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Structural and serological characterization of the O-chain polysaccharide of *Aeromonas salmonicida* strains A449, 80204 and 80204-1

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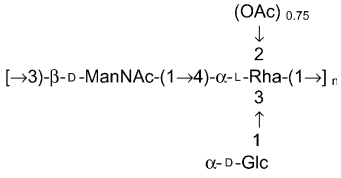
Abstract—The O-chain polysaccharide (O-PS) of *Aeromonas salmonicida* was studied by a combination of compositional, methylation, CE-ESMS and one- and two-dimensional NMR analyses. It was found to be a branched polymer of trisaccharide-repeating units composed of L-rhamnose (Rha), D-glucose (Glc), 2-acetamido-2-deoxy-D-mannose (ManNAc) and *O*-acetyl group (OAc) and having the following structure:



CE-ESMS analysis of *A. salmonicida* cells from strains A449, 80204 and 80204-1 grown under different conditions confirmed that the O-PS structure was conserved. ELISA-based serological study with native LPS-specific antisera performed on the native O-PS and its O-deacetylated and periodate-oxidized derivatives confirmed the importance of the O-PS backbone structure as an immunodominant determinant.

Keywords: *Aeromonas salmonicida*; Lipopolysaccharide; O-chain polysaccharide; NMR; CE-ESMS; ELISA

1. Introduction

Aeromonas salmonicida is the causative agent of furunculosis, an infectious disease principally found in salmonid fish, which can also cause diverse pathology in freshwater and marine fishes.[1] Since the annual worldwide losses of farmed fish to diseases involve millions of dollars, this species has been the subject of considerable investigation.[2,3] Gram-negative *A. salmonicida* strains possess the outer-membrane lipopolysaccharide (LPS), which is partially exposed on the cell surface, appears to mediate host-cell invasion and also plays an important structural role in the assembly and maintenance of the

Abbreviations: CE-ESMS, capillary electrophoresis-electrospray mass spectrometry; COSY, correlated spectroscopy; 1D, 2D, one- and two-dimensional; dHexOAc, O-acetylated deoxy hexose; ELISA, enzyme-linked immunosorbent assay; GLC, gas–liquid chromatography; Glc, glucose; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; LPS, lipopolysaccharide; ManNAc, 2-acetamido-2-deoxy-mannose; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; OAc, *O*-acetyl; O-PS, O-chain polysaccharide; Hex, hexose; HexNAc, 2-acetamido-2-deoxy-hexose; Rha, rhamnose; TOCSY, total correlated spectroscopy; TSA, Tryptic Soy Agar; TSB, Tryptic Soy Broth

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surface protein array of *A. salmonicida* virulent strains.[4] Similar to another important virulence factor, the surface layer (S-layer) also known as A-layer, the surface-exposed regions of *A. salmonicida* LPS are considered to be responsible for the serological cross-reactivity of *A. salmonicida* strains.[5] The study of serological relatedness between *A. salmonicida* and other bacteria, including *Edwardsiella*, *Pseudomonas*, *Vibrio* and *Yersinia*, revealed that *A. salmonicida* LPS was highly specific as compared with the cell-surface proteins.[6] Therefore, structural and immunochemical studies of *A. salmonicida* LPS are important for understanding the basis for classification of the genus *Aeromonas* and provide immunochemical rationale for understanding the antigenic relationships between *Aeromonas* and other bacteria at the molecular level. Previous studies have elucidated the structure of O-chain polysaccharide and the core oligosaccharide region of LPS from *A. salmonicida* strain SJ-15.[7,8] Recently we have described the structure of the capsular polysaccharide from *A. salmonicida* strain 80204-1.[9] In this study, we report the structural and serological characterization of the O-chain polysaccharide (O-PS) of *A. salmonicida* strains grown under different conditions and demonstrate that the structure of O-PS is conserved among the different strains of *A. salmonicida*.

