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Immunochemical studies of the lipopolysaccharides of *Hafnia alvei* PCM 1219 and other strains with the O-antigens containing D-glucose 1-phosphate and 2-deoxy-2-[(*R*)-3-hydroxybutyramido]-D-glucose

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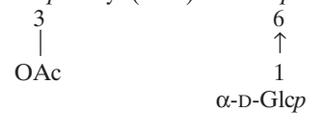
Abstract

Introduction: *Hafnia alvei* is the only species of the genus *Hafnia*, which belongs to the family of *Enterobacteriaceae*. These Gram-negative bacteria are commonly distributed in the natural environment and are often the cause of human opportunistic infections. Their lipopolysaccharides (LPSs) are important surface antigens which are responsible for the serological specificity and numerous cross-reactions with other enterobacterial genera. So far, 29 different O-polysaccharide (OPS, O-antigen) structures in *Hafnia* LPSs have been established and for some of them the molecular basis of the serological activity has been elucidated.

Materials and Methods: OPS from *H. alvei* strain PCM 1219 was obtained by mild acid hydrolysis of the LPS followed by gel permeation chromatography of carbohydrate material on Sephadex G-50 column. The polysaccharide structure was determined using chemical methods as well as ^{13}C NMR and ^{1}H NMR spectroscopy. For serological studies, SDS-PAGE, immunoblotting, and passive hemagglutination tests were used.

Results: The serological studies revealed a cross-reactivity of the LPSs of *H. alvei* PCM 1219 and a group of *H. alvei* strains with an O-antigen containing D-glucose 1-phosphate and [(*R*)-3-hydroxybutyramido]-D-glucose. The following structure of the OPS was established: →2)-α-D-Glc*p*-(1-PO_4-6)-α-D-Glc*p*NAcyl-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→



where Acyl stands for (*R*)-3-hydroxybutyryl and the degree of *O*-acetylation is ~70%. The structure of the core oligosaccharide was found to be typical of the genus *Hafnia*.

Conclusions: Based on the OPS structure and serological results it was concluded that *H. alvei* strain PCM 1219 should be classified in the same serogroup as the *H. alvei* type strain ATCC 13337 and five other strains containing D-glucose 1-phosphate and 2-deoxy-2-[(*R*)-3-hydroxybutyramido]-D-glucose in their O-antigens.

Key words: *Hafnia alvei*, enterobacteria, O-antigen, lipopolysaccharide, bacterial polysaccharide structure, serological cross--reactivity.

Abbreviations: COSY – correlation spectroscopy, OPS – O-polysaccharide, HMQC – heteronuclear multiple-quantum coherence, HSQC – heteronuclear single-quantum coherence, LPS – lipopolysaccharide, ROESY – rotating frame NOE spectroscopy, TOCSY – total correlation spectroscopy.

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INTRODUCTION

Hafnia microorganisms are typical representatives of the *Enterobacteriaceae* family and show close serological relationship with such species and genera as *Escherichia coli*, *Enterobacter cloaceae*, *Salmonella*, *Klebsiella*, and *Citrobacter*. The genus *Hafnia* contains only one species, *Hafnia alvei,* which is a rarely occurring but important opportunistic human pathogen found in many incidents of nosocomial infection [11, 18, 23]. In the natural environment, *Hafnia* bacteria widely exist in soil, flowing waters, and sewage. According to the serological classification, the genus *Hafnia* includes 39 O-serotypes [1]. Chemical studies carried out on *Hafnia* lipopolysaccharides (LPSs), besides their purely scientific significance, can explain the molecular basis of the serological classification of *Hafnia* [14, 15, 21]. Structural investigation of O-specific polysaccharides (O-antigens) will also help to understand the serological cross-reactivity of *Hafnia* with different species of *Enterobacteriaceae*. Most of the polysaccharides examined are neutral or acidic hexosaminoglycans, some of them containing rarely occurring monosaccharides and non-sugar 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, phosphoethanolamine, N-linked 3-hydroxybutanoyl, and formyl groups [21]. Several polysaccharides with phosphodiester linkages resemble the teichoic acids of Gram-positive bacteria. The repeating units of *H. alvei* O-polysaccharides (OPSs) are linear or branched oligosaccharides which range in size from two [17] to eight [7, 13] monosaccharide residues, the most common being penta- or hexa-saccharides.

Here we report the structure of the OPS isolated from *H. alvei* strain PCM 1219, which belongs to a group of strains represented by the type strain ATCC 13337, which contain [(*R*)-3-hydroxybutyramido]-D-glucose in their O-antigens.

