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The structure of the exocellular polysaccharide produced by the Archaeon *Haloferax gibbonsii* (ATCC 33959)

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Abstract

The structure of the neutral exocellular polysaccharide isolated from the Archaeon *Haloferax gibbonsii* (ATCC 33959) has been determined using acid hydrolysis, methylation analysis and NMR spectroscopy. The polysaccharide contained D-Man, D-Glc, D-Gal and L-Rha in the ratios 2:1:3:1. The substitution patterns of the sugar residues were deduced from the methylation analysis which indicated the polymer to be composed of a heptasaccharide repeating unit containing two branches. The ^{1}H and ^{13}C NMR resonances of the component sugars were assigned using COSY, HOHAHA, HMQC, and HMQC-TOCSY 2D NMR experiments and the sequence of the sugars in the repeating unit was determined from NOESY and HMBC experiments. The structure can be written as:

→4)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→
3 2
↑ ↑
1 1
α-D-Gal*p*-(1→2)-α-L-Rha*p* α-D-Glc*p*

Keywords: Archaeon; *Haloferax gibbonsii*; Polysaccharide; NMR spectroscopy

1. Introduction

The Archaea (previously known as the archaebacteria [1]) are divided into the three Kingdoms of Euryarchaeota (containing the Families Methanogenaceae and Halobacteriaceae), Crenarchaeota, and the newly proposed Korarchaeota (which contains hyperthermophilic organisms that have been identified from 16S RNA sequences but are not yet represented by laboratory cultures) [2].

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Ribosomal RNA sequence information, amongst other data, has shown that, despite being prokaryotes, Archaea are as distant from bacteria as they are from eukaryotes. The Halobacteriaceae are extremely halophilic organisms that inhabit aquatic hypersaline environments such as marine solar salterns and typically require concentrations of 1–5.2 M NaCl for growth [3]. There are nine genera within the Halobacteriaceae, i.e. *Halobacterium*, *Halorubrum*, *Haloarcula*, *Haloferax*, *Halobaculum*, *Natrialba*, *Halococcus*, *Natronococcus*, and *Natronobacterium* [4]. Exopolysaccharide (**EPS**) production, while not common among the Archaea, is, however, found in some species within the genera *Haloferax* and *Haloarcula*. The structure of the **EPS** of *Haloferax mediterranei* [5] has been reported and that of *Haloferax denitrificans* is under investigation. We now report on the structural elucidation of the **EPS** of *Haloferax gibbonsii* ATCC 33959.

2. Results and discussion

Production and isolation of the polysaccharide.—*Haloferax gibbonsii* cells are short, highly pleomorphic rods that produce orange-red colonies when grown on solid media. The pigmentation of these colonies varies slightly with salt concentration and the mucoid nature of the colonies is ascribed to the exopolysaccharide which is exuded into the medium. *H. gibbonsii* ATCC 33959 was grown in liquid culture [6] containing a mixture of marine salts (25%) and after attaining the stationary phase the culture was killed by the addition of formaldehyde. The culture was centrifuged and the supernatant was filtered, concentrated and dialysed and the **EPS** was precipitated from the solution by the addition of ethanol [7]. The recovered material was purified by passage through an ion-exchange resin. Purification of the **EPS** was effected by passage through an ion-exchange gel, lipidic material was removed by centrifugation after treating the **EPS** with mild aqueous acid, and contaminating proteins were removed by Pronase treatment. Finally the **EPS** was chromatographed on Sephacryl S-500.

Composition and NMR spectra.—Complete acid hydrolysis of the **EPS** and GLC-MS of the derived alditol acetates identified the monosaccharides Rha:Man:Glc:Gal in the molar ratios 1:2:1:3, while methylation analysis indicated terminal Glc, terminal Gal, 4-substituted Gal, 2-substituted Rha, 4-substituted Man, 3,4-disubstituted Man, and 2,3-disubstituted Gal. Man, Glc and Gal were shown to be D, and Rha to be L, by GLC analysis of the derived acetylated (−)-2-octyl glycosides [8].