2. Experimental

2.1. Bacterial culture and isolation of LPS

A. salmonicida strains 80204-1, 80204 and A449 were obtained from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, NS, Canada). The bacteria were cultured on Tryptic Soy Agar (TSA) or Tryptic Soy Broth without glucose (TSB) at 18 or 25 °C for 48–72 h. The cells were killed with 1% (w/v) phenol soln (22 °C, 4 h), washed with 0.01 M phosphate buffered saline pH 7.4 and harvested by low-speed centrifugation (3000*g*, 25 min). The cells were washed with 2.5% saline soln (w/v), digested enzymatically and extracted by the method of Westphal and Jann.[10] Phenol and water layers were collected separately, dialyzed against tap water and lyophilized. The lyophilizates were then dissolved in 1% saline soln (w/v), subjected to ultracentrifugation (105,000*g*, 4 °C, 16 h) and the LPS pellets were redissolved in water and lyophilized.

2.2. Preparation of the O-PS and its O-deacetylated and periodate-oxidized derivatives

(a) Mild acid hydrolysis of LPS: LPS (60 mg) was hydrolyzed with 0.2 M AcOH (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-PS was further purified on a Bio-Gel P-10 column (Bio-Rad).

(b) O-Deacetylation of the O-PS:[11] O-PS (10 mg) was dissolved in 5% ammonium hydroxide (2 mL) and incubated at 37 °C overnight. The soln was lyophilized and the product purified by gel-permeation chromatography using the Bio-Gel P-10, resulting in a yield of 7 mg.

(c) Periodate oxidation and Smith degradation:[12] The O-PS (10 mg) was oxidized with 0.05 M sodium metaperiodate (1.6 mL) at 4 °C in the dark for 6 days. Then ethylene glycol (1 mL) was added to stop the reaction. The mixture was stirred at 22 °C for 30 min, followed by reduction with sodium borohydride at 22 °C for 18 h and neutralized with 10% (v/v) AcOH. The soln was dialyzed and lyophilized. Smith-type hydrolysis of the periodate-oxidized and reduced polymer was effected with 0.5 M trifluoroacetic acid (TFA) at 22 °C for 48 h, the sample was purified on the Bio-Gel P-2 column and the sugar composition of the product was determined.

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 °C per 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.[13] 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.

2.4. Methylation analysis

The O-PS was methylated according to the method of Ciucanu and Kerek.[14] The permethylated polysaccharide was subjected to hydrolysis as described by Stellner et al.[15] and analyzed according to previously reported conditions for partially methylated alditol acetates.[16]

2.5. NMR spectroscopy

NMR spectra were performed on Varian INOVA 500 and 600 MHz spectrometers using standard software. All NMR experiments were performed at 50 °C using a 5 mm indirect detection probe with the ^{1}H coil nearest to the sample. The observed ^{1}H chemical shifts are reported relative to external acetone (2.225 ppm), and

the ^{13}C chemical shifts are quoted relative to the methyl group of external acetone (31.07 ppm).

Standard homo- and heteronuclear correlated 2D techniques were used for general assignments of the O-PS and the O-deacetylated O-PS: COSY, TOCSY, NOESY, HSQC and HMBC.[17]

2.6. CE-ESMS

All experiments were performed as described previously in detail.[18] Briefly, a Crystal Model 310 CE instrument (ATI Unicam, Boston, MA, USA) was coupled to an API 3000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) via a microIonspray interface. Sheath soln (isopropanol–MeOH, 2:1) 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 soln to the end of the capillary column. The separations were obtained on ca. 90 cm length bare fused-silica capillary using 10 mM ammonium acetate 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.

2.7. Direct CE-ESMS analysis of *A. salmonicida* cells

Bacterial cells, 2.5×10^{11} cfu, were washed with 2.5% (w/v) saline, and the pellet recovered by low-speed centrifugation (3000*g*, 4 °C, 10 min) and lyophilized. The lyophilized pellet was treated with RNase and DNase to release LPS (final concentration 10 μg/mL in 0.02 M ammonium acetate, pH 7.5, 37 °C, 2 h) and lyophilized following low-speed centrifugation (yield, 27 mg dry weight). It was treated with proteinase K as described above and the product was recovered by low-speed centrifugation. Lyophilized sample was treated with 1% AcOH (100 °C, 1 h), desalted using a centrifugal filter device (Pall Corporation, Novato, CA, USA) and analyzed directly by a Crystal Model 310 CE instrument coupled to an API 3000 mass spectrometer.

In addition, lyophilized cells were subjected to sugar composition and methylation analyses as described above for purified polysaccharide samples.

