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Note

Structure of the O-polysaccharide from the lipopolysaccharide of *Hafnia alvei* strain PCM 1529

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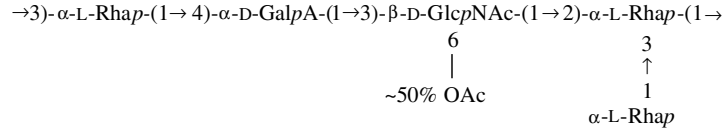
Abstract—The following structure of the pentasaccharide repeating unit of an acidic O-polysaccharide of *Hafnia alvei* PCM 1529 was established by sugar and methylation analyses along with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy:



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

1. Introduction

Hafnia alvei is a typical member of the *Enterobacteriaceae* family. This Gram-negative bacterium is an opportunistic pathogen, which has been found in many nosocomial infections including wounds, enteric, urinary and respiratory tract disorders.[1] According to the serological scheme based on the O-antigens (O-polysaccharides, OPSs) this species is divided into 39 O-serotypes.[2] By now structures of more than 25 different *H. alvei* O-antigens have been established.[3–6] Now we report on the structure of a new acidic OPS from the lipopolysaccharide (LPS) of *H. alvei* strain PCM 1529, which has been recently reclassified based on genetic data and moved from the genus *Citrobacter* to the genus *Hafnia*.[7]

The LPS of *H. alvei* PCM 1529 was isolated from dry bacterial mass by phenol–water extraction and recovered from the water phase in a yield 2.4%. Mild acid hydrolysis cleaved the lipid A portion of the LPS, and a water-soluble carbohydrate portion was fractionated by GPC on Sephadex G-50 to give four fractions: P_1 (a high molecular-mass material; OPS), P_2 (a core oligosaccharide substituted with a short OPS chain), P_3 (an unsubstituted core oligosaccharide) and P_4 (a low molecular-mass material containing 3-deoxyoct-2-ulosonic acid) in yields 28%, 19%, 30% and 23%, respectively, of the total material eluted from the column.

The elution profile of the carbohydrate-containing material and the yield of fraction P_2 were similar to those obtained previously from the LPS of *H. alvei* PCM 1222. In contrast, the yield of fraction P_2 was much higher as compared to other *H. alvei* strains. In SDS-PAGE, the LPS of *H. alvei* PCM 1529 showed a ladder-like pattern typical of smooth (S)-type character but different from that of the LPS of *H. alvei* PCM 1222 (Fig. 1).

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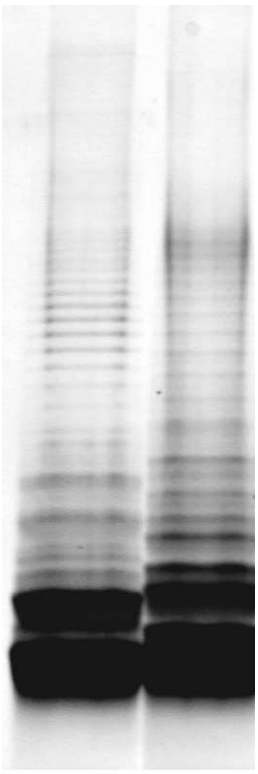

Figure 1. Silver-stained SDS-PAGE of LPS of *H. alvei* PCM 1529 (lane 1) and *H. alvei* PCM 1222 (lane 2).

Using colorimetric methods, OPS was found to contain mainly 6-deoxyhexose (46.5%; 2.8 μmol mg^{-1}), uronic acid (11%; 0.6 μmol mg^{-1}) and *O*-acetyl groups (0.73 μmol mg^{-1}). Uronic acid was identified as GalA using a sugar analyser. Sugar analysis by GLC–MS of the alditol acetates obtained after hydrolysis of OPS with 2 M CF_3CO_2H (120 °C, 2 h) revealed the presence of Rha and GlcN in molar ratio 3.0:0.63, and similar analysis of carboxyl-reduced OPS revealed Rha, Gal and GlcN in molar ratios 3.0:1.2:0.74, thus confirming the *galacto* configuration of hexuronic acid. The absolute configuration of L-Rha was established by GLC of the (*S*)-oct-2-yl glycosides and that of D-GalA using D-galactose oxidase for determination of D-Gal (10%) in the carboxyl-reduced OPS. The D configuration of GlcN was determined in OPS hydrolysate (4 M HCl, 105 °C, 18 h) with hexokinase, which in the presence of ATP completely phosphorylated this sugar. It was confirmed by GLC analysis, which revealed the lack of GlcN in the hexokinase-treated sample contrary to the control sample treated with heat-inactivated enzyme (see Experimental).

