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The structure of the exopolysaccharide produced by the halophilic Archaeon *Haloferax mediterranei* strain R4 (ATCC 33500)

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Abstract

The halophilic Archaeon *Haloferax mediterranei* exudes into the growth medium a high molecular weight sulfated polysaccharide. The structure of the repeating unit of this polymer was determined by a combination of glycose, methylation, and sulfate analysis, periodate oxidation, and 1D and 2D NMR spectroscopic analysis of the native and periodate-oxidised/reduced polysaccharides. The location of the sulfate group was established from the ^{1}H and ^{13}C NMR data. The structure of the repeating unit of the polysaccharide may be written as

→4)-β-D-Glc*p*NAcA-(1→6)-α-D-Man*p*-(1→4)-β-D-Glc*p*NAcA-3-O-SO_3^--(1→

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1. Introduction

The members of the Domain Archaea (formerly archaebacteria) [1] are prokaryotic organisms phylogenetically distant from the Bacteria (formerly eubacteria). The halophilic Archaea require extremely high concentrations of NaCl to grow and survive, and they can be found in large numbers in the NaCl-saturated water of solar salterns, where they are virtually exclusive inhabitants [2]. The halophilic Archaea maintain the highest concentrations of inorganic ions in their cytoplasm, 5 M intracellular K^+, instead of the organic compatible solutes that some moderately halophilic Bacteria accumulate. *Haloferax mediterranei* is an extremely halophilic Archaeon that produces considerable amounts of an exocellular polymeric substance (EPS) which gives the colonies a typical mucous character.This has been partially characterised as an acidic exopolysaccharide [3]. The present report describes the structural elucidation of the EPS of *Haloferax mediterranei*.

2. Results and discussion

Isolation, purification, and composition of the polysaccharide EPS.—H. mediterranei was grown in liquid culture under constant temperature, aeration, and pH, with stirring, until the stationary phase was reached ($OD_{600} = 5.5$) [4]. The culture was centrifuged and the supernatant solution was subjected to tangential filtration to remove the remaining cells, concentrated, and dialysed. The exopolysaccharide (0.3 g/L) was obtained from the resulting solution by precipitation with either ethanol or acetone and passed through an Amberlite IR-120 (Na^+) column [5]. After this step about 70–80% of the sample was recovered and finally purified by ion-exchange chromatography on DEAE-Sepharose using a linear NaCl gradient [6]. The elution profile indicated that the polymer was charged.

The EPS was hydrolysed with aq 4 M trifluoroacetic acid and the hydrolysate was converted into alditol acetates. GLC analysis showed only mannitol hexaacetate, indicating that mannose was the only neutral sugar in the polymer. A further portion of the EPS was methanolysed, the methyl esters were reduced, and the products were hydrolysed and converted into alditol acetates as before. GLC analysis showed mannitol hexaacetate and 2-acetamido-2-deoxyglucitol pentaacetate in the molar ratio 1.0:1.1, indicating that the acidic sugar present in the EPS is 2-amino-2-deoxyglucuronic acid. The sugars were both shown to be D by GLC analysis of their derived acetylated (−)-2-octyl glycosides [7].

The ^1H NMR spectrum of the Na^+ salt of the EPS in D_2O (Fig. 1) showed three resonances for anomeric protons (one α and two β linkages) at δ 5.23, 4.72, and 4.59, as well as a resonance at δ 4.49 (broad triplet) emanating from a ring proton, and two resonances for the CH_3 of acetamido sugars at δ 2.06 and 2.02. In the ^{13}C NMR spectrum (Fig. 2) resonances for anomeric carbons were observed at 102.40, 101.46, and 100.98 ppm, for carbons bearing N at 55.07 and 55.90 ppm, for carboxyl carbons at 175.48 ppm, and for CH_3 at 23.49 and 23.21 ppm. A DEPT [8] spectrum indicated that the signal at 68.98 ppm was for a CH_2 and can therefore be assigned to C-6 of a

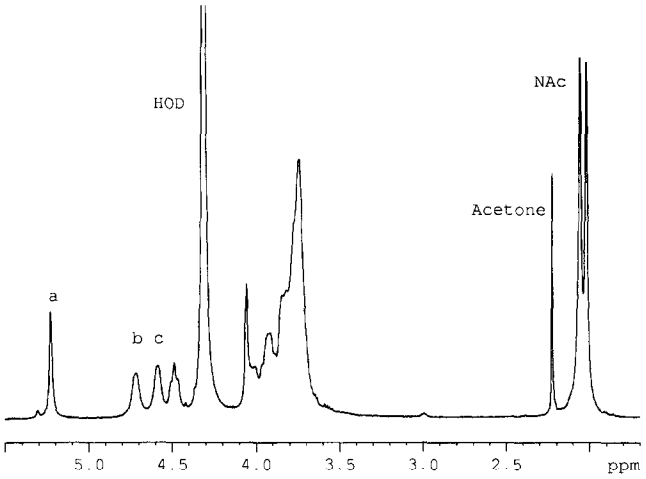


Fig. 1. ^{1}H NMR spectrum of the EPS in D_2O at 70 °C. For **a**, **b**, **c**, see text.