2.8. Serological methods[19]

2.8.1. Rabbit polyclonal sera production. New Zealand White rabbits (Charles River Canada Inc., St-Constant, Quebec) were given seven injections of LPS from *A. salmonicida* strain A449 mixed 1:1 with Incomplete Freunds Adjuvant (IFA) on days 14, 28, 46, 66, 81 and 101. The amounts varied from 50 to 200 μg of LPS per injection. After sufficient titre, the rabbits were exsanguinated and the sera were pooled, aliquoted and frozen at −80 °C for further study.

2.8.2. Inhibition ELISA. The wells of a microtiter plate (ICN, Costa Mesa, CA) were coated with 10 μg/mL of *A. salmonicida* strain A449 LPS in 10 mM PBS, pH 7.4 (37 °C, 3 h). Following the wash with PBS, the plates were blocked with 1% (w/v) BSA in PBS (37 °C, 1 h). Serial dilutions of the three inhibitors, O-PS, O-deacetylated O-PS and O-chain backbone polysaccharide were prepared in separate test tubes and mixed with a previously determined dilution of rabbit sera to give an OD_{450} of 1. This mixture was incubated at 22 °C for 15 min and transferred to the original microtiter plate blocked with LPS and incubated at 37 °C for another 2 h. The plates were washed with PBS. The second antibody, a goat anti-rabbit IgG horseradish peroxidase conjugate (Caltag, So. San Francisco, CA) was added and the plates were incubated at rt for 1 h. Following a final washing step, 3,3′,5,5′-tetramethylbenzidene (TMB) (Kirkgaard and Perry, Gaithersburg, MD), substrate was added and the reaction was stopped with 1 M phosphoric acid. The absorbance was determined at 450 nm using a microtiter plate reader (Dynatech, Chantilly, VA). The percentage inhibition was then calculated according to the following formula: % inhibition = 100% × [(OD_{450} with inhibitor − OD_{450} without inhibitor)/OD_{450} without inhibitor].

3. Results and discussion

3.1. Structural studies of the O-PS of *A. salmonicida*

LPS was extracted from enzymatically digested cells of *A. salmonicida* strains A449 and 80204-1 using the hot aqueous phenol method and purified by ultracentrifugation. Mild acid hydrolysis of LPS with hot diluted AcOH and gel chromatography on Bio-Gel P-2 and Bio-Gel P-10 afforded O-PS and core oligosaccharide fractions.

Acid hydrolysis of the O-PS followed by GLC–MS analysis of resultant monosaccharides as alditol acetates revealed that it was composed of glucose (Glc), rhamnose (Rha) and 2-amino-2-deoxy-mannose (ManN) in the approximate molar ratio of 1.0:0.7:0.6, together with other minor components corresponding to the core oligosaccharide: galactose, 2-amino-2-deoxy-galactose and L-*glycero*-D-*manno*-heptose. In addition, GLC–MS analysis of the acetylated derivatives of the (*R*)-(−)-2-butyl glycosides confirmed that Glc and ManN had the D- and Rha had the L-configuration.

The methylation analysis of the O-PS revealed the presence of 2,3-di-*O*-methylrhamnose, 2-*O*-methylrhamnose, 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido) mannose and 2,3,4,6-tetra-*O*-methylglucose (Table 1),

Table 1. Methylation analysis of O-PS of *A. salmonicida* A449

Sugar linkage	Rt_{GM}[a] (min)	Relative molar ratios[b]
4-Substituted Rha	5.22	0.08
Terminal Glc	6.12	1.00
3,4-Substituted Rha	7.09	0.86
3-Substituted ManNAc	34.90	0.62

[a] Retention time of the derived alditol acetate derivative relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol-1-*d*.
[b] Based on the detector response (total ion count).

suggesting that the O-PS contained 3,4-substituted Rha, 3-substituted ManNAc and terminal Glc. In addition, partially methylated disaccharide was also obtained and identified as 4,6-di-*O*-methyl-2-*N*-(methylacetamido)-2-deoxy-mannosyl-(1→4)-2-*O*-methyl-rhamnitol-1-*d* based on its electron impact mass spectrum (data not shown).