Based on the results of methylation analysis of OPS and carboxyl-reduced OPS (Table 1), it was concluded that the pentasaccharide repeating unit of OPS consists of terminal, 3-substituted and 2,3-disubstituted Rha, 4-substituted GalA and 3-substituted GlcNAc.

The ^{13}C NMR spectrum of OPS (Fig. 2B) showed a structural heterogeneity, most likely owing to nonstoichiometric O-acetylation (there was a signal for an *O*-acetyl groups at δ 21.7). After O-deacetylation, the ^{13}C NMR spectrum demonstrated a regular polysaccharide having a pentasaccharide repeating unit (Table 2, Fig. 2A). It contained signals for five anomeric carbons at δ 102.0–104.2, one $HOCH_2$–C group (C-6 of GlcN) at δ 61.5, three CH_3–C groups (C-6 of Rha) at δ 17.9–18.0, one nitrogen-bearing carbon (C-2 of GlcN) at δ 55.8, other sugar-ring carbons in the region δ 69.6–82.8, one *N*-acetyl group at δ 23.6 and two CO groups (C-6 of GalA and NAc) at δ 175.0 and 175.6. The ^1H NMR spectrum of the O-deacetylated OPS (Table 2) showed, inter alia, signals for five anomeric protons at δ 4.57–5.29, three CH_3–C groups (C-6 of Rha) at δ 1.24–1.31 and one *N*-acetyl group at δ 1.99.

Therefore, the O-deacetylated OPS has a pentasaccharide repeating unit containing three residues of L-Rha and one residue each of D-GlcNAc and D-GalA. The monosaccharide residues were designated as **A**–**E** according to their sequence in the repeating unit (see below).

The ^1H and ^{13}C NMR spectra of the O-deacetylated polysaccharide were assigned using 2D COSY, TOCSY, ROESY and H-detected ^1H,^{13}C HSQC experiments (Table 2). The TOCSY spectrum showed correlations for H-6 of Rha and H-1 of each sugar residue with all other protons within each sugar spin system, whose

Table 1. Methylation analysis data

Partially methylated monosaccharide	Relative retention time	Relative GLC detector response			
		OPS		Carboxyl-reduced polysaccharide	
		A	B	A	B
2,3,4-Me_3Rha	0.66	0.6	0.3	0.6	0.8
2,4-Me_2Rha	0.92	1.0	1.2	0.8	1.0
4-MeRha	1.10	1	1	1	1
2,3,6-Me_3Gal	1.24			0.9	1.3
4,6-Me_2GlcNMeAc	1.91	0.4	1.3	1.1	1.8

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). The following were the hydrolysis conditions: A, 2 M CF_3CO_2H, 120 °C, 2 h; B, 10 M HCl, 80 °C, 0.5 h.

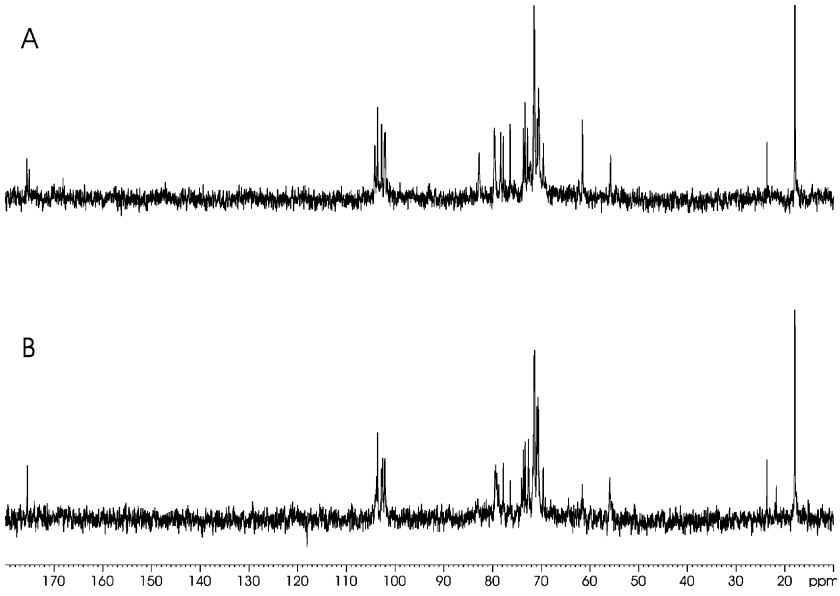

Figure 2. 125 MHz ^{13}C NMR spectra of O-deacetylated OPS (A) and OPS (B) of *H. alvei* PCM 1529.