The ^{1}H NMR spectrum of the **EPS** at 47 °C contained, inter alia, seven signals in the anomeric region at δ 5.569, 5.262, 5.116, 5.085, 4.849, 4.796, and 4.750 and a signal at δ 1.34 (3 H) which could be assigned to the methyl group of Rha. The broad signals were consistent with the high viscosity of the sample. The ^{13}C NMR spectrum contained, inter alia, seven signals for anomeric carbons (94–102 ppm) and a signal for CH_3 of Rha at 17.34 ppm. These results, together with the methylation data, are consistent with a heptasaccharide repeating unit for the **EPS**.

2D NMR spectroscopy of the EPS.—The residues in the **EPS** were labelled **a**–**g** in order of decreasing chemical shift of their anomeric protons. The ^{1}H and ^{13}C resonances for each residue (Table 1) were assigned using a combination of 2D NMR techniques which included COSY [9], HOHAHA [10], HMQC [11], HMQC-TOCSY [12], HMBC [13], and NOESY [14] experiments. A section of the HMQC contour plot is shown in Fig. 1 and is only partly annotated due to the amount of crowding of the cross-peaks. The identity of the residues followed from comparison of the chemical shifts with those of literature values [15] and from observation of the coupling patterns. The $^1J_{C1,H1}$ values, which are indicative of the anomeric configurations of the monosaccharides [16], were measured from a ^{13}C–^{1}H HMQC spectrum acquired without proton decoupling during acquisition and indicate that the repeating unit contains four α- and three β-linked monosaccharides. The assignments were made as follows.

Residue ***a***. [α-D-Glc*p*]: H-2 was assigned from the COSY while H-2,3,4,5,6 could be traced in the H-1 track of the HOHAHA spectra recorded at two different mixing times. The ^{13}C resonances were then assigned from the ^{13}C–^{1}H HMQC experiment while the HMQC-TOCSY and HMBC confirmed the assignments of C-1,2,3,5. An H-1/H-2 NOE confirmed the anomeric configuration as α as did the $^1J_{C1,H1}$ value of 180 Hz.

Residue ***b***. [→4)-α-D-Gal*p*]: H-2 was obtained from the COSY while the HOHAHA spectra showed the resonances for H-2,3,4 in the H-1 track and for H-5 in the H-4 track. In the NOESY spectrum H-1/H-2 and H-4/H-5 NOEs were consistent

Table 1
^1H and ^{13}C chemical shift data[a] for *H. gibbonsii* polysaccharide

Residue		H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a,b/C-6
α-D-Glc	**a**	5.569 97.93	3.514 72.31	3.657 74.1	3.497 70.09	4.014 71.84	3.83 61–62
→4)-α-D-Gal	**b**	5.262 94.24	3.95 70.45	3.936 68.94	4.245 **77.18**[b]	4.04 71.82	61–62
→2)-α-L-Rha	**c**	5.116 94.02	4.054 **77.35**	4.142 70.45	3.478 73.48	4.429 69.43	1.34 17.34
α-D-Gal	**d**	5.085 98.68	3.849 69.14	3.951 69.17	4.037 70.07	3.933 71.91	61–62
→4)-β-D-Man	**e**	4.849 101.59	4.204 70.65	3.814 72.4	3.832 **78.1**	3.485 75.64	61–62
→3,4)-β-D-Man	**f**	4.796 101.39	4.318 67.47	3.942 **75.02**	3.849 **73.81**	3.675 73.29	61–62
→2,3)-β-D-Gal	**g**	4.750 100.97	3.929 **75.2**	3.927 **71.82**	4.281 67.66	3.688 75.81	61–62

[a] Chemical shifts in ppm relative to acetone at δ 2.23 for ^1H and 31.07 for ^{13}C, at 47 °C.
[b] Linkage carbons are indicated in bold.

