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Structural studies on the acidic exopolysaccharide from *Haloferax denitrificans* ATCC 35960

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Abstract

The structure of a linear, acidic exopolysaccharide isolated from the Archaeon *Haloferax denitrificans* ATCC 35960 has been determined using NMR spectroscopy. The sugar residues in the repeating unit of the polysaccharide were identified as Gal and GlcA2,3NAc after the assignment of the ^{1}H and ^{13}C resonances using COSY, HOHAHA, HMQC and HMQC-TOCSY experiments. The sequence of the residues in the polysaccharide was established from the inter-residue connectivities observed in the HMQC-NOESY plot. The only sugar released on acid hydrolysis was shown to be D-Gal by GLC analysis, while the absolute configuration of the acidic sugars was shown to be D by comparison of the carbon chemical shifts with those of model compounds. Partial acid hydrolysis yielded a tetrasaccharide, terminated by D-Gal at the reducing end, whose structure confirmed that of the repeating unit of the polysaccharide as →4)-β-D-Glc*p*A2,3NAc-(1→4)-β-D-Glc*p*A2,3NAc-(1→4)-α-D-Glc*p*A2,3NAc-(1→3)-α-D-Gal*p*-(1→, where D-Glc*p*A2,3NAc is 2,3-diacetamido-2,3-dideoxy-D-glucopyranosiduronic acid.

Keywords: Archaeon; *Haloferax denitrificans*; Polysaccharide; Structure; NMR spectroscopy

1. Introduction

Haloferax denitrificans ATCC 35960 is a member of the Archaea Domain and is classified as belonging to the family Halobacteriaceae, order Halobacteriales [1]. These Archaea are extremely halophilic organisms which grow in salt concentrations of between 1.5 and 4.5 M and are aerobic, highly pleomorphic and produce orange–red colonies. Growth can occur up to 55 °C with the optimum pH being 6.7. *H. denitrificans* ATCC 35960 was first isolated from salterns in the southern part of San Francisco Bay using techniques selective for extreme halophiles capable of nitrate reduction. It was the first organism of the Halobacteriaceae family in which anaerobic growth by dissimilatory nitrate reduction was unambiguously shown [2]. Like other members of the genus *Haloferax*, viz. *mediterranei* [3] and *gibbonsii* [4], this organism also produces exocellular polysaccharide material, the structural characterisation of which we now report.

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2. Results and discussion

Production of exopolysaccharide.—*H. denitrificans* ATCC 35960 was grown in liquid culture containing a mixture of marine salts (25%) and glucose as the sole carbon source [5]. On reaching the stationary phase, the cells were killed by the addition of formaldehyde and ultracentrifuged. The pellet was reserved for further processing while the supernatant was filtered, concentrated and dialysed. The exopolysaccharide (EPS) was precipitated from the solution by the addition of alcohol and NaOAc, passed through Amberlite IR-120(Na) and lyophilised [6]. The material was treated with aqueous 2% HOAc to remove a small amount of lipidic material, followed by treatment with Pronase, and then chromatographed on Sephacryl S-500 to yield purified EPS.

Composition and NMR spectra.—The ^{1}H NMR spectrum of the EPS (Na salt) at 70 °C (Fig. 1) showed signals, inter alia, at δ 5.170 (1 H), 5.046 (1 H), 4.654 (1 H) and 4.606 (1 H) in the anomeric region of the spectrum (δ 4.5–5.5) as well as signals for methyl groups (18 H) of acetamido sugars at δ 1.95–1.97. The ^{13}C NMR spectrum (Fig. 2) contained signals at 94.55, 99.25, 102.35 and 102.53 ppm in the anomeric region of the spectrum, signals for ring carbons at 51–80 ppm of which six were in the region for carbons adjacent to nitrogen (51.18, 52.62, 54.53, 54.62, 54.74, and 55.79 ppm) and one in the region for unsubstituted hydroxymethyl groups (61.54). Signals for methyl groups of acetamido sugars (22.49–23.08 ppm), and signals for C=O from 174.85–175.79 ppm were also observed. No signals occurred in the region 83–88 ppm,

