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The O-acetylation patterns in the O-antigens of *Hafnia alvei* strains PCM 1200 and 1203, serologically closely related to PCM 1205

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Abstract—Serological tests revealed immunochemical similarities between the lipopolysaccharides of *Hafnia alvei* strains PCM 1200, 1203 and 1205. Immunoblotting and ELISA showed cross-reactions between the strains. NMR spectroscopy showed that the O-deacetylated O-specific polysaccharides isolated from lipopolysaccharides of *H. alvei* strains PCM 1200 and 1203 possessed the same composition and sequence as the O-deacetylated O-specific polysaccharide of *H. alvei* strain PCM 1205, that is a glycerol teichoic-acid-like polymer with a repeating unit of the following structure:

α-D-Glc*p*
1
↓
4
→3)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Qui*p*4NAc-(1→1)-Gro-(3-P→
2
↑
1
β-D-Glc*p*NAc

NMR spectroscopic studies of the polysaccharides concluded that O-3 of the side chain β-D-Glc*p*NAc is partially O-acetylated (50–80%) in both investigated strains. In strain PCM 1203 an additional O-acetyl group (50–80%) is linked to O-6 of the chain →3)-α-D-Glc*p*NAc-(1→ residue. The structural features of the isolated O-specific polysaccharides were also the same as those of the O-specific polysaccharides on the bacterial cells directly observed by the HR-MAS NMR technique.

Keywords: *Hafnia alvei*: lipopolysaccharide; O-acetylation; O-antigen; High-resolution magic angle spinning; HR-MAS; Serology

1. Introduction

Hafnia alvei, a Gram-negative bacterium and a member of the family *Enterobacteriaceae*, is a rare but important pathogen that is often responsible for urinary tract, respiratory tract and wound infections, that is the most common nosocomial infections. It has been found that multidrug resistance is widespread among enteric bacteria including *Escherichia coli* and *H. alvei*. A sharing of virulence-associated properties at the phenotypic and genetic levels between those enteropathogenic bacteria has also been reported, thus *H. alvei* should be considered as an important diarrhogenic pathogen.[1–7]

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The serotyping scheme of *H. alvei* includes 39 O-serotypes[8] and preliminary chemical analyses of lipopolysaccharides (LPS) isolated from 33 *H. alvei* strains have previously been reported.[9] In on-going structural studies of the *H. alvei* LPS the O-specific polysaccharides from a number of serologically different strains were elucidated.[10–15]

For the definition of the O-serotypes, the O-antigens were analysed in slide agglutination on saline suspensions of bacteria.[16] Most *H. alvei* O-antigens were determined by appropriately diluted nonabsorbed antisera. However, serologically closely related antigens, as in strains PCM 1200, 1203 and 1205, were only assigned to different O-serotypes with the use of monospecific absorbed antisera.

As the LPS of all three *H. alvei* strains show immunochemical similarities, we now report on serological and structural studies of O-specific polysaccharides of *H. alvei* strains PCM 1200 and 1203 in relation to that of strain PCM 1205.[17] The NMR spectra of the isolated O-specific polysaccharides and the spectra obtained by high-resolution magic angle spinning (HR-MAS) NMR from whole bacteria were also compared.

2. Experimental

2.1. Bacteria

The bacteria *Hafnia alvei* strain PCM 1200, 1203 and 1205 were obtained from the collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The bacteria were grown and harvested as described previously.[13]

2.2. Immunisation procedure and serological methods

Antisera against the O-antigens of *H. alvei* PCM 1200 and 1203 were obtained by intravenous immunisation of rabbits with PBS suspensions of killed bacterial cells as previously described.[18] Enzyme-linked immunosorbent assay (ELISA), using LPS as solid-phase antigen, was performed according to the method described by Voller et al.[19] with modifications.[20] Immunoblotting was done as previously described.[13] A goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) was used as the second antibody in both immunoassays. *p*-Nitrophenyl phosphate and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium were applied as detection systems for ELISA and immunoblotting, respectively.