Table 2. 500 MHz ^{1}H NMR and 125 MHz ^{13}C 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-L-Rha*p*-(1→	(**A**)	5.13	4.14	3.84	3.46	3.75	1.24	
→4)-α-D-Gal*p*A-(1→	(**B**)	5.29	3.90	4.00	4.39	4.28		
→3)-β-D-Glc*p*NAc-(1→	(**C**)	4.57	3.81	3.75	3.69	3.44	3.76	3.85
→2,3)-α-L-Rha*p*-(1→	(**D**)	5.21	4.14	3.94	3.42	3.81	1.27	
α-L-Rha*p*-(1→	(**E**)	5.05	4.04	3.77	3.49	3.71	1.31	
		C-1	C-2	C-3	C-4	C-5	C-6	
→3)-α-L-Rha*p*-(1→	(**A**)	102.2	71.5	79.5	72.8	70.4	17.9	
→4)-α-D-Gal*p*A-(1→	(**B**)	102.0	69.6	71.4	78.3	72.4	175.0	
→3)-β-D-Glc*p*NAc-(1→	(**C**)	104.2	55.8	82.8	71.6	76.4	61.5	
→2,3)-α-L-Rha*p*-(1→	(**D**)	102.8	79.6	77.7	73.7	70.8	17.9	
α-L-Rha*p*-(1→	(**E**)	103.6	71.4	71.5	73.3	70.5	18.0	

Signals for NAc are at δ_H 1.99, δ_C 23.6 (Me) and 175.6 (CO).

assignment was fulfilled using the COSY spectrum. The five spin systems were identified based on characteristic chemical shifts and $^{3}J_{H,H}$ coupling constant values, which were estimated from the 2D NMR spectra. The H-1 chemical shift of δ 4.57 and a relatively large $^{3}J_{1,2}$ value of ~7 Hz showed that GlcNAc is β-linked, which was confirmed by H-1,H-3 and H-1,H-5 correlations in the ROESY spectrum. The other anomeric signals were broadened singlets, which gave intense H-1,H-2 with no H-1,H-3 and H-1,H-5 correlations in the ROESY spectrum and thus belonged to the α-linked sugar residues (Rha and GalA). The α configuration of all Rha residues was confirmed by the C-5 chemical shifts of δ 70.4–70.8, which are typical of an α-rhamnopyranose, whereas C-5 of a β-rhamnopyranose would resonate at a lower field by ~3 ppm.[8,9]

The modes of glycosylation of the monosaccharides were determined by significant downfield displacements of the signals for C-3 of Rha **A** and GlcNAc **C**, C-4 of GalA **B** and C-2 and C-3 of Rha **D** by 4–10 ppm, as compared with their positions in the corresponding nonsubstituted monosaccharides.[8,9] The ^{13}C NMR chemical shifts of C-2–C-5 of Rha **E** were close to those in the nonsubstituted α-Rha*p*[8] and, hence, this residue occupies the terminal position in the side chain, whereas Rha **D** is at the branching point of the main chain.

The ROESY spectrum showed strong interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: Rha **A** H-1,GalA **B** H-4; GalA **B** H-1,GlcNAc **C** H-3; GlcNAc **C** H-1,Rha **D** H-2; Rha **D** H-1,Rha **A** H-3; and Rha **E** H-1,Rha **D** H-3. In the ^{1}H,^{13}C HMBC spectrum, there were correlations between the anomeric protons and the corresponding linkage carbons and vice versa. The ROESY and HMBC data were in full agreement with each other, as well as with the glycosylation pattern determined by the ^{13}C NMR chemical shift data (see above), and defined the monosaccharides sequence in the repeating unit.

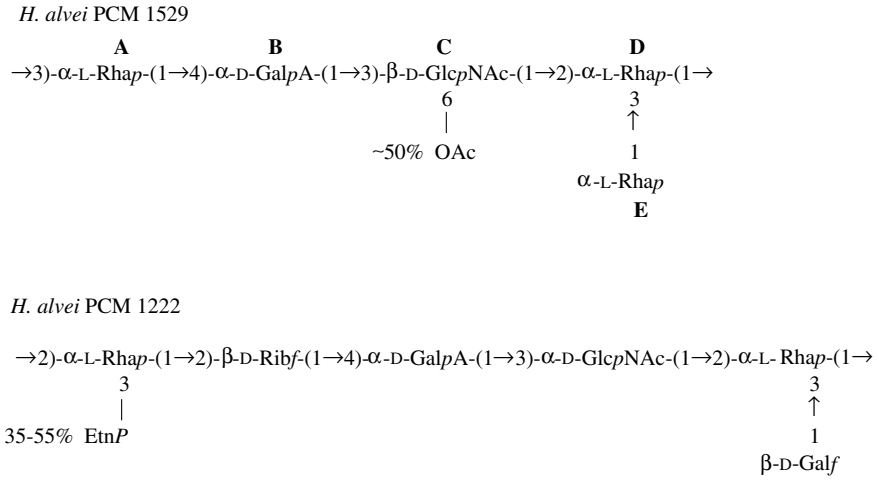

Figure 3. Structures of OPSs of *H. alvei* PCM 1529 (this work) and *H. alvei* PCM 1222 (Ref. 3).

