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Structural studies of the core region of *Aeromonas salmonicida* subsp. *salmonicida* lipopolysaccharide

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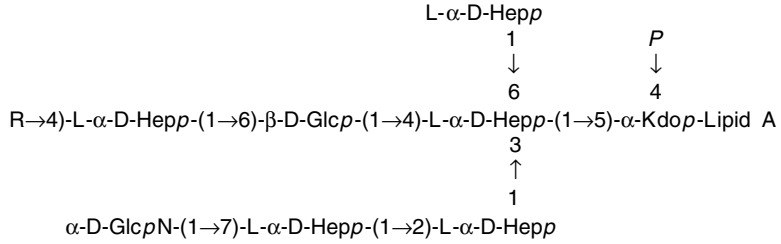
Abstract—The core oligosaccharide structure of the in vivo derived rough phenotype of *Aeromonas salmonicida* subsp. *salmonicida* was investigated by a combination of compositional, methylation, CE-MS and one- and two-dimensional NMR analyses and established as the following:



where R = α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→ or α-D-Gal*p*-(1→ (approx. ratio 4:3).
Comparative CE-MS analysis of *A. salmonicida* subsp. *salmonicida* core oligosaccharides from strains A449, 80204-1 and an in vivo rough isolate confirmed that the structure of the core oligosaccharide was conserved among different isolates of *A. salmonicida*.

Keywords: *Aeromonas salmonicida*; Lipopolysaccharide; Core oligosaccharide; NMR; CE-MS

1. Introduction

Aeromonas salmonicida subsp. *salmonicida* has been recognized as a pathogen of fish for over 100 years and its geographical distribution is almost worldwide. It is the causative agent of furunculosis, an infectious disease principally in salmonid fish, and has also been associated with clinical or covert disease in a variety of non-salmonid species.[1] Since the annual worldwide losses of farmed fish to diseases involve millions of dollars, this pathogen has been subjected to considerable investigation.[2,3] Lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria, is the most immunoreactive cell surface antigen[4] and is considered to be an important virulence factor involved in the pathogenesis of bacterial infections.[5] In order to understand the interactions between pathogen and host factors contributing to the disease state, the complete and detailed structure of LPS should be defined. Previous studies have characterized structures of O-chain polysaccharide and the core oligosaccharide region of LPS from *A. salmonicida* strain SJ-15.[6–8] Recently, we have established structures of the capsular polysaccharide and the O-chain polysaccharide from *A. salmonicida*.[9,10] Here we describe the structural elucidation of the core oligosaccharide region of the *A. salmonicida* subsp. *salmonicida* LPS from the in vivo rough isolate and isolate of strains A449 and 80204-1.

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2. Experimental

2.1. Bacterial culture and growth conditions

The in vivo derived rough phenotype of *A. salmonicida* subsp. *salmonicida* and strains A449 and 80204-1 were obtained from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, Nova Scotia). The bacteria were cultured in Tryptic Soy Broth (TSB) at 18 °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 (PBS, pH 7.4) and harvested by low-speed centrifugation (3000*g*, 25 min).

2.2. LPS isolation

Bacterial cells were washed successively with 2.5% saline (w/v), EtOH, acetone and ethyl ether (anhyd), and the cells were recovered by centrifugation. LPS from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* was extracted from dried cells with 5:5:8 phenol–$CHCl_3$–petroleum ether at 60–95 °C and precipitated as reported previously,[11,12] while LPS from strains A449 and 80204-1 was extracted by the method of Westphal and Jann.[13]

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with a mini-slab gel apparatus (Bio-Rad) using the method of Laemmli.[14] LPS samples were prepared at a concentration of 0.1% (w/v) in a sample buffer (62.5 mM Tris/HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol), equivalent amounts loaded in each lane and stained according to Tsai and Frasch.[15]

2.4. Mild acid hydrolysis of LPS and preparation of the N-acetylated core oligosaccharide

The purified LPS from the in vivo rough phenotype of *A. salmonicida* subsp. *salmonicida* (60 mg) was hydrolyzed with 2% AcOH (60 mL) at 100 °C for 2 h. The soln was cooled down on ice, and the precipitated lipid A removed by low-speed centrifugation. The supernatant was lyophilized and water-soluble components were fractionated by gel permeation chromatography on a Sephadex G-25 column (1.6 cm × 95 cm) (Sigma–Aldrich, St. Louis, Mo, USA) and the core oligosaccharide fraction eluted with distilled water (OS-1). LPS from *A. salmonicida* strains A449 and 80204-1 were purified and delipidated as described previously[10] using Sephadex G-25 and the resultant core oligosaccharide fractions subjected to CE-MS analysis.