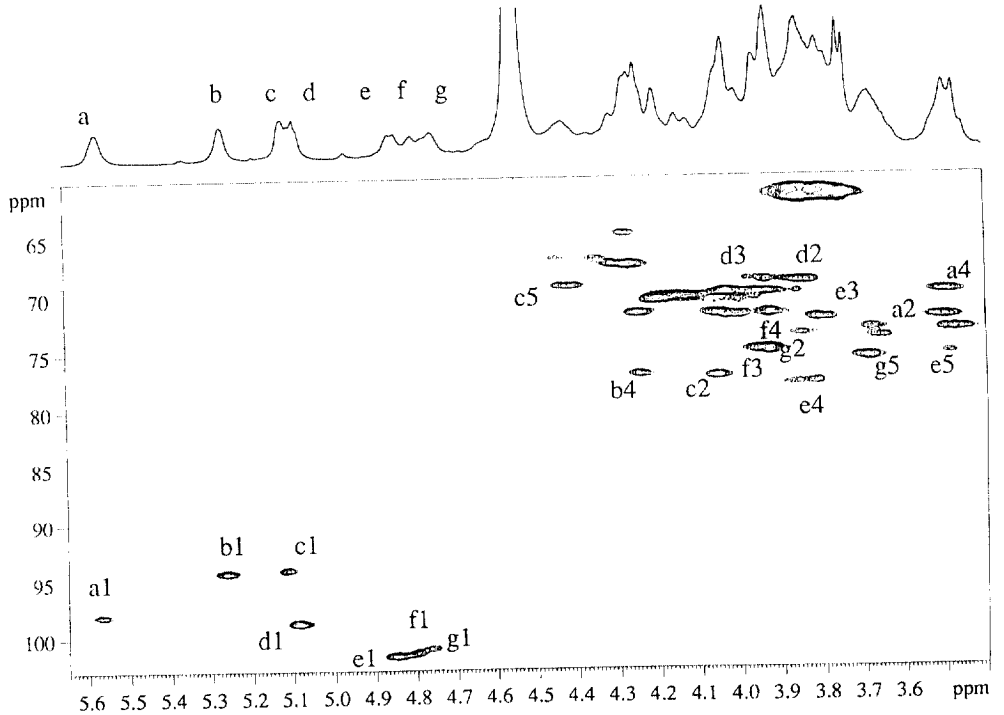

Fig. 1. Partial contour plot of an HMQC experiment on *H. gibbonsii* **EPS**.

with an α-Gal residue. The HMQC-TOCSY and HMBC spectra allowed the assignment of C-2,3,5, and C-4 followed from the HMQC spectrum. $^1J_{C1,H1}$ was 173 Hz.

Residue ***c***. [→2)-α-L-Rha*p*]: H-2,3,4,5,6 could be traced in the COSY spectrum and were confirmed from the HOHAHA experiments. The ^{13}C resonances followed from the HMQC spectrum and were confirmed by the HMQC-TOCSY experiment. An H-1/H-2 NOE confirmed that H-2 was equatorial and a $^1J_{C1,H1}$ value of 172 Hz indicated the α-configuration.

Residue ***d***. [α-D-Gal*p*]: H-2 was obtained from the COSY while H-1/H-2,3,4 and H-4/H-5 correlations were observed in the HOHAHA experiments. The HMQC-TOCSY gave the H-1/C-2,3 and C-1/H-2,3,4 correlations while H-1/C-5 came from the HMBC experiment. A H-1/H-2 NOE and

a $^1J_{C1,H1}$ value of 174 Hz were consistent with the α-configuration.

Residue ***e***. [→4)-β-D-Man*p*]: A very small H-1,2 cross-peak was seen in the COSY spectrum and H-2,3 was obtained from the HOHAHA experiment. H-1/H-2,3,5 and H-2/H-3 NOEs confirmed the *manno* configuration while the assignments of C-2,3,4,5 were made from the HMQC-TOCSY (C-5/H-4,3 correlation) and HMBC (H-1/C-2 correlation) experiments. $^1J_{C1,H1}$ was 164 Hz.

Residue ***f***. [→3,4)-β-D-Man*p*]: A small H-1/H-2 cross-peak was seen in the COSY spectrum and H-3,4,5 were assigned from the correlations seen in the H-2 track in the HOHAHA plot. The NOESY spectrum gave the expected H-1/H-2,3 NOEs and the ^{13}C resonances were assigned from the HMQC spectrum. A $^1J_{C1,H1}$ value of 164 Hz confirmed the β-configuration.

Residue ***g***. [→2,3)-β-D-Gal*p*]: The COSY experiment gave H-2 and the HOHAHA plot allowed the assignment of H-2,3,4 from the correlations observed in the H-1 track, and H-5 from the H-4 track. It was clear that H-2 and H-3 overlapped. In the NOESY spectrum NOEs were observed for H-1/H-3,5 and H-4/H-5. The ^{13}C resonances followed from the HMQC and HMQC-TOCSY experiments and $^1J_{C1,H1}$ was 163 Hz.

