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Structural Determination of the O-Chain Polysaccharide from the Lipopolysaccharide of the Haloalkaliphilic *Halomonas pantelleriensis* Bacterium

M. Michela Corsaro,*[a] Agata Gambacorta,[b] Alfonso Iadonisi,[a] Rosa Lanzetta,[a] Teresa Naldi,[a] Barbara Nicolaus,[b] Ida Romano,[b] Salvatore Ummarino,[a] and Michelangelo Parrilli[a]

Dedicated to the memory of Professor Gaspare Barone

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The structural determination of the O-chain repeating unit of the lipopolysaccharide from the haloalkaliphilic *Halomonas pantelleriensis* bacterium is described. The structure of the repeating unit was suggested on the basis of chemical analysis and NMR and MS data. The 4-*O*-[(*S*)-1-carboxyethyl]-D-GlcA residue has been found for the first time in a lipopolysaccharide, being previously only found in capsular polysaccharides. A comparison of the O-chain structures of *Halomonas magadiensis* and *H. pantelleriensis* is also reported. The results show that both bacteria present lipopolysaccharides containing a high number of carboxylate groups whose salification might determine a protective buffer effect on bacterium against extreme life conditions.

Introduction

Extremophiles are microorganisms that live in extreme environments and develop a strong ability to survive a wide range of environmental stresses. They include, for example, thermophiles, halophiles, alkaliphiles and psychrophiles and are currently found between the domains of *Bacteria* and *Archaea*. *Halomonas pantelleriensis* is a Gram-negative haloalkaliphilic rod belonging to the *Halomonadaceae* family. It can be isolated from Venus' Mirror Lake on Pantelleria island (Sicily, Italy) and is classified as an haloalkaliphilic phenotype.[1] The family *Halomonadaceae* belongs to the γ-subclass of *Proteobacteria*. It was proposed by Franzmann et al.,[2] in accord with the 16S rDNA cataloguing technique, to accommodate the moderately halophilic and marine bacteria of the genera *Halomonas* and *Deleya*. Today this family comprises several genera, including *Halomonas*. Polyphasic approaches were used to determine the natural taxonomic position of species belonging to the genus *Halomonas*, including 16S rRNA and 23S rRNA genes. Several conclusions can be extracted from these phylogenetic analyses. First, the genus is not monophyletic and two phylogenetic groups are distinguishable.[3] Secondly, the members of group 1 shared relatively low values of the 16S rRNA sequence similar to the members of group 2. These results are in agreement with the phenotypic heterogeneity reported for species of the genus *Halomonas*. Finally, there are features that clearly support the current classification of species of the genus *Halomonas*. The range of C + G content of DNA changes from 52 to 68 mol-%, too wide (more than 10%) for the accepted definition of species.[4]

Haloalkaliphilic strains show optimal growth in high salt concentrations (3–15% w/v), displaying optimal growth between pH 9 and 10. To survive in these harsh conditions extremophiles have developed several strategies that allow the microorganisms to thrive. These adaptive strategies involve the outer membrane of the bacteria, a barrier that regulates exchanges with the environment. In such a context, lipopolysaccharides (LPSs), which are among the major constituents of the Gram-negative outer membrane, are thought to contribute to the restrictive membrane permeability properties. In addition, since there is only a little information on the structure–function of LPSs from extremophiles, we recently began a study of LPSs from these microorganisms and the O-specific chain structure of the two components from the lipopolysaccharide fraction of *Halomonas magadii* 21 M1 has been investigated.[5,6] LPSs are complex macromolecules made up of three covalently

[a] Department of Organic Chemistry and Biochemistry, University Federico II, Complesso Universitario Monte S. Angelo, Via Cintia 4, 80126 Napoli, Italy
Fax: +39-081674149
E-mail: corsaro@unina.it
[b] Institute of Biomolecular Chemistry ICB-CNR, Via Campi Flegrei 34, 80078 Pozzuoli, Italy

linked regions, genetically and structurally distinct: the O-specific polysaccharide (O-chain, O-antigen), the core oligosaccharide and a glycolipid portion, termed lipid A, which anchors the molecule to the outer layer of the bacterial outer membrane. Depending on the carbohydrate size, two kinds of lipopolysaccharides exist: smooth and rough types (S-LPS and R-LPS). Both structures are built up of lipid A covalently bonded to the core, but the smooth-type LPS core region is in turn covalently linked to the O-specific chain. Both forms are present in wild-type Gram-negative bacteria, the core–lipid A moiety being the minimum structural unit required for bacterial life.

In this paper we report the O-chain polysaccharide structure from the LPS of *Halomonas pantelleriensis*. The general features of *H. magadiensis* and *H. pantelleriensis* are compared as well as the properties of the two LPSs.