For N-reacetylation, the core oligosaccharide was treated with Ac_2O (0.1 mL) in aq satd soln of sodium bicarbonate (1 mL) (22 °C, 30 min)[16] and the products purified on a Sephadex G-25 column (Sigma–Aldrich, St. Louis, Mo, USA) to give the N-acetylated core oligosaccharide (OS-2).

2.5. Deacylation of LPS and preparation of backbone oligosaccharides

LPS from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* (80 mg) was treated with 4 M KOH (4 mL) at 125 °C for 20 h according to Holst.[17] Following neutralization and purification on a Sephadex G-25 column, the oligosaccharide fraction was further fractionated by high-performance anion-exchange chromatography (HPAEC) using a linear gradient of 0.1–0.8 M sodium acetate in 0.1 M NaOH, followed by desalting on a Sephadex G-25 column to give two fractions, F 1 (5 mg) and F 2 (14 mg). F 1 was pure and contained a backbone oligosaccharide (LPS-OH-1), while F 2 was shown to be a mixture of two backbone oligosaccharides, LPS-OH-1 and LPS-OH-2.

2.6. Compositional analysis

Core oligosaccharide samples (0.5 mg) were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 18 h and analyzed as their alditol acetates as previously described.[18]

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. Peracetylated heptitol derivative was found to have the L-*glycero*-D-*manno* (or D-*glycero*-L-*manno*) configuration by comparison of its GLC retention time with that of an authentic standard. The L-*glycero*-D-*manno* absolute stereochemistry was assumed on biosynthetic grounds.[19] The hexose residues were determined to have the D-configurations by capillary GLC of their acetylated (−)-2-butyl glycosides, according to the method of Leontein et al.[20]

2.7. Methylation analysis

The core oligosaccharide samples were methylated according to the method of Ciucanu and Kerek.[21] The permethylated polysaccharide was subjected to hydrolysis as described by Stellner et al.[22] and analyzed according to previously reported conditions for partially methylated alditol acetates.[23]

2.8. NMR spectroscopy

NMR spectra were performed on Varian INOVA 500 MHz spectrometer using standard software. All NMR experiments were performed at 25 °C using a 5 mm indirect detection probe with the ^1H coil nearest to the sample. The methyl resonance of acetone was

used as an internal reference at δ 2.225 ppm for ^{1}H spectra and 31.07 ppm for ^{13}C spectra.

Standard homo- and heteronuclear correlated 2D techniques were used for general assignments: COSY, TOCSY, NOESY, HSQC and HMBC.[24] Spectra were assigned with the help of a computer program PRONTO.[25]

^{31}P NMR experiments were performed on a Varian INOVA 200 MHz spectrometer, chemical shifts are given relative to the external 85% H_3PO_4 (δ_P 0.0 ppm). The 2D ^{1}H–^{31}P HMQC experiment was acquired on a Varian 400 MHz spectrometer with the coupling constant at 11 Hz.

2.9. Capillary electrophoresis-mass spectrometry (CE-MS)

All experiments were performed as described previously in detail.[26] 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. The sheath soln (2:1 isopropanol–MeOH) was delivered at a flow rate of 1 μL/min. An electrospray stainless steel needle (27-gauge) was butted against the low dead vol tee and enabled the delivery of the sheath soln to the end of the capillary column. The separations were performed according to Li et al.[27]

3. Results and discussion

The in vivo derived rough phenotype of *A. salmonicida* subsp. *salmonicida* was grown in flasks and LPS isolated by a modified phenol–chloroform–light petroleum (PCP) extraction of dried bacterial cells and purified by ultracentrifugation. The purified LPS was analyzed by SDS-PAGE and silver staining confirmed that it lacked the typical high-molecular-mass smear pattern as compared with the smooth-form LPS from *A. salmonicida* strain A449 (Fig. 1). The core oligosaccharides of *A. salmonicida* strains A449, 80204-1 and an in vivo derived rough isolate were subjected to CE-MS analysis. Comparison of their CE-MS profiles confirmed that core oligosaccharides from all three strains exhibited similar MS profiles suggesting the presence of the identical glycoforms differing only in their relative distribution (Fig. 2).

LPS from the in vivo derived rough isolate was delipidated by treatment with mild AcOH and purified by gel permeation chromatography on Sephadex G-25 column affording a core oligosaccharide OS-1. Composition analysis of OS-1 revealed the presence of Glc, Gal, GalN, LD-Hep and an unhydrolyzed disaccharide in the approximate molar ratio of 1:1:0.5:4:1. The disaccharide was determined by GLC–MS analysis of alditol