The positions of linkage of the sugar residues were readily identified by the downfield location (β-effect) of the signals of the linkage carbons as C-4 of **b**, C-2 of **c**, C-4 of **e**, C-3,4 of **f**, and C-2,3 of **g**; **a** and **d** were identified as terminal residues. These are in agreement with the results of the methylation analysis.

The sequence of the residues **a**–**g** in the repeating unit was established primarily by NOESY and HMBC experiments. The interresidue NOEs (Table 2) and the three-bond ^{13}C–^1H correlations (Table 3) from the anomeric protons to the carbons across the glycosidic linkage, when considered together with the methylation results, support unambiguously the following partial sequences:

Table 2
NOE data for *H. gibbonsii* **PS**

Proton at δ		NOE to							
5.569	**a1** [a]	3.51	**a2**,	3.93	**g2**				
5.262	**b1**	3.95	**b2**,	4.28	**g4**				
5.116	**c1**	4.05	**c2**,	3.94	**f3**,	4.32	**f2**		
5.085	**d1**	3.85	**d2**,	4.05	**c2**				
4.849	**e1**	4.2	**e2**,	3.81	**e3**,	3.49	**e5**,	4.25	**b4**
4.796	**f1**	4.32	**f2**,	3.94	**f3**,	3.83	**e4**		
4.750	**g1**	3.93	**g3**,	3.69	**g5**				
4.245	**b4**	4.04	**b5**						
4.204	**e2**	3.81	**e3**						
4.281	**g4**	3.69	**g5**						

[a] **a1** denotes H-1 of residue **a**, etc.

d-(1→2)-**c**-(1→3)-**f**-(1→4)-**e**-(1→4)-**b**- and
a-(1→2)-**g**.

The linkage between these two sequences was established from the interresidue NOE which was observed between H-1 of **b** and H-4 of **g**. The position of this linkage, however, is not consistent with the methylation results which show unit **g** to be 2,3- and not 2,4-disubstituted. This apparent anomaly is explained by previous observations [17,18] that the NOE to H-4 of a 3-substituted *galacto* aglycon is stronger than that to H-3 and occurs because of a preferred conformation which locates the anomeric proton of the α-linked glycon closer to H-4 of the aglycon than to H-3. In the case of the *H. gibbonsii* **EPS** the weaker NOE to H-3 of **g** was not observed.

No correlations were observed between unit **g** and **f**. However, as unit **f** was shown by methylation analysis and by the downfield location of C-3 and C-4 to be 3,4-disubstituted and is substituted by residue **c** in position 3, it follows that the only possible sequence for the repeating unit is as shown below.

The above data allow the following structure to be written for the repeating unit of *H. gibbonsii* polysaccharide.

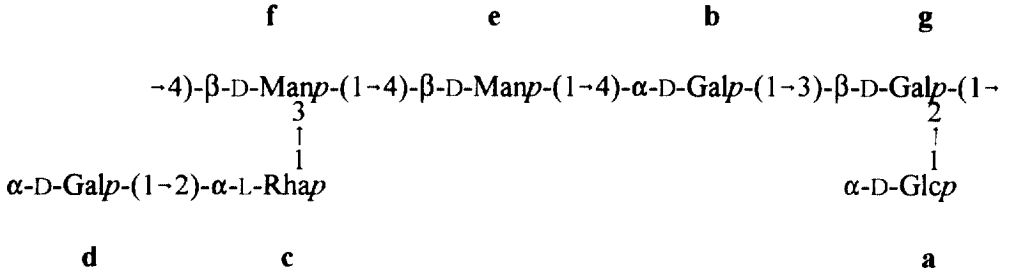

Table 3
Two- and three-bond ^{13}C–^{1}H correlations for *H. gibbonsii* **PS**

Proton at δ		Long-range correlation to carbon at			
5.57	**a1**[a]	74.1	**a3**,	71.8	**a5**
5.26	**b1**	70.5	**b2**,	71.8	**b5**
5.12	**c1**	75.0	**f3**		
5.09	**d1**	71.9	**d5**,	77.4	**c2**
4.85	**e1**	70.7	**e2**,	77.2	**b4**
4.80	**f1**	78.1	**e4**		

[a] **a1** denotes H-1 (or C-1, respectively) of residue **a**, etc.