MATERIALS AND METHODS

Bacterial strains, isolation and degradation of the LPS

H. alvei strain PCM 1219 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 earlier [22]. The LPS was isolated from dry bacterial mass by phenol-water extraction [28] and purified by gel-permeation chromatography on Sepharose 2B as described [20]. LPS from other strains were obtained earlier [21]. The LPS from *H. alvei* 1219 was degraded with aq 1% HOAc at 100°C for 45 min or with 0.1 M sodium acetate buffer, pH 4.2, at 100°C for 4 h, the lipid A precipitate was removed by centrifugation, and the carbohydrate portion was fractionated by gel-permeation chromatography on a column (2×100 cm) of Sephadex G-50 equilibrated with 0.05 M pyridine/acetic acid, pH 5.7, to give fractions P_1 (subfractions 1-5), P_2 (subfractions 6,7), P_3 (fraction 8), and P_4 (fraction 9) with yields of 41, 12.8, 30.8, and 15.4%, respectively. Fraction P_3 (core oligosaccharide) was further fractionated on a Bio-Gel P-2 column (2×100 cm) using the same buffer to give fractions P_{3a} and P_{3b}. Elution was monitored by the phenol-sulfuric acid method [4]. Dephosphorylation of the OPS (fraction P_1) and core oligosaccharide (fraction P_{3b}) was performed with aqueous 48% HF at 4°C for 48 h. *O*-deacetylation of the OPS was carried out by treatment with 12% aqueous ammonia at 20°C for 16 h.

Chemical analyses

For sugar analysis, the OPS and core oligosaccharide were hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h) or 10 M HC1 (80°C, 30 min). D-GalN was identified after hydrolysis of the OPS with 4 M HCl at 105°C for 18 h. The monosaccharides were reduced with $NaBH_4$, peracetylated as described [24], and the resultant alditol acetates were analyzed by GLC-MS using a Hewlett-Packard 5971A chromatograph equipped with an HP-1 glass capillary column (12 m×0.2 mm) and a temperature program of 150→270°C at 8°C/min. Paper chromatography was performed on Whatman l paper in a butanol/pyridine/water (6:4:3, v/v) solvent system and alkaline silver nitrate solution was used for staining. The D configurations of glucose, galactosamine, and glucosamine were established using D-glucose oxidase [2], D-galactose oxidase [6], and hexokinase in the presence of ATP [25], respectively. *O*-acetyl groups were determined by the method of Hestrin [10], sialic acid by the resorcinol method [26], Kdo with thiobarbituric acid [12], phosphorus according to Chen et al. [3], and 3-hydroxybutyric acid by the procedure [29] of Williamson and Mellanby.

Methylation was performed according to the procedure of Gunnarsson [9], the methylated products were purified by extraction with chloroform/water (1:1, v/v), hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h) or 10 M HCl (80°C, 30 min), reduced with $NaBD_4$, acetylated with acetic anhydride in pyridine, and analyzed by GLC-MS as above.

NMR spectroscopy

Prior to the measurements, the samples were lyophilized twice from D_2O. The ^1H- and ^{13}C-NMR spectra were run on a Bruker DRX-500 spectrometer (Germany) in 99.96% D_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). Mixing times of 100 and 200 ms were used in 2D total correlation spectroscopy (TOCSY) and rotating frame NOE spectroscopy (ROESY) experiments, respectively.

SDS-PAGE and serological assays

Preparation of rabbit serum against whole cells of *H. alvei* PCM 1187 and PCM 537, SDS-PAGE, staining

of the gels, and immunoblotting were performed as described [14, 19, 27]. A passive hemagglutination test was performed as described previously [8]. LPSs (1 mg/10 ml PBS) were heated (100°C, 2 h) and used to coat horse red blood cells (0.2 ml of packed cells) at 37°C for 1 h. The test was carried out with 1% erythrocytes and 10-fold dilutions of serum, all in PBS, at 37°C for 2 h. Results were expressed as the reciprocal titers of the serum dilutions. Horse erythrocytes were obtained from the Agricultural Academy, (Wrocław, Poland).

