Structural characterization of the core region from the lipopolysaccharide of the haloalkaliphilic bacterium *Halomonas alkaliantarctica* strain CRSS

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Halophilic and halotolerant Gram-negative bacteria are microorganisms which thrive in high salt environments. LPS are the major components of their outer leaflet, nevertheless very little is known about the role of this molecules in the adaptation mechanisms of extremophiles. Recently we determined the O-chain repeating unit structure of the LPS from *Halomonas alkaliantarctica* strain CRSS, an haloalkaliphilic Gram-negative bacterium isolated from salt sediments of a saline lake in Cape Russell in Antarctic continent. The polysaccharide is constituted of the trisaccharidic repeating unit: →3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→. In this paper we report the complete core LPS structure from this bacterium. The LPS was hydrolyzed both under mild acid and strong alkaline conditions. The MALDI spectra showed the presence of two glycoforms. The most abundant was recovered after HPAEC purification of the alkaline hydrolyzed product and was characterized by means of 2D-NMR spectroscopy. A comparison of the MALDI-PSD spectra of the two glycoforms suggested that the branched heptose was not stoichiometrically substituted.

Introduction

Extremophiles are microorganisms able to survive in environment on Earth where the conditions are too harsh to support most life forms. These conditions range from scalding hot springs, to pH 1.5 acid mine drainage, to high pressure vents thousands of feet below the ocean's surface, to Antarctic ice fields, to salt flats. It has been found that extremophilic organisms belong to all three domains, *Archaea*, *Bacteria* and *Eukarya*, although to date the most extreme conditions are inhabited by *Archaea*.[1]

Halophiles include organisms which thrive in high salt environments and are able to survive osmotic stress. Halophilic and halotolerant microorganisms survive this stressed conditions by accumulating osmolytes or by maintaining cytoplasmic salt concentration (KCl) close to that of the surrounding medium.[2,3] As a result, proteins from halophiles present negative charged amino acid residues on their surface in order to manage the high salt concentration. Many enzymes isolated from halophiles have been exploited for their application in aqueous/organic and non-aqueous media.[4]

To maintain the turgor of the cells halophiles enhance the production of anionic phospholipids[5,6] and alter the fatty acid composition of the membrane lipids.[7] The Gram-negative bacteria outer-membrane forms a barrier for the cell and it is constituted by phospholipids, outer membrane proteins (OMP) and lipopolysaccharides (LPS).

Lipopolysaccharides are amphiphilic molecules contained in the outer leaflet of the external membrane of Gram-negative bacteria. They are anchored in the membrane with the lipid part (Lipid A) which is covalently linked to an oligosaccharide (core) in turn bonded to a polysaccharide (*O*-antigen, O-side chain). Bacteria that lack the O-chain in their LPS are of the rough type (R form) and the molecules are named lipooligosaccharides (LOSs). Due to their outward location, the LPSs are involved in the interaction mechanisms with the surrounding.[8] Despite of the fact that Gram-negative bacteria colonize very different organisms and environments, LPSs show a common architectural structure.[9] To date, much emphasis has been put on the elucidation of the core region structures from Gram-negative pathogenic bacteria, due to their immunogenic properties.[10] Very recently *Halomonas* species have been isolated from dialyzate coming from dialysis machines in hospital,[11,12] making the medical community aware of the pathogenic potential of the genus *Halomonas*. Therefore, complete structural analyses of the core region of LPS from halophiles and the comparison of its structures with that of common pathogenic Gram-negative bacteria are of high importance for a better understanding of LPS action.

Recently we determined the O-chain repeating unit structure of the LPS of *Halomonas alkaliantarctica* strain CRSS,[13] an haloalkaliphilic Gram-negative bacterium isolated from salt sediments of a saline lake in Cape Russell in Antarctic continent.[14] The polysaccharide is constituted of the trisaccharidic repeating unit: →3)-β-L-Rha*p*-(1→4)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→.

