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Structural Characterization of the Core Region of the Lipopolysaccharide from the Haloalkaliphilic *Halomonas pantelleriensis*: Identification of the Biological O-Antigen Repeating Unit

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Dedicated to Prof. Matteo Adinolfi on the occasion of his 70th birthday

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Halomonas pantelleriensis is an extremophile, haloalkaliphilic microorganism that requires strictly aerobic conditions for growth. It is able to optimally grow in media containing 3–15 % (w/v) total salt at a pH value between 9 and 10. To survive in these harsh conditions the extremophiles have developed several strategies that allow the microorganism to thrive. These adaptative strategies probably concern the bacteria outer membrane, which is a barrier regulating the exchange with the environment. In such a context, the lipopolysaccharides (LPSs), which are among the major constituent of the Gram-negative outer membrane, are thought to contribute to restrictive membrane permeability properties. Previous studies concerning the structure of the O-chain repeating unit of the lipopolysaccharide from this bacterium showed that it is constituted of a tetrasaccharidic repeating unit containing a high number of acidic monosaccharides. It was hypothesized that the carboxylate groups might serve as a protective buffer for bacterium under the extreme life conditions. To provide insight into the relationship and interactions between the environmental factors and microbial life, the core structure was also characterized. The LPS was hydrolyzed under both mild acid and strong alkaline conditions. This last treatment was the best one to obtain the whole core backbone and to gain information about the phosphates position. Moreover, the strong alkaline treatment product allowed us to identify the linkage between the O-chain and the core structure. Two core oligosaccharides were found and their structures were determined by FTICR MS and NMR spectroscopy. To the best of our knowledge, this represents the first description of the core structure of a lipopolysaccharide of an extremophile bacterium.

Introduction

Microorganisms are well known for their ability to occupy a wide variety of environmental niches. They can be isolated, for example, from environments with extremely low concentrations of inorganic ions up to saturated brines, and this last kind of environment represents a well-recognized example of a so-called "extreme" environment inhabited by extreme and halotolerant microorganisms belonging to different taxa.[1] This is a considerable feat of adaptation not only to the increasing salt concentration, but also to the inevitable osmotic stresses that it imposes. Physiological adaptation to osmotic stress appears to be a capability of all microorganisms that are able to physiological adjustment to fluctuations in the water activity of aqueous environments as a prerequisite for growth and survival.[1–3]

It is not surprising that the cell envelope displays adaptive changes in the face of salinity, particularly in its lipid composition.[1,4–6] In addition, most microorganisms subjected to water stress accumulate organic solutes to control their internal water activity, to maintain the appropriate cell volume and turgor pressure, and to protect intracellular macromolecules.[1,4,7] We investigated the accumulation of osmoprotectants and lipid pattern modulation in *Halomonas pantelleriensis*, a Gram-negative haloalkaliphilic bacterium isolated from the sand of the volcanic Venus mirror

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lake, which is close to the seashore in the Pantelleria island in the south of Italy. It is able to optimally grow in media containing 3–15% (w/v) total salt and at pH 9–10.[4,8]

Gram-negative bacteria are characterized by an outer membrane, whose main component are lipopolysaccharides (LPSs). In their complete form, these are complex macromolecules composed of three different regions termed lipid A, core oligosaccharide, and O-specific polysaccharide (O-antigen, O-chain), and they differ in their structure, genetics, biosynthesis, and function.[9,10] Bacteria that lack the O-chain in their LPS are of the rough type (R form). The determination of the LPS primary structure is the mandatory starting point for the comprehension of the biological role played by these molecules.

Recently,[11] we determined the O-chain repeating unit structure of the LPS of this bacterium, and it is composed of a tetrasaccharidic repeating unit →4)-β-D-Glc*p*A-(1→4)-α-D-Gal*p*NAcA-(1→3)-β-L-Qui*p*NAc-(1→2)-β-D-[4-*O*-(*S*)-1-carboxyethyl]-Glc*p*A-(1→.

In this paper we report the complete core oligosaccharide structure of *Halomonas pantelleriensis* LPS, which, to the best of our knowledge, represents the first description of the core structure of an LPS of an extremophile bacterium. In addition, the sugar that links the O-chain to the core oligosaccharide was identified.

Results and Discussion

Isolation and Purification of the LPS

The LPS was recovered from dried bacteria by extraction with phenol/chloroform/light petroleum as described.[12] The sample was free from nucleic acids as confirmed by sugar analysis and UV spectroscopy, after DNase and RNase treatment. DOC-PAGE (deoxycholate polyacrylamide gel electrophoresis) of the purified LPS showed a ladder-banding pattern typical for smooth LPS relative to that of a standard LPS from *Escherichia coli* O55:B5 serotype. The electrophoresis profile confirmed the presence of few O-chain repeating units for the LPS of *H. pantelleriensis*, as previously reported.[11]

Compositional Analysis

The LPS, after reduction of the carboxylic groups with $NaBD_4$, was hydrolyzed and acetylated to give alditol acetates; the analysis by GC–MS revealed the presence of the sugars D-Glc (glucose), D-GlcN (deoxyglucose), and L,D- and D,D-Hep (D-*glycero*-D-*manno*-heptose), in addition to L-QuiN (2-amino-2,6-dideoxyglucose), D-GlcA (glucuronic acid), D-GalNA (2-amino-2-deoxygalacturonic acid), and 4-*O*-(1-carboxyethyl)-D-GlcA belonging to the O-chain repeating unit.[11]

All sugar derivatives were identified by comparison of the EI-MS and GC retention times of the samples to those of authentic standards.