Results

Isolation and Purification of the LPS Fraction

Halomonas pantelleriensis (*Hp*) cells were extracted by the phenol/water method to obtain crude LPS extract from the aqueous phase. In order to eliminate nucleic acids this sample was treated with RNase since the presence of only RNA was established by both GC–MS analysis of the sugars (ribose content) and by agarose gel electrophoresis. As this treatment was not sufficient to completely eliminate nucleic acids the LPS fraction was submitted to hydrophobic chromatography on a Butyl Sepharose column (Pharmacia). A fraction eluted in the absence of 2-propanol showed, by GC–MS analysis of sugars, the sample to still contain ribose while containing a very small amount of LPS. Another sample, eluted with 2-propanol (40%), was shown to be completely devoid of ribose and therefore the structural analysis was performed on this fraction.

DOC-PAGE analysis of this fraction (Figure 1, lane A) showed the typical ladder of LPS by comparison with a standard from *Escherichia coli* (lane B). Moreover the electrochromatographic profile suggested that the LPS of *Hp* bacterium has a semi-rough nature.

Figure 1. DOC-PAGE analysis of the LPS fraction from *Halomonas pantelleriensis* (lane A) and *Escherichia coli* serotype 055:B5 (lane B).

Compositional Analysis

The fatty-acid composition of LPS was determined by GC–MS analysis of the lipid methyl esters after treatment with 1 M HCl/CH_3OH and extraction with hexane. The GC column profile showed the presence of C12:0 and C12:0 (3-OH). Sugar analysis by GC–MS of acetylated methyl glycosides indicated, by comparison with standards, the presence of QuiNAc, Glc, GlcNAc, two different heptoses and traces of GlcA and Kdo. Moreover two signals that give the same EI-MS spectra were present. The fragmentation patterns of these spectra were very similar to those of uronic acids, but it was not easily interpretable. Accordingly, the LPS sample was shown to be highly positive towards the *m*-diphenyl test (uronic acid assay).

In order to identify this sugar the LPS sample was first treated with 1 M HCl/CH_3OH, then deuterium-carboxy reduced and finally totally hydrolysed. After carbonyl reduction the alditol mixture was acetylated and analysed by GC–MS. The GC elution profile (Figure 2, a) confirmed the presence of QuiNAc (a), Glc (c) and GlcNAc (d) and indicated the presence of two heptose units (f and g). All these sugars were identified from both their EI-MS spectra and their GC column retention times by comparison with those of authentic samples. The EI-MS spectrum of Glc showed the typical isotopic pattern indicating the presence of Glc and GlcA in the native LPS. In addition two more peaks were present. The first, at a retention time of 37.2 min (e), was attributed to a 2-amino-2-deoxyhexose on the basis of its fragmentation pattern. In particular, this was identified, from its retention time, as GalNAc, which is clearly derived from the galactosaminuronic acid (GalNAcA). The second (h) gave the EI-MS spectrum reported in Figure 2 (b). From the fragment analysis and by comparison with pertinent EI-MS spectra[7] the species was identified as a 4-*O*-hydroxypropylhexose, which is derived from a 4-*O*-(1-carboxyethyl)uronic acid.

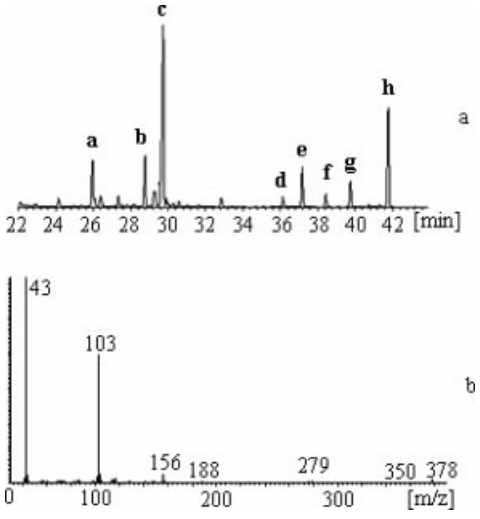


Figure 2. GC–MS profile of alditol acetate derivatives obtained from the *Halomonas pantelleriensis* O-chain (a) and the EI-MS spectrum of peak h (b). Peaks were identified by their mass spectra and by comparison of retention times with standards. Peak a, quinovosamine; peak b, inositol as internal standard; peak c, glucose and glucuronic acid; peak d, glucosamine; peak e, galactosaminuronic acid; peak f, heptose; peak g, heptose; peak h, 4-*O*-(1-carboxyethyl)uronic acid.