In this paper we now report the complete core oligosaccharide structure of *Halomonas alkaliantarctica* LPS, that was obtained after deacylation in mild acid and alkaline conditions. The obtained products were both analysed by MALDI-TOF mass spectrometry, but only that recovered after alkaline hydrolysis was purified by HPAEC and characterized by means of 2D-NMR spectroscopy giving the following structure:

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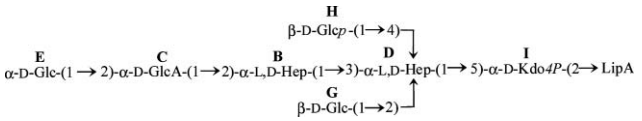


Results and discussion

Halomonas alkaliantarctica cells were grown and the LPS was extracted by PCP method.[15] The LPS was acid hydrolysed under mild conditions and the lipid A was removed by centrifugation. The supernatant was fractionated on a Biogel P-10 column. Fraction **a**, eluted in the void volume, was characterized as the O-chain.[13] Fractions **b** and **c** mainly contained the core oligosaccharide. The sugar analysis performed on these fractions showed the presence of D-glucose (Glc), D-glucuronic acid (GlcA), L-*glycero*-D-*manno*-heptose (L,D-Hep) and 2-keto-3-deoxy octulosonic acid (Kdo). In addition fraction **b** still contained rhamnose (Rha) belonging to the O-chain, therefore its analysis was not convenient for the core structural elucidation. As the previous glycosyl analysis performed on the LPS[13] indicated also the presence of D-*glycero*-D-*manno*-heptose (D,D-Hep), we hydrolysed a sample of LPS and analysed the monosaccharides as alditol acetates by GC-MS. Only the signal attributable to L,D-heptose was found, thus definitively excluding the presence of D,D-heptose in the LPS of *H. alkaliantarctica*.

The methylation analysis of fraction **c** was performed after dephosphorylation and identified the substitution pattern of the core region, *i.e.* 2-substituted GlcA, terminal Glc, 2-substituted Hep, and 2,3,4-trisubstituted Hep.

The fraction **c** was analysed by both MALDI-TOF MS and ^{1}H NMR. The reflectron positive ions MALDI-TOF spectrum showed the presence of only two species. The most abundant pseudomolecular ion $[M+Na+K]^+$ (**P1**) at *m/z* 1326.6, was attributed to the following composition: $Glc_3GlcAHep_2Kdo$*anhydro* (calculated molecular mass 1327.32 u). The second one (**P2**) occurred at *m/z* 1164.7, 162 u lower than **P1** and it was then attributed to a molecular species containing one glucose less (calculated molecular mass 1165.27 u). The ^{1}H NMR spectrum of the same fraction appeared to be very complex, probably due to the reducing end Kdo present as *anhydro*[16] forms. Therefore the NMR analysis was performed on the deacylated LPS.

Fully deacylation of the LPS was performed by treating the LPS first with hydrazine and then with KOH.[17] The obtained product was purified on a Sephadex G10 in order to remove the salts and then partially purified on a Biogel P-10 column. The most abundant fraction (**A**) contained the core oligosaccharide linked to a large number of O-chain repeating units, while the second one (**B**) was mainly constituted by the core oligosaccharides. The reflectron negative ions MALDI-TOF spectrum. of this fraction (Fig. 1) displayed a pseudomolecular ion $[M+K-H]^-$ at *m/z* 1883.8 (**M1**, calculated molecular mass 1884.37 u) which revealed the composition $GlcA(Glc)_3(Hep)_2Kdo(GlcN)_2P_3K$ (GlcN = 2-deoxy-2-amino-glucose; $P = PO_4^{3-}$). The signal at *m/z* 1785.9 (calculated molecular mass 1786.53 u) suggested the presence of a fragment ion which lacks a phosphoric acid molecule, as its mass difference respect to the species **M1** is 98 u.[18] In addition a less intense signal at 162 u lower masses (**M2**) indicated a molecular species with one glucose less, in agreement with the data obtained from the MALDI-TOF analysis of fraction **c**. Fraction **B** was then purified

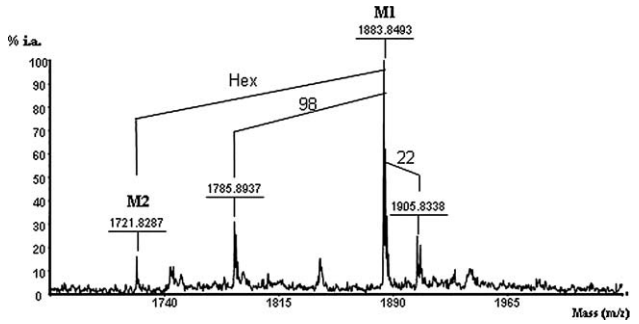


Fig. 1 Reflectron MALDI-TOF mass spectrum of fraction **B**. The spectrum was acquired in negative ions mode. (M1: $GlcA(Glc)_3(Hep)_2Kdo(GlcN)_2P_3K$; M2: $GlcA(Glc)_2(Hep)_2Kdo(GlcN)_2P_3K$; i.a.: ionic abundance).