2.3. Isolation of LPS and polysaccharide fractions

LPS was extracted from bacterial cells by the hot phenol–water method,[21] then purified by repeated ultracentrifugation (100,000*g*, 6 h) and freeze-dried. The O-specific polysaccharide was released by treatment with 1.5% AcOH containing 2% SDS at 100 °C for 15 min. SDS was removed from freeze-dried hydrolysate by extraction with 96% ethanol and the residue suspended in water and centrifuged. The supernatant was fractionated by size-exclusion chromatography on a column (1.6 × 100 cm) of Bio-Gel P-10, equilibrated with 0.05 M pyridine/acetic acid buffer, pH 5.6. Eluates were monitored by a Knauer differential refractometer and a polysaccharide fraction was collected and analysed by ^{1}H and ^{13}C NMR spectroscopy.

2.4. O-deacetylation

Native polysaccharide (3–6 mg) was treated with aq 12.5% NH_3 soln (2 mL) overnight at room temperature followed by dilution with water and lyophilisation.

2.5. NMR spectroscopy

NMR spectra of the polysaccharides were obtained for D_2O solns at 30 °C on a Bruker DRX 600 spectrometer, using acetone (δ_H 2.225 ppm, δ_C 31.05 ppm) as internal reference. The polysaccharide preparations were repeatedly exchanged with D_2O with intermediate lyophilisation before the NMR experiments. One- and two-dimensional spectra (COSY, TOCSY, NOESY, HMBC and HSQC-DEPT) were acquired and processed using standard Bruker software. In the TOCSY experiments the mixing times were 30 and 100 ms. The delay time in the HMBC was 60 ms and the mixing time in the NOESY experiments 200 ms. The processed spectra were assigned with help of the SPARKY program.[22] The amounts of O-acetyl groups were determined by integration of the signals from 1D ^{1}H NMR spectra obtained for the O-specific polysaccharides with a 30°-pulse angle and a relaxation delay of 5 s.

^{1}H NMR spectra of bacterial cells and lipopolysaccharides in D_2O suspensions were obtained by the HR-MAS technique.[23] The bacteria (~3 mg) were placed into the rotor and suspended in 12 μL of D_2O. All HR-MAS NMR experiments were carried out at 5 kHz spin rate at 23 °C with a Bruker 4 mm HR-MAS probe and a ZrO_2 rotor. One dimensional ^{1}H spectra of bacteria and LPS were acquired with a Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence[24] [90°–(τ–180°–τ)$_n$-acquisition (total delay time 12 ms)] as T_2-filter to remove the broad signals from semi-solid bacterial components. All HR-MAS NMR spectra were obtained with acetone in D_2O as a reference (δ_H 2.225 ppm) in a separate experiment just before the actual run.

3. Results and discussion

The antigenic relationship between the *H. alvei* strains PCM 1200, 1203 and 1205 lipopolysaccharides (LPS)

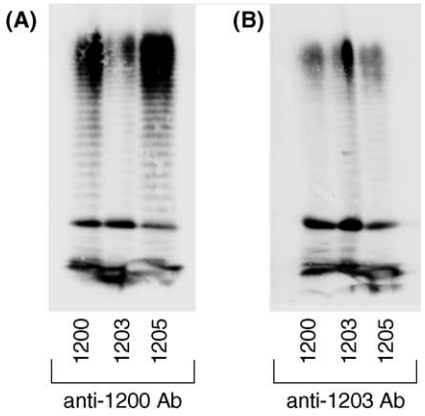

Figure 1. Reaction of polyclonal antibodies against *H. alvei* strain PCM 1200 (A) and *H. alvei* strain PCM 1203 (B) with *H. alvei* strain PCM 1200, 1203 and 1205 LPS, separated on SDS-PAGE and transblotted onto nitrocellulose.