The results of CE-ESMS were consistent with those of compositional and methylation analyses. Presence of the fragment ion at *m*/*z* 392.5 suggested that HexNAc was attached to RhaOAc and a fragment ion at *m*/*z* 554.5 was consistent with the consecutive addition of Hex (Fig. 1). Other fragment ions corresponding to the consecutive addition of sugar residues were also observed (*m*/*z* 757.5, 946.5 and 1108.5). In order to confirm that the observed fragment ions generated through in-source collision-induced dissociation originated from the native O-PS, they were subjected to MS/MS analysis and were shown to correlate with the sugar sequence of the O-PS (data not shown). The sequence of constituent glycoses, the position of linkage and the location of *O*-acetyl group were further confirmed by 1D and 2D NMR anal-

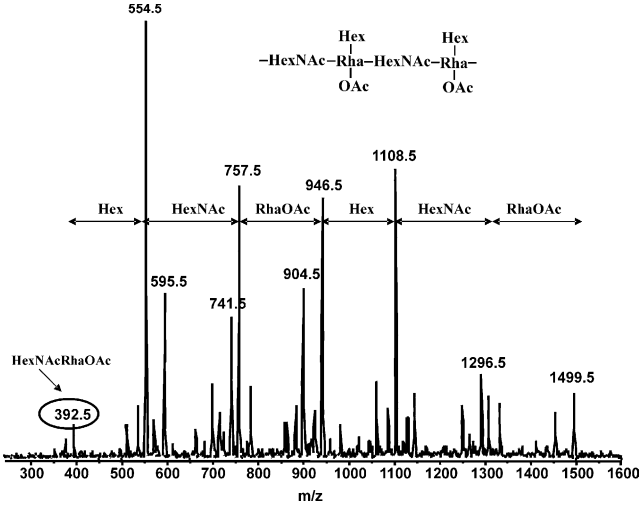


Figure 1. CE-ESMS spectrum (positive ion mode, orifice voltage: 200 V) of the O-PS of *A. salmonicida*.

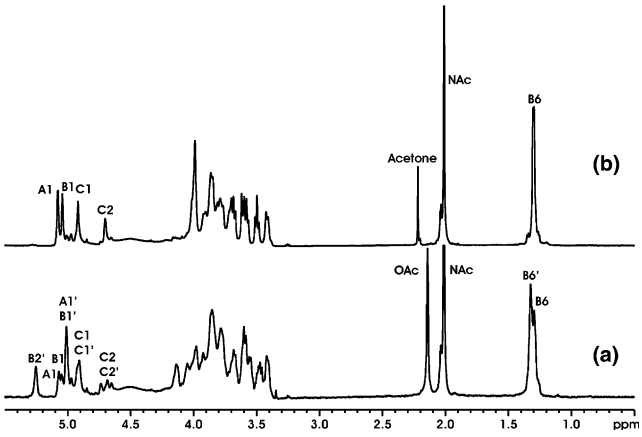


Figure 2. ^{1}H NMR spectrum of: (a) the O-PS; (b) the O-deacetylated O-PS.

yses carried out on the native O-PS and the O-deacetylated O-PS.

The ^{1}H NMR spectrum of the O-PS (Fig. 2) showed significant structural heterogeneity due to non-stoichiometric O-acetylation substitution pattern (δ_H = 2.15 ppm, δ_C = 21.5 ppm). Following O-deacetylation, both 1D ^{1}H NMR and 2D ^{1}H–^{13}C heteronuclear correlation spectra became homogeneous and typical of a regular polymer, showing correlations for three anomeric signals at δ 4.92 ppm (^{1}H)/100.5 ppm (^{13}C), 5.04 ppm (^{1}H)/98.2 ppm (^{13}C) and 5.08 ppm (^{1}H)/95.0 ppm (^{13}C), one CH_3 group at δ 1.30 ppm (^{1}H)/18.4 ppm (^{13}C) (C-6 of Rha), one nitrogen-bearing carbon at δ 4.70 ppm (^{1}H)/51.2 ppm (^{13}C) (C-2 of ManNAc), and one *N*-acetyl group (CH_3 at δ 2.01 ppm, ^{1}H/23.2 ppm, ^{13}C, CO at δ 174.9 ppm, ^{13}C).