by HPAEC obtaining a main oligosaccharide, named OS. The ^{1}H NMR spectrum of OS (Fig. 2) showed eight proton anomeric signals, assigned to residues **A**-**H**, at δ 5.55–4.36 (Table 1). The assignment of ^{1}H resonances was achieved by tracing the spin-connectivities, delineated in the DQF-COSY and TOCSY contour maps, from the anomeric and some other diagnostic ring protons, such as axial and equatorial protons (H-3) of Kdo residue at δ 1.92 and δ 2.18. In addition, the spatial proximity of some protons was recognized by NOE contacts measured in a ROESY spectrum. Carbon chemical shifts were assigned utilizing ^{1}H,^{13}C-DEPT-HSQC (Fig. 3) and ^{1}H,^{13}C-HMBC.

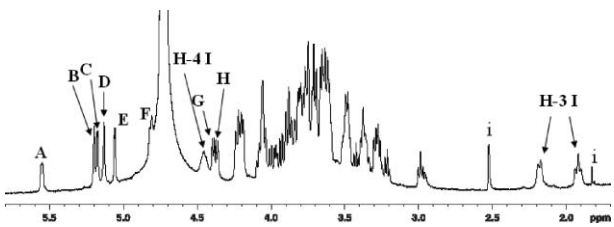


Fig. 2 ^{1}H NMR of OS performed at 25 °C. The spectrum was recorded in D_2O at 600 MHz. The letters refer to the residues as described in Table 1. Impurities are indicated by letter i.

Monosaccharide residues were recognised based on their ^{1}H and ^{13}C chemical shifts, which were in agreement with published data of their respective pyranosides,[19,20] and on the basis of coupling constant values and *inter*-residue NOE contacts. Anomeric configurations of the *gluco* configured residues, Glc, GlcA and GlcN were suggested from their $^3J_{H\text{-}1,H\text{-}2}$ coupling constant values in the ^{1}H NMR spectrum experiment and confirmed by measuring the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants in a 2D *F2*-coupled HSQC experiment (Table 1). The α anomeric configuration for heptose residues were deduced from the value of the $^1J_{C\text{-}1,H\text{-}1}$ coupling constants of 178 Hz and 173 Hz for **B** and **D**, respectively. The difference ($\Delta = 0.26$) between the proton chemical shifts of H-3ax and H-3eq (Table 1) of the Kdo residue indicated the α-configuration for this monosaccharide.[21] Glucosamine residues **A** and **F** were identified by the correlation of their H-2 protons, at δ 3.29 and δ 2.99, with the nitrogen-bearing carbons at δ 55.9 and δ 56.9, respectively.

All the ^{1}H and ^{13}C chemical shifts of both residues **A** and **F** were in agreement with the presence of the lipid A skeleton disaccharide structure β-GlcN-(1→6)-α-GlcN. The $^3J_{H\text{-}1,H\text{-}2}$ of both residues

Table 1 ^{1}H, ^{13}C NMR assignments of fraction OS. All the chemical shifts values are referred to acetone as internal standard (δ_H 2.225; δ_C 31.45). $^{3}J_{H-1,H-2}$ -$^{1}J_{C-1,H-1}$ are reported in parentheses and given in Hertz. Spectra were recorded in D_2O at 25 °C

	H-1 ($^{3}J_{H-1,H-2}$)	H-2/H3ax	H-3/H-3eq	H-4	H-5	H-6a	H-7a/H-6b	H-8/H7b
Residue	C-1 ($^{1}J_{C-1,H-1}$)	C-2	C-3	C-4	C-5	C-6	C-7	C-8
A →6)-α-GlcN*p*(1→	5.55 (3.2)	3.29	3.80	3.37	4.07	4.23	3.80	
	92.1 (177)	55.9	71.6	70.9	73.6	70.8		
B →2)-α-Hep*p*(1→	5.19 (<2)	4.19	3.87	3.80	3.71	3.97	3.89	
	102.8 (178)	80.1	72.3	68.8	74.5	69.9	64.7	
C →2)-α-GlcA*p*(1→	5.17 (3.0)	3.60	3.81	3.39	3.92	—		
	99.7 (173)	78.5	72.5	73.9	75.1	177.7		
D →2,3,4)-α-Hep*p*(1→	5.13 (<2)	4.06	4.24	3.99	4.05	3.75	3.83	3.65
	99.6 (173)	80.0	75.6	75.0	69.6	70.8	64.9	
E t-α-Glc*p*(1→	5.05 (3.5)	3.48	3.71	3.38	3.85	3.71	3.75	
	98.3 (173)	72.9	74.5	70.9	73.1	61.9		
F →6)-β-GlcN*p*(1→	4.81 (8.1)	2.99	3.77	3.71	3.62	3.49	3.62	
	100.9 (171)	56.9	74.1	73.2	75.5	63.9		
G t-β-Glc*p*(1→	4.39 (8.0)	3.28	3.46	3.48	3.61	3.62		
	103.4(165)	74.1	76.3	72.9	78.7	63.8		
H t-β-Glc*p*(1→	4.36 (7.6)	3.26	3.31	3.22	3.35	3.89	3.66	
	104.3 (166)	74.1	77.1	71.8	77.9	63.2		
I →5)-α-Kdo*p4P*(2→	—	1.92	2.18	4.45	4.20	3.77	4.05	n.d.
	n.d.	n.d.	35.6	70.8	74.5	73.4	73.1	n.d.

