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Carbohydrate Research 343 (2008) 483–488
Carbohydrate RESEARCH

Structural characterization of the O-chain polysaccharide of *Aeromonas caviae* ATCC 15468 lipopolysaccharide

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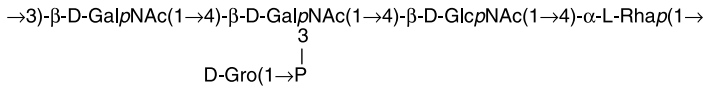
Received 1 October 2007; received in revised form 7 November 2007; accepted 12 November 2007
Available online 22 November 2007

Abstract—The O-chain polysaccharide produced by a mild acid degradation of *Aeromonas caviae* ATCC 15468 lipopolysaccharide was found to be composed of L-rhamnose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose and phosphoglycerol. Subsequent methylation and CE–ESIMS analyses and 1D/2D NMR (^{1}H, ^{13}C and ^{31}P) spectroscopy showed that the O-chain polysaccharide is a high-molecular-mass acidic branched polymer of tetrasaccharide repeating units with a phosphoglycerol substituent having the following structure:



where Gro represents glycerol and P represents a phosphate group.

Keywords: *Aeromonas caviae*; Lipopolysaccharide; NMR; CE–ESIMS

1. Introduction

Aeromonas species have been recognized as fish pathogens and are associated with furunculosis in salmonids and other freshwater species.[1] Little is known about the role of mesophilic *Aeromonas* species in gastrointestinal disease, wound infections and septiacemia.[2] *Aeromonas caviae* belongs to the family *Vibrionaceae* and is associated with gastrointestinal disease in adults and acute, severe gastroenteritis in children.[3,4] Although previous reports have described the recovery of *A. caviae* from the stools of diarrhoeic patients, mostly children,[5–8] its role as a definitive agent of diarrohea remains to be established. A number of putative virulence factors have been identified for *A. caviae*, including polar flagella,[9] pili[10] and cytotoxin.[7] Both flagella[9] and pili[10] have been implicated in the adherence of *A. caviae* to human epithelial cells in vitro. Some strains of *A. caviae* are able to form biofilms on inert surfaces, a phenomenon attributed to a hyperpiliation of the cells through type IV pili.[11] Less is known about the role of LPS in these processes, although the *flmA* and *flmB* genes of *A. caviae* Sch3N have been implicated in LPS O-chain biosynthesis.[12] As a major cell-surface component, LPS of *A. caviae* has been implicated in the adherence to human epithelial cells and biofilm formation, but the role of LPS in the pathogenesis of *A. caviae*-induced gastroenteritis is not well understood. Comprehensive structural and genetic studies of LPS are essential to determine its etiological role in pathology of the disease. In this study, we report the structural characterization of the O-chain polysaccharide of *A. caviae* ATCC 15468.

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0008-6215/$ - see front matter
doi:10.1016/j.carres.2007.11.016

2. Experimental

2.1. Bacterial culture and isolation of LPS

A. caviae ATCC 15468 was obtained from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, NS, Canada). The bacteria were cultured in 25-L fermentor in tryptic soy broth (TSB) at 37 °C for 20 h. The cells were killed with 1% (w/v) phenol solution (14 °C), washed with 0.01 M phosphate-buffered saline, pH 7.4 and harvested by low-speed centrifugation (3000*g*, 25 min). The recovered bacterial cell pellet was washed with 2.5% saline (w/v), digested with lysozyme, RNAse, DNAse and trypsin and extracted by the method of Westphal and Jann.[13] Phenol and water layers were collected separately, dialyzed against tap water and lyophilized. The lyophilizates were then dissolved in 1% saline (w/v), subjected to ultracentrifugation (105,000*g*, 4 °C, 16 h), and the recovered LPS pellets were redissolved in water and lyophilized. The crude LPS pellet (630 mg) was dissolved into a NaOAc buffer solution (30 mL, pH 5.0) containing RNase (10 mg). The solution was dialyzed against the same buffer for 24 h at 4 °C, followed by dialysis against distilled water for 3d at 4 °C. The dialyzate was lyophilized[14] to yield purified LPS (460 mg).