6-substituted sugar. These data in combination with those from the hydrolysis studies indicated that the EPS is composed of a regular repeating trisaccharide unit containing one mannose and two 2-acetamido-2-deoxyglucuronic acid moieties. The low value for GlcNAcA seen in the GLC analysis is explained by the known resistance of the glycosidic linkage of acidic and amino sugars to hydrolysis.

The presence of ester sulfate in the EPS was previously demonstrated by IR spectroscopy and chemical analyses [3]. A strong absorption band for S=O stretching in sulfate esters was observed at 1240 cm^{-1} as well as an absorption at 830 cm^{-1} attributable to sulfate ester in a secondary equatorial conformation [9]. The amount of sulfate present was estimated using the sodium rhodizonate method [10] and found to be 0.98 mol per mol of trisaccharide repeating unit.

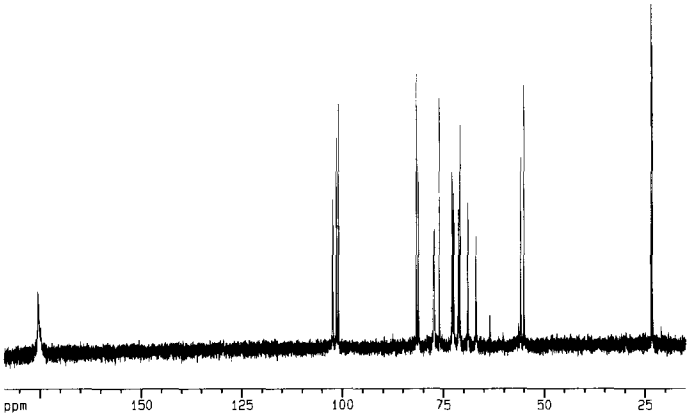


Fig. 2. ^{13}C NMR spectrum of the EPS in D_2O at 70 °C.

Table 1
Chemical shift data [a] for *Haloferax mediterranei* polysaccharide (EPS)

Proton or carbon	Residue **a**	**b**	**c**
	→ 6)-α-D-Man*p*-	→ 4)-β-D-Glc*p*NAcA-3-O-SO_3^--	→ 4)-β-D-Glc*p*NAcA-
H-1	5.23	4.72	4.59
C-1	101.46	100.98	102.40
H-2	4.06	3.91	3.79
C-2	70.92	55.07	55.90
H-3	3.75	4.49	3.74
C-3	71.30	81.69	72.82
H-4	3.74	3.95	3.79
C-4	67.01	**76.02**	**81.21**
H-5	3.74	3.84	3.77
C-5	72.46	77.27	77.46
H-6	4.03 3.83		
C-6	**68.98**		

[a] Chemical shifts in ppm, recorded at 400 MHz and 70 °C, relative to internal acetone at 2.23 and 31.07 ppm for ^1H and ^{13}C, respectively. Data for linkage carbons are in boldface type.

2D NMR spectroscopy on the EPS.—The structure of the trisaccharide repeating unit was determined by a series of 2D NMR experiments. The residues were labelled **a**–**c** reflecting the decreasing value of their H-1 chemical shifts. The ^1H and ^{13}C resonances of the residues were assigned using COSY [11], HMQC [12], HMQC-TOCSY [13] with three different mixing times, and NOESY [14] experiments. The data are collected in Table 1. The resonances for H-1/3 of **a**, H-1/5 of **b**, and H-1/2 of **c** could be traced in the COSY plot. The HMQC-TOCSY experiment allowed the assignment of the resonances for H-1/6 and C-1/6 of unit **a** and showed that the resonances for H-4 and H-5 of this unit coincided. The C-2 track of unit **b**, in the HMQC-TOCSY spectrum, showed connectivities for H-2/5, while C-2/5 could be traced in the H-1 and H-3 tracks. The H-1 track of unit **c** showed connectivities to C-2/5. All ^{13}C resonances were then confirmed from the C–H correlation experiment (HMQC). The NOESY experiment confirmed the assignment of the anomeric configurations of the residues as well as their identities, namely, H-1/H-2 NOE for the α-linked sugar, and H-1/H-3 and H-1/H-5 NOEs for the β-linked sugars. The NOESY experiment also allowed the sequence of the residues in the repeating unit to be established. NOE contacts were observed between H-1 of **a** and H-2 of **a**, and H-4 of **b**; between H-1 of **b** and H-3 and H-5 of **b**, and H-4 of **c**; and between H-1 of **c** and H-3 and H-5 of **c**, and H-6a and H-6b of **a**. The sites of linkage were confirmed by the strong downfield shifts observed for C-4 of **b** and **c**, and C-6 of **a**. It was evident from the ^{13}C chemical shifts that all the sugars are present in the pyranoid form [15]. The position of substitution of the sulfate group was evident from the strong downfield shift of the C-3 and H-3 resonances of unit **b**, placing it at O-3 of this unit. The above information allows the structure of the trisaccharide repeating unit to be written as follows:

→4)-β-D-Glc*p*NAcA-(1→6)-α-D-Man*p*-(1→4)-β-D-Glc*p*NAcA-3-O-SO_3^--(1→ **1**

c **a** **b**

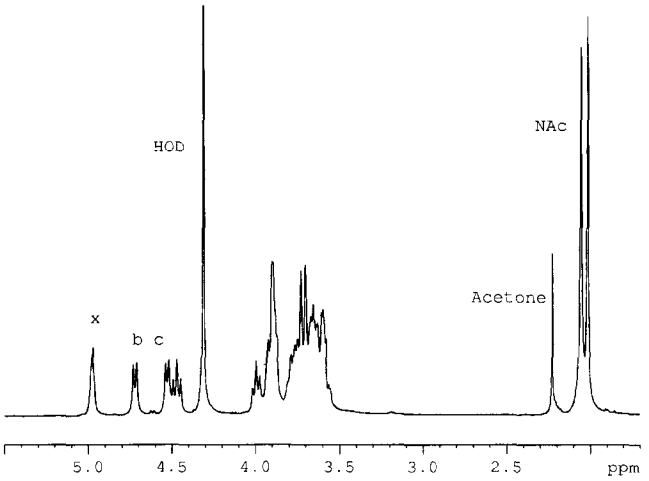


Fig. 3. ^{1}H NMR spectrum of the EPS-OX in D_2O at 70 °C. For **x**, **b**, **c**, see text.

Partial hydrolysis of EPS.—The polysaccharide was treated with fuming HCl [16] for 30 min at 20 °C and the resulting oligosaccharides were separated by GPC. A trisaccharide fraction was analysed by FAB-MS in the negative ion mode and showed

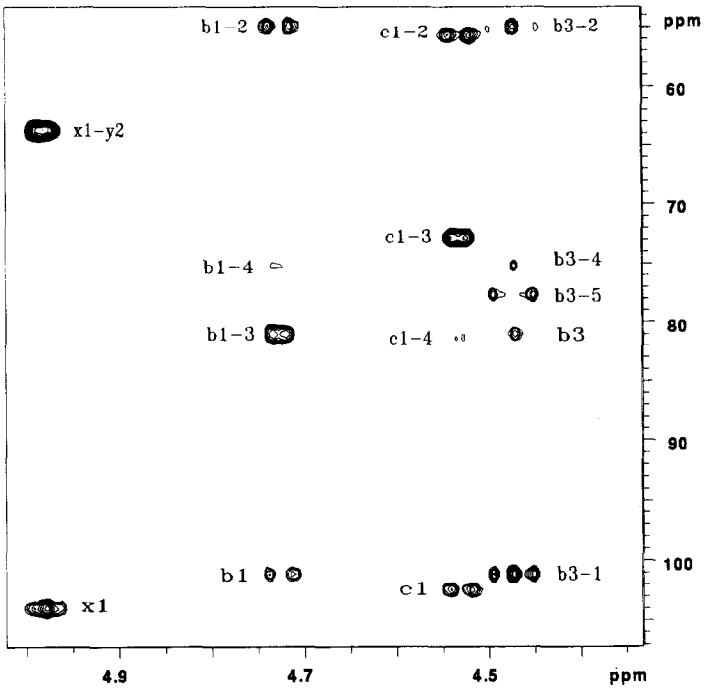
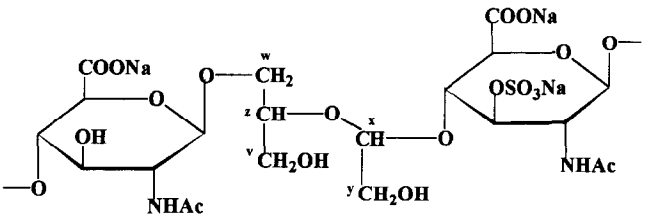