Isolation and Characterization of the O-Chain

Mild hydrolysis of the LPS sample revealed, as expected for a semi-rough bacterium, a very low content of the O-chain and a much more abundant core fraction. The O-chain glycosyl composition confirmed the presence of all the sugars described above, including the heptoses, which are characteristic of the core fraction, suggesting the presence of only a few repeat units in the O-chain. In accord with this result is the negative-ion MALDI reflectron spectrum reported in Figure 3 which shows signals of only four repeating units. In this spectrum the mass difference between the signals at *m*/*z* = 845.23, 1673.23, 2501.14, 3329.0 and 4156.81 was always 828 a.m.u. and suggests a repeating unit made up of four sugars. This difference corresponds to the sum of the following contributions: GlcA (176 a.m.u.), GalNAcA (217 a.m.u.), QuiNAc (187 a.m.u.) and 4-*O*-(1-carboxyethyl)uronic acid (248 a.m.u.).

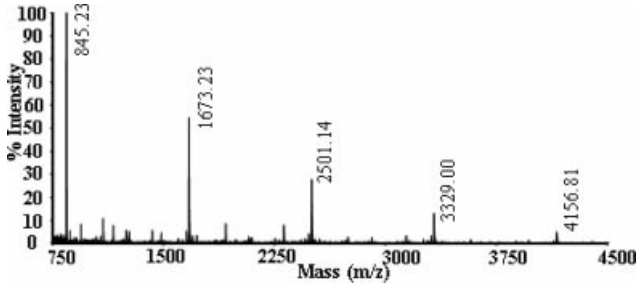


Figure 3. Negative-ion MALDI-TOF spectrum of the O-chain from *Halomonas pantelleriensis*.

Methylation analysis indicated the presence of 4-linked GlcA, 3-linked QuiNAc and 2-linked 4-*O*-(1-carboxyethyl)-uronic acid, in addition to the signals of the core sugars. No traces of GalNAcA could be found.

NMR Spectroscopy of the O-Chain Fraction

Complete assignment of all the proton and carbon signals (Table 1) of the O-chain repeating unit was achieved by 2D (COSY, TOCSY, HSQC, HMBC and NOESY) and 1D (HOHAHA and selective ROE) NMR experiments. The low-field region of the ^{1}H NMR spectrum (Figure 4, a) exhibited six signals of which only four showed connectivities to anomeric carbon atoms. In particular, the anomeric proton signals at δ = 5.14, 4.71, 4.66 and 4.53 ppm, assigned to residues **A**–**D**, respectively, were identified by their correlation with the carbon signals at δ = 98.0, 100.8, 102.2 and 103.2 ppm, respectively (Figure 4, b). The anomeric configurations were determined on the basis of the values of the $^3J_{H1,H2}$ coupling constants and/or the chemical shifts of the anomeric protons.

Starting with the signals of these protons each residue was identified by COSY and TOCSY experiments. The signal at δ = 5.14 ppm (d, 3.1 Hz, H1) of residue **A** is correlated to the signal at δ = 4.15 ppm (dd, 3.1 and 12.0 Hz, H2), which, in turn, is correlated to the carbon signal at δ = 50.1 ppm. It was then deduced that **A** is a 2-amino-2-deoxy residue. The H2 signal is correlated to the signal at δ = 4.01 ppm (br. d, 12.0 Hz, H3), and this, in turn, is correlated to the broad singlet at δ = 4.58 ppm (H4) and this last to the signal at δ = 5.07 ppm (H5), which also appears as a singlet. Taking into account the values of both the chemical shifts and the coupling constants, residue **A** was easily assigned to the α-Gal*p*NAcA unit. The identification of residue **C** as that of β-Qui*p*NAc was obtained starting from both the anomeric (δ = 4.66 ppm; d, 8 Hz) and the 6-methyl signals (δ = 1.29; d, 5.8 Hz). The *gluco* configuration was deduced from the large value (8 Hz) of the ^3J(H,H) coupling constants for both H2 (δ = 3.87 ppm) and H4 (δ = 3.21 ppm) in the ^{1}H NMR spectrum (Figure 4, a). The anomeric signals of **B** (δ = 4.71 ppm; d, 6.9 Hz) and **D** (δ = 4.53 ppm; d, 6.8 Hz) were assigned to two β-GlcA residues on the basis of the proton multiplicities obtained by HOHAHA experiments. In particular, the appearance of the H5 atom as doublets (10.0 Hz) suggests the presence of a carboxy group at C6. This was confirmed by the heterocorrelation of H5 with carboxy signals in the HMBC spectrum. The presence of the 1-carboxyethyl fragment was evidenced by the doublet methyl signal at δ = 1.45 ppm (7.0 Hz) and the quadruplet signal at δ = 4.36 ppm (7.0 Hz) in the ^{1}H NMR spectrum and by their correlation to the carboxy signal at δ = 178.3 ppm in the HMBC spectrum. The location of the 1-carboxyethyl substituent was indicated by the dipolar and the hetero long-range scalar couplings that its methine proton showed with H4 and H3 and with C4 of residue **B**, respectively.