was investigated by serological methods. The cross-reactions of the LPS of *H. alvei* strains PCM 1200, 1203 and 1205 were investigated by immunoblotting and ELISA experiments, using anti-*H. alvei* PCM 1200 and anti-*H. alvei* PCM 1203 antibodies. Rabbits were immunised with the PBS suspension of bacterial cells from strains PCM 1200 and 1203 and sera with polyclonal antibodies against the O-antigens of *H. alvei* PCM 1200 and 1203 were obtained. The cross-reactions between the LPS of *H. alvei* strains PCM 1200, 1203 and 1205 were studied by immunoblotting experiments (Fig. 1) and ELISA (Fig. 2). Both tests demonstrated that the antibodies against the O-antigens of *H. alvei* PCM 1200 reacted strongly with homologous LPS and those of strains PCM 1203 and 1205 (Figs. 1A and 2A). Similarly, the antibodies against the O-antigens of *H. alvei* PCM 1203 showed reactions with homologous LPS and cross-reactions with LPS of strains PCM 1200 and 1205 (Figs. 1B and 2B).

The intensities of reactions of anti-bacterial sera with different LPS in ELISA were almost identical. However, immunoblotting tests showed some differences in the intensities of those reactions. The antibodies against *H. alvei* PCM 1200 reacted strongly with the LPS of homologous strain and strain PCM 1205, but the reaction with the LPS of strain PCM 1203 was weaker. When the anti-serum against *H. alvei* PCM 1203 was used, the strongest reaction was observed for the homologous LPS and clear but weaker reactions for the LPS of strains PCM 1200 and 1205.

The LPS of *H. alvei* strain PCM 1200, 1203 and 1205 were isolated by phenol–water extraction and they showed on SDS/PAGE patterns typical for LPS with extended O-specific polysaccharide chains. The lipid A part was cleaved off by mild acidic hydrolysis and the recovered O-specific polysaccharide fraction was separated from the core oligosaccharide fraction by gel permeation chromatography on Bio-Gel P-10. The fractions containing O-specific polysaccharide were studied by NMR spectroscopy.

1D ^{1}H NMR (Fig. 3) and 2D HSQC-DEPT spectra, recorded for the O-specific polysaccharides from *H. alvei* strains PCM 1200 and 1203, showed rather complex spectra due to nonstoichiometric O-acetylation. After O-deacetylation both polysaccharides gave the same NMR spectra consistent with a pentasaccharide repeating unit. ^{1}H and ^{13}C signals (Table 1) were assigned by a combination of different 2D NMR spectra and the spin systems for each monosaccharide determined by starting with the respective signals for the