2.2. Preparation of the O-chain polysaccharide and dephosphorylated O-chain polysaccharide

(a) *Mild acid hydrolysis of LPS*: LPS (60 mg) was hydrolyzed with 0.2 M HOAc (100 °C, 2 h). The reaction mixture was cooled down on ice, and the insoluble lipid A was removed by centrifugation. The water-soluble part was lyophilized and purified by gel chromatography on a Bio-Gel P-2 column (Bio-Rad). The fraction containing crude O-chain polysaccharide was further purified on a Bio-Gel P-10 column (Bio-Rad) and lyophilized, affording purified O-chain polysaccharide (23 mg).

(b) *Dephosphorylation with aqueous hydrofluoric acid*:[15] The O-chain polysaccharide (5 mg) was treated with cold 48% HF (100 μL, 48 h, 4 °C). The solution was concentrated to dryness by flushing with a stream of N_2, and the residue was dissolved in water, desalted with Microcon-3K and lyophilized, affording dephosphorylated O-chain polysaccharide (~3 mg).

2.3. Compositional analysis

LPS samples (0.5 mg) were hydrolyzed with 2 M TFA at 100 °C for 18 h and analyzed as their alditol acetates by GLC using a Hewlett–Packard chromatograph equipped with a 30 m DB-17 capillary column [190 °C (32 min), 16°/min to 270 °C (32 min)] and by GC–MS in the electron impact mode (EI) recorded using a Varian Saturn 2000 mass spectrometer.

The absolute configuration of glycoses was established by capillary GLC of their acetylated (−)-2-butyl glycosides, according to the method of Leontein et al.[16] The identity of each glycose derivative was established by comparison of its GLC retention time and mass spectrum with that of an authentic reference sample. The absolute configuration of glycerol was determined by the previously reported method.[17]

2.4. Methylation analysis

The O-chain polysaccharide was methylated according to the method of Ciucanu and Kerek.[18] Permethylated polysaccharide was subjected to hydrolysis as described by Stellner et al.[19] and analyzed according to previously reported conditions for partially methylated alditol acetates.[20]

2.5. NMR spectroscopy

NMR spectra were determined on Varian INOVA 500 MHz spectrometer using standard software. All NMR experiments were performed at 50 °C using a 5 mm indirect detection probe with the ^1H coil nearest to the sample. The observed ^1H chemical shifts are reported relative to external acetone (δ 2.225), and the ^{13}C chemical shifts are quoted relative to the methyl group of external acetone (δ 31.07).

Standard homo- and heteronuclear correlated 2 D techniques were used for general assignments: COSY, TOCSY, NOESY, HSQC and HMBC.[21] ^{31}P NMR experiments were performed on a Varian INOVA 200 MHz spectrometer, and chemical shifts are given relative to an external 85% H_3PO_4 reference (δ_p 0.0). The 2D ^1H–^{31}P HMQC experiment was acquired on a Varian 400 MHz spectrometer with the coupling constant at 11 Hz.

2.6. Capillary electrophoresis–electrospray-ionization mass spectrometry (CE–ESIMS)

All experiments were performed using a Crystal Model 310 capillary electrophoresis (CE) instrument (ATI Unicam, Boston, MA, USA) coupled to an API 3000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a microionspray interface. Sheath solution (2:1 2-PrOH–MeOH) was delivered at a flow rate of 1 μL/min. An electrospray stainless steel needle (27-gauge) was butted against the low dead volume tee and enabled the delivery of the sheath solution to the end of the capillary column. Separations were obtained on ca. 90 cm length bare fused-silica capillary using 10 mM NH_4OAc in deionized water, pH 9.0, containing 5% MeOH. A voltage of 25 kV was typically applied at

the injection. The outlet of the capillary was tapered to ca. 15 μm i.d. using a laser puller (Sutter Instruments, Novato, CA, USA). Mass spectra were acquired with an orifice voltage of 200 V.[22]

3. Results and discussion

A. caviae ATCC 15468 was grown in a 25-L fermentor in tryptic soy broth (TSB), and the lipopolysaccharide (LPS) was extracted from enzyme-digested cells by the hot aqueous phenol method[13] and purified by ultracentrifugation. LPS was found to be contaminated by RNA (30%, based on the presence of RNA derived ribitol) and was further purified by additional digestion with RNase. The O-chain polysaccharide was obtained by mild acid hydrolysis of LPS with 2% HOAc, and purified by gel-permeation chromatography on Bio-Gel P-2 and Bio-Gel P-10 columns.