Comparison of the $^{1}H,^{13}C$ HSQC spectra of the initial and O-deacetylated polysaccharides revealed a significantly lower-field position in both dimensions of about half of the H-6a,C-6 and H-6b,C-6 cross-peaks of GlcNAc (δ 4.31/64.0 and 4.40/64.0 in the former vs δ 3.76/61.5 and 3.85/61.5 in the latter). This distinction was due to a deshielding effect of the *O*-acetyl group and indicated O-acetylation of about half of the GlcNAc residues at position 6.

On the basis of the data obtained, it was concluded that OPS of *H. alvei* PCM 1529 has the structure shown in Figure 3. The structure of OPS of *H. alvei* PCM 1529 shows some similarity to that of *Hafnia* PCM 1222,[3] the OPSs containing a similar tetrasaccharide fragment in the main chain, including a →4)-GalA-(1→3)-GlcNAc disaccharide (Fig. 3).

2. Experimental

2.1. General methods

SDS-PAGE analysis was performed according to Laemmli[10] and silver staining of the gels[11] as described. GPC was carried out on a column (2×100 cm) of Sephadex G-50 in pyridinium acetate buffer pH 5.6; sugar content in 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}.

2.2. Bacterial strain, isolation and degradation of the lipopolysaccharide

H. alvei strain PCM 1529 (strain Balt 6331-50,[7] formerly *Citrobacter* O5:63; IHE Be 56/57) was from the collection of the L. Hirszfeld Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were harvested from a liquid medium[12] and LPS was obtained from acetone-dried bacterial mass by phenol–water extraction.[13,14] LPS was hydrolysed with aq 1% HOAc (100 °C, 90 min) and after removal of a lipid sediment the carbohydrate-containing material (65% of the LPS weight) was fractionated by GPC on Sephadex G50 to give fractions P_1–P_4.

2.3. Sugar and methylation analysis

The OPS and carboxyl-reduced OPS were hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) and the monosaccharides obtained were converted into the alditol acetates[15] and analysed by GLC–MS. GalA was identified using a Biotronik LC-2000 sugar analyser on an Chromex DA×8-11 column at 70 °C in 0.04 M KH_2PO_4 buffer pH 2.4. Methylation was performed with methyl iodide in the presence of solid base according to Gunnarsson;[16] the methylated compounds were hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h) or 10 M HCl (80 °C, 0.5 h), converted into the partially methylated alditol acetates and analysed by GLC–MS as in sugar analysis.

In order to determine the absolute configuration of GlcNAc, the OPS (1 mg) was hydrolysed with 4 M HCl (105 °C, 18 h) and acid removed in dessicator over NaOH. The OPS hydrolysate (0.5 mg) was treated with hexokinase in the presence of ATP[17] in 0.2 M ammonium acetate pH 8.0 (1.5 mL) at 20 °C for 3 h or with heat-inactivated (100 °C, 5 min) enzyme. Internal standard of galactose (0.1 mg) was added to the sample and to the control prior to the incubation with enzyme. The lack of peracetylated glucosaminitol showed by GLC analysis of hexokinase-treated OPS hydrolysate subjected to $NaBH_4$ reduction and peracetylation gives an evidence for D-configuration (complete phosphorylation) of GlcN. The absolute configuration of Rha was established by GLC of the acetylated (*S*)-oct-2-yl gly-

cosides[19] and that of GalA using D-galactose oxidase[18] after hydrolysis of the polysaccharide and the carboxyl-reduced OPS, respectively, with 2 M CF_3CO_2H (120 °C, 2 h). *O*-Acetyl groups were quantified by the Hestrin procedure[20], rhamnose was determined according to Dische[21] and hexuronic acid as described.[22]

Carboxyl reduction of OPS (10 mg) was carried out according to Taylor et al.,[23] and the reduced polysaccharide (7.4 mg) was recovered by dialysis against distilled water and lyophilisation. The O-deacetylation of OPS was performed with aq 12% ammonia (20 °C, 16 h) followed by lyophilisation.

2.4. 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 30 °C. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45). A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively.

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