Fig. 4. Partial contour plot of the HMQC-TOCSY experiment on EPS-OX. **b1–3** connotes the cross-peak observed between H-1 and C-3 of unit **b**, etc. **c1** connotes the cross-peak observed between H-1 and C-1 of unit **c**, etc.

peaks consistent with a mixture of a sulfated and a non-sulfated trisaccharide. Peaks were observed at *m/z* 613 $[M-1]^-$ and 635 $[M-2+Na]^-$ for GlcNAcA–GlcNAcA–Man, and *m/z* 693 $[M-1]^-$ for $GlcNAcASO_3$–GlcNAcA–Man. Peaks were also observed for $GlcNAcASO_3$–GlcNAcA and $GlcNAcASO_3$.

Periodate oxidation of the EPS and NMR studies of the EPS-OX.—In order to confirm the structure of the repeating unit of the EPS a periodate oxidation of the polymer was carried out. The oxidised polymer was reduced with $NaBH_4$ and purified by dialysis to produce EPS-OX. Hydrolysis of the new polymer after carboxyl reduction followed by GLC analysis of the derived alditol acetates showed only GlcNAc, indicating that the Man units had been oxidised. The 1D ^1H NMR spectrum (Fig. 3) of the EPS-OX indicated that the chemical shifts for the anomeric signals of the two β-linked GlcNAcA units were largely unchanged from those in the spectrum of the EPS and that, in the place of the signal for the α-Man, a new signal had appeared, a multiplet at δ 4.98, resonating 0.25 ppm upfield of that for H-1 of the α-Man in the spectrum of the EPS. This confirmed that only the 6-substituted Man had been oxidised. A series of 2D ^1H and ^{13}C experiments allowed all the ^1H and ^{13}C signals of the EPS-OX to be assigned. These included COSY, HMQC, and HMQC-TOCSY, part of the contour plot of which is shown in Fig. 4. The linkage sites and sequence of the units were again established by the NOESY experiment. The data are collected in Tables 2 and 3, and are consistent with the 6-substituted Man unit having been oxidised to a five-carbon fragment leaving the two 4-substituted GlcNAcA moieties intact. The structure of the EPS-OX repeating unit can be written as:



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The above data allow the structure of the repeating unit of the EPS of *Haloferax mediterranei* to be written as

→4)-β-D-Glc*p*NAcA-(1→6)-α-D-Man*p*-(1→4)-β-D-Glc*p*NAcA-3-O-SO_3^--(1→

This polymer is a highly charged molecule as previously described [3] and this is the first study to be reported of a polysaccharide produced by halophilic Archaea. Among the Archaea there are several examples in which charged polysaccharides form part of the cell wall, and these have been studied in detail. However there are few structural studies of charged polysaccharides derived from Archaea. *Halococcus* has a highly sulfated complex heteropolysaccharide consisting of neutral and amino sugars, uronic

Table 2
Chemical shift data[a] for *Haloferax mediterranei* periodate-oxidised/reduced polysaccharide (EPS-OX)

Proton or carbon	Residue **b**	**c**								
	→ 4)-β-D-Glc*p*NAcA-3-O-SO_3^--	→ 4)-β-D-Glc *p*NAcA-	–CH–[x]	$-CH_2^yOH$		–CH–[z]	$-CH_2^yOH$		$-CH_2-^w$	
				a	b		a	b	a	b
H-1	4.72	4.53	4.98	3.61	3.59	3.90	3.70	3.66	3.63	3.92
C-1	101.08	102.38	103.93		63.802	79.21		62.46		**70.02**
H-2	3.91	3.76								
C-2	55.01	55.80								
H-3	4.47	3.71								
C-3	81.02	72.82								
H-4	4.00	3.79								
C-4	**75.16**	**81.34**								
H-5	3.89	3.72								
C-5	77.65	77.50								

[a] Chemical shifts in ppm, recorded at 400 MHz and 70 °C, relative to internal acetone at 2.23 and 31.07 ppm for ^1H and ^{13}C, respectively. Data for linkage carbons are in boldface type.
[x,y,z,v,w] See structure **2**.