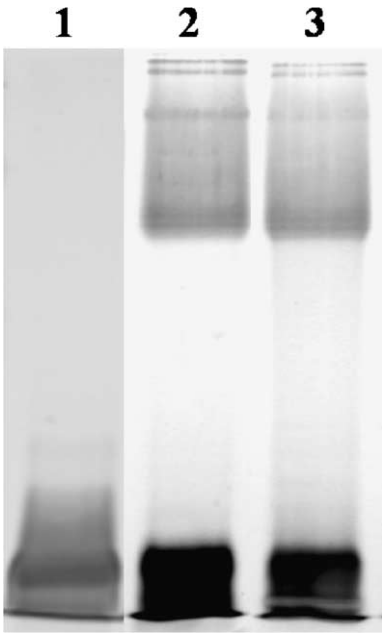


Figure 1. Silver stained SDS-PAGE (12% acrylamide) of LPS from *A. salmonicida* subsp. *salmonicida*: lane 1, in vivo rough isolate; lane 2, strain A449; lane 3, strain 80204-1.

acetates as 7-*O*-(2-amino-2-deoxy-glucopyranosyl)-L-*glycero*-D-*manno*-heptose previously identified by Shaw et al.[7] and also isolated from the core oligosaccharide of *Aeromonas hydrophila*.[28] Composition analysis of the N-acetylated core oligosaccharide derivative (OS-2) showed, in addition, the presence of GlcN, consistent with the presence of glucosamine-containing disaccharide residue in the native core of *A. salmonicida* LPS. GLC–MS analysis of the acetylated derivatives of their derived (*R*)-(−)-2-butyl glycosides confirmed that Glc, Gal, GalN and GlcN had the D-configuration.

In order to determine the linkage positions of sugar residues, methylation analysis was carried out on both OS-1 and OS-2. The results indicated the presence of terminal Gal (**Y**), 6-substituted Glc (**I**), terminal Hep (**H**), 2-substituted Hep (**F**), 4-substituted Hep (**J**), terminal GlcN (**M**), 7-substituted Hep (**G**), 4-substituted GalNAc (**Z**) and 3,4,6-trisubstituted Hep (**E**) in the approximate molar ratio of 1:1:0.9:1:1:0.8:1:0.5:0.8. Isomeric monosaccharides (such as Gal/Glc, GalN/GlcN) were identified according to GLC retention times of their partial methylated alditol acetate derivatives (Table 1). Methylation analysis of the purified core oligosaccharides from *A. salmonicida* strains A449 and 80204-1 was consistent with the above data (Table 1).

CE-MS analysis of OS-1 (Fig. 2) has indicated that it was a complex mixture, owing to mutarotation of the reducing end 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue and its existence in multiple forms, including 4,7- and 4,8-anhydro forms,[29,30] which resulted from the partial elimination of the phosphate group of Kdo

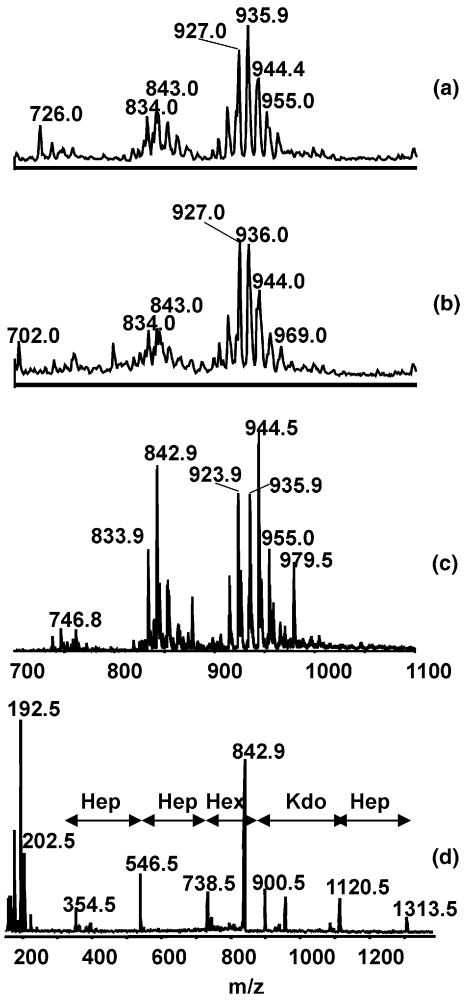


Figure 2. CE-MS and CE-MS/MS analysis (+ion mode) of the core oligosaccharide (OS-1) from *A. salmonicida* subsp. *salmonicida*: (a) strain A449; (b) strain 80204-1; (c) in vivo rough isolate; (d) MS/MS spectrum of a doubly charged molecular ion at *m*/*z* 842.9.

4-phosphate (see below) present in the native LPS. Additionally, CE-MS analysis of OS-1 revealed the presence of mainly two series of doubly charged positive ions, observed at *m*/*z* 842.9 and *m*/*z* 944.5, respectively. MS/MS analysis of the doubly charged ion at *m*/*z* 842.9 indicated that it corresponded to the backbone oligosaccharide fragment $Hex_2HexNHexNAcHep_5Kdo_1$ (Fig. 2 and Table 2), which was consistent with its sugar composition. The doubly charged ion at *m*/*z* 944.5 corresponded to a glycoform containing an additional *N*-acetyl hexosamine (HexNAc) residue and was consistent with the presence of additional GalNAc residue according to the composition analysis.