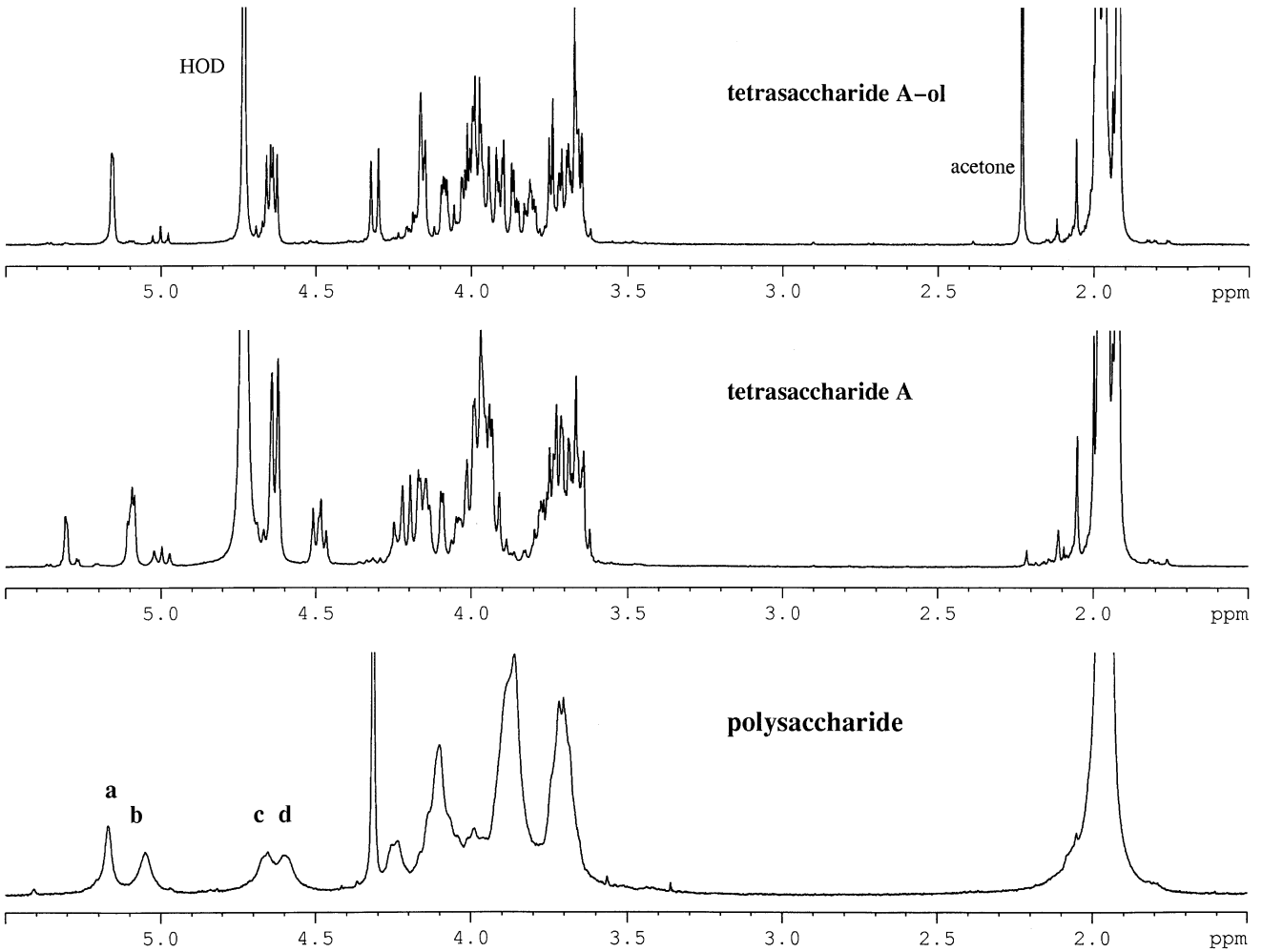

Fig. 1. ^{1}H NMR spectra for *H. denitrificans* EPS and derived oligosaccharides **A** and **A-ol**.