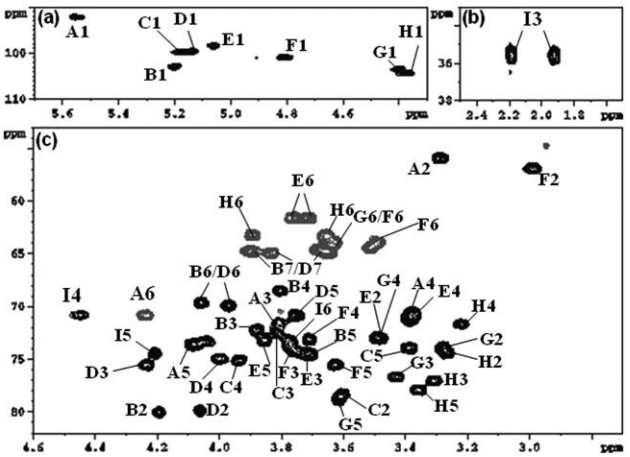


Fig. 3 Anomeric region (a), deoxy-group of Kdo (b) and primary and secondary alcohol groups region (c) of ^{1}H,^{13}C DEPT-HSQC spectrum of OS. Spectrum was performed at 600 MHz in D_2O at 25 °C. The letters refer to the residues as described in Table 1.

as well as the NOE contact between the anomeric proton of **F** and both H-6 protons of **A** confirmed this structure. The phosphorylation at position *O*-1 of **A** and at position *O*-4 of **F** were inferred from the multiplicity of the anomeric proton signal ($^{3}J_{H,P}$ = 6.6 Hz) of **A** and from both the C-4 and H-4 downfield shift of **F**, respectively.[22]

Heptose **B** with C-1/H-1 signals at δ 102.8/5.19 showed a C-2 downfield glycosylation shift of about 8 ppm, thus indicating that this position was substituted. The assignment of 2,3,4-trisubstituted heptose to residue **D** was deduced by the downfield shift of its C-2, C-3 and C-4, respectively. Residue **C** with C-1/H-1 signals at δ 99.7/5.17 was attributed to 2-substituted glucuronic acid as its C-2 resonance was shifted downfield. The residues **E**, **G** and **H** were assigned to terminal non-reducing glucose units as none of their carbon atoms were shifted downfield. Phosphorylation at position *O*-4 of Kdo was suggested by its H-4 chemical shift (δ 4.45) and by the downfield shifted C-4 at δ 70.8. Finally, its glycosylation at *O*-5 position was indicated by the shift of its C-5 carbon signal at δ 74.5 respect to the value of δ 67.4 for an unsubstituted Kdo residue.[23]

The ^{31}P NMR spectrum of OS confirmed the presence of three phosphates as it displayed (Fig. 4) three resonances at δ 2.67, 3.87 and 4.02, in agreement with the results obtained by MALDI-TOF mass spectrum of the oligosaccharide mixture. The positions of phosphate groups were confirmed by ^{1}H, ^{31}P HMQC experiment. More in detail a ^{31}P signal at δ 2.67 showed a correlation with H-1 of **A** while the ^{31}P signal at δ 3.87 was correlated with H-4 of **F**, thus confirming that both glucosamine residues of lipid A were phosphorylated. Finally the phosphorylation at position *O*-4