In the high-field region of the ^{1}H NMR spectrum the two methyl singlet signals at δ = 2.03 and 1.98 ppm, correlated to the two carbon signals at δ = 23.6 and 23.5 ppm in the HSQC spectrum and to 175.0 and 174.5 ppm in the HMBC spectrum, respectively, were assigned to the acetamido groups of QuiNAc and GalNAcA.

The sequence of the four residues and the attachment points of the glycosidic linkages of the repeating oligosaccharide unit **1** (Scheme 1) were deduced from HMBC experiments which indicated the following correlation: H1/C1 of **D** with C4/H4 of **A**, C1 of **A** with H3 of **C**, H1 of **C** with C2 of **B** and H1/C1 of **B** with C4/H4 of **D**. The low-field ^{13}C chemical shifts of the glycosylated carbon atoms supported the identification of the positions involved in the glycosidic linkages.

Interresidue NOE contacts (Figure 5 and arrows in Scheme 1) were observed between H1 of **D** and H4 of **A**, H1 of **A** and H3 of **C**, H1 of **C** and H2 of **B**, H1 of **B** and H4 of **D**, thus confirming the monosaccharide sequence of **1**.

The intraresidue NOEs of H1 with H3 and H5 supported the β configuration for the **C**, **B** and **D** residues whereas that between H1 and H2 of **A** supported the α configuration for this last residue.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ) measured at pD 2 (uncorrected) and 16 °C in D_2O at 400 MHz.

Sugar residue	Atom		Atom		Ref.[c]
4-α-D-Gal*p*NAcA[a]	**H1**	5.14	**C1**	98.0	
A	**H2**	4.15	**C2**	50.1	
	H3	4.01	**C3**	66.9	
	H4	4.58	**C4**	78.8	
	H5	5.07	**C5**	70.4	
			C6	172.8	
3-β-L-Qui*p*NAc[b]	**H1**	4.66	**C1**	102.2	
C	**H2**	3.87	**C2**	56.3	
	H3	3.64	**C3**	78.5	
	H4	3.21	**C4**	74.1	
	H5	3.42	**C5**	72.6	
	H6	1.29	**C6**	17.8	
4-β-D-Glc*p*A	**H1**	4.53	**C1**	103.2	
D	**H2**	3.42	**C2**	72.6	
	H3	3.67	**C3**	73.1	
	H4	3.82	**C4**	79.8	
	H5	4.09	**C5**	73.8	
			C6	170.6	
2-β-[4-O-((*S*)-1-carboxyethyl)]-D-Glc*p*A	**H1**	4.71	**C1**	100.8	103.6
B	**H2**	3.51	**C2**	80.2	74.0
	H3	3.62	**C3**	75.3	76.2
	H4	3.61	**C4**	79.7	81.4
	H5	4.02	**C5**	73.2	75.1
			C6	173.0	175.0
$CH_3CHOHCOOH$			**C1**	178.3	174.6
	H2	4.36	**C2**	78.5	77.8
	H3	1.45	**C3**	19.4	19.1
Methyl 4-*O*-[(*S*)-(1-hydroxyisopropyl)-β-D-Glc*p*	**H1**	4.80	**C1**	100.7	
	H2	3.57	**C2**	72.8	
	H3	3.69	**C3**	74.6	
	H4	3.45	**C4**	77.8	
	H5	3.65	**C5**	72.5	
	H6	3.84/3.88	**C6**	61.9	
$CH_3CHOHCH_2OH$	**H1/1'**	3.53/3.62	**C1**	66.8	
	H2	3.89	**C1**	79.4	
	H3	1.19	**C3**	17.7	

[a] Chemical shifts for the *N*-acetyl groups: 2.03 (CH_3), 23.6 (CH_3) and 175.0 ppm (*C*O). [b] Chemical shifts for the *N*-acetyl groups: 1.98 (CH_3), 23.5 (CH_3) and 174.5 ppm (*C*O). [c] Values from ref.[7] and corrected relative to internal acetone at δ = 31.45 ppm.

By means of the GC octyl glycosides method the absolute configuration of the Glc*p*A and Gal*p*NAcA residues was defined as D, whereas that of Qui*p*NAc was determined to be L from the specific rotation of its methyl α-glycoside. As for the configurations of the 4-*O*-(1-carboxyethyl)-Glc*p*A unit, the lack of any reference compound and the very small amount available prevented us from using either of the above approaches. The D configuration for the glucuronic acid was suggested on the basis of the expected differences in the ^{13}C chemical shifts of the two diastereoisomeric disaccharide fragments L-QuiNAc-(1→2)-4-*O*-(1-carboxyethyl)-D-Glc*p*A and L-QuiNAc-(1→2)-4-*O*-(1-carboxyethyl)-L-Glc*p*A.[8,9] The value of –0.9 ppm, obtained from the difference between the chemical shift of the C3 atom of