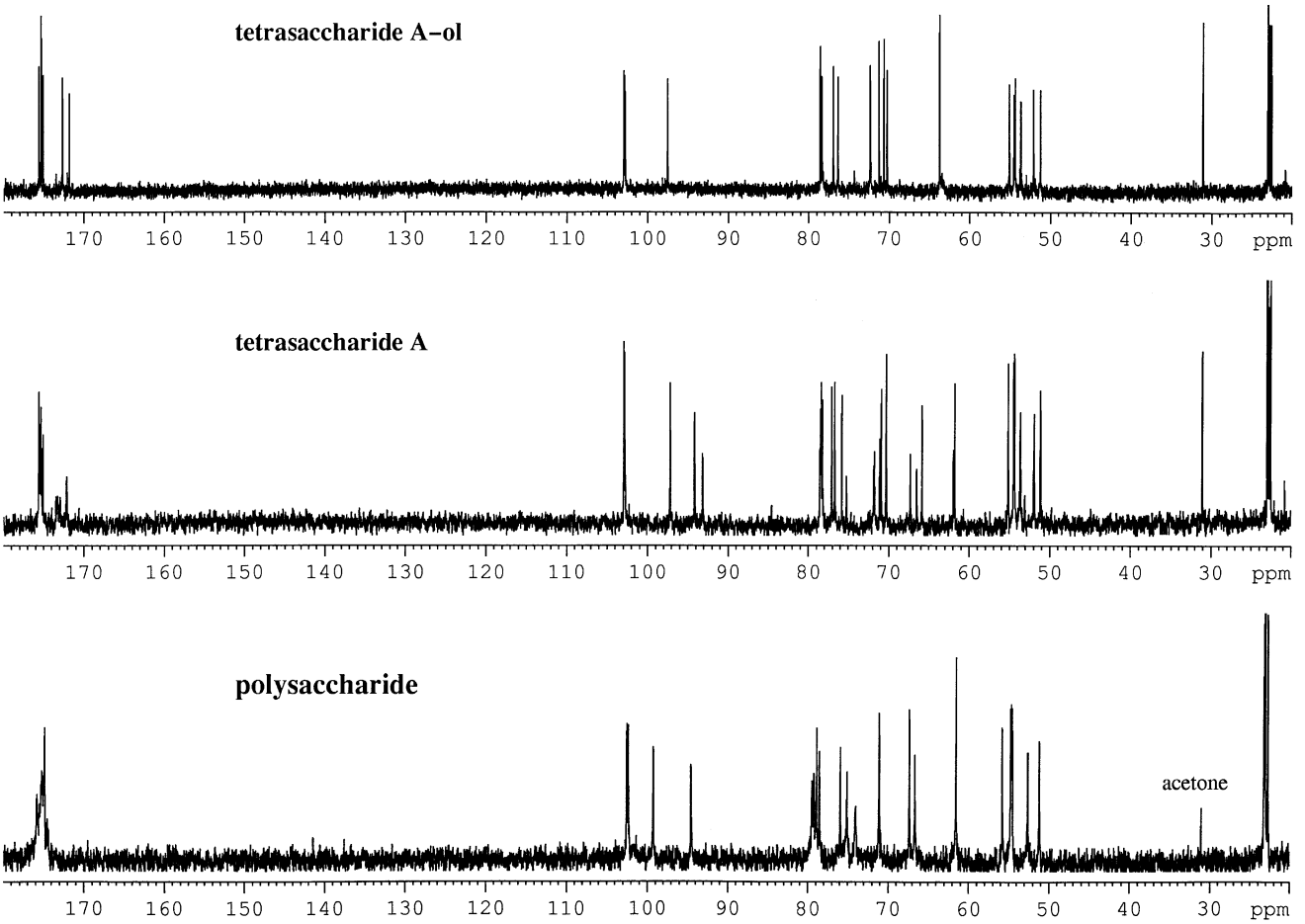

Fig. 2. ^{13}C NMR spectra for *H. denitrificans* EPS and derived oligosaccharides **A** and **A-ol**.

indicating that all sugars were in the pyranose configuration [7]. The data suggest a tetrasaccharide repeating unit for the EPS containing di- or tri-acetamido sugars. The only sugar released by acid hydrolysis of the EPS was Gal, which was shown to have the D-configuration by GLC of the acetylated (−)-2-octyl glycosides [8].

2D NMR spectroscopy.—The identity of the sugars, their positions of substitution, and their sequence in the repeating unit of the EPS were determined by 2D NMR experiments at 70 °C which included ^{1}H-^{1}H COSY [9], HOHAHA [10], ^{1}H-^{13}C HMQC [11], HMQC-TOCSY [12] and HMQC-NOESY experiments. The residues in the repeating unit were labelled **a**–**d** in order of decreasing chemical shift of their anomeric protons. The ^{1}H and ^{13}C resonances were assigned in the following manner and the identification of the residues followed from comparison of the chemical shifts with literature values [7,13]. The data are collected in Table 1. A partial plot of the HMQC data is shown in Fig. 3.