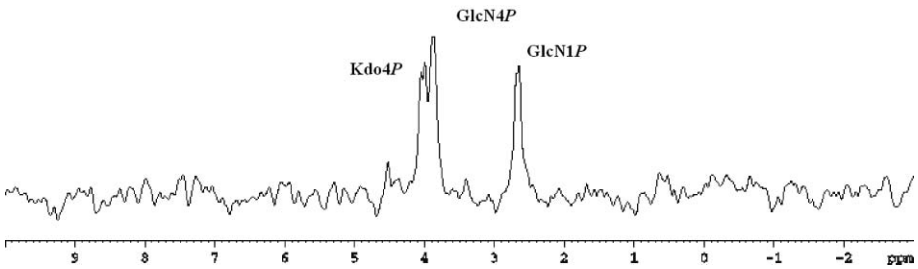


Fig. 4 ^{31}P NMR spectrum of OS. Spectrum was recorded at 400 MHz (25 °C) in D_2O at pD ~12. H_3PO_4 was used as external standard.

of the Kdo residue was confirmed by the correlations between its H-4 and the ^{31}P signal at δ 4.02.

The sequence of the monosaccharides and the linkage positions were deduced based on the HMBC and the ROESY experiments. The HMBC revealed the following *inter*-residue correlations: H-1 of **D** with C-5 of Kdo, H-1 of **B** with C-3 of **D**, H-1 of **C** with C-2 of **B**, H-1 of **E** with C-2 of **C**, H-1 of **G** with C-2 of **D**, and H-1 of **H** with C-4 of **D**. In agreement with these results were the *inter*-residue NOE contacts obtained from the ROESY spectrum (Fig. 5, Table 2). In particular dipolar couplings were observed between H-1 of **E** and H-2 of **C**, H-1 of **C** and H-2 of **B**, H-1 of **B** and H-3 of **D**, H-1 of **G** and H-2 of **D**, H-1 of **H** and H-4 of **D**, H-1 of **D** and H-5 of Kdo. The Kdo residue did not show any diagnostic NOE interactions or heteronuclear multiple-bond correlations, which may result from the conformation of the Kdo(2→6)GlcN(**F**) glycosidic linkage.[18] Because the C-6 chemical shift of **F** was in agreement with reported data,[24] the Kdo moiety was placed at C-6 of **F**. Finally the NOE interaction between H-5 of **D** and H-3ax of Kdo suggests a D configuration for Kdo.[25]

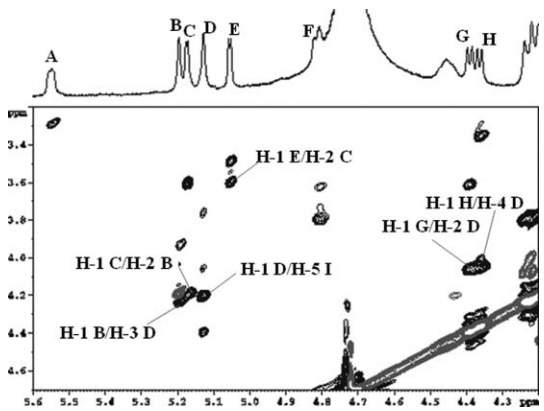


Fig. 5 Anomeric region of ^{1}H-^{1}H ROESY spectrum of OS. Spectrum was recorded at 600 MHz in D_2O (25 °C). The letters refer to the residues as described in Table 2.

Table 2 Nuclear Overhauser Enhancement *intra*- and *inter*-residual connectivities (ROESY) for the anomeric protons of fraction OS

H-1 of sugar residue	*Intra*-residue NOE	*Inter*-residue NOE
→6)-α-GlcN*p*(1→ A	H-2	
→2)-α-Hep*p*(1→ B		**D** H-3, H-4
→2)-α-GlcA*p*(1→ C	H-2	**E** H-1
		B H-2
→2,3,4)-α-Hep*p*(1→ D	H-2	**I** H-5, H-6
		G H-1
t-α-Glc*p*(1→ E	H-2	**C** H-1,H-2
→6)-β-GlcN*p*(1→ F	H-3, H-5	**A** H-6a,b
t-β-Glc*p*(1→ G	H-3, H-5	**D** H-1, H-2
t-β-Glc*p*(1→ H	H-2, H-3,H-5	**D** H-4

From all these data it was possible to depict the structure of the main core oligosaccharide for the LPS of *H. alkaliantarctica*, which corresponds to the **M1** species (Scheme 1).

Nevertheless the MALDI-TOF spectra of the products obtained after mild acid hydrolysis and alkaline hydrolysis showed the presence of two glycoforms, the second of which lacking one glucose unit. This glycoform was recovered in very small amount after HPAEC, precluding its NMR characterization.