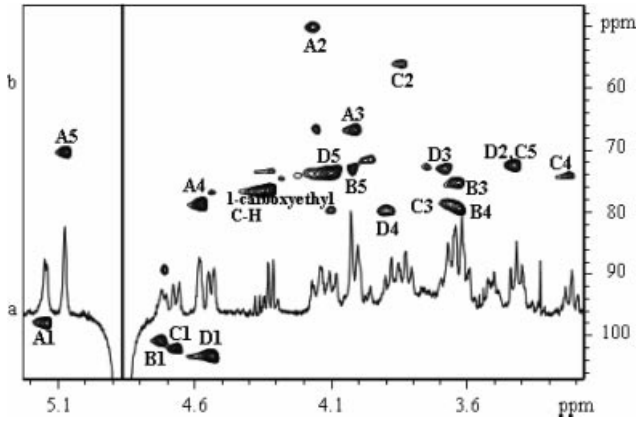


Figure 4. Partial ^{1}H NMR (a) and HSQC spectra (b) of the O-chain from *Halomonas pantelleriensis*. The spectra were recorded at 400 MHz, in D_2O, at pD 2 (uncorrected), at 16 °C. The letters refer to the carbohydrate spin systems as described in the text and shown in Table 1. The numerals next to the letters indicate the protons/carbons in the respective residues.

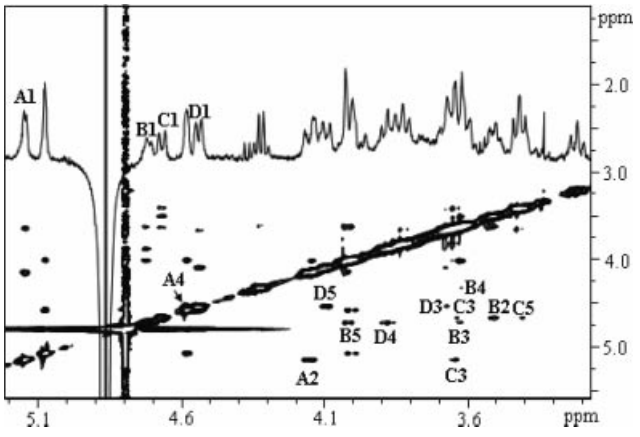
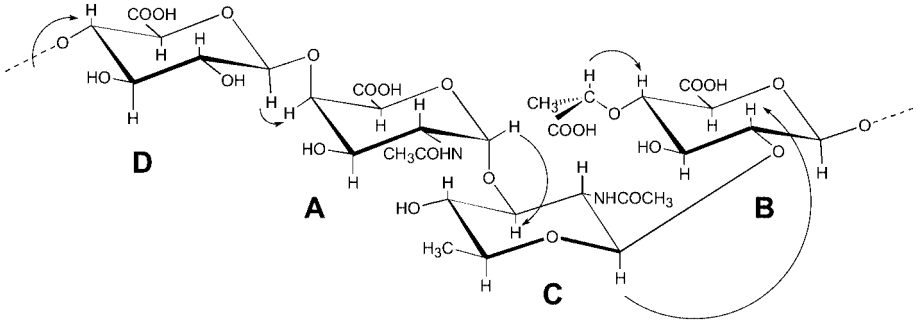


Figure 5. Partial NOESY and ^{1}H NMR spectra of the O-chain from *Halomonas pantelleriensis*. The spectra were recorded at 400 MHz, in D_2O, at pD 2 (uncorrected), at 16 °C. The letters refer to the carbohydrate spin systems as described in the text and shown in Table 1. The numerals next to the letters indicate the protons in the respective residues.

the 4-*O*-(1-carboxyethyl)-Glc*p*A in the *Hp* O-chain and the same carbon atom of the same monosaccharide reported as the terminal unit in ref.[7], both given in Table 1, was particularly diagnostic. This value is closer to the value of –1.3 ppm found for the LD dyad than the value of 0 ppm expected for the LL dyad. In order to determine the absolute configuration of the 1-carboxyethyl substituent the diastereoisomers of methyl 4-*O*-(1-hydroxyisopropyl)-D-glucopyranoside (2*R* and 2*S*) were synthesized. These products were obtained form the known methyl 2,3,6-tri-*O*-benzyl-glucopyranoside which was initially treated with (*S*)-2-chloropropionic or (*R*)-2-bromopropionic acid in the presence of sodium hydride. The resulting products were then debenzylated by transfer hydrogenolysis. The details of these procedures will be reported elsewhere. The deprotected 4-*O*-(1-carboxyethyl) derivatives were then submitted to the standard sequence of methanolysis, carboxy-group reduction and acetylation. These compounds were compared, by GC–MS, with the corresponding product obtained from the O-chain after the analogous sequence of methanolysis, reduction and acetylation. The (*S*) configuration for the 1-carboxyethyl substituent of the natural product was deduced by comparison of the intensities of its mass spectrum fragments with those of the corresponding synthesized diastereoisomer (Figure 6). These results definitively confirmed that the acidic sugar is 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid and that it is identical to that found in *Klebsiella* K22 and K37 capsular polysaccharides.[7,10]