Methanolysis of the LPS was performed, followed by extraction with hexane to separate the lipid and saccharide portions. The former fraction was analyzed by GC–MS, which revealed the presence of decanoic, dodecanoic, and 3-hydroxydodecanoic acids. The latter fraction was dried and acetylated to give the acetylated methyl glycosides; its analysis by GC–MS confirmed the above data.

Because in untreated LPS Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) could not be found, the LPS was first dephosphorylated with 48% aqueous HF and then subjected to methanolysis and acetylation. The thus identified Kdo suggested its phosphorylation in native LPS.

Acid Hydrolysis of the LPS

The LPS was hydrolyzed with 1% aqueous AcOH (100 °C, 6 h). The supernatant containing the oligosaccharide portion of the LPS was fractionated on a column of Sephadex G-50 (Pharmacia, Figure 1). Six fractions were collected, and each one contained a different number of O-

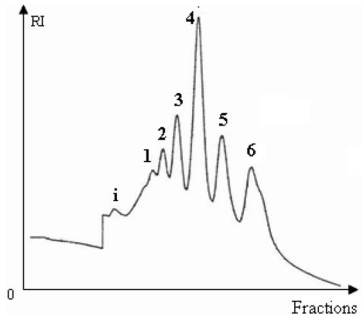


Figure 1. Sephadex G50 chromatogram of the polysaccharide portion obtained after mild acid hydrolysis of the LPS from *Halomonas pantelleriensis*. The letter **i** indicates impurities.

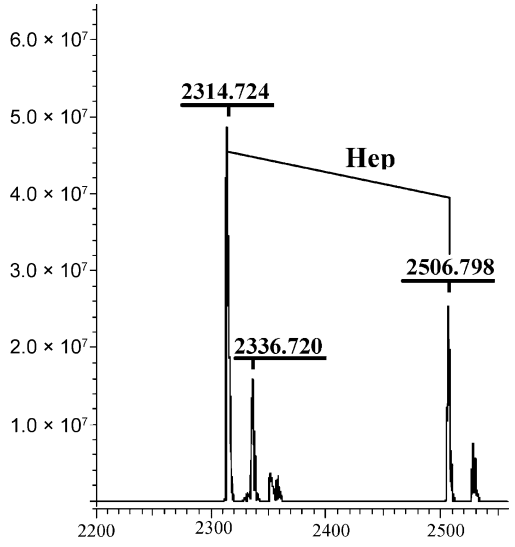


Figure 2. Charge deconvoluted ESI FTICR mass spectrum of the main fraction isolated after Sephadex G-50 chromatography of the oligosaccharide mixture obtained after mild acid hydrolysis of the LPS from *Halomonas pantelleriensis* bacterium. The spectrum was acquired in negative ion mode.

chain repeating units (O-chain RU). The most abundant fraction was analyzed by electrospray ionization Fourier-transform ion cyclotron resonance mass spectrometry (ESI FTICR MS). The spectrum (Figure 2) showed the presence of two main peaks differing for one heptose residue at *m*/*z* = 2506.798 and 2314.724.

On the basis of sugar analysis, the following composition for the oligosaccharide corresponding to the signal at *m*/*z* = 2506.798 was deduced: $(O\text{-ChainRU})_1(Glc)_3(Hep)_4(GlcNAc)_1(Kdo)$-18 (GlcNAc = 2-acetamido-2-deoxyglucose). The long hydrolysis time required to cleave the Kdo-lipid A linkage and the almost exclusive presence of the anhydrous form of Kdo suggested that the LPS contained Kdo-4*P*.[13,14]

Methylation analysis of this sample identified the substitution pattern of the core region, that is, terminal Glc, 4-linked Glc, 6-linked Glc, terminal Hep, 2-linked Hep, 2,6-linked Hep, 7-linked Hep, 3,4-linked Hep, 4-linked GlcNAc, and 3-linked QuiNAc (2-acetamido-2,6-dideoxyglucose).

Deacylation of the LPS

O-Deacylation was performed with anhydrous hydrazine, and the negative mode ESI-FTICR mass spectrum of the product (LPS-OH) identified five main species (Figure 3). The signal at *m*/*z* = 896.410 was attributed to the *O*-deacylated lipid A (lipid A-OH) containing two *N*-linked 3-hydroxydodecanoic fatty acids.

The mass peaks with higher mass units were attributed to LPS-OH according to the sugar composition. In particular, the species of 2676.933 Da could be attributed to the following composition $(Glc)_3(Hep)_4GlcNAc(GlcN)_2KdoP_3(C12{:}3OH)_2Na$ according to the calculated mass of 2676.913 Da. Moreover, the species of 3527.156 Da was consistent with a LPS-OH species containing one O-chain repeating unit, as the difference to the signal of 2676.933 Da corresponded to the mass of the repeating unit (828.228 Da).[11] Finally, the species of 2484.864 and 3335.085 Da confirmed the presence of two core glycoforms differing for one heptose unit.

The presence of 4-substituted uronic acids in the O-chain repeating units allowed the isolation of a phosphorylated core structure lacking most of the O-chain repeating unit because of the β-degradation that occurred during *N*-deacylation under strong alkaline conditions with 4 M KOH.