In order to identify the non stoichiometric glucose, MALDI-PSD experiments were performed. The best results were obtained on the oligosaccharides released after mild acid hydrolysis. Both the pseudomolecular ions **P1** and **P2** were selected for PSD analysis, obtaining fragment ions for which the Domon and Costello nomenclature was used.[26] In both the spectra (Fig. 6a, b) the same signals corresponding to the fragment Glc-GlcA-Hep were displayed at *m/z* 553.2 and 552.9 (B ions) and at *m/z* 571.2 and 571.0 (C ions) for **P1** and **P2** respectively, indicating that the glycosylation at GlcA was stoichiometric.

Moreover the ions at *m/z* 907.8 in Fig. 6a and at *m/z* 745.7 in Fig. 6b indicated that **P2** species contained one Glc less in the inner core. This was also confirmed by the presence of the fragment ions at *m/z* 597.7 in Fig. 6b and at *m/z* 435.0 in Fig. 6a.

From these data we suggest that the branched heptose is not stoichiometrically substituted.

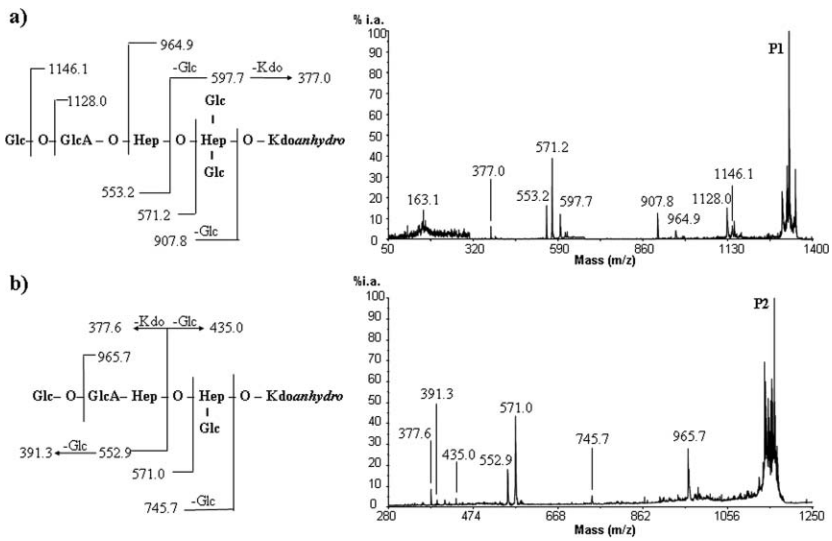
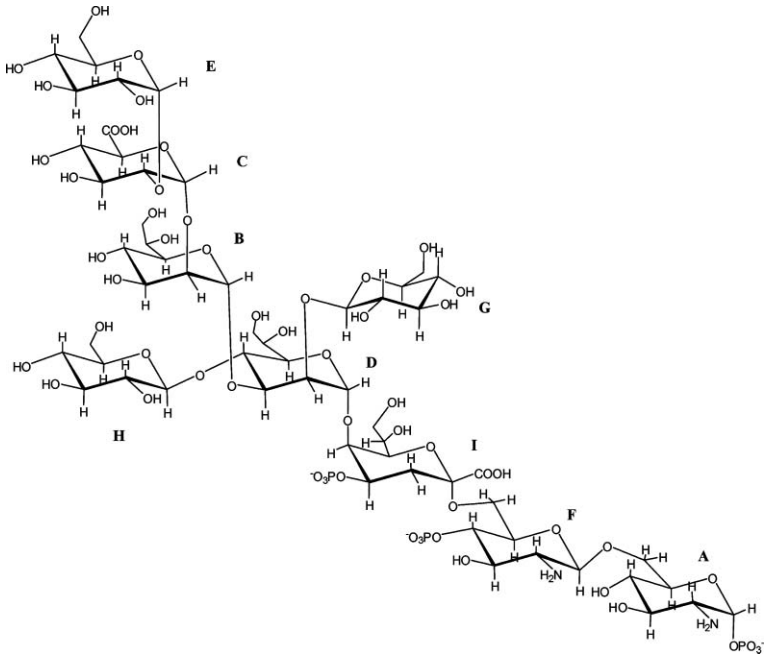


Fig. 6 Positive ions MALDI-PSD spectra of **P1** (a) and **P2**(b). i.a. stands for ionic abundance.