Table 3
NOE contacts for EPS-OX

Proton	δ	NOE contact to, δ
x [a]	4.98	4.00 **b4**, 3.90 **z**, 3.61 **ya**
b1 [b]	4.72	4.47 **b3**, 3.79 **c4**
c1	4.53	3.63 **wa**, 3.92 **wb**, 3.90 **z**

[a] **x**, **w**, **z**, etc, see structure **2**.
[b] **b1** connotes H-1 of residue **b**, etc.

acids, an aminouronic acid, and acetyl and glycinyl groups [17]; *Natronococcus* possesses a cell wall sacculi polymer containing GlcN, uronic acids, Glc, Gal, and Man [18]; and *Halobacterium salinarium* [18] and *Haloferax volcanii* [19] have glycoprotein S-layers containing charged oligosaccharides with uronic acids and sulfate. Of the methanogenic Archaea known to have polysaccharides, the *Methanosarcina* species contain a thick cell-wall consisting of a chondroitin-like polymer [20] while *Sulpholobus*, a hyperthermophilic Archaeon which has a cell wall with an S-layer, produces a sulfated polysaccharide containing Glc, Gal, Man, and GlcN [21].

3. Experimental

General methods.—Analytical GLC and GLC-MS were performed as previously described [22]. Ion-exchange chromatography was performed on a column containing 50 mL of DEAE-Sepharose using a gradient of 0–3 M NaCl in 0.001 M Tris-HCl buffer, pH 7.0 [6]. Fractions were assayed for carbohydrate by the phenol–H_2SO_4 reagent [23]. Polysaccharide samples were hydrolysed with 4 M CF_3CO_2H for 1 h at 125 °C. Alditol acetates were prepared as described previously [22]. Samples were methanolysed by refluxing with methanolic 3% HCl for 16 h. Methoxycarbonyl groups formed during methanolysis were reduced with $NaBH_4$ in dry MeOH for 16 h at room temperature. Acetylated (−)-2-octyl glycosides were prepared as described by Leontein et al. [7].

Production of polysaccharide.—Haloferax mediterranei strain R4 (ATCC 33500) was grown in a Braun Biostat ED fermentor with an effective vol of 15 L. A 1-L preculture of the archaeon was inoculated into a growth medium containing a mixture of salts (25%) [4] with D-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 solution was then subjected to tangential filtration using a Millipore Pellicon system (0.45 μm) to remove remaining cells. The EPS solution was concentrated, dialysed (1×10^5 MW cut off), and concentrated again by dialysis against PEG 6000 (4 °C, 2–3 days). The EPS was precipitated from the solution by the addition of CH_3CO_2Na and 2–3 vol of cold ethanol or acetone [5], redissolved and reprecipitated, and finally lyophilised. The lyophilised samples were passed through Amberlite IR-120 (Na^+) cation-exchange resin before chromatography on DEAE-Sepharose. Fractions were collected, dialysed, and lyophilised.

Partial hydrolysis of the EPS.—Polysaccharide (50 mg) was treated with fuming HCl at 20 °C for 30 min, neutralised with NaOH (0.1 M), and chromatographed on Bio-Gel P-2. A fraction (5 mg) eluting in the trisaccharide region was analysed by negative ion FAB-MS on a JEOL SX-102 instrument at a resolution of 3000, using triethanolamine as matrix.

Periodate oxidation of EPS.—Polysaccharide (100 mg) in water (30 mL) was added to a solution (10 mL) of $NaIO_4$ (320 mg) and $NaClO_4$ (980 mg), and kept in the dark at 4 °C. After 96 h, $(CH_2OH)_2$ was added to quench remaining $NaIO_4$ (stirring, 1 h), followed by the addition of $NaBH_4$ (400 mg, 4 h). The solution was finally dialysed against running water (3 days), and lyophilised (72 mg). The material was then reoxidised to ensure complete oxidation, reduced, dialysed, and lyophilised (yield 51 mg). A portion (4 mg) was methanolysed, reduced with $NaBH_4$ in dry MeOH, hydrolysed, and converted into the alditol acetates. A further portion (30 mg) was prepared for NMR spectroscopy.

NMR spectroscopy.—Polysaccharide samples were deuterium-exchanged by lyophilising several times from 99.6% D_2O and then examined as the Na^+ salt in 99.99% D_2O containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded at 70 °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) phase-sensitive NOESY using TPPI with presaturation during relaxation delay and mixing time (200 ms); (c) HMQC using TPPI with presaturation during relaxation delay and GARP decoupling during acquisition; (d) HMQC-TOCSY as for HMQC, with MLEV-17 pulse sequence for mixing (44, 68, and 84 ms). The data matrices for (a) and (b) were 256×2048 points, zero-filled in both dimensions, and a sine window function for (a), and a sine squared function for (b), applied prior to Fourier transformation; matrices for (c) and (d) were 256×4096 points, zero-filled in both dimensions and a sine squared window function applied prior to transformation.

Acknowledgements

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