The ^{1}H NMR and ^{13}C NMR spectra of the O-deacetylated O-PS were completely assigned using 2D COSY, TOCSY, HSQC, HMBC and NOESY experiments (Table 2). Briefly, the ^{1}H NMR chemical shifts were assigned by tracing connectivities in the COSY and TOCSY spectra from the anomeric and some other isolated ring protons, such as H-2 of ManNAc at δ 4.70 ppm and H-6 of Rha at δ 1.30 ppm. The carbon chemical shifts of the O-deacetylated O-PS were assigned through its HSQC and HMBC spectra. Based on the ^{1}H NMR and ^{13}C NMR chemical shift data, which were in agreement with the literature values for their respective pyranosides,[20,22–24] three observed spin systems were attributed to Glc (residue **A**), Rha (residue **B**) and ManNAc (residue **C**). The $J_{1,2}$ coupling constant value of <2 Hz for H-1 signal at δ 5.08 ppm showed that residue **A** was α-linked. Anomeric configurations of residue **B** and **C** could not be deduced from the $J_{1,2}$ coupling constant values in the ^{1}H NMR spectrum because of their *manno* configuration. The α configuration of residue **B** and the β configuration of residue **C** were confirmed by comparison of their ^{1}H and ^{13}C NMR chemical shift values and the values of one-bond

Table 2. ^{1}H and ^{13}C chemical shifts of the native O-PS and O-deacetylated O-PS of *A. salmonicida* strain A449[a]

Residue[b]	Atom no.	Chemical shifts[c] (ppm)					
		^{1}H			^{13}C		
		OAc^{+}	OAc^{-}	$\Delta\delta_H$	OAc^{+}	OAc^{-}	$\Delta\delta_C$
A/A′	1	5.01	5.08	−0.07	95.1	95.0 (171.2)[d]	0.1
	2	3.55	3.61	−0.06	72.1	72.3	−0.2
	3	3.61	3.68	−0.07	75.1	75.0	0.1
	4	3.48	3.50	−0.02	70.7	70.7	0
	5	3.69	3.71	−0.02	73.5	73.4	0.1
	6	3.80, 3.85	3.80, 3.85	0	61.9	61.9	0
B/B′	1	5.01	5.04	−0.03	96.0	98.2 (170.5)[d]	−2.2
	2	5.26	3.99	1.27	68.4	67.0	1.4
	3	4.14	3.98	0.15	72.7	74.9	−2.2
	4	3.86	3.86	0	77.6	77.6	0
	5	4.04	4.00	0.04	68.9	68.9	0
	6	1.32	1.30	0.02	18.4	18.4	0
	OAc	2.15			21.5		
	CO				172.5		
C/C′	1	4.91	4.92	0	100.5	100.5 (162.9)[d]	0
	2	4.74–4.65	4.70	−0.005	51.2	51.2	0
	3	3.77	3.77	0	78.0	78.0	0
	4	3.58	3.58	0	66.0	66.4	−0.4
	5	3.41	3.41	0	77.3	77.3	0
	6	3.86	3.86	0	61.8	61.8	0
	NAc	2.01	2.01		23.2	23.2	
	CO				174.9	174.9	

[a] Recorded at 50 °C.
[b] A, B, C correspond to individual sugar residues in O-deacetylated repeating units and A′, B′, C′ correspond to individual sugar residues in O-acetylated repeating units.
[c] OAc^{+} corresponds to sugar residues in O-acetylated repeating units; OAc^{-} corresponds to sugar residues in O-deacetylated repeating units.
[d] Values of $^{1}J_{C,H}$ in parentheses are in Hertz (Hz).

^{13}C, ^{1}H-coupling constants ($^{1}J_{C,H}$) with the literature data.[21–25] The observed H-1, H-2 intraresidue connectivities for residue **B**, and H-1, H-3 and H-1, H-5 intraresidue connectivities for residue **C** in the NOESY spectrum of the O-deacetylated O-PS (Fig. 3) confirmed these results.