Discussion and Conclusions

Halomonas pantelleriensis was enclosed by Duckworth et al.[11] in the same group (group 2 of the genus *Halomonas*) as *H. magadii* 21 MI (now *magadiensis*). Arahal et al.[3] demonstrated that *H. pantelleriensis* did not fall clearly into either of the two groups mentioned above and did not form a group itself. Recently, Mata et al.[12] grouped, on the basis of its phenotypic characteristics, the genus *Halomonas* into three phenons. Although *H. magadiensis* and *H. pantelleriensis* share many common features they fall into two different phenons; while *H. magadiensis* is in the phenon B,



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Scheme 1.

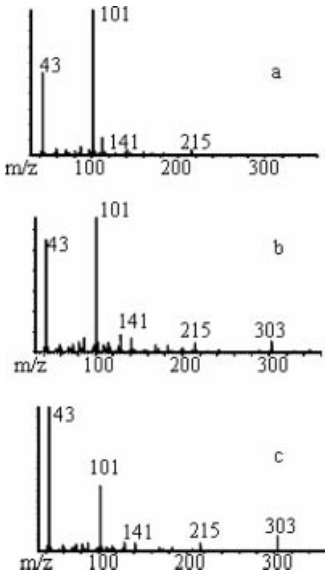

Figure 6. EI-MS spectra of acetylated methyl 4-*O*-[(*R*)-1-hydroxyisopropyl]-D-glucopyranoside (a), methyl 4-*O*-[(*S*)-1-hydroxyisopropyl]-D-glucopyranoside (b) and methyl 4-*O*-(1-hydroxyisopropyl)-D-glucopyranoside isolated from *H. pantelleriensis* (c).

H. pantelleriensis is in phenon A. *H. magadiensis* and *H. pantelleriensis* showed little difference with regard the temperature range of growth and their halophilicity. In fact, although *H. magadiensis* grew in the presence of up to 20% salt (*H. pantelleriensis* is less halophilic, 15% NaCl at a maximum) it did not grow with a NaCl concentration of less than 1.2%. Both produced poly-β-hydroxybutyrate (PHB) and had similar lipid patterns at the level of the major component, phosphatidyl glycerol, diphosphatidyl glycerol and phosphoethanolamine. Phosphatidyl glycerol phosphate and a small amount of an unknown glycolipid were also present in *H. magadiensis*, but absent in *H. pantelleriensis*. The latter also accumulated osmoprotectants, whose composition depended on the growth conditions.[13] No reports of these molecules in *H. magadiensis* have been made.

The LPS fraction was obtained from the aqueous layer of a phenol/water extraction of *Hp* cells. Purification of the LPS fraction required nuclease digestion of the extract followed by hydrophobic chromatography, which gave a very pure LPS sample. In order to obtain a complete hydrolysis of the LPS it was necessary to use SDS; the O-chain was isolated after gel chromatography. The repeating unit of the O-chain of *Hp* was established from chemical and methylation analyses and from NMR spectroscopic and mass spectrometric investigations and was found to be that reported in Scheme 1. This structure contains three acidic sugars together with a 1-carboxyethyl group as a substituent. The 4-*O*-[(*S*)-1-carboxyethyl]-D-glucuronic acid is an unusual sugar hitherto found only in capsular polysaccharides from *Klebsiella* serotypes. Therefore, to our knowledge, this is the first time this acidic sugar has been found in lipopolysaccharide structures.

Other O-chain structures from haloalkaliphilic bacteria have been published.[5,6] In particular, the O-chain repeating unit [→4)-α-L-Gul*p*NAcA-(1→6)-α-D-Glc*p*-(1→4)-α-L-Gul*p*NAcA-(1→] containing acidic sugars was found for *Halomonas magadiensis*. It is tempting to speculate that salification of the carboxylate groups might determine a protective buffer effect on both *Halomonas* species against extreme life conditions.

Finally, note that nevertheless LPS fractions were obtained from both bacteria from the aqueous phases of phenol/water extraction *H. magadiensis* gave a smooth LPS, while *Hp* produced a semi-rough LPS.

Experimental Section

Abbreviations: COSY, correlation spectroscopy; DOC-PAGE, deoxycholate-polyacrylamide gel electrophoresis; EI-MS, electron ionisation mass spectrometry; Gal*p*NAcA, 2-acetamido-2-deoxygalactopyranuronic acid; GC–MS, gas chromatography–mass spectrometry; Glc, glucose; Glc*p*A, glucopyranuronic acid; GlcNAc, 2-acetamido-2-deoxyglucose; HMBC, heteronuclear multiple bond correlation; HOHAHA, homonuclear Hartmann–Hahn; HSQC, heteronuclear single quantum coherence; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser-desorption ionisation time of flight; NOESY, nuclear Overhauser enhancement spectroscopy; Qui*p*NAc, 2-acetamido-2,6-dideoxyglucopyranose; ROE, rotating-frame nuclear Overhauser enhancement; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; TOCSY, total correlation spectroscopy.