Composition analysis of the O-chain polysaccharide revealed that it was composed of rhamnose (Rha), 2-amino-2-deoxy-glucose (GlcN) and 2-amino-2-deoxy-galactose (GalN) in the approximate molar ratio of 0.9:1.0:1.5, respectively, together with other minor components corresponding to the core oligosaccharide: glucose, galactose and L-*glycero*-D-*manno*-heptose. GLC–MS analysis of the acetylated derivatives of the (*R*)-(−)-2-butyl glycosides confirmed that GlcN and GalN had the D- and Rha had the L-configuration. The methylation analysis of the O-chain polysaccharide showed the presence of 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactose, 2,3-di-*O*-methylrhamnose, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)galactose and 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose in the approximate molar ratio of 1.1:0.9:0.3:1.0, respectively, suggesting that the O-chain polysaccharide contained 3-substituted GalNAc, 4-substituted Rha, 4-substituted GalNAc and 4-substituted GlcNAc.

The ^{31}P NMR spectrum of the O-chain polysaccharide showed a signal at δ −0.17, suggesting the presence of a phosphate group. The O-chain polysaccharide was dephosphorylated with 48% HF. Composition analysis of the resultant product revealed the presence of Rha, GlcN and GalN in the approximate molar ratio of 0.8:1.0:1.9, respectively. A noticeable increase in GalN content was observed in the dephosphorylated O-chain polysaccharide as compared with the native O-chain polysaccharide, suggesting that the phosphate group was attached to GalN residue. Methylation data was consistent with the above conclusions and revealed the presence of two GalN residues: 4-substituted GalN and 3-substituted GalN; however, the content of the 4-substituted GalN was much lower than that of other sugar residues, indicating it was phosphorylated.

CE–ESIMS (positive-ion mode) analysis of the native O-chain polysaccharide (Fig. 1a) was consistent with the results of the compositional and methylation analyses. The observed fragment ions were generated through in-source collision-induced dissociation. This technique allows in-source fragmentation of the O-chain polysaccharide, thus confirming its sequence.[22] Presence of the fragment ion at *m*/*z* 358.2 suggested that HexNAc was substituted with a phosphoglycerol (GroP) group. The corresponding fragment ion was also observed in the negative-ion detection mode at *m*/*z* 355.9. Subsequent MS/MS analysis of the fragment ion at *m*/*z* 355.9 gave rise to ions at *m*/*z* 153.0, confirming the presence of GroP group (data not shown). As shown in Figure 1a, the fragment ion at *m*/*z* 561.3 was consistent with the consecutive addition of HexNAc to GroP-HexNAc. Fragment ions corresponding to the consecutive additions of other sugar residues in the repeating unit were also observed at *m*/*z* 707.4, *m*/*z* 764.4 and *m*/*z* 910.6 and *m*/*z* 1113.7, indicating the monosaccharide sequence of the O-chain polysaccharide to be GroP-HexNAc-HexNAc-HexNAc-Rha or GroP-HexNAc-HexNAc-Rha-HexNAc. MS/MS analysis of each fragment ion was consistent with the proposed monosaccharide sequence. CE–ESIMS analysis of the dephosphorylated O-chain polysaccharide (Fig. 1b) revealed the sugar sequence similar to that of the native O-chain polysaccharide except for the presence of HexNAc instead of GroP-HexNAc, further establishing the presence of GroP in the native O-chain polysaccharide. In addition,