Significant downfield chemical shifts were observed for C-3 and C-4 of residue **B** (δ 74.9 and 77.6 ppm) and C-3 of residue **C** (δ 78.0 ppm), as compared with their respective chemical shifts in the spectra of the corresponding non-substituted monosaccharides at 71.3–72.4 ppm, indicating the linkage position for each sugar residue. Interresidue connectivities observed in the NOESY spectrum (Fig. 3) confirmed these findings. The interresidue connectivities between δ_H 5.08 ppm/3.99 ppm, 5.04 ppm/3.77 ppm and 4.92 ppm/3.86 ppm were attributed to the anomeric protons and glycosidically linked protons H-1**A**/H-3**B**; H-1**B**/H-3**C** and H-1**C**/H-4 **B**, respectively. Consistent with these data, the HMBC spectrum showed the cross peaks between C-3**B** and H-1**A**, C-3**C** and H-1**B** at δ_C 74.9 ppm/δ_H 5.08 ppm and δ_C 78.0 ppm/δ_H 5.04 ppm, respectively. These data confirmed that the monosaccharide sequence

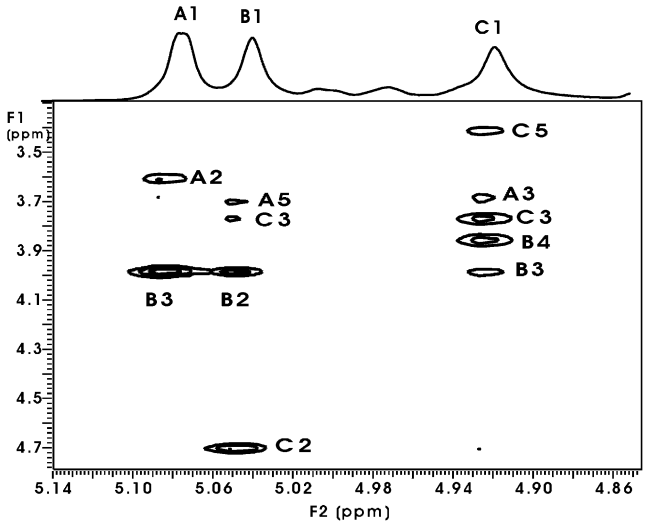


Figure 3. A portion of NOESY spectrum of the O-deacetylated O-PS with assignments.

and position of linkages of the O-deacetylated O-PS were →3)-**C**-(1→4)-[**A**-(1→3]-**B**-(1→. Comparison of the ^{1}H and ^{13}C chemical shift data of the native and

O-deacetylated O-PS showed that ^{1}H and ^{13}C chemical shifts for residues **A** and **C** in the native O-PS were similar to the ones obtained for its O-deacetylated derivative (Table 2) suggesting that they were not O-acetylated in the native O-PS. A significant downfield chemical shift of H-2**B**/C-2**B** cross-peak from δ_H 3.99 ppm/δ_C 67.0 ppm to δ_H 5.26 ppm/δ_c 68.4 ppm was observed in the HSQC spectrum of the native O-PS (Fig. 4) as compared with that of the O-deacetylated O-PS and was caused by a deshielding effect of the *O*-acetyl group thus revealing that the residue **B** carried an *O*-acetyl group at position *O*-2 in a non-stoichiometric amount. Based on relative intensities of the signals for the *O*-acetyl and *N*-acetyl groups in the native O-PS, the degree of O-acetylation was ~75% (Fig. 2). This was consistent with the observed upfield ^{1}H and ^{13}C chemical shifts exhibited by adjacent neighbouring atoms: H-1**B**/C-1**B** partially shifted upfield from δ_H 5.04 ppm/δ_C 98.2 ppm to δ_H 5.01 ppm/δ_C 96.0 ppm, and H-3**B**/C-3**B** partially shifted from δ_H 3.98 ppm/δ_C 74.9 ppm to δ_H 4.14 ppm/δ_C 72.7 ppm.

The combined chemical and NMR evidence established that the structure of the native O-PS of *A. salmonicida* is constituted of a branched trisaccharide repeating unit:

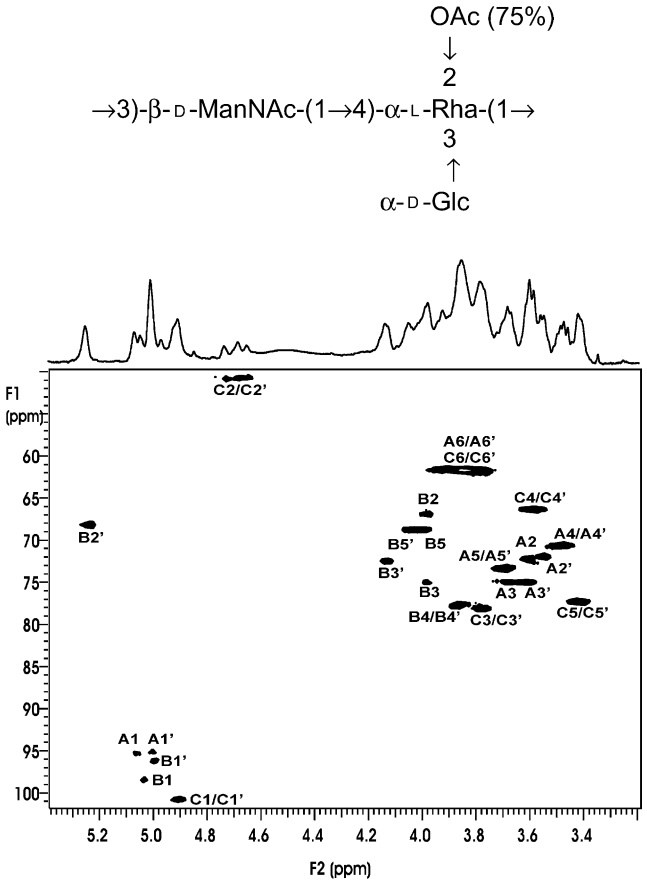


Figure 4. HSQC spectrum of the O-PS of *A. salmonicida* LPS with assignments.

3.2. Microscale CE-ESMS analysis of O-chain polysaccharide from *A. salmonicida* strains on bacterial cells

In order to detect any structural variations in the O-PS from *A. salmonicida* strains cultured under different conditions, we have developed a microscale CE-ESMS-based method for analysis of *A. salmonicida* LPS directly on bacterial cells. This method involves pretreatment of bacterial cells (approx 10^{10} cells) with proteinase K, RNase and DNase followed by delipidation with mild AcOH and CE-ESMS analysis. Using this method, *A. salmonicida* strains 80204, 80204-1 and A449 were analyzed when grown on TSA or TSB. The effect of growth temperature was also investigated and experiments were carried out on cells grown at 18 and 25 °C. Identical fragmentation pattern in the CE-ESMS spectra (Fig. 5) was obtained for all *A. salmonicida* strains used in the present investigation and cultured under different conditions, suggesting that O-PS structure was conserved and independent of growth conditions and consisted of a polymer of trisaccharide repeating units containing Hex (Glc), HexNAc (ManNAc) and dHexOAc (RhaOAc) as identified above.

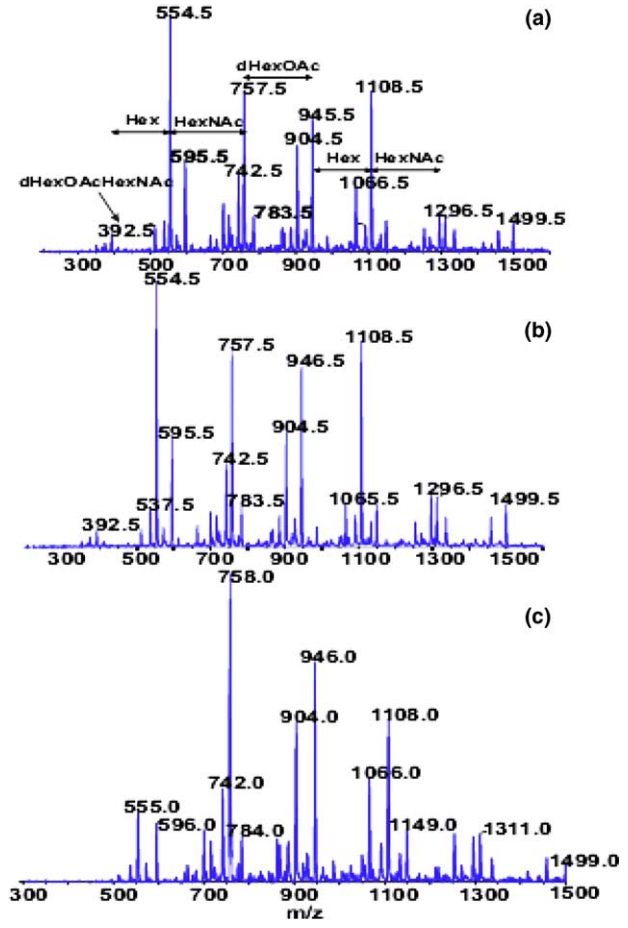


Figure 5. CE-ESMS analysis performed on the whole cells of *A. salmonicida*: (a) strain 80204-1; (b) strain 80204; (c) strain A449.