Bacterial Strain and Growth Conditions: *Halomonas pantelleriensis* was isolated from the hard sand of the lake of Venere on Pantelleria island (Italy) as previously reported.[13] The bacterium was aerobically grown at 35 °C on the following enrichment media (g/L): yeast extract, 10; NaCl, 100; Na_3 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; distilled water, pH 9.0 (Na_2CO_3 and NaCl were autoclaved separately). Cultures of *H. pantelleriensis* were routinely grown in 2 L flasks with 500 mL of medium in a rotary shaker incubator (Innova 4300) whilst stirring at 75 rpm. The inoculum was 1% of the total volume. Cell growth was monitored by measuring the turbidity at 540 nm. The cultures were grown until the late exponential phase, harvested by centrifugation (*g* 8000) and washed once with a saline solution.

LPS Extraction and Purification: Dry cells (16 g) were suspended in ultrapure Milli-Q water (90 mL) and extracted with phenol according to the phenol/water method.[14] The resulting aqueous phase was dialysed (cut-off 3500) for 4 days. Contents of the tube were lyophilised (700 mg); the residue was dissolved in ultrapure Milli-Q water and treated with RNase (Sigma, 36 mg) for 3 h at 37 °C. After this time the sample was dialysed as above and lyophilised to yield "crude" LPS (593 mg, yield 3.7% of dried cells). Then,150 mg of this fraction were purified by hydrophobic chromatography on a Butyl Sepharose column (Pharmacia). The column (47 × 2.5 cm) was equilibrated with 200 mM NaOAc (pH 4.7) eluting initially with the same buffer (160 mL) and then with a linear gradient NaOAc/*n*-PrOH (50:50 v/v, 450 mL).[15] Fractions (3 mL) were collected and monitored for carbohydrates (phenol/sulfuric acid test: A at 490 nm) and nucleic acids (A at 260 nm). On the basis of the chromatographic profile the eluted fractions were pooled, dialysed and freeze-dried. Fractions A and B of 54 and 52 mg, respectively, were obtained. The analysis was carried out on fraction B because it did not contain nucleic acids, as shown by the glycosyl analysis and the UV test.

Polyacrylamide Gel Electrophoresis (DOC-PAGE): PAGE was performed by using the system of Laemmli and Favre[16] as modified by Komuro and Galanos[17] with deoxycholate as detergent. The separation gel contained final concentrations of 14% acrylamide, 0.5% sodium deoxycholate and 375 mM Tris/HCl (pH 8.8); the stacking gel contained 4% acrylamide, 0.5% sodium deoxycholate and 125 mM Tris/HCl (pH 6.8). LPS samples were prepared at a concentration of 0.05% in the sample buffer [0.25% deoxycholate and 175 mM Tris/HCl (pH 6.8), 20% glycerol]. Bromophenol blue (0.003% in sample buffer) was used as the tracking dye. All the concentrations were expressed as a percentage of mass/vol. The electrode buffer was composed of sodium deoxycholate (2.5 g/L), glycine (14.4 g/L) and Tris (3.0 g/L). Electrophoresis was performed at a constant current of 30 mA. Gels were fixed in an aqueous solution of 40% ethanol and 5% acetic acid. LPS bands were visualised by silver staining as described previously.[18]

Hydrolysis of LPS: A sample (48 mg) of the LPS fraction was hydrolysed with 1% AcOH containing 0.1% of SDS at 100 °C for 4 h. After cooling the sample was centrifuged (10000*g*) for 20 min. The supernatant (25 mg) was applied to a Sephacryl S-300 HR column (Pharmacia, 109 × 1.5 cm, flow rate 14 mL/h, volume fraction 2.5 mL) and eluted with 50 mM NH_4HCO_3 buffer to obtain the O-chain fraction (5 mg) and a core fraction (16 mg).

Glycosyl Analysis: A sample of LPS (1 mg) was dried under vacuum over P_2O_5 for 16 h and then subjected to methanolysis by adding 1 M HCl/MeOH (1 mL) for 20 h at 80 °C. The methyl glycosides obtained were acetylated with pyridine (200 μL) and acetic anhydride (100 μL) for 30 min at 100 °C and analysed by GC–MS. Another sample of LPS (1 mg) was subjected to methanolysis as above. The sample was evaporated under a gentle stream of air and treated with Super-deuteride (lithium triethylborodeuteride, Aldrich, 300 μL) for 16 h at 20 °C. After destroying the Super-deuteride by adding some drops of acetic acid the sample was evaporated and hydrolysed with 2 M TFA at 120 °C for 2 h, reduced with $NaBD_4$, acetylated and analysed by GC–MS. The LPS fraction was also tested for uronic acid content by using the *m*-diphenyl test.[19] Briefly: to a sample (0.2 mL) containing the LPS fraction (100 μg) 0.0125 M sodium tetraborate in concentrate sulfuric acid (1.2 mL) was added. The sample was refrigerated in crushed ice, shaken in a Vortex mixer and heated in a water bath at 100 °C for 5 min. After cooling in a water–ice bath, the *m*-hydroxydiphenyl reagent (20 μL) was added and the mixture was shaken. The absorbance was measured after 5 min at 520 nm.