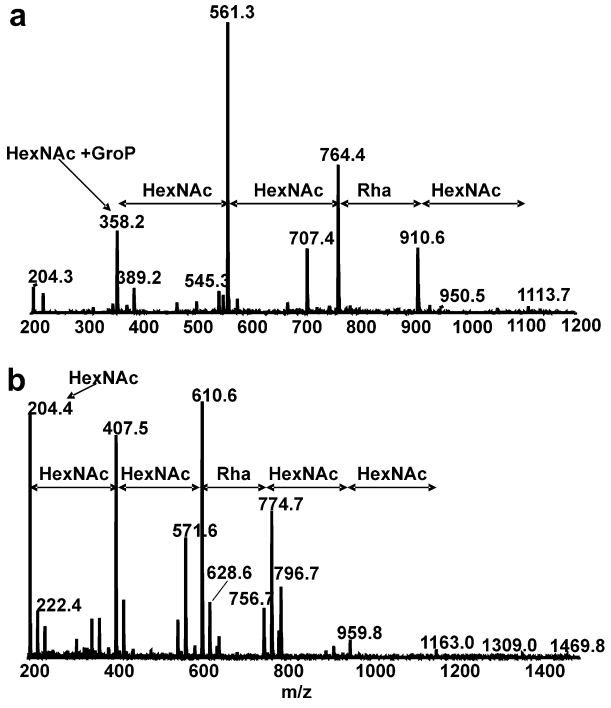


Figure 1. CE–ESIMS and CE–ESIMS/MS analysis (positive-ion mode) of the O-chain polysaccharide (a) and the dephosphorylated O-chain polysaccharide (b) from *A. caviae* ATCC 15468. The orifice potential was set at 200 V.

Table 1. ^{1}H- and ^{13}C NMR chemical shifts of the O-chain polysaccharide of *A. caviae* ATCC 15468 at 50 °C

Residue	Nucleus	1	2	3	4	5	6	CH_3CO	
→3)β-D-GalNAc(1→	^{1}H	4.74	4.11	3.76	3.96	3.69	3.66,3.85	2.06	
A	^{13}C	103.08	52.48	79.15	68.45	75.70	60.90	23.06	175.20
→4)α-L-Rha(1→	^{1}H	4.88	3.80	3.90	3.60	3.81	1.30		
B	^{13}C	102.75	71.41	71.56	81.00	68.56	17.90		
→4)β-D-GalNAc(1→	^{1}H	4.57	3.99	4.25	4.31	3.75	3.71, 3.86	2.08	
C	^{13}C	102.41	54.10	75.58	75.16	74.57	61.69	23.18	174.52
→4)β-D-GlcNAc(1→	^{1}H	4.77	3.72	3.88	3.59	3.50	3.78, 3.78	2.08	
D	^{13}C	102.24	56.12	71.02	80.51	75.51	61.65	23.18	175.01
D-Gro(1→P	^{1}H	3.85, 3.94	3.73	3.60, 3.67					
	^{13}C	67.25	73.49	63.04					

the results of the CE–ESIMS analysis of the dephosphorylated O-chain polysaccharide suggested that tetrasaccharide repeating units in the native O-chain polysaccharide were not joined through GroP linkages and that the GroP moiety was a lateral substituent. The sequence of the constituent glycoses, the position of linkages and the location of GroP group were further confirmed by 1D and 2D NMR analyses performed on the O-chain polysaccharide.

The ^{1}H NMR and ^{13}C NMR spectra of the O-chain polysaccharide of *A. caviae* ATCC 15468 were fully assigned using 2D COSY, TOCSY, HSQC, HMBC and NOESY experiments (Table 1). The ^{1}H NMR spectrum showed resonances for four anomeric protons at δ 4.88, 4.77, 4.74 and 4.57. The assignment of ^{1}H NMR resonances was achieved by tracing connectivities in the COSY and TOCSY spectra from the anomeric and some other isolated ring proton resonances, such as H-6 of Rha at δ 1.30. Some of the vicinal protons were also assigned by their correlations in the NOESY spectrum (Fig. 2a). The ^{13}C NMR chemical shifts of the O-chain polysaccharide were fully assigned by the HSQC (Fig. 3) and HMBC spectra (Fig. 2b), which showed correlations for four anomeric carbon resonances at δ 4.74 (^{1}H)/103.08 (^{13}C), 4.88 (^{1}H)/102.75 (^{13}C), 4.57 (^{1}H)/102.41 (^{13}C) and 4.77 (^{1}H)/102.24 (^{13}C), one CH_3 group at 1.30 (^{1}H)/17.90 (^{13}C) (C-6 of Rha). The GalNAc and GlcNAc residues were confirmed by the presence of three nitrogen-bearing carbons at δ 4.11 (^{1}H)/52.48 (^{13}C), 3.99 (^{1}H)/54.10 (^{13}C) and 3.72 (^{1}H)/56.12 (^{13}C). Based on the ^{1}H NMR and ^{13}C NMR chemical shift data, which were in agreement with literature values for their respective pyranosides,[23–26] four observed spin systems were attributed to GalNAc (residue **A**), Rha (residue **B**), GalNAc (residue **C**) and GlcNAc (residue **D**), respectively (Table 1). The remaining carbon resonances belonged to glycerol and were assigned by correlation with the corresponding proton resonances in the HSQC spectrum, which showed correlations at δ 3.85, 3.94 (H-1,1′)/67.25 (C-1), 3.73 (H-2)/73.49 (C-2) and 3.60, 3.67 (H-3,3′)/63.04 (C-3) (Fig. 3, Table 1). The absolute configuration of the glycerol group was determined to be D according to the method of Rundlöf and Widmalm.[17]

