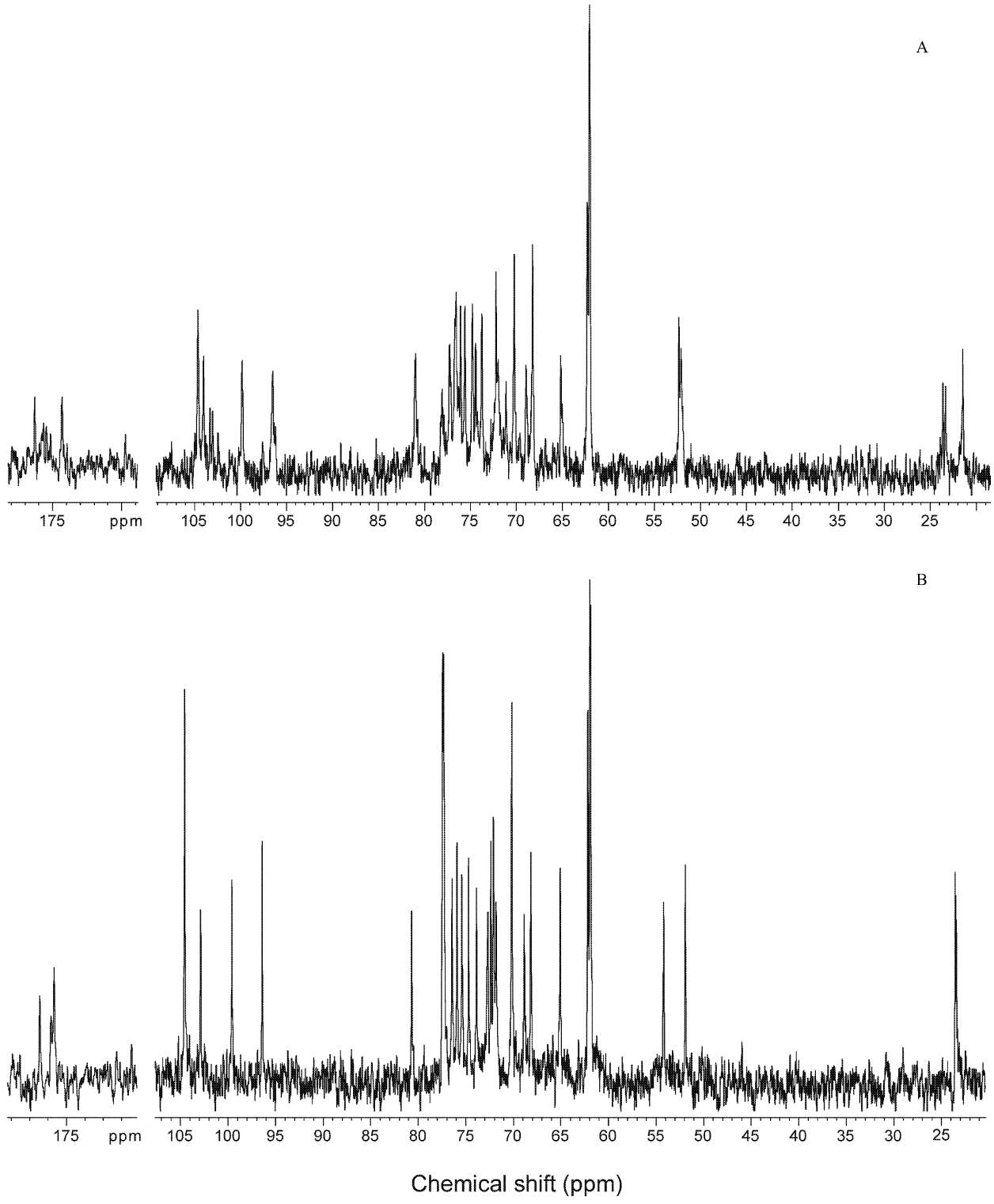


Fig. 2. One hundred and twenty-five megahertz ^{13}C NMR spectra of the O-polysaccharide (A) and O-deacetylated polysaccharide (B) of *H. alvei* PCM 1546.

from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Bacteria were harvested from a liquid medium[13] and the LPS obtained from acetone-dried bacterial mass by phenol–water extraction.[14,15]. The LPS was hydrolysed with aq 1% HOAc (100 °C, 40 min.), and, after removal of a lipid sediment, the carbohydrate-containing material (50% of the LPS mass) was fractionated by GPC on Sephadex G-50 to give fractions P_1–P_3 and a Kdo-containing material.