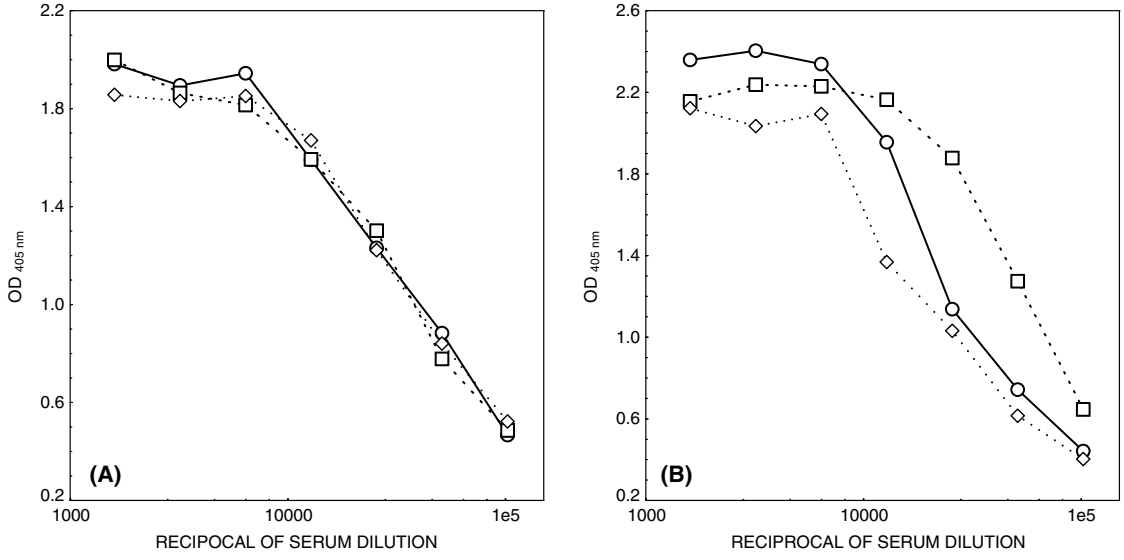

Figure 2. The reaction of polyclonal antibodies against *H. alvei* strain PCM 1200 LPS (A) and strain PCM 1203 LPS (B) with the LPS of *H. alvei* strains PCM 1200 (○), 1203 (□) and 1205 (◊) as solid phase antigens in ELISA.