The analysis of the core oligosaccharide from the deacylated LPS was performed by CE-MS in the negative mode which revealed the presence of several doubly and triply charged ions, consistent with the presence of two glycoforms, LPS-OH-1 and LPS-OH-2 (Table 2). CE-MS of LPS-OH-2 afforded two major species, a doubly charged ion at *m*/*z* 1122.4 and a triply charged ion at *m*/*z* 747.6. Their fragmentation pattern was consistent with the core oligosaccharide composition of $Hex_2Hep_5HexN_3Kdo_1P_3$ (Table 2), as confirmed in a separate MS/MS experiment. Presence of the doubly and triply charged negative ions at *m*/*z* 1203.5 and *m*/*z* 801.6, respectively, was consistent with the core oligosaccharide glycoform LPS-OH-1 (Table 2). This glycoform contained an additional HexN residue as compared with LPS-OH-2, consistent with the presence of GalN in the native core oligosaccharide as determined by composition analysis. MS/MS analysis of the doubly charged negative ion at *m*/*z* 1203.5 afforded a singly charged fragment ion at *m*/*z* 499.1, corresponding to 1,4′-bisphosphorylated GlcN-(1→6)-GlcN (**B**→**A**) disaccharide backbone of lipid A (Table 2). Based on relative molecular ion intensities, the molar ratio of glycoforms LPS-OH-1 to LPS-OH-2 was estimated as 4:3.

In order to confirm the sequence of constituent glycoses and the position of linkages in the core oligosaccharide of *A. salmonicida* subsp. *salmonicida* LPS, 1D and 2D NMR studies were carried out on LPS-OH-1 which gave homogeneous spectra.

The ^1H and ^{13}C NMR spectra of LPS-OH-1 were completely assigned using 2D COSY, TOCSY, HSQC, HMBC and NOESY experiments based on the previously reported methods[24] (Table 3). The ^1H NMR spectrum of LPS-OH-1 showed resonances for eleven anomeric protons at δ 4.53–5.74 ppm. The assignment

Table 1. Methylation analysis of core oligosaccharides of *A. salmonicida* subsp. *salmonicida* strains A449, 80204-1 and the in vivo rough isolate

Sugar residues	OS-1 strain 80204-1	OS-1 strain A449	OS-1 in vivo rough isolate	OS-2 in vivo rough isolate
Terminal Gal	1.0	1.0	1.0	1.0
6-Substitued Glc	1.2	1.1	1.1	1.0
Terminal Hep	0.7	0.8	0.8	0.9
2-Substituted Hep	1.1	1.1	1.0	1.0
4-Substituted Hep	0.8	0.9	0.9	1.0
Terminal GlcNAc	0.2	0.1	0.2	0.8
7-Substituted Hep	0.2	0.4	0.3	1.0
3,4,6-Substituted Hep	0.8	0.7	0.7	0.8
4-Substituted GlcNAc	0.8	0.7	0.6	0.5
Disaccharide[a]	0.5	0.6	0.6	

[a] The disaccharide was determined by GLC–MS analysis of alditol acetates as 7-*O*-(2-amino-2-deoxy-glucopyranosyl)-L-*glycero*-D-*manno*-heptose.

Table 2. CE-MS data and proposed compositions for O-deacylated LPS (LPS-OH-1 and LPS-OH-2) and core oligosaccharide (OS-1) derived from LPS of the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida*

Products	Observed ions (*m/z*)		Molecular mass (Da)		Proposed composition
	$[M-3H]^{3-}$	$[M-2H]^{2-}$	Observed	Calculated	
LPS-OH-1[a]	801.6	1203.5	2407.8	2407.9	$Hex_2{\cdot}HexN_4{\cdot}Hep_5{\cdot}P_3{\cdot}Kdo$
LPS-OH-2[a]	747.6	1122.4	2246.8	2246.7	$Hex_2{\cdot}HexN_3{\cdot}Hep_5{\cdot}P_3{\cdot}Kdo$
		$[M+2H]^{2+}$			
OS-1[b]		944.5	1887.0	1887.7	$Hex_2{\cdot}HexNAc{\cdot}HexN{\cdot}Hep_5{\cdot}Kdo$
		935.4	1868.8	1869.6	$Hex_2{\cdot}HexNAc{\cdot}HexN{\cdot}Hep_5{\cdot}$anhKdo
		923.9	1845.8	1846.6	$Hex_2{\cdot}HexN_2{\cdot}Hep_5{\cdot}Kdo$
		842.9	1683.8	1684.5	$Hex_2{\cdot}HexN{\cdot}Hep_5{\cdot}Kdo$
		833.9	1865.8	1666.5	$Hex_2{\cdot}HexN{\cdot}Hep_5{\cdot}$anhKdo