Table 1. Passive hemagglutination of *H. alvei* LPS with anti-*H. alvei* PCM 1187 serum

LPS from strain	Reciprocal titer
1219	1280
537	1280
1187	10240
1194	640
744	5120
1221	5120

RESULTS AND DISCUSSION

The LPS was extracted from *H. alvei* strain PCM 1219 by the phenol/water procedure in a yield of 6.5% of dry bacterial mass. SDS-PAGE of the LPS showed a ladder-like pattern of bands typical of smooth strains, which was observed also in immunoblotting with LPS--specific rabbit antiserum (Fig. 1). Immunoblotting revealed strong cross-reactivity with LPS of *H. alvei* type strain PCM 537 (ATCC 13337), but not with several other *Hafnia* LPSs (not shown). In the passive hemagglutination test, cross-reactivity was observed only for a group of strains related to *H. alvei* strain PCM 537 (Table 1). In order to establish the molecular basis for the serological relationship in this heterogeneous group of strains, the detailed structure of the OPS of *H. alvei* PCM 1219 was examined.

The LPS of *H. alvei* PCM 1219 was degraded by mild acid hydrolysis with 1% HOAc (100°C, 1 h), and the supernatant was fractionated on Sephadex G-50 to give nine fractions, indicating the depolymerization of the OPS. When the LPS was degraded with 0.1 M sodium acetate buffer, pH 4.2, at 100°C for 4 h, only four fractions were obtained, including a longer-chain (OPS) and shorter-chain polysaccharides (P_1 and P_2, respectively), a 3-deoxyoctulosonic acid (Kdo)-containing oligosaccharide (LPS core, P_3), and free Kdo (P_4). Fraction P_3 was further fractionated on Bio-Gel P-2 to give fractions P_{3a} and P_{3b}.

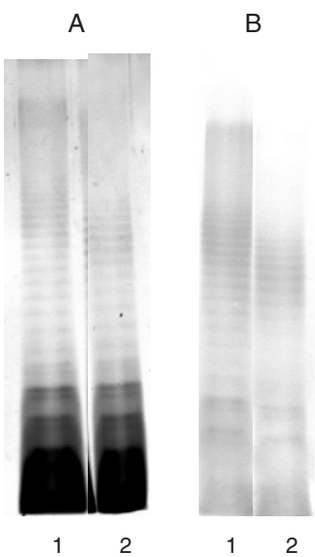

Fig. 1. Silver-stained SDS-PAGE (**A**) and immunoblotting with anti-*H. alvei* PCM 537 serum (**B**) of the LPS from *H. alvei* PCM 1219 (lane 1) and *H. alvei* PCM 537 (lane 2).

The search for an acid-labile component (Kdo, sialic acid, glycosyl phosphate) revealed the presence of phosphorus (2.3%) in the OPS. Sugar analysis of the OPS by GLC-MS after hydrolysis with 10 M HCl showed the presence of glucose and galactosamine in a molar ratio of 2.5:2.0. After dephosphorylation of the OPS with 48% HF (4°C, 48 h), glucosamine was additionally detected, the molar ratios of glucose, glucosamine, and galactosamine being 2.8:0.8:2.0.

Methylation analysis of the OPS by GLC-MS showed the presence of terminal glucose, 2-substituted glucose, and 3-substituted and 4,6-disubstituted galactosamine residues (Table 2). After dephosphorylation of the OPS followed by methylation analysis including acetylation, the major derivative of terminal glucosamine (3,4,6-Me_3GlcNAc) was also identified together with a trace amount of an N-(3-hydroxybutyryl) derivative (3,4,6-Me_3GlcNAcyl). Therefore, the glucosamine derivative in the OPS is phosphorylated and, at least partially, N-acylated with the 3-hydroxybutyryl group. The content of 3-hydroxybutyric acid in the OPS (0.8 μM/mg) was determined by an enzymatic method after hydrolysis of the OPS with 4 M HC1 (100°C for 2.5 h). In addition, the OPS was also found to contain *O*-acetyl groups (0.38 μM/mg) and, hence, is non-stoichiometrically *O*-acetylated.

The structural features of the O-antigen of *H. alvei* PCM 1219, such as the content and substitution pattern of the monosaccharides as well as the presence of a glycosyl phosphate and phosphorylated GlcNAcyl, are similar to those reported earlier for the OPS of *H. alvei* type strain PCM 537 (ATCC 13337) [21]. The similarity was confirmed by methylation analysis data (Table 2) performed on the dephosphorylated OPS from *H. alvei* strains PCM 1219 (P_1 1219_{HF}), PCM 537 (P_1 537_{HF}), and PCM 1187 (P_1 1187_{HF}).