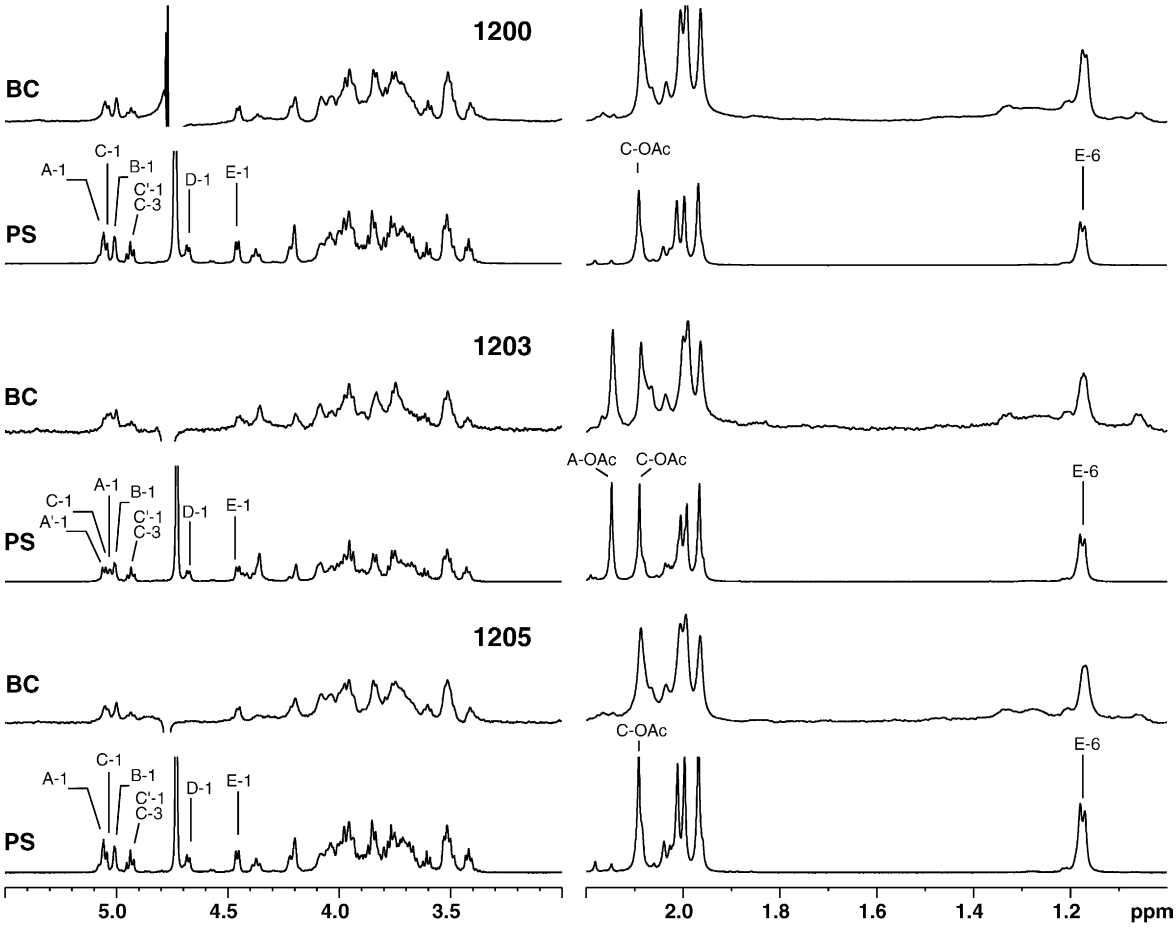

Figure 3. ^{1}H NMR spectra of the isolated O-specific polysaccharides (PS) and of the O-antigens obtained directly on bacteria cells (BC) of *H. alvei* strains PCM 1200, 1203 and 1205. Spectra on suspensions of bacteria cells in D_2O at 23 °C were obtained by the HR-MAS NMR technique. (The primed A′ and C′ residues are from nonacetylated repeating units.)

Table 1. NMR data for the O-deacetylated O-specific polysaccharides of *H. alvei* strains PCM 1200 and 1203 in D_2O solns at 30 °C

	Sugar residue	Chemical shifts (ppm) [$J_{H1,H2}$ (Hz)]							
		H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a/H-6b C-6	NAc	CO
A	→3)-α-D-Glc*p*NAc-(1→	5.08 [<3]	4.03	3.97	3.68	4.22	3.88/3.80	2.04	
		97.9	52.6	80.7	68.5	71.9	60.8	23.4	174.7
B	α-D-Glc*p*-(1→	5.01 [<3]	3.52	3.77	3.49	3.93	3.95[a]/3.82[a]		
		101.1	72.9	73.5	70.2	73.0	60.8		
C	β-D-Glc*p*NAc-(1→	4.93 [8]	3.77	3.48	3.39	3.42	3.94/3.75	2.08	
		100.7	56.3	76.2	71.0	77.1	62.0	23.3	175.8
D	→2,3,4)-β-D-Gal*p*-(1→	4.69 [8]	3.98	4.37	4.19	3.76	3.90/3.88		
		100.4	75.1	78.4	77.7	75.7	60.8		
E	→3)-β-D-Quip4NAc-(1→	4.46 [8]	3.43	3.70	3.73	3.52	1.17	1.97	
		103.5	73.3	78.1	57.4	72.1	17.3	22.9	174.7
	→1)-Gro-(3-P→	4.01/3.71	4.08	4.03/3.99					
		71.8	70.3	67.1					

Chemical shifts (ppm) are given using internal acetone (δ_H 2.225 and δ_C 31.05) as reference.
[a] Tentatively assigned signals.

anomeric atoms. The chemical shifts were in good agreement ($|\Delta\delta|$<0.03 ppm for ^{1}H signals and $|\Delta\delta|$ < 0.2 ppm for ^{13}C signals) with the corresponding values for the previously studied O-deacetylated polysaccharide of *H. alvei* strain 1205.[17] The positions of the O-acetyl groups in the O-specific polysaccharides were determined by comparison of ^{1}H and ^{13}C NMR spectra obtained for the polysaccharides and the O-deacetylated

Table 2. NMR data for the monosaccharides influenced by O-acetylation of the *H. alvei* strain PCM 1200 and 1203 O-specific polysaccharides

	Residue	Atom no	Chemical shifts and O-acetylation shifts (ppm)							
			1200		1203		1200		1203	
			^{1}H	$\Delta\delta_H$	^{1}H	$\Delta\delta_H$	^{13}C	$\Delta\delta_C$	^{13}C	$\Delta\delta_C$
A	→3)-α-D-Glc*p*NAc-(1→	1	5.06	−0.02	5.04	−0.04	98.0	0.1	98.2	0.3
		2	4.04	0.01	4.09	0.06	52.8	0.2	52.9	0.3
		3	3.96	−0.01	3.97	0	80.3	−0.4	80.2	−0.5
		4	3.67	−0.01	3.67	−0.01	68.4	−0.1	68.6	0.1
		5	4.20	−0.02	4.42	−0.20	71.9	0	70.0	−1.9
		6	3.88/3.80	0/0	4.36/4.36	0.48/0.56	60.8	0	63.9	3.1
B	α-D-Glc*p*-(1→	1	5.00	−0.01	5.01	0	101.1	0	101.1	0
C	β-D-Glc*p*NAc-(1→	1	5.05	0.12	5.06	0.13	100.3	−0.4	100.4	−0.3
		2	3.85	0.08	3.86	0.09	54.7	−1.6	54.7	−1.6
		3	4.93	1.45	4.94	1.46	77.9	1.7	78.0	1.8
		4	3.60	0.21	3.62	0.23	68.7	−2.3	68.7	−2.3
		5	3.52	0.10	3.52	0.10	76.9	−0.2	76.9	−0.2
		6	3.92/3.73	−0.02/−0.02	3.94/3.75	0/0	62.0	0	62.0	0
D	→2,3,4)-β-D-Gal*p*-(1→	1	4.67	0	4.69	0.02	100.8	0.4	100.6	0.2
E	→3)-β-D-Qui*p*4NAc-(1→	1	4.45	−0.01	4.46	0	103.5	0	103.6	0.1