[a] LPS-OH-1 and LPS-OH-2 were analyzed in the negative ion detection mode.
[b] OS-1 was analyzed in the positive ion detection mode. Average mass units used for calculation of the molecular mass based on a proposed composition are as follows: Hex, 162.14; HexNAc, 203.08; HexN, 161.16; Hep, 192.17; Kdo, 220.18; *P*, 79.98; Ac, 42.04; H_2O, 18.02.

Table 3. ^1H and ^{13}C NMR chemical shifts (in ppm) of LPS-OH-1 of the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida*

Residue	Nucleus	1	2 (3ax)	3 (eq)	4	5	6 (6a)	7 (6b)	8a (7b)	8b
GlcN	^1H	5.64	3.37	3.90	3.48	4.17	3.89	4.34		
A	^{13}C	90.9	54.8	70.1	70.1	72.4	69.8			
GlcN	^1H	4.88	3.08	3.85	3.82	3.71	3.56	3.70		
B	^{13}C	99.5	55.8	73.0	73.5	74.4	62.7			
Kdo	^1H		1.95	2.24	4.48	4.31	3.82	3.75	3.75	3.95
C	^{13}C			35.0	68.7	70.8	72.5	69.7	63.9	
Hep	^1H	5.20	4.20	3.98	4.24	4.34	4.16	4.01	4.22	
E	^{13}C	99.1	70.4	73.0	74.3	72.9	77.9	61.2		
Hep	^1H	5.74	4.19	3.89	3.89	3.66	4.11	3.70	3.79	
F	^{13}C	98.8	80.3	70.9	66.9	71.6	68.9	63.4		
Hep	^1H	5.13	3.99	3.89	3.75	3.84	4.23	3.80	3.95	
G	^{13}C	101.7	70.7	70.9	66.5	72.5	69.3	69.5		
Hep	^1H	5.39	4.09	3.90	3.85	3.83	4.09	3.72	3.79	
H	^{13}C	99.0	71.0	70.9	66.8	72.4	69.0	63.5		
Glc	^1H	4.53	3.49	3.45	3.57	3.49	3.83	4.05		
I	^{13}C	103.4	73.6	77.3	70.1	74.7	65.0			
Hep	^1H	4.95	4.19	3.95	4.13	3.90	4.03	3.71	3.82	
J	^{13}C	99.3	69.9	69.1	76.0	69.7	69.4	63.4		
GlcN	^1H	5.15	3.50	3.82	3.64	3.64	3.81	3.88		
M	^{13}C	95.0	54.1	70.5	69.5	73.6	60.9			
Gal	^1H	4.99	3.86	3.95	4.03	4.34	3.72	3.72		
Y	^{13}C	100.5	68.8	69.2	69.4	71.4	61.0			
GalN	^1H	4.65	3.08	3.72	4.04	3.87	3.88	3.96		
Z	^{13}C	102.0	53.8	72.0	76.8	75.9	60.7			

Spectra recorded at 25 °C in D_2O. The observed ^1H and ^{13}C chemical shifts are reported relative to external acetone (δ_H 2.225 ppm, δ_C 31.07 ppm).

of ^1H resonances was achieved by tracing the spin-connectivities delineated in the COSY and TOCSY contour maps from the anomeric and some other isolated ring protons, such as axial and equatorial protons (H3) of Kdo residue at δ 1.95 and 2.24 ppm. In addition, some of the vicinal protons were assigned by their correlations in the NOESY spectrum.

The COSY spectrum of LPS-OH-1 enabled differentiation between protons within each spin system. The carbon chemical shifts of LPS-OH-1 were assigned through HSQC (Fig. 3) and HMBC spectra.

Monosaccharide residues were identified based on their ^1H and ^{13}C NMR chemical shifts and vicinal coupling constants, which were in agreement with literature values for their respective pyranosides.[31–34] Anomeric configurations of the *gluco* and *galacto* sugar residues, Glc, Gal, GalN and GlcN were deduced based on their $J_{1,2}$ coupling constant values in the ^1H NMR spectrum and those of Hep residues and Kdo were confirmed by their typical ^1H and ^{13}C NMR chemical shifts as compared with the literature data.[25,31–35] Amino sugars were identified by correlation of their H-2 protons at the nitrogen-bearing carbon to the corresponding C-2 carbons at δ 3.37/54.8 ppm for **A**, δ 3.08/55.8 ppm for **B**, δ 3.08/53.8 ppm for **Z** and δ 3.50/54.1 ppm for **M**.