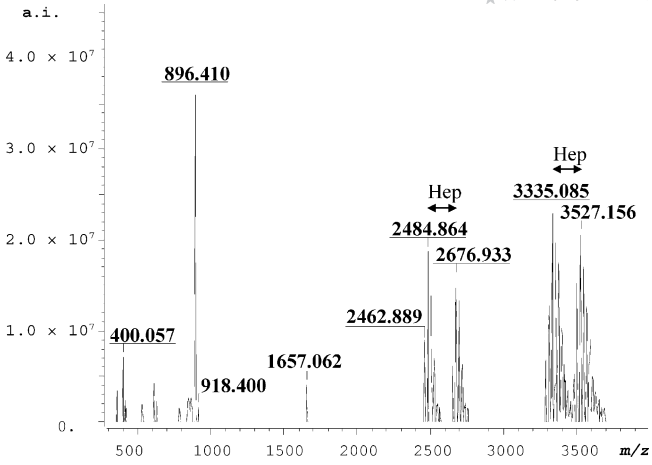


Figure 3. Charge deconvoluted ESI FTICR mass spectrum of the *O*-deacylated LPS from *Halomonas pantelleriensis*. The spectrum was acquired in negative ion mode.

The ESI-FTICR mass spectrum of the fully deacylated LPS identified six oligosaccharides termed M^1–M^6 (Figure 4), and their compositions are summarized in Table 1.

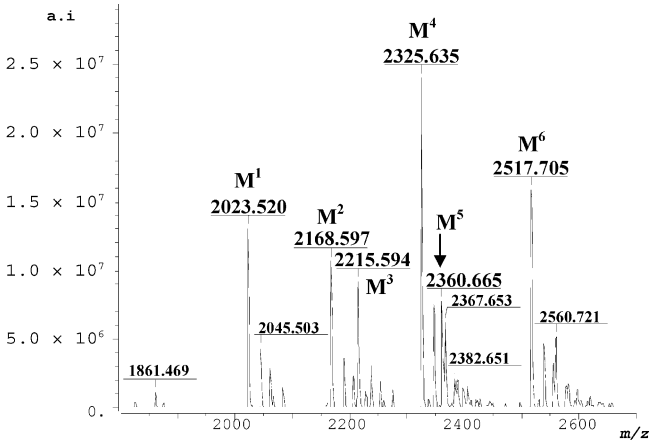


Figure 4. Charge deconvoluted ESI FTICR mass spectrum of the fully deacylated LPS from *Halomonas pantelleriensis*. The glycosyl composition of M^1–M^6 is reported in Table 1. The spectrum was acquired in negative ion mode.

By taking into account that the QuiN was linked to the GalNA in the O-chain structure[11] and because oligosaccharides M^4 and M^6 still possessed QuiN and a β-degraded

Table 1. Composition of the main species present in the ESI-FTICR mass spectrum of deacylated LPS from *Halomonas pantelleriensis* (Figure 3).

Glycoform	Observed Mass [Da]	Calculated Mass [Da]	Oligosaccharide composition
M^1	2023.520	2023.520	$(Glc)_3$ $(GlcN)_3$ $(Hep)_3$ Kdo P_3
M^2	2168.597	2168.594	[a]QuiN $(Glc)_3$ $(GlcN)_3$ $(Hep)_3$ Kdo P_3
M^3	2215.594	2215.583	$(Glc)_3$ $(GlcN)_3$ $(Hep)_4$ Kdo P_3
M^4	2325.635	2325.631	[a]ΔHexNA QuiN $(Glc)_3$ $(GlcN)_3$ $(Hep)_3$ Kdo P_3
M^5	2360.665	2360.657	[a]QuiN $(Glc)_3$ $(GlcN)_3$ $(Hep)_4$ Kdo P_3
M^6	2517.705	2517.694	[a]ΔHexNA QuiN $(Glc)_3$ $(GlcN)_3$ $(Hep)_4$ Kdo P_3

[a] QuiN and ΔHexNA arise from the O-chain degraded structure.

amminouronic acid (ΔHexNA), QuiN was unambiguously identified as the sugar that links the O-chain to the core region.

The oligosaccharide mixture was then partially purified by HPAEC, and the chromatogram obtained under alkaline conditions identified three fractions (Figure 5). ESI-FTICR mass spectra of these fractions (data not shown) showed that each contained two molecular species differing by one heptose unit, that is, fraction A contained the two core oligosaccharides M^1 and M^3, fraction B contained M^2 and M^5, and fraction C contained M^4 and M^6.

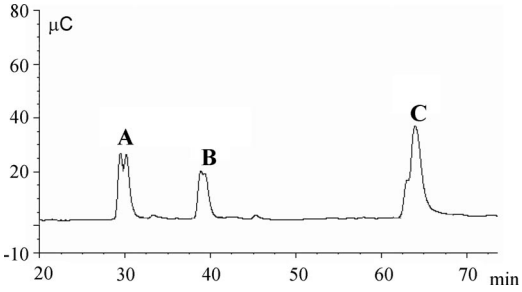


Figure 5. HPAEC chromatogram of the fully deacylated LPS from *Halomonas pantelleriensis*. Each fraction contained two species. In particular, fraction A contained M^1 and M^3, fraction B contained M^2 and M^5, and fraction C contained M^4 and M^6.