polysaccharides (Table 2) as O-acetyl groups cause significant shifts for both the proton and carbon signals at and adjacent to the substitution position.[25] Chemical shift differences, $\Delta\delta_H$ and $\Delta\delta_C$ (Table 2), between signals in spectra of native and O-deacetylated polysaccharides of *H. alvei* strain PCM 1200 and 1203 were compared with corresponding $\Delta\delta$-values of mono-O-acetylated methyl α- and β-D-glycosides.[25] For strain PCM 1200 significant differences were only observed for the signals of residue **C** [β-D-Glc*p*NAc-(1→] where downfield shifts were observed for the H-3 ($\Delta\delta_H$ 1.45 ppm) and C-3 ($\Delta\delta_C \sim 1.7$ ppm) signals. These shifts demonstrate that O-3 of residue **C** is acetylated and this is also supported by the shifts of the signals from H-2, H-4, C-2 and C-4[25] of the same residue. For *H. alvei* strain PCM 1203 similar shifts for signals of residue **C** were observed, demonstrating acetylation at O-3. However, there were also downfield shifts of the C-6 and the H-6 signals ($\Delta\delta_C$ 3.1 ppm; $\Delta\delta_H$ 0.48 and 0.56 ppm) from the chain residue **A** [→3)-α-D-Glc*p*NAc-(1→], demonstrating acetylation of O-6 of this residue. Further support for the positions of the acetyl groups and assignments of the O-acetyl signals were obtained by the two- and three-bond heteronuclear connectivities, observed as cross-peaks in the HMBC spectra. Connectivities were found between H-3 of **C** (δ_H 4.94 ppm), the acetyl carbonyl carbon (δ_{CO} 174.8 ppm) and the acetyl methyl protons (δ_H 2.09 ppm) and between H-6 of residue **A** (δ_{H6} 4.29 ppm), the carbonyl carbon (δ_{CO} 175.1 ppm) and the acetyl methyl protons (δ_H 2.18 ppm).

These results show that the O-specific polysaccharide of *H. alvei* strain PCM 1200 is acetylated at O-3 of the

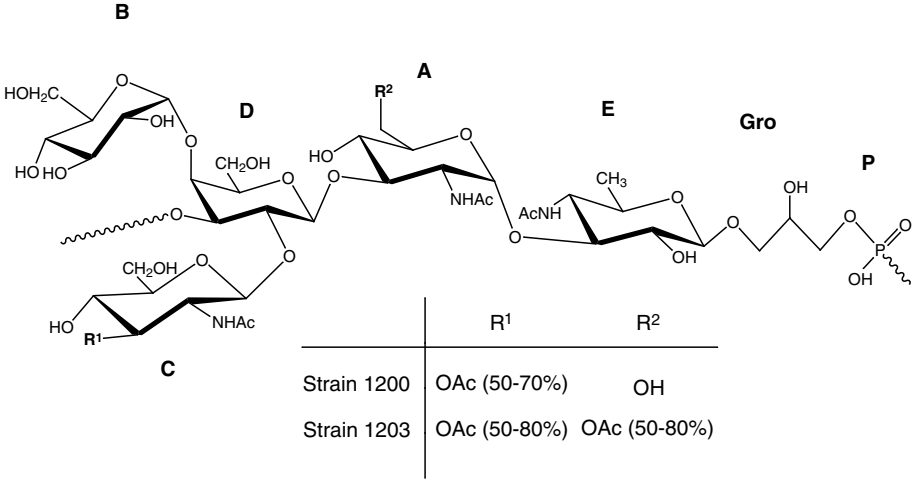

Chart 1. Structures of the repeating units of the *H. alvei* strains PCM 1200 and 1203 O-specific polysaccharides.