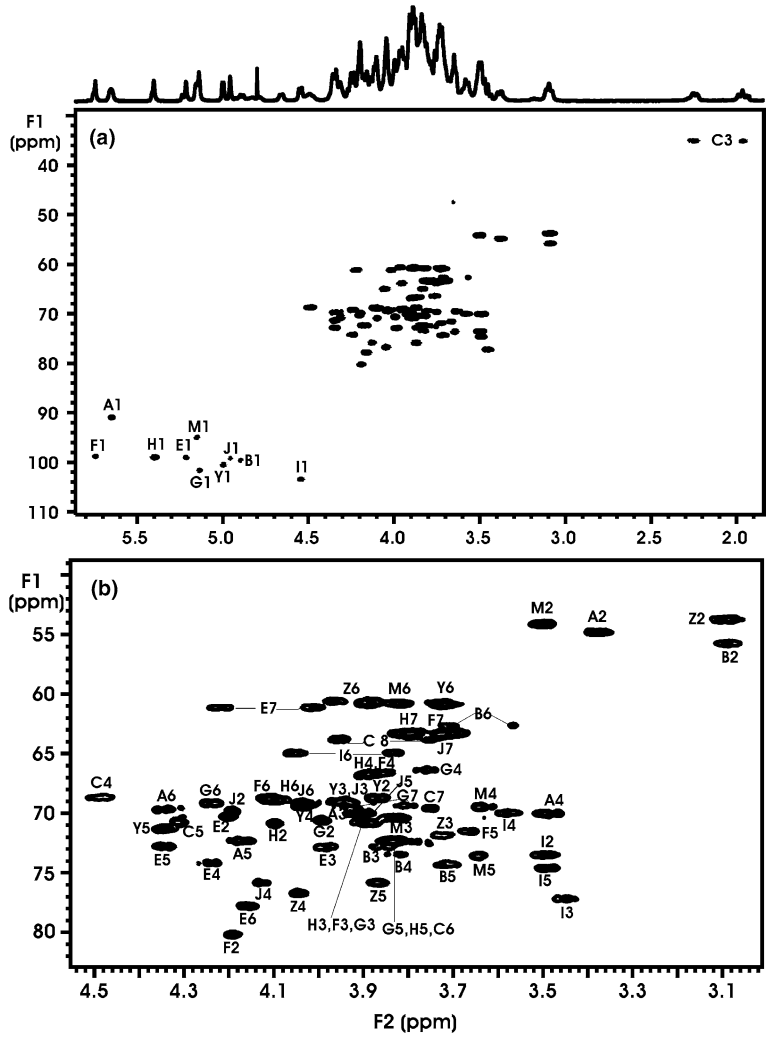


Figure 3. Complete ^{1}H NMR and 2D ^{1}H, ^{13}C HSQC spectra of LPS-OH-1 from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* in (a) and partial 2D ^{1}H, ^{13}C HSQC spectrum of LPS-OH-1 showing correlations for the ring oxygen bearing carbons in (b). Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Table 3.

The sequence of monosaccharides and the linkage positions in LPS-OH-1 were determined through 2D NOESY and HMBC experiments as shown in Figure 4. The following interresidue correlations between the anomeric protons and glycosidically linked protons in the NOESY spectrum were established: **E1C5**, **F1E3**, **G1F2**, **H1E6**, **M1G7**, **I1E4**, **J1I6**, **Y1Z4** and **Z1J4** at δ 5.20/4.31, 5.74/3.98, 5.13/4.19, 5.39/4.16, 5.15/3.80,3.95, 4.53/4.24, 4.95/3.83,4.05, 4.99/4.04, 4.65/4.13 ppm, respectively. In agreement with these results, the HMBC spectrum showed the following cross peaks between the anomeric protons and glycosidically linked carbons: **E1C5**, **F1E3**, **G1F2**, **H1E6**, **J1I6** and **Y1Z4** at δ 5.20/70.8, 5.74/73.0, 5.13/80.3, 5.39/77.9, 4.95/65.0, 4.99/76.8 ppm, respectively. Taken together these results confirmed the linkage position for each sugar residue and their sequence in LPS-OH-1. Moreover, significant downfield ^{13}C chemical shifts of the glycosidically linked carbons confirmed their linkage position for each sugar residue, as compared with their respective chemical shifts in the ^{13}C NMR spectra of the corresponding nonsubstituted monosaccharides at 71.3–72.4 ppm.[36] In addition, the NOESY spectrum also showed a number of other long-range interresidue correlations, such as **E1C7**, **G1I4**, **H1I1**, **F1G1**, **F1G5**, **F1G7**, **H1E4** and **I1E6**, indicating that the core OS has a tightly packed spatial structure.