NMR Spectroscopy of Fraction C

Because the 1D ^{1}H NMR spectrum of the product obtained after acid hydrolysis appeared to be very complex, the NMR spectroscopic analysis was performed on the deacylated LPS. The ^{1}H NMR spectrum of fraction C was recorded at two different temperatures to reduce overlapping peaks. The anomeric regions of the spectra recorded at 300 and 313 K are depicted in Figure 6a,b. Besides a very minor component, there were two major species as suggested by the nonstoichiometric intensities of residues **E**, **F**, and **G** relative to the other anomeric signals. It turned out that singlet **E** was diagnostic for the amount of OS1, whereas the intensities of **F** and **G** were diagnostic for that of OS2. The anomeric regions of the proton spectra also included a downshift signal at δ = 5.86 ppm, which is diagnostic for 4-H of a *threo*-hex-4-enuronoaminopyranose residue.

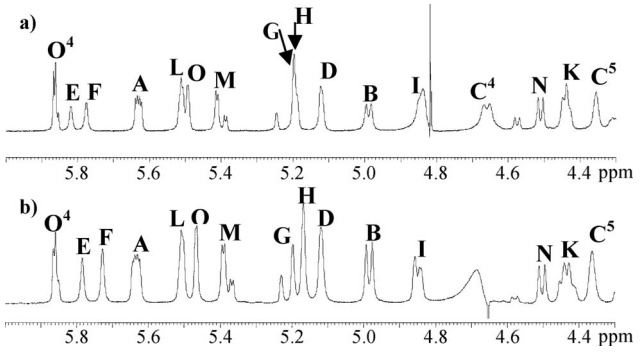
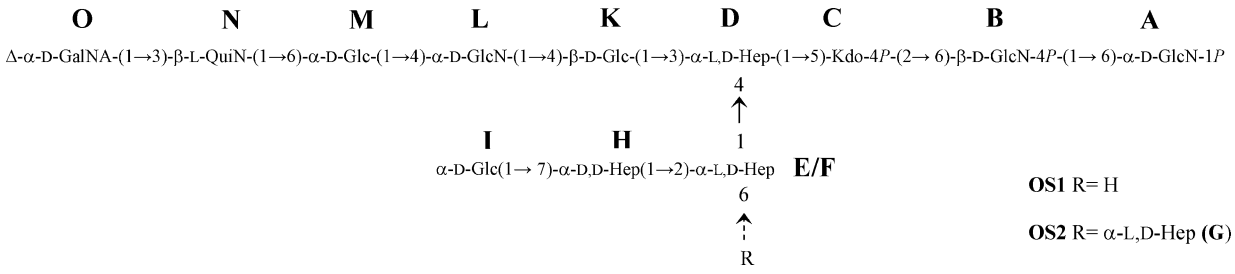


Figure 6. ^{1}H NMR anomeric region of the fully deacylated LPS from *Halomonas pantelleriensis* performed at 300 K (a) and 313 K (b). The spectra were recorded in D_2O at 500 MHz. The letters refer to the residues as described in Table 2.

2D NMR spectroscopic experiments (COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating-frame nuclear Overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; HSQC-TOCSY, heteronuclear single quantum coherence; 2D *F2*-coupled HSQC) determined the complete structure of the two core oligosaccharides OS1 and OS2, in which all sugars were pyranoses (Scheme 1, Tables 2 and 3). Residue **A** was attributed to the 6-substituted α-Glc*p*N-1*P* residue of lipid A on the basis of the multiplicity of the anomeric proton signal due to its phosphorylation ($^3J_{H,P}$ = 6.6 Hz), of the C-2 chemical shift at δ = 55.4 ppm, which indicated a nitrogen-bearing carbon atom, and of the C-6 chemical shift, which was downfield at δ = 70.1 ppm due to its glycosylation.

Residue **B** with C-1/1-H signals at 100.6/4.98 ppm ($J_{1\text{-H},2\text{-H}}$ = 8.4 Hz) was attributed to the 6-substituted β-Glc*p*N-4*P* residue as a result of its C-2 chemical shift at δ = 56.5 ppm and its linkage to C-6 of **A**. This structural feature was inferred by the interresidual connectivity between 1-H of **B** and both C-6 and $6_{a,b}$-H of **A** identified in the HMBC and ROESY experiments. Moreover the 4-H and C-4 downfield shifts are diagnostic for the presence of a phosphate group linked to O-4.

Residues **D**, **E**, **F**, **G**, and **H** were identified as five *manno*-configured α-heptopyranoses owing to their small $J_{1\text{-H},2\text{-H}}$ and $J_{2\text{-H},3\text{-H}}$ values. The anomeric configuration for all these residues was confirmed to be α by measuring $^1J_{1\text{-H},C\text{-}1}$ in a



Scheme 1.

Table 2. ^{1}H and ^{13}C NMR assignments of OS1 and OS2. All the chemical shifts values are referred to acetone as internal standard (^{1}H, 2.225 ppm; ^{13}C, 31.45 ppm). $^{1}J_{C-1,H-1}$ are reported in parentheses and given in Hertz. Spectra were recorded at 313 K.