GC–MS Analyses: All the analyses were performed on a Hewlett–Packard 5890 instrument equipped with an RTX-5 capillary column (Restek, 30 m × 0.25 mm i.d., flow rate 1 mL/min, helium as carrier gas). Analysis of acetylated methyl glycosides was performed with the following temperature program: 150 °C for 5 min, 150→250 °C at 3 °C/min, 250 °C for 3 min. Analysis of the alditol acetates was performed using the following temperature program: 150 °C for 5 min, 150→330 °C at 3 °C/min. For partially methylated alditol acetates the temperature program was: 90 °C for 1 min, 90→140 °C at 25 °C/min, 140→200 °C at 5 °C/min, 200→280 °C at 10 °C/min, 280 °C for 10 min. Analysis of the acetylated octyl glycosides was performed as follows: 150 °C for 5 min, 150→240 °C at 6 °C/min, 240 °C for 5 min.

NMR Spectroscopy: NMR spectra were recorded with a Bruker DRX400 Avance spectrometer using a 5 mm multinuclear inverse Z-grad probe. ^{13}C and ^{1}H chemical shifts were measured in D_2O using acetone (δ = 2.222 and 31.45 ppm for ^{1}H and ^{13}C NMR, respectively) as the internal standard. Two-dimensional homo- and heteronuclear experiments (COSY, HMBC, HSQC, NOESY, TOCSY) and one-dimensional homonuclear experiments (HOHAHA and selective ROE) were performed using standard pulse sequences available in the Bruker software.

Mass Spectrometry: The MALDI-TOF mass spectrum was obtained with the Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF Optics. Calibration was carried out in positive mode on a peptide (Glu-fibrinopeptide) and also used in negative mode. The energy of the laser was set to 4900 in MS mode which corresponds to 20 μJ. 2,5-Dihydroxybenzoic acid at 25 mg/mL in 20% aqueous acetonitrile was used as the matrix. Spectra were acquired in negative mode.

Determination of the Absolute Configurations of Monosaccharides: In order to determine the absolute configuration of glucuronic acid and galactosaminuronic acid a sample of LPS (2 mg) was treated with 1 M HCl/MeOH (500 μL) for 20 h at 80 °C. After neutralization the sample was evaporated and reduced with Super-hydride (lithium triethylborohydride, Aldrich, 500 μL) as above. After the usual work up the sample was hydrolysed with a mixture of 2 M H_2SO_4/AcOH and derivatised as acetylated octyl glycosides as previously described.[20] The mixture was analysed by GC–MS. The absolute configuration of quinovosamine was established by measuring the specific rotation of its methyl α-glycoside. Briefly a sample of the O-chain (4 mg) was treated with 1 M HCl/MeOH (1 mL) as above, neutralised and *N*-acetylated with 50 μL of acetic anhydride for 2 h at 20 °C. After evaporation of the solvent the sample was treated with Super-hydride (500 μL) for 20 h at 25 °C. The mixture was then purified by HPLC using a Nucleosil C18 Nautilus column (Macherey–Nagel) and a 9:1 mixture of ultrapure water and methanol as eluent. Methyl 2-acetamido-2,6-dideoxy-α-glucopyranoside was isolated and its specific rotation was measured: $[a]_D$ = –102 (*c* = 0.1, H_2O).[21] Methyl 4-*O*-(1-hydroxyisopropyl)-β-glucopyranoside was also isolated, identified by NMR spectroscopy (see Table 1), acetylated and injected into the GC–MS.

Methylation Analysis: An O-chain sample (1 mg) was methylated by a modification of the Hakomori procedure[22,23] and worked up as follows: water and $CHCl_3$ was added to the crude reaction product. After centrifugation at 3000 rpm for 3 min the organic layer was collected and the water was extracted two more times with $CHCl_3$. All the organic layers were pooled together and the solvents evaporated. The methylated polysaccharide was then reduced with Super-deuteride (300 μL) and, after desalting on Dowex 50W-X8 (H^+), hydrolysed with 2 M CF_3COOH at 120 °C for 2 h. The partially methylated products in the hydrolysates were reduced with $NaBD_4$, acetylated, and analysed by GC–MS.

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