Compared with the above structure, the previously reported structure of the O-PS from *A. salmonicida* strain SJ-15[7] displayed several discrepancies: an *O*-acetyl group was located on the position *O*-4 of D-ManNAc and an extra 4-linked Glc was present in the side chain. In addition, we established that when encapsulated, *A. salmonicida* strain 80204-1 produced a novel O-PS having a structure identical to that of its capsular polysaccharide (CPS).[9] These exceptions suggest a possibility of dividing this species into more than one serological group, although *A. salmonicida* strains were considered as serologically homogeneous.[26–28]

Furthermore, compositional and methylation analyses carried out on bacterial cells revealed that when grown on TSB without glucose, all *A. salmonicida* strains produced a large quantity of a polymeric material, which was identified as a branched α-(1→4)-linked glucan with α-(1→6)-linked branches occurring every four glucose units. A similar glycogen-type structure was previously found in *Vibrio* species[29] (data not shown). No glucan was produced when *A. salmonicida* strains were cultured on TSA.

3.3. Serological studies

Pyle et al.[6] have reported that rabbit polyclonal antisera produced to purified *A. salmonicida* LPS reacted strongly with *A. salmonicida* isolates, but did not react with the isolates of six other bacteria, suggesting that *A. salmonicida* LPS was species specific. The importance of immunodeterminants in the O-PS for its serological specificity was established by ELISA and the inhibition ELISA using the purified O-PS, O-deacetylated O-PS and its periodate-oxidized derivative which contained the polysaccharide backbone only. Comparison of the polysaccharides in the inhibition test ELISA showed that the O-PS, its O-deacetylated derivative and the backbone O-PS possessed almost identical inhibitory activity (Fig. 6).

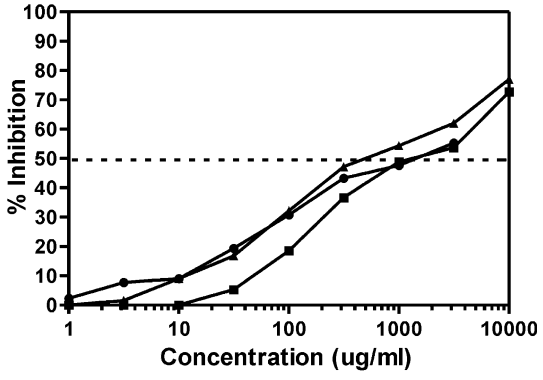


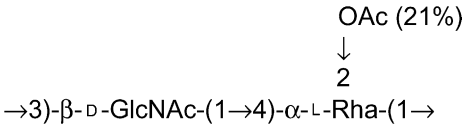
Figure 6. Inhibition curves for binding of a rabbit anti-*A. salmonicida* LPS sera to *A. salmonicida* polysaccharides: the O-PS (■); the O-deacetylated O-PS (▲); the O-PS backbone (●).

4. Conclusion

In the present investigation we have established the structure of the O-PS from *A. salmonicida* strains A449, 80204 and 80204-1 and have shown by composition and methylation analyses, two-dimensional NMR spectroscopy and mass spectrometry that it is a polymer of branched trisaccharide repeating units.

The serological specificity of O-PS was established by ELISA-based serological study with native LPS-specific antisera, which revealed that neither *O*-acetyl group nor glucose residue in the side chain were important for the serological specificity of the O-PS of *A. salmonicida* LPS, whereas, the presence of the intact O-chain backbone structure was essential.

This conclusion was also supported by the report of Dooley et al.[5] who described that the cells of both *A. hydrophila* and *A. salmonicida* species were recognized by polyclonal antisera raised against *A. salmonicida* LPS, suggesting the presence of the common structural epitope. The structure of the O-PS of *A. hydrophila* LL1 is shown below:[30]



Similarly, the *O*-acetyl group in the O-PS from *Citrobacter freundii* O38 and *Proteus vulgaris* OX2 LPS was also regarded as having no importance for their serological specificity.[29,31]

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