Table 3
Five hundred megahertz ^{1}H NMR data of the O-deacetylated polysaccharide (δ, ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→3)-α-D-Gal*p*-(1→ (**A**)	5.08	3.87	3.77	4.19	3.84	3.70	3.70
→3)-β-D-Gal*p*NAc-(1→ (**B**)	4.52	4.08	3.82	4.16	3.66	3.75	3.84
→6)-α-D-Glc*p*-(1→ (**C**)	5.44	3.46	3.65	3.40	3.73	3.84	4.07
→4)-β-D-Glc*p*A-(1→ (**D**)	4.65	3.45	3.75	3.77	3.80		
→4)-β-D-Gal*p*NAc-(1→ (**E**)	4.60	4.02	3.81	4.14	3.67	3.73	3.73

Signals for NAc are at δ 2.02 and 2.07.

1.3. Sugar and methylation analysis

The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 0.5 h), and the monosaccharides were converted into the alditol acetates[16] and analysed by GLC. Methylation was performed according to Gunnarsson;[17] the methylated compounds were hydrolysed as in sugar analysis, converted into the partially methylated alditol acetates and analysed by GLC–MS.

The absolute configuration of Glc and GlcA was determined with D-glucose oxidase[18] after hydrolysis of the polysaccharide and carboxyl-reduced polysaccharide with 2 M CF_3CO_2H (120 °C, 2 h). The absolute configuration of Gal and GalNAc was established with D-galactose oxidase[19] after hydrolysis of the polysaccharide with 2 M CF_3CO_2H (120 °C, 2 h) and 4 M HCl (105 °C, 18 h), respectively. *O*-Acetyl groups were determined by the Hestrin procedure,[20] and hexuronic acid according to Blumenkrantz and Asboe-Hansen.[21]

Carboxyl reduction of the polysaccharide (8 mg) was carried out as described,[22] and the reduced polysaccharide (4 mg) was recovered by dialysis against distilled water. The O-deacetylation of the polysaccharide was performed with aq 12% NH_3 (20 °C, overnight) followed by freeze-drying.

1.4. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying three times from D_2O, and examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 40 °C. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45). A mixing time of 200 ms was used in 2D TOCSY and NOESY experiments.

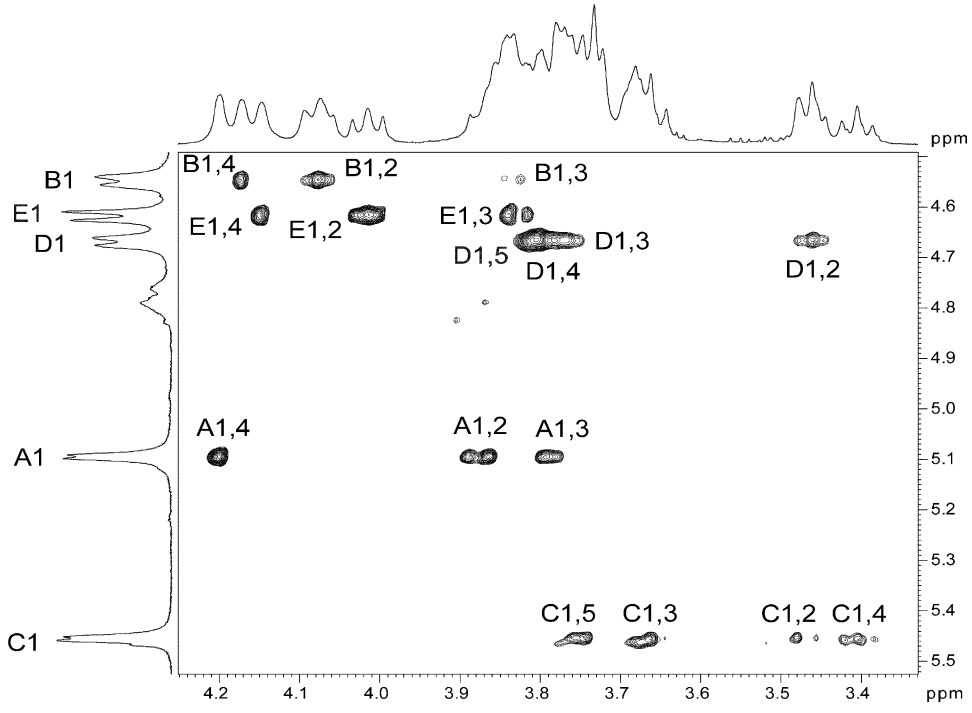


Fig. 3. Five hundred megahertz 2D TOCSY spectrum of the O-deacetylated polysaccharide of *H. alvei* PCM 1546. Arabic numerals refer to sugar residues denoted by letters as indicated in Table 3.

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