Residue	1	2	3	4	5	6	7	8
6-α-GlcN*1P*	5.63 (178)	3.36	3.89	3.43	4.17	4.26, 3.89		
A	91.8	55.6	72.9	71.06	73.8	70.1		
6-β-GlcN*4P*	4.98 (169)	3.07	3.87	4.04	3.72	3.61, 3.61		
B	100.6	56.5	73.8	74.0	75.3	62.5		
5-α-Kdo*4P*	nd	nd	1.92, 2.25	4.69	4.37	3.91	3.70	3.75, 3.95
C			35.9	69.3	72.6	72.9	70.6	64.9
3,4-α-Hep	5.12 (178)	4.14	4.11	3.99	nd	nd	3.69, 3.79	
D	100.4	71.3	76.3	73.6			64.2	
2-α-Hep	5.72 (182)	4.14	3.91	3.92	nd	4.09	nd	
E	99.5	80.8	71.1	67.5 / 67.0		69.7		
2,6-α-Hep[a]	5.78 (181)	4.13	3.92	3.92	nd	4.15	3.75	
F	99.0	80.8	71.1	67.5 / 67.0		74.8	62.5	
t-α-Hep[a]	5.20 (175)	3.87	4.01	3.82	3.68	nd	nd	
G	100.3	72.0	70.3	67.1	73.9			
7-α-Hep	5.17 (174)	3.98	3.68	3.77	3.77	4.22	3.80, 3.84	
H	102.9	71.6	73.3	67.0	73.6	69.9	71.3	
t-α-Glc	4.85 (174)	3.72	3.61	3.49	nd	nd		
I	100.3	73.9	70.7	70.9				
4-β-Glc	4.44 (166)	3.55	3.72	3.61	3.60	3.97, 3.82		
K	103.9	74.4	71.9	79.0	76.1	62.1		
4-α-GlcN	5.50 (178)	3.31	4.08	3.71	nd	nd		
L	98.97	55.4	71.9	78.1				
6-α-Glc	5.39 (176)	3.62	3.90	3.69	3.46	4.17, 3.85		
M	101.1	72.7	73.1	70.6	70.6	69.8		
3-β-QuiN	4.49 (166)	2.92	3.75	3.34	3.54	1.33		
N	103.4	57.4	84.6	74.5	73.03	18.0		
α-ΔHexA	5.46 (174)	3.34	4.39	5.86				
O	99.1	55.6	65.9	108.3	146.0	nd		

[a] Monosaccharides belonging to OS2; nd: not determined.

Table 3. Nuclear Overhauser enhancement interresidual connectivities (ROESY) for the anomeric protons of OS1 and OS2.

1-H of sugar residue	NOE correlations
GlcN **B**	**A** 6a,b-H
Hep **D**	**C** 5-H, 6-H
Hep **E**	**D** 4-H
Hep **F**	**D** 4-H
Hep **G**	**F** 6-H
Hep **H**	**F/E** 1-H, 2-H, 3-H
Glc **I**	**H** 7a,b-H
Glc **K**	**D** 3-H, 4-H
GlcN **L**	**K** 3-H, 4-H
Glc **M**	**L** 3-H, 4-H
QuiN **N**	**M** 6a,b-H
ΔHexNA **O**	**N** 2-H, 3-H

2D *F2*-coupled HSQC spectrum (>170 Hz for α and <168 Hz for β, see Table 2). Downfield-shifted carbon signals indicated substitutions at O-3 and O-4 of residue **D** (C-3 at 76.3 ppm and C-4 at 73.6 ppm), at O-2 of **E** (C-2 at δ = 80.8 ppm), at O-2 and O-6 of residue **F** (C-2/C-6 at δ = 80.8/74.8 ppm), and at O-7 of **H** (C-7 at δ = 71.3 ppm). Residue **G** was assigned to a terminal heptose unit, as none of its carbon atoms were shifted downfield. All these residues were found to be L,D configured except **H**. For this residue, a D,D configuration was suggested, as its diagnostic C-6 chemical shift value occurring at δ = 72.0 ppm[15] was upfield shifted (δ = 69.9 ppm) as a result of β glycosylation at the O-7 position. Residue **O** with C-1/1-H signals at 99.1/5.46 ppm ($J_{1-H,2-H}$ = 2.0 Hz) was identified as Δ-α-Hex*p*NA on the basis of the 4-H/C-4 chemical shift values in the sp^2 region at 5.86 and 108.3 ppm, respectively, and 2-H/C-2 chemical shifts at 3.34/55.6 ppm.

Residue **N** with C-1/1-H signals at 103.4/4.49 ppm ($J_{1-H,2-H}$ = 8.8 Hz) was assigned to a 3-substituted β-L-Qui*p*N on the basis of the TOCSY spectrum, which showed a methyl proton signal at δ = 1.33 ppm, 2-H/C-2 chemical shifts at 2.92/57.4 ppm, and a strongly downfield-shifted C-3 resonance at δ = 84.6 ppm. The other four residues, that is, **M**, **K**, **L**, and **I**, were attributed to 6-substituted α-Glc