Large $J_{1,2}$ coupling constant values of 7–8 Hz were observed for H-1 signals at δ 4.74, 4.57 and 4.77, confirming that residues **A** (GalNAc), **C** (GalNAc), **D** (GlcNAc) were β-linked. Since residue **B** (Rha) was present

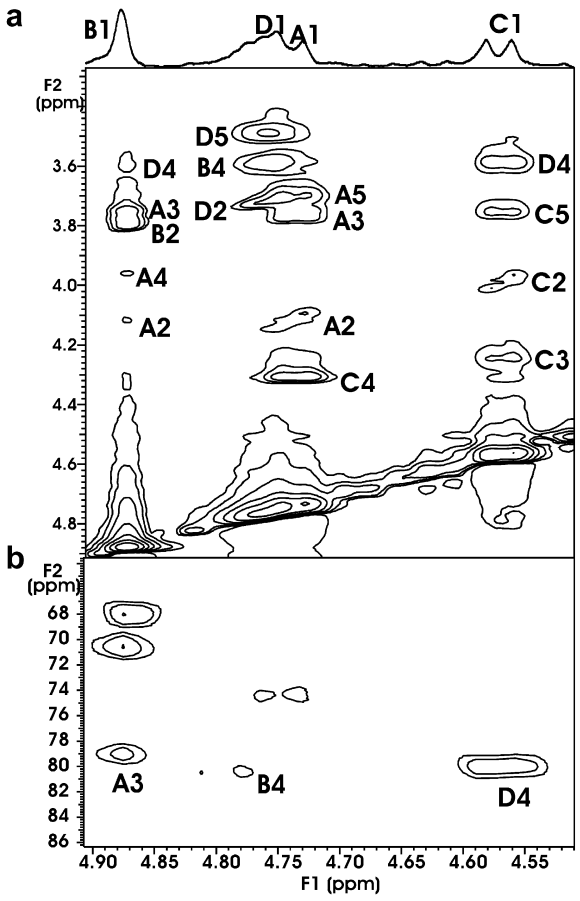


Figure 2. Part of a NOESY spectrum (a) and HMBC spectrum (b) showing correlations for anomeric protons of the O-chain polysaccharide from *A. caviae* ATCC 15468. Arabic numerals refer to the atoms in sugar residues denoted by letters as shown in Table 1.

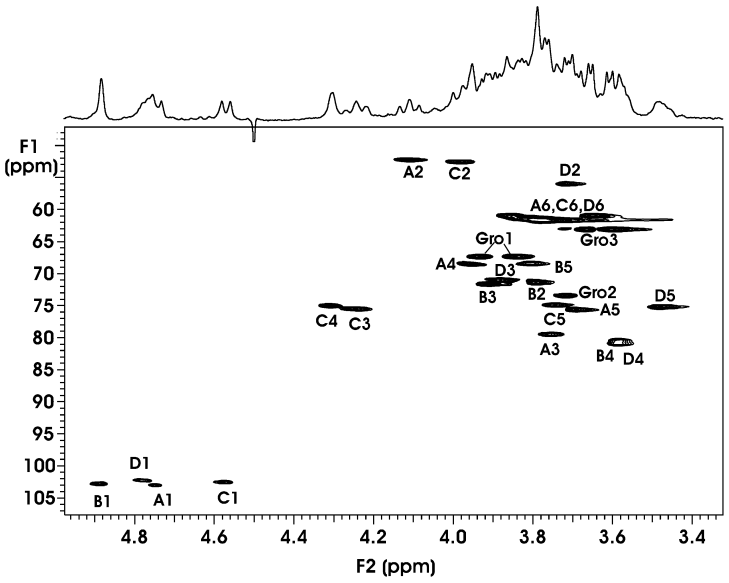
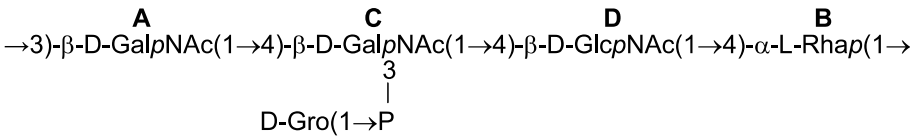