Scheme 1

Conclusions

Up to now, only the core oligosaccharide structure from the haloalkaliphilic *Halomonas pantelleriensis* has been elucidated,[27] and a comparison of this structure with that of *Halomonas alkaliantarctica* revealed that both show in the inner-core only one Kdo, which is *O*-4 phosphorylated. This feature is also in common with the majority of core structures from marine LPSs.[28]

Moreover, in *H. alkaliantarctica* the core oligosaccharide has an additional negative charge due to the presence of a glucuronic acid, whereas in *H. pantelleriensis* the negative charges belonged to the O-chain. Such structural elements contribute to the tightness of the outer-membrane and decrease the ion permeability, due to the association of LPS molecules through divalent cations (Ca^{2+} and Mg^{2+}).[28,29] This adaptation mechanism matches the well known accumulation of osmolytes inside the cytoplasm,[5,6] and it is in agreement with the tendency of halophiles to increase the proportion of negative charged phospholipids.[7,30]

During the preparation of this manuscript the core oligosaccharide structure of new *Halomonas* phylogenetically close to *alkaliantarctica* species was elucidated.[31] Although this new strain expresses a Rough LPS, its core structure shares some features with the object of this paper. More in details, the 2,3,4-trisubstituted heptose and the glucuronic acid confirm the proximity in the phylogenetic tree of the genus. Finally it would be interesting to know if the Kdo residue is *O*-4 phosphorylated, since it seems to be a common feature of *Halomonas* LPSs.

Experimental Section

1. Growth of bacteria, isolation and purification of the LPS

Halomonas alkaliantarctica strain CRSS was isolated from salt sediments in a saline lake of Cape Russell, Antarctica as previously reported.[14] It was routinely grown at 30 °C in a rotary shaker incubator with stirring at 100 rpm in 5 L flasks filled with 1000 cm^3 of the following enrichment medium (g L^{-1}): yeast extract 10, NaCl 100, trisodium-citrate 3, KCl 2, $MgSO_4 \cdot 7H_2O$ 1, $MnCl_2 \cdot 4H_2O$ 0.00036, $FeSO_4$ 0.050, Na_2CO_3 3 in distilled water, pH 9.0 (Na_2CO_3 and NaCl were autoclaved separately). The inoculum was 5% of total volume and aerobic condition was used. Cell growth was monitored by measuring the turbidity at 540 nm. The cultures were grown until the late exponential phase and harvested by centrifugation (10000 *g*). The cells were washed once with a saline solution and lyophilised.

Dried bacteria cells (21 g) were extracted by using PCP (a mixture of 90% Phenol, Chloroform, Petroleum ether in 2 : 5 : 8 ratio), obtaining 396 mg of LPS (yield 1.9%) as reported.[13,15]

2. Sugar analysis

Monosaccharides were analysed as acetylated methyl glycosides obtained from the LPS (0.5 mg) as follows: the sample was first dephosphorylated with 48% HF (100 µL), then the methanolysis was performed in 1 M HCl/MeOH (0.5 cm^3, 80 °C, 20 h). The obtained product was extracted twice with hexane and the methanol layer was dried and acetylated with Ac_2O (50 µL) and Py (50 µL) at 100 °C for 30 min. Alditol acetates were obtained from the LPS (1 mg). The LPS was hydrolysed with 2 M trifluoroacetic acid (120 °C, 2 h), reduced with $NaBD_4$ and acetylated with Ac_2O and pyridine. The monosaccharides were identified by EI mass spectra and GC retention times by comparison with those of authentic standards.

The absolute configuration of the sugars was determined by gas-chromatography of the acetylated (*S*)-2-octyl glycosides.[32]

All of these sugar derivatives were analysed on a Agilent Technologies gas chromatograph 6850A equipped with a mass selective detector 5973 N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 cm^3 min^{-1}, He as carrier gas). Acetylated methyl glycosides and alditol acetates were

analyzed accordingly with the following temperature program: 150 °C for 3 min, 150 °C→240 °C at 3 °C min^{-1}. Analysis of acetylated octyl glycosides was performed at 150 °C for 5 min, then 150 °C→240 °C at 6 °C min^{-1}, 240 °C for 5 min.