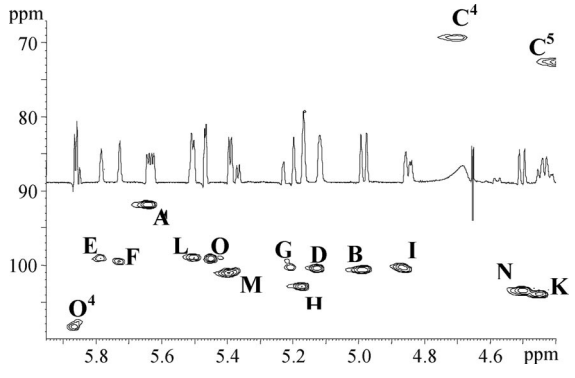
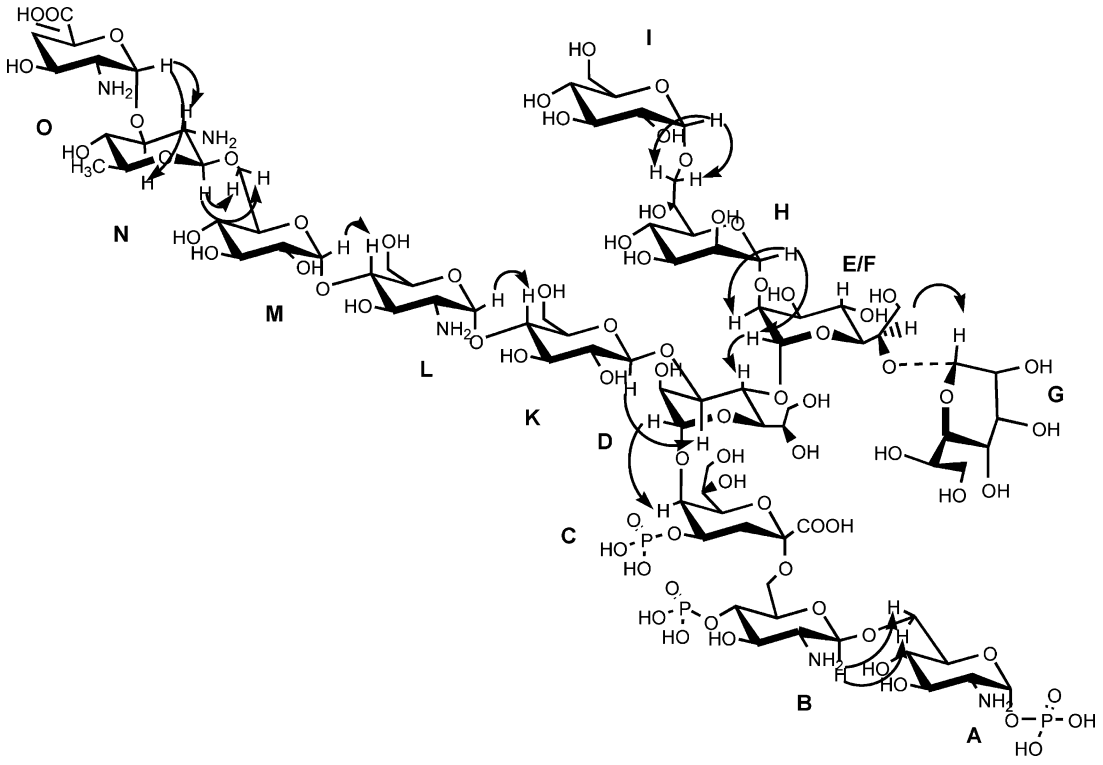


Figure 7. Anomeric region of ^{1}H and ^{1}H–^{13}C DEPT-HSQC spectra of the fully deacylated LPS from *Halomonas pantelleriensis* performed at 500 MHz in D_2O at 313 K. The letters refer to the residues as described in Table 2.



Scheme 2. OS core structures from *Halomonas pantelleriensis*. Only the main interresidual NOE are indicated; for more details see Table 3. Dotted lines indicate nonstoichiometric substitution of residue **G**.

(O-chain R.U.)n(1→6)-α-D-Glc-(1→4)-α-D-GlcNAc-(1→4)-β-D-Glc-(1→3)-α-L,D-Hep-(1→5)-Kdo-4*P*-(2→ 6)-β-D-GlcN-4*P*-(1→ 6)-α-D-GlcN-1*P*

4 ↑

α-D-Glc(1→ 7)-α-D,D-Hep-(1→2)-α-L,D-Hep

6 ↑

R

OS1 R= H

OS2 R= α-L,D-Hep

(**M**), 4-substituted α-GlcN (**L**), 4-substituted β-Glc (**K**), and terminal α-Glc (**I**) on the basis of their proton and carbon atom chemical shifts and their $J_{1\text{-H},2\text{-H}}$ coupling constants.

In the ^{1}H NMR spectrum recorded at 313 K (Figure 6b) an additional resonance was present under the water signal that was resolved in the spectrum recorded at 300 K, and it was assigned to 4-H of Kdo-4*P* (residue **C**), on the basis of the downfield shift of the 4-H and C-4 signals at 4.69 and 69.3 ppm, respectively (Figure 7).

The monosaccharide sequences in OS1 and OS2 were deduced from ROESY (Table 3, Scheme 2) and ^{1}H–^{13}C HMBC NMR experiments. Heptose **D** was linked to O-5 of the Kdo unit, as revealed by a strong NOE between 1-H of **D** and 5-H of **C**. Residues **E** in OS1 and **F** in OS2 were both linked to O-4 of **D**, as both their anomeric proton signals gave a NOE contact with 4-H **D** at δ = 3.99 ppm. Strong NOE contacts between 1-H **H** and both 1-H and 2-H of **E/F**, together with a long-range correlation between 1-H **H**/C-2 **E/F** and the NOE contact 1-H **I**/7-H **H** indicated the sequence α-Glc*p*-(1→7)-α-Hep*p*-(1→2)-α-Hep*p*. Long-range scalar connectivities 1-H **K**/C-3 **D**, 1-H **L**/C-4 **K**, and 1-H **M**/C-4 **L** and the NOE contacts reported in Table 3 allowed the assignment of the sequence of these residues in both OS1 and OS2. Noteworthy was the NOE contact 1-H **N**/6a,b-H **M** and the HMBC correlation 1-H **N**/C-6 **M**, which identified the linkage between the O-chain and the core region.