The ^{31}P NMR spectrum of LPS-OH-1 showed resonances for three phosphate groups: one at δ 2.29 ppm and two at δ 3.87 ppm. The positions of phosphate groups were determined by ^{1}H–^{31}P HMQC experiment (Fig. 5), which showed three-bond correlations for ^{31}P resonances with H-1 of residue **A**, H-4 of residue **B** and H-4 of residue **C** at δ 2.29/5.64, 3.87/3.82 and 3.87/4.48 ppm, respectively.

The combined chemical and NMR evidence established the structure of the core oligosaccharide region of *A. salmonicida* subsp. *salmonicida* LPS from an

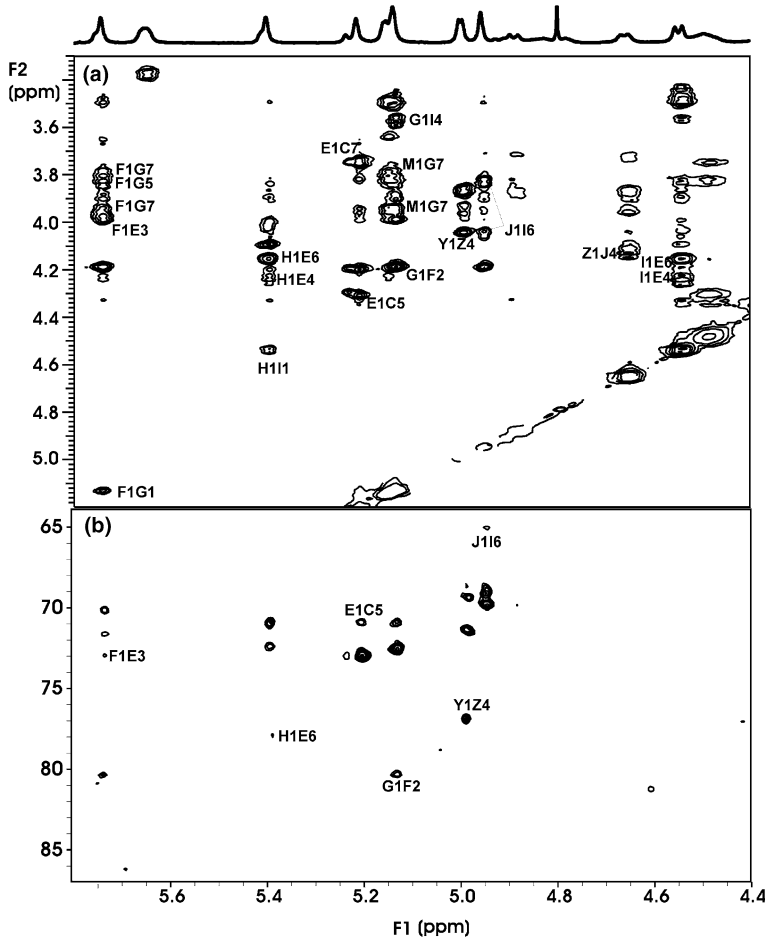


Figure 4. Partial NOESY in (a) and HMBC in (b) spectra showing correlations for anomeric protons of LPS-OH-1 from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida*. Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Table 3.

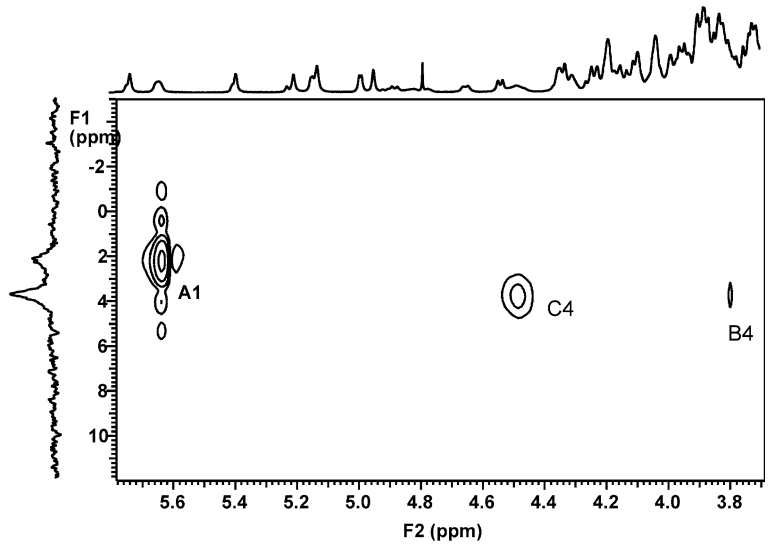


Figure 5. 2D ^{31}P–^{1}H NMQC spectrum of LPS-OH-1 of the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* showing correlations between the ^{31}P resonances (F1) and ^{1}H resonances (F2). Arabic numerals refer to atoms in sugar residues denoted by letters as shown in Table 3.