The full structure of the OPS from *H. alvei* PCM 1219 was established using ^{1}H-, ^{13}C- (Fig. 2), and ^{31}P-NMR spectroscopy, including two-dimensional ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C heteronuclear single-quantum coherence (HSQC), and ^{1}H,^{31}P het-

Table 2. Methylation analysis of the OPS of *H. alvei* PCM 1219, PCM 537, and PCM 1187

Methylated sugar derivative	T_R[a]	Substitution	Molar ratio			
			$P_1$1219	P_1 1219$_{HF}$[b]	$P_1$537$_{HF}$[b]	P_1 1187$_{HF}$[b]
2,3,4,6-Me_4Glc	1.00	tGlc	1.00	1.00	1.00	0.13
3,4,6-Me_3Glc	1.20	→2Glc	0.90	0.34	0.23	0.04
3,4,6-Me_3GlcNAc	1.68	tGlcNAc	–	1.06	1.50	1.00
3,6-Me_2GalNAc	1.89	→4GalNAc	–	–	–	0.67
4,6-Me_2GalNAc	1.95	→3GalNAc	0.40	0.46	0.75	0.38
3-MeGalNAc	2.13	→4,6GalNAc	0.21	0.58	1.08	–
3,4,6-Me_3GlcNAcyl[c]	2.15	tGlcNAcyl	–	+[d]	–	–

[a] 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-M_4Glc). Content of the methylated derivatives of monosaccharides is given as GLC-MS detector response.
[b] P_1 1219$_{HF}$, P_1 537$_{HF}$, P_1 1187$_{HF}$, dephosphorylated OPS.
[c] Acyl stands for the 3-hydroxybutyryl group.
[d] Compound present in trace amount.
Methylated samples were hydrolyzed with 10 M HCl at 80°C for 30 min.

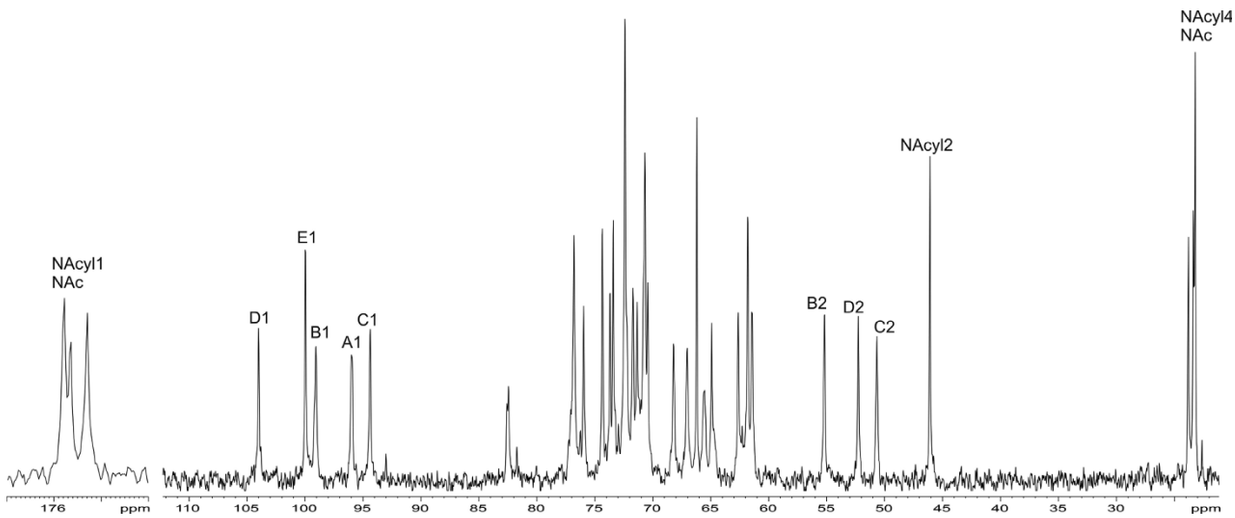

Fig. 2. ^{13}C NMR spectrum of the O-polysaccharide of *H. alvei* PCM 1219. Numerals refer to carbons in the sugar residues denoted as shown in Table 3. NAc, N-acetyl groups; NAcyl, N-3-hydroxybutyryl groups.

eronuclear multiple-quantum coherence (HMQC) experiments according to the published methodology [5]. Based on the assigned chemical shifts given in Table 3, the glycosylation and phosphorylation patterns in the *O*-deacetylated polysaccharide were defined using H-detected ^1H,^{13}C HSQC and ^1H,^{31}P HMQC data. The sequence of the monosaccharides in the repeating unit was determined by a ROESY experiment, which showed correlations between the anomeric protons and protons at the linkage carbons. Finally, the presence of an *O*-acetyl group at position 3 of GlcNAcyl was established by a comparison of the ^1H,^{13}C HSQC spectra of the initial OPS and *O*-deacetylated polysaccharide and, based on integral intensities ratios of ^1H-NMR signals of the nonacetylated and 3-*O*-acetylated GlcNAcyl residues, the degree of *O*-acetylation was estimated as ~70%