Conclusion

Two main-core oligosaccharide structures were established for *Halomonas pantelleriensis* bacterium (OS1 and OS2), and they differ in one heptose residue. When present, this sugar was linked to O-6 of the 2-substituted α-L,D-Hep*p*. This was proved by chemical-shift values of residues **E** and **F** and from the fact that 1-H of the 7-substituted α-D,D-Hep*p* gave an intense NOE correlation with both 2-H of the 2-substituted and the 2,6-substituted α-L,D-Hep*p* residues. Consistent with this data were the long-range scalar connectivities and the results of the methylation analysis. It is noteworthy that this investigation allowed us to determine the biological O-chain repeating unit by identifying that Qui*p*NAc was linked to the outer core. This result was

in agreement with the proposed mechanism for the biosynthesis of O-chain heteropolysaccharides for which the chain extension occurs at the reducing terminus with the nascent chain being transferred from its undecaprenyl carrier to the nonreducing terminus of the "new" undecaprenyl-linked O-repeating unit.[16]

Experimental Section

Growth of Bacteria and Isolation of LPS: *Halomonas pantelleriensis* (DSM 9661) was isolated from the sand of the lake Venere on the Pantelleria island (Italy),[8] and it was cultivated as reported previously.[17] Bacterial dried cells (11 g) were extracted by using phenol/chloroform/light petroleum (2:5:8) as described.[11] The product (240 mg) was then incubated in phosphate buffer (pH 8, 30 mL) with RNase and DNase for 2 h at 37 °C and then with Protease K for 1 h at 68 °C to purify the LPS from the nucleic acids. The reaction mixture was then dyalized and lyophilized to yield the crude LPS (200 mg, 1.8% of dried cells). DOC-PAGE was performed as already reported.[11]

Sugar Analysis: Monosaccharides were analyzed as alditol acetates as well as acetylated methyl glycosides. Acetylated alditols were obtained from the LPS (1 mg). Briefly, LPS was treated with 0.5 M MeOH/HCl (0.5 mL, 85 °C, 45 min), and after the usual workup the methanol layer was dried and treated with $NaBD_4$. The reaction mixture was then hydrolyzed with 2 M trifluoroacetic acid (120 °C, 2 h), reduced with $NaBD_4$, and acetylated.

Acetylated methyl glycosides were obtained from the crude LPS (0.5 mg). The LPS was dephosphorylated with 48% aqueous HF (4 °C, 16 h) then dried under vacuum over NaOH. Methanolysis was performed in 0.5 M MeOH/HCl (0.5 mL, 85 °C, 45 min), and the sample was extracted twice with hexane. The methanol layer was concentrated, and the residue was dried and acetylated.

The linkage positions of the monosaccharides were determined by methylation analysis. Briefly, the product obtained from acid hydrolysis of the LPS (1 mg) was reduced with $NaBH_4$. Methylation was performed with CH_3I in DMSO and NaOH (2.5 h). The product was then hydrolyzed with 4 M trifluoroacetic acid (100 °C, 4 h), reduced with $NaBD_4$, and then acetylated.

The absolute configuration of the sugars was determined by gas chromatography of the acetylated (*S*)-2-octyl glycosides.[18]

Acetylated octyl glycosides were analyzed with a Agilent Technologies gas chromatograph 6850A equipped with a mass-selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m × 0.25 mm i.d., flow rate 1 mL min^{-1}, He as carrier gas). Acetylated methyl glycosides and alditol acetates were analyzed accordingly with the following temperature programs: 150 °C for 3 min, 150 °C→240 °C at 3 °C min^{-1} and 150 °C for 3 min, 150 °C→310 °C at 3 °C min^{-1}. For partially methylated alditol acetates the temperature program 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. 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.

Mild Acid Hydrolysis of the LPS: The LPS (50 mg) was hydrolyzed with 1% aqueous CH_3COOH (5 mL, 100 °C, 6 h). The obtained suspension was then centrifuged (10000 *g*, 4 °C, 30 min). The pellet was washed twice with water, and the supernatant layers were combined and lyophilized (polysaccharide, 34 mg). The precipitate (lipid A) was also lyophilized (13 mg). The polysaccharide portion was then fractionated on a column (4.9 × 700 mm) of Sephadex G-50 (superfine, flow rate 36 mL h^{-1}, fraction volume 4 mL) and eluted with water buffered (pH 4.3) with 0.4% (v/v) pyridine and 1% (v/w) sodium acetate.

Deacylation of the LPS: The LPS (60 mg) was first dried with phosphorus anhydride under vacuum and then it was incubated with hydrazine (2 mL, 37 °C, 1.5 h). Cold acetone was then added to precipitate the *O*-deacylated LPS. The pellet was recovered after centrifugation (4 °C, 8000 *g*, 30 min), washed three times with acetone, and finally dissolved in water and lyophilized (35 mg).