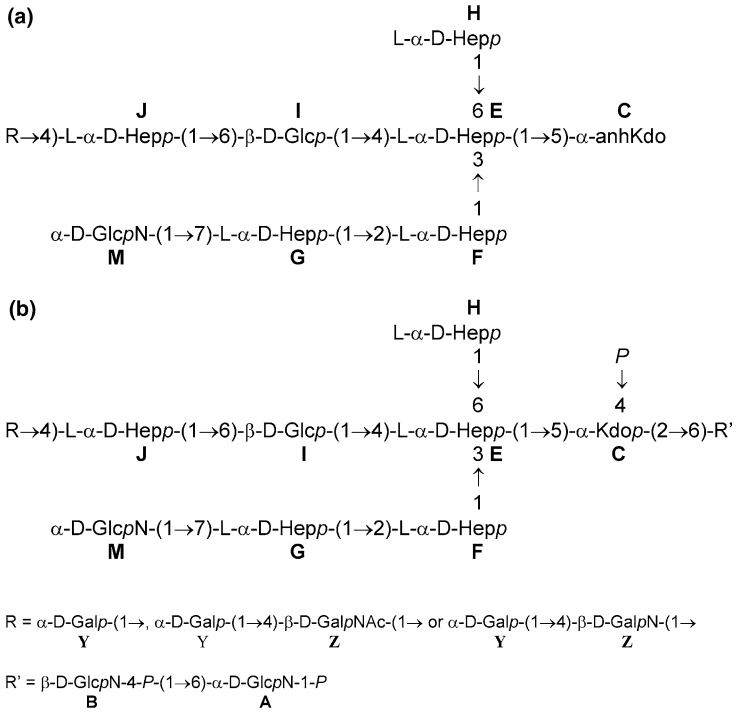


Figure 6. Structure of the core oligosaccharide of *A. salmonicida* subsp. *salmonicida* following mild acid hydrolysis (OS-1) in (a) and alkaline degradation (LPS-OH-1, LPS-OH-2) in (b).

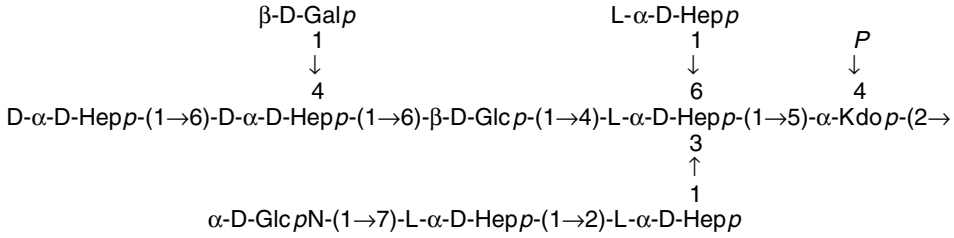
in vivo rough phenotype as shown in Figure 6. Based on the results of CE-MS analysis carried out on the purified LPS from *A. salmonicida* strains A449 and 80204-1 (Fig. 2) and results of the compositional and methylation analyses performed on their respective core oligosaccharide fractions (Table 1), this structure was conserved in LPS from all three strains of *A. salmonicida* subsp. *salmonicida*.

Recently, the core oligosaccharide structure of the spontaneous phage-resistant deep-rough mutant of *A. salmonicida* strain 438 (strain SJ-83) LPS was reinvestigated using electrospray quadrupole time of flight (ES-qTOF) tandem MS.[8,30] In this instance, structural information, such as sequence patterns of branched oligosaccharides or differentiation of isomeric hexosyl units, has been obtained using tandem mass spectrometry approach only and MS analysis was performed on the core oligosaccharide generated through the mild acid treatment of LPS resulting in the cleavage of the Kdo moiety.

The present study confirms irrevocably the absence of the furanoid form of Kdo in *A. salmonicida* core, suggested earlier by Shaw et al.[7] as one of the unique features of *A. salmonicida* LPS, and is consistent with the deep inner core LPS structure proposed by Banoub et al.,[28] which also confirmed the phosphate substitution at the O-4 position of Kdo residue.

Interestingly, the structure of the core oligosaccharide of *A. salmonicida* subsp. *salmonicida* is also consistent with the recently established core structure of a rough strain of *A. hydrophila*:[16]

Both structures are identical with respect to their inner and outer core regions differing only in the outer core extremities with D-α-D-Hep*p*-(1→6)-[β-D-Gal*p*-(1→ 4)]-D-α-D-Hep*p*-(1→ trisaccharide fragment being present in the outer core of *A. hydrophila* and α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→4)-L-α-D-Hep*p*-(1→ trisaccharide fragment present in the outer core region of *A. salmonicida*. These results suggest the presence of the conserved core fragment in *Aeromonas* species.



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