3. Experimental

General methods.—Analytical GLC and GLC-MS were performed as previously described [19]. Ion-exchange chromatography was performed on a column (2.6×40 cm) of DEAE-Sepharose CL-6B using a gradient of 0–1 M NaCl in 0.01 M Tris-HCl buffer, pH 8.5. Fractions were assayed for carbohydrate by the phenol–H_2SO_4 reagent [20]. Gel permeation chromatography was performed on a column (1.6×68 cm) of Sephacryl S-500 in 0.1 M NaOAc buffer, pH 5.0. Fractions were monitored by refractive index. Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125 °C. Alditol acetates were prepared as described previously [19]. Acetylated (−)-2-octyl glycosides were prepared from a hydrolysate of the **EPS** as described by Leontein et al. [8]. The **EPS** was methylated according to a modified Hakomori method using potassium methylsulfinyl anion and CH_3I in Me_2SO [21], and the methylated **EPS** was hydrolysed and converted to alditol acetates prior to analysis by GLC-MS.

Production of polysaccharide.—*H. gibbonsii* ATCC 33959 was grown in a Braun Biostat ED fermenter with an effective vol of 15 L. A 1 L preculture of the Archaeon was inoculated into a growth medium containing a mixture of marine salts (25%) [6] with glucose as carbon source (1%). The sources of phosphate (KH_2PO_4) and carbon were sterilised separately before being added to the autoclaved mixture of salts. On reaching the stationary phase CH_2O (0.15%, v/v) was added to prevent contamination and the culture was centrifuged (23 000*g*, 1 h, 4 °C). The supernatant was then subjected to tangential filtration using a Millipore Pellicon system (0.45 μm) to remove remaining cells. The **EPS** solution was concentrated and dialysed (1×10^5 MW cut off) and the **EPS** was precipitated from the solution by the addition of NaOAc and 2–3 vol cold EtOH or acetone [7], redissolved and reprecipitated, and finally lyophilised. The lyophilised samples were passed through Amberlite IR-120 (Na^+) cation-exchange resin before chromatography on DEAE-Sepharose CL-6B. Crude polysaccharide (83 mg) was suspended in aq 2% HOAc and heated for 1.5 h at 100 °C. After cooling the lipidic material was removed by centrifugation at low speed and the supernatant was lyophilised. The residue was dissolved in 0.18 M phosphate buffer (pH 7.5) and treated with Pronase (20 mg, Boehringer Mannheim) at 37 °C for 24 h after which the temperature was raised to 60 °C for 10 min. The solid material was removed by centrifugation and the supernatant dialysed, lyophilised and finally chromatographed on Sephacryl S-500 (44 mg).

NMR spectroscopy.—The polysaccharide sample was deuterium exchanged by lyophilising several times from 99.6% D_2O and was then examined as a solution (15 mg/mL) in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^{1}H and 31.07 for ^{13}C). Spectra were recorded at 47 °C on a Bruker AMX-400 spectrometer using UXNMR software. The 2D pulse programmes were as follows: (a) COSY-45 with presaturation during relaxation delay; (b) HOHAHA using the MLEV-17 pulse sequence for mixing, two mixing times of 86 and 171 ms, respectively, presaturation during relaxation delay and mixing; (c) phase-sensitive NOESY using TPPI with presaturation during relaxation delay and mixing time of 150 ms; (d) HMQC using TPPI with presaturation during relaxation delay and GARP decoupling during acquisition; (e) HMQC-TOCSY as for HMQC, with MLEV-17 pulse sequence for mixing (43 and 86 ms); (f) HMQC using TPPI without decoupling during acquisition; (g) HMBC with presaturation during relaxation delay and a mixing delay of 60 ms. The data matrices for (a) and (c) were 256×2048 points, zero filled in both dimensions to 1024×2048, and a sine window function for (a), and a sine squared function for (c), applied prior to Fourier transformation; matrices for (b), (d–f) and (g) were 256×4096 points, zero filled in both dimensions to 1024×2048, and a sine squared window function applied prior to transformation.

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