3. Linkage analysis

The linkage positions of the monosaccharides were determined by methylation analysis. Briefly, a sample of the oligosaccharides, obtained after mild acid hydrolysis and removal of the lipid A by centrifugation (2 mg), was treated with 48% HF (0.1 cm^3) for 20 h at 22 °C, in order to eliminate the phosphate groups. Methylation was performed with CH_3I in DMSO and NaOH (20 h).[33] The methylated sample was carboxymethyl reduced with $NaBD_4$, then totally hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2 h), reduced with $NaBD_4$ and finally acetylated. The temperature program used was: 90 °C for 1 min, 90 °C →140 °C at 25 °C min^{-1}, 140 °C→200 °C at 5 °C min^{-1}, 200 °C →280 °C at 10 °C min^{-1}, 280 °C for 10 min.

4. Mild acid hydrolysis of the LPS

The LPS (58 mg) was hydrolysed with 1% aqueous CH_3COOH, the lipid A was removed by centrifugation and the supernatant was fractionated on a Biogel P-10 column (Biorad, 1.5 × 100 cm, flow rate 14 cm^3 h^{-1}, fraction volume 2 cm^3), eluted with water buffered (pH 4.3) with 0.4% (v/v) pyridine and 1% (v/w) sodium acetate. Three fractions were obtained: **a** (32 mg), **b** (1.8 mg) and **c** (1.3 mg).

5. Deacylation of the LPS

The LPS (180 mg) was first dried over phosphoric anhydride under vacuum and then it was incubated with hydrazine (6.0 cm^3, 37 °C, 1.5 h). Cold acetone was then added to precipitate the *O*-deacylated LPS. The pellet was recovered after centrifugation (4 °C, 10.000 *g*, 30 min.), washed 3 times with acetone and finally dissolved in water and lyophilized (125 mg).

The *O*-deacylated LPS was dissolved in 4 M KOH (2.0 cm^3) and incubated at 120 °C for 16 h. KOH was neutralized with 4 M HCl until pH 6 and the mixture was extracted three times with $CHCl_3$. The water phase was recovered and desalted on a column (1.5 × 100 cm) of Sephadex G-10 (15 cm^3 h^{-1}, fraction volume 2 cm^3) eluted with water. The eluted oligosaccharide mixture was lyophilized (60 mg) and partially purified on a Biogel P-10 (Biorad, 1.5 × 100 cm, flow rate 14 cm^3 h^{-1}, fraction volume 2 cm^3), eluted with water. Two fractions were obtained **A** (52 mg) and **B** (6 mg).

6. HPAEC Analysis

Separation of the oligosaccharides mixture obtained after deacylation of the LPS (Fraction B, 6 mg) was performed by HPAEC-PAD on a semi-preparative column (9 × 250 mm) of Carbopac PA-100 eluted with the following gradient: 25%→31% of B over 55 min, 31%→50% of B over 30 min (A: 0.1 M NaOH and B: 2 M NaOAc/0.1 M NaOH) at 1 cm^3 min^{-1}. The main fraction was desalted on a column (1.5 × 100 mm) of Sephadex G-10 (Amersham Biosciences, 15 cm^3 h^{-1}, fraction volume 2 cm^3, eluent water) giving OS (0.9 mg).

7. NMR spectroscopy

For structural assignments of OS 1D and 2D ^1H and ^{13}C NMR spectra were recorded at 25 °C using a Bruker 600 MHz spectrometer equipped with a cryo-probe. All two-dimensional homo- and heteronuclear experiments (COSY, TOCSY, ROESY, HSQC-DEPT, *F2*- coupled HSQC and HMBC) were performed using standard pulse sequences available in the Bruker software. Chemical shifts were measured in D_2O using acetone as internal standard (δ 2.225 and 31.45 for CH_3 proton and carbon, respectively).^{31}P NMR spectra were recorded on a Bruker DRX-400 spectrometer. Phosphoric acid was used as an external reference (δ 0.00) for ^{31}P NMR spectroscopy. NaOD was added to the sample prior to one- and two-dimensional ^{31}P spectroscopy as described.[23]

8. Mass spectrometry analysis

Negative ions reflectron MALDI-TOF mass spectra were acquired on a Voyager DE-PRO instrument (Applied Biosystems) equipped with a delayed extraction ion source. Ion acceleration voltage was 20 kV, grid voltage was 17 kV, mirror voltage ratio 1.12 and delay time 200 ns. Samples were irradiated at a frequency of 5 Hz by 337 nm photons from a pulsed nitrogen laser. Mass calibration was obtained with a hyaluronan oligosaccharides mixture. A solution of 2,5-dihydroxybenzoic acid in 20% CH_3CN in water at a concentration of 25 mg cm^{-3} was used as the MALDI matrix. Spectra were calibrated and processed under computer control by using the Applied Biosystems Data Explorer software.

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