Figure 3. Part of 2D ^{1}H–^{13}C HSQC spectra of the O-chain polysaccharide from *A. caviae* ATCC 15468.

in the *manno* configuration, its anomeric configuration could not be unambiguously assigned from the $J_{1,2}$ coupling constant value in the ^{1}H NMR spectrum. The absence of an H-1/H-5 cross-peak for residue **B** in the NOESY spectrum of the O-chain polysaccharide (Fig. 2a) confirmed its α configuration. This was also in agreement with the ^{1}H and ^{13}C NMR chemical shift data for residue **B**.[26–28]

Significant downfield chemical shifts were observed for C-3 of residue **A** (δ 79.15), C-4 of residue **B** (δ 81.0), C-3 and C-4 of residue **C** (δ 75.58 and 75.16, respectively), C-4 of residue **D** (δ 80.51) and C-1 of glycerol (δ 67.25) as compared with their positions in the spectra of corresponding non-substituted monosaccharides and glycerol,[21,29] indicating the linkage position for each monosaccharide and glycerol residues. These results were confirmed by interresidue connectivities observed in the NOESY spectrum of the O-chain polysaccharide (Fig. 2a). The interresidue connectivities between δ_H 4.74/4.31, 4.88/3.76, 4.57/3.59 and 4.77/3.60 were attributed to the following anomeric and glycosidically linked protons: H-1**A**/H-4**C**; H-1**B**/H-3**A**, H-1**C**/H-4**D** and H-1**D**/H-4**B**, respectively. In addition, the HMBC spectrum (Fig. 2b) also showed the cross peaks between C-3**A** and H-1**B**, C-4**D** and H-1**C**, C-4**B** and H-1**D** at δ_C 79.15/δ_H 4.88, δ_C 80.51/δ_H 4.57 and δ_C 81.00/δ_H 4.77, respectively. The combined evidence allowed the sequence of monosaccharides and position of linkages in the O-chain polysaccharide to be established as →3)-**A**-(1→4)-**C**-(1→4)-**D**-(1→4)-**B**-(1→.

As mentioned above, the ^{31}P NMR spectrum of the O-chain polysaccharide showed the presence of one phosphate group. The location of the phosphate group was determined by a ^{1}H–^{31}P HMQC experiment which showed a three-bond correlation for the ^{31}P NMR resonance with H-1,1′ of the glycerol moiety and the H-3 of residue **C** at δ −0.17/3.85, 3.94 and 4.25, respectively. This indicated that the phosphate group was present as a monophosphodiester group that was attached to residue **C** and glycerol. The combined chemical, CE–ESIMS and NMR evidence permitted the structure of the O-chain polysaccharide of *A. caviae* ATCC 15468 to be established as the following:



Previously determined structure of the O-chain polysaccharide from *A. caviae* strain 11212[30] bears no resemblance to the above structure and suggests the possibility of dividing this species into more than one serological group.

Interestingly, the phosphoglycerol moiety identified in the structure of the O-chain polysaccharide of *A. caviae* ATCC 15468 was previously found in the O-chain polysaccharides of *Citrobacter* O16,[31] *Hafnia alvei* strain PCM1207[32] and *Proteus* species[33] as well as in the exopolysaccharide produced by *Lactobacillus sake* 0-1[34] and

the specific capsular polysaccharide of *Streptococcus pneumoniae* type 45.[35] It is recognized as an immunodominant epitope, and the cross-reactions between the LPS of *Citrobacter* O16[31] and *H. alvei* strain PCM1207 could be attributed to the presence of this shared epitope in their respective O-specific polysaccharide structures.[32]

Acknowledgements

This work was supported by the National Research Council's Genomics and Health Initiative (GHI). The authors thank Dr. Laura L. Brown from the Institute of Marine Biosciences, National Research Council of Canada for providing *A. caviae* stock cultures.

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