side-chain β-D-Glc*p*NAc-(1→ residue, **C**, whereas the O-polysaccharide of *H. alvei* strain PCM 1203 contains acetyl groups in both the **A** and **C** residues. Previous studies on the O-specific polysaccharide of *H. alvei* strain PCM 1205 showed O-acetylation at O-3 but also O-6 in the side chain β-D-Glc*p*NAc-(1→ residue.[17] By integration of the NMR signals for two batches the amounts of O-acetyl groups were determined to 50–70% in strain PCM 1200 and 50–80% in both positions in strain PCM 1203 (Chart 1).

Studies of the O-acetylation of O-specific polysaccharides are normally done on the isolated polysaccharides, generated by acid hydrolysis under mild conditions of the Kdo linkage to lipid A. During the isolation procedures the O-acetyl groups can be cleaved off or migrate to neighbouring positions. Thus the distribution of the O-acetyl groups found in the isolated O-specific polysaccharides was compared to that found on bacterial cells by HR-MAS NMR analysis. This technique allows for observation of the flexible O-specific polysaccharide part of the LPS bound to the cell wall.[26] All the O-acetyl groups determined in the isolated O-specific polysaccharides were also identified in the HR-MAS NMR spectra (Fig. 3) recorded on suspensions of the bacteria. The same pattern obtained for signals from the O-acetyl groups in the δ 2.0–2.2 region indicated the same O-acetyl substitution in the O-specific polysaccharides in both isolated and native form.

4. Conclusion

The immunochemical similarities shown by the *H. alvei* strains PCM 1200, 1203 and 1205 could be explained by the structural similarities identified in this study. The repeating unit of the O-specific polysaccharides from all strains consists of the same pentasaccharide including the side-chain structural element [3-*O*-Ac-β-D-Glc*p*NAc-(1→]. All LPS used in serological studies showed different O-acetylation patterns. In the LPS of strains PCM 1200 and 1205 the O-acetyl groups are situated only to the lateral β-D-Glc*p*NAc of the O-specific polysaccharide, whereas the LPS of strain PCM 1203 is additionally O-acetylated at O-6 of the →3)-α-D-Glc*p*NAc-(1→ chain residue.

The LPS of strains PCM 1200 and 1205 reacted similarly with anti-*H. alvei* PCM 1200 and anti-*H. alvei* PCM 1203 antibodies but differently than the LPS of strain PCM 1203. These data suggest the presence of at least two different populations of antibodies in the antisera, that is the antibodies that recognise the epitope common to LPS of all the investigated strains and the population of antibodies present only in the anti-*H. alvei* PCM 1203 serum, recognising the 6-O-acetylated →3)-α-D-Glc*p*NAc-(1→ residue of strain PCM 1203 LPS as a part of the epitope.

Structural data are in agreement with the strong serological cross-reactions of LPS of *H. alvei* strains PCM 1200, 1203 and 1205 with antisera raised against the different strains. Most of the anti-LPS antibodies are produced against the common pentasaccharide, which explains the observed cross-reactivity. However, those strains could not be defined by the use of nonabsorbed anti-bacterial sera alone, as only the repeatedly absorbed sera, that is containing monospecific antibodies, could recognise different epitopes present in the O-specific chains. The presence of different epitopes in the O-specific polysaccharide of *H. alvei* strains PCM 1200, 1203 and 1205 could be explained by a variation in the number of O-acetyl groups and their location.

Acknowledgements

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