The data obtained showed that the O-antigen of *H. alvei* 1219 has the same structure as those of *H. alvei* PCM 537 (ATCC 13337) and 114/60, including the position and degree of *O*-acetylation (structure 1 in Fig. 3). The O-antigens from related strains, including *H. alvei* 1194, 744, and PCM 1221, differ in the absence of *O*-acetyl groups (structure 2). The O-antigen of yet another, more distantly related strain, *H. alvei* PCM 1187, is devoid of both *O*-acetyl groups and the lateral glucose residue (structure 3). Therefore, *H. alvei* PCM 1219 is already the seventh representative of the group of strains with identical or similar O-antigen structures (Fig. 3) and all these strains should be classified in the same serogroup.

As for the majority of *H. alvei* strains studied so far [16, 21], methylation analysis of the core oligosaccharide fractions P_{3a} and P_{3b} from *H. alvei* PCM 1219 revealed terminal and 3-substituted glucose as well as terminal heptose residues, and after dephosphorylation also 3-

Table 3. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of the *O*-deacetylated polysaccharide from *H. alvei* PCM 1219

	Residue	Atom						
		H-1	H-2	H-3	H-4	H-5	H-6	H-6’
A	→2)-α-D-Glc*p*-1-*P*-(O→[a]	5.79	3.63	3.85	3.53	3.84	3.91	3.70
B	-6)-α-D-Glc*p*NAcyl-(1→[b]	4.99	4.01	3.87	3.65	4.31	4.18	4.05
C	→4)-α-D-Gal*p*NAc-(1→[c] 6 ↑	5.15	4.32	3.93	4.17	4.03	3.85	3.66
D	→3)-β-D-Gal*p*NAc-(1→[c]	4.72	4.11	3.82	4.09	3.65	3.83	3.83
E	α-D-Glc*p*-(1→	4.88	3.59	3.68	3.48	3.66	3.87	3.79
		C-1	C-2	C-3	C-4	C-5	C-6	
A	→2)-α-D-Glc*p*-1-*P*-(O→[b]	96.0	82.4	73.9	70.6	72.7	62.7	
B	-6)-α-D-Glc*p*NAcyl-(1→[d]	99.2	55.2[c]	71.9	71.0	72.5	65.8	
C	→4)-α-D-Gal*p*NAc-(1→[e] 6 ↑	94.6	50.8	68.4	76.9	71.6	67.4	
D	→3)-β-D-Gal*p*NAc-(1→[e]	104.0	52.5	76.1	65.2	76.9	61.7	
E	α-D-Glc*p*-(1→	100.2	72.7	74.5	70.8	73.6	62.0	

Letters **A–E** refer to formula 1 in Fig. 3.
[a] A signal for ^{31}P is at –0.7 ppm.
[b] Signals for 3-hydroxybutyryl (Acyl) are at 2.53 (H-2), 4.23 (H-3), and 1.30 (H-4) ppm.
[c] Signals for NAc are at 2.03 and 2.05 ppm.
[d] Signals for 3-hydroxybutyryl (Acyl) are at 176.1 (C-1), 46.3 (C-2), 66.2 (C-3), and 23.3 (C-4) ppm.
[e] Signals for NAc are at 23.8, 24.0 (both CH_3), 175.6, and 176.1 (both CO) ppm.

H. alvei PCM 537 (ATCC 13337), 114/60, PCM 1219

A **B** **C** **D**
→2)-α-D-Glc*p*-1-*P*-(O→6)-α-D-Glc*p*NAcyl-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→ **1**
3 6
| ↑
(~70%) OAc α-D-Glc*p*1
E

H. alvei 1194, 744, PCM 1221

→2)-α-D-Glc*p*-1-*P*-(O→6)-α-D-Glc*p*NAcyl-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→ **2**
6
↑
α-D-Glc*p*1

H. alvei PCM 1187

→2)-α-D-Glc*p*-1-*P*-(O→6)-α-D-Glc*p*NAcyl-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→ **3**

Fig. 3. Structures of the OPS from *H. alvei* PCM 1219 (this work) and related O-polysaccharides [21]. Acyl stands for the 3-hydroxybutyryl group.

-substituted and 3,7-disubstituted heptose residues. The presence of the typical *H. alvei* LPS core is in accordance with the classification of strain PCM 1219 within the genus *Hafnia*.

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