The *O*-deacylated LPS was dissolved in 4 M KOH (1.5 mL), kept at 20–22 °C under an atmosphere of nitrogen for 15 min and then 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 CH_2Cl_2. The water phase was recovered and then desalted on a column (1.5 × 800 mm) of Sephadex G-10 (30 mL h^{-1}, fraction volume 1 mL) eluted with 10 mM ammonium hydrogen carbonate. The eluted oligosaccharide mixture was then lyophilized (12 mg).

HPAEC Analysis: Separation of the oligosaccharides mixture obtained after deacylation of the LPS (7 mg) was performed by HPAEC-PAD on a semipreparative column (9 × 250 mm) of Carbopac PA-100 eluted with a gradient of 30–45% 1 M NaOAc/0.1 M NaOH at 2 mL min^{-1} over 90 min to yield fraction A (0.5 mg), B (1 mg), and C (3 mg). Fractions were desalted on a column (1.5 × 800 mm) of Sephadex G-10 (30 mL h^{-1}, fraction volume 1 mL, eluant 10 mM NH_4HCO_3).

NMR Spectroscopy: For structural assignments, 1D and 2D ^1H and ^{13}C NMR spectra were recorded at 313 K with a Varian Inova 500 spectrometer. Chemical shifts were measured in D_2O by using acetone as an internal standard (δ = 2.225 and 31.45 ppm for CH_3 protons and carbon, respectively). All 2D homo- and heteronuclear experiments (COSY, TOCSY, ROESY, HSQC-DEPT, HMBC, HSQC-TOCSY, and 2D *F2*-coupled HSQC) were performed by using standard pulse sequences available in the Varian software.

Mass Spectrometry Analysis: FTMS was performed in the negative ion mode with an APEX II-Instrument (Bruker Daltonics, Billerica, MA, USA) equipped with an actively shielded 7-Tesla magnet and an Apollo II ESI ion source. Mass spectra were acquired by using standard experimental sequences as provided by the manufacturer. Samples (≈ 10 ng μL^{-1}) were dissolved in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine (TEA). The latter was added step-by-step to exclude that pH 9 was exceeded, as a higher concentration of TEA might otherwise cause the cleavage of O-linked fatty-acid residues. The samples were sprayed at a flow rate of 2 μL min^{-1}. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 180 °C. The spectra, which showed several charge states for each component, were charge-deconvoluted by using the XMASS-6.1 software, and mass numbers given refer to the monoisotopic molecular masses. Each spectrum is an average of at least 20 transients composed of 1-M data points.

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[1] A. Oren, *Halophilic Microorganisms and Their Environments*, Kluwer Academic Publishers, Dordrecht, **2002**.

[2] S. Mikkat, E. A. Galinski, G. Berg, A. Minkwitz, A. Schoor, *Syst. Appl. Microbiol.* **2000**, *23*, 31–40.
[3] N. J. Russell, *J. Bioenerg. Biomembr.* **1989**, *21*, 93–113.
[4] I. Romano, B. Nicolaus, L. Lama, D. Travaso, G. Caracciolo, A. Gambacorta, *Syst. Appl. Microbiol.* **2001**, *24*, 342–352.
[5] R. H. Vreeland, R. Anderson, R. G. Murray, *J. Bacteriol.* **1984**, *160*, 879–883.
[6] R. L. Adams, N. J. Russell, *Can. J. Microbiol.* **1992**, *38*, 823–827.
[7] M. S. da Costa, H. Santos, E. A. Galinski, *Adv. Biochem. Eng. Biotechnol.* **1998**, *61*, 117–153.
[8] I. Romano, B. Nicolaus, L. Lama, M. C. Manca, A. Gambacorta, *Syst. Appl. Microbiol.* **1996**, *19*, 326–333.
[9] C. Alexander, E. T. Rietschel, *J. Endotoxin. Res.* **2001**, *7*, 167–202.
[10] M. Caroff, D. Karibian, *Carbohydr. Res.* **2003**, *338*, 2431–2447.
[11] M. M. Corsaro, A. Gambacorta, A. Iadonisi, R. Lanzetta, T. Naldi, B. Nicolaus, I. Romano, S. Ummarino, M. Parrilli, *Eur. J. Org. Chem.* **2006**, 1801–1808.
[12] C. Galanos, O. Lüderitz, O. Westphal, *Eur. J. Biochem.* **1969**, *9*, 245–249.
[13] J. Banoub, A. El Aneed, A. Cohen, P. Martin, *Eur. J. Mass Spectrom.* **2004**, *10*, 715–730.
[14] S. Kondo, Y. Haishima, K. Hisatsune, *Carbohydr. Res.* **1992**, *231*, 55–64.
[15] J. R. Brisson, E. Crawford, D. Uhrín, N. H. Khieu, M. B. Perry, W. B. Severn, J. C. Richards, *Can. J. Chem.* **2002**, *80*, 949–963.
[16] C. R. H. Raetz, C. Whitfield, *Annu. Rev. Biochem.* **2002**, *71*, 635–700.
[17] M. M. Corsaro, A. Gambacorta, R. Lanzetta, B. Nicolaus, G. Pieretti, I. Romano, M. Parrilli, *Carbohydr. Res.* **2007**, *342*, 1271–1274.
[18] K. Leontein, B. Lindberg, J. Lönngren, *Carbohydr. Res.* **1978**, *62*, 359–362.

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