Residue **a**: →3)-α-D-*Gal*p. H-1,2 were assigned from the COSY with the signal at δ 3.862 (H-2) showing a connectivity to δ 4.097. This signal was shown to belong to H-4 from the evidence in the HMQC-TOCSY contour plot and from the HOHAHA plot which showed only connectivities from H-1 to δ 3.862 and δ 4.097 in the H-1 track. Thus H-2 and H-3 overlapped. The HMQC-TOCSY experiment showed the connectivity from H-1 to C-1,2,3 and the C-2 track showed H-4 leading to the assignment of C-4. The HMQC-NOESY experiment confirmed the assignments of the first four H/C pairs and allowed the assignment of H-5,6 and C-5,6 with the observation of the cross peaks for H-4/C-5, H-5/C-4,6, C-4/H-5,6 and H-6/C-5. The cross peaks for C-5/H-3 and C-3/H-4 further confirmed the overlap of H-2 and H-3.

Residue **b**: →4)-α-*Glc*p*A2,3NAc*. The COSY spectrum gave H-1,2 while the H-1 track of the HOHAHA gave the positions of H-2 and H-3. The HMQC-TOCSY spectrum showed the cross peaks for C-2/H-2,3,4,5, which were then confirmed from the HOHAHA (H-4,3,2 in the H-5 track). The remaining C resonances were assigned from the HMQC experiment.

Residue **c**: →4)-β-*Glc*p*A2,3NAc*. The resonances for H-1,2,3,4,5 could be traced from the COSY plot, while the HOHAHA spectrum showed H-1/H-2,3 cross peaks in the H-1 track. The HMQC-TOCSY experiment showed the cross peaks for H-1/C-2,3 and C-3/H-2,3,4,5 while the HMQC-NOESY experiment indicated connectivities between H-1 and C-3, C-1 and H-3,5, and C-2 and H-4. The assignment of the remaining C resonances came from the HMQC experiment.

Residue **d**: →4)-β-*Glc*p*A2,3NAc*. H-1,2,3 could be traced in the COSY spectrum. The resonance at δ 3.886 showed a cross peak to δ 3.726 which, from other evidence, was shown to be H-5, thus indicating that H-3 and H-4 overlapped. In the HOHAHA experiment acquired with a long mixing time only three cross peaks were observed in the H-1 track (for H-2, H-3/4 and H-5). The HMQC-TOCSY spectrum confirmed the overlap showing the cross peaks for H-1/C-2,3,4 and the HMQC experiment then confirmed the assignment of H-2,3,4,5. The HMQC-NOESY experiment showed cross peaks for H-1/C-5 and H-3/C-5.

The *gluco* configuration for the acidic sugars was suggested by the NOEs observed between H-1 (and/or C-1) and H-3 and H-5 (and/or C-3 and C-5) in the HMQC-NOESY experiment (see Table 2), and by comparison of the chemical shifts with those of similar residues in *P. aeruginosa* O:6 O-specific polysaccharide and oligosaccharides [13].

The positions of substitution of the sugars were clear from the downfield locations of the resonances of C-3 of **a**, and C-4 of **b**, **c** and **d**. The sequence of the residues in the repeating unit was obtained from the interresidue NOEs observed in the HMQC-NOESY experiment (Table 2) and indicate the sequence

→3)-**a**-(1→4)-**c**-(1→4)-**d**-(1→4)-**b**-(1→

The absolute configurations of the acidic sugars **b**, **c** and **d** were determined on the basis of the magnitudes of the effects of glycosylation, which were obtained by comparison of the chemical shifts of the ^{13}C resonances of the sugars in the repeating unit with those of the corresponding residues of α-Gal and α- and β-GlcA2,3NAc [13,14]. The relatively large negative β-effects of glycosylation on C-2 (−2 ppm) and C-4 (−3.7 ppm) of the →3)-α-D-Gal unit (residue **a**) indicated that it was substituted by a sugar having the same absolute configuration [14], i.e. →4)-α-D-GlcA2,3NAc (residue **b**). Residue **c** has the D configuration by virtue of the negative β-effect of glycosidation on C-3 (−1.0 instead of +0.1 if substituted by an L sugar) by the substitution of α-D-Gal at C-4 [14]. The negative β-effect of glycosidation on C-3 of **d** is also large (−2.3) indicating substitution by a sugar having the same absolute configuration, i.e., D [14]. The structure of the repeating unit of the EPS can thus be written as

Table 1
^{1}H and ^{13}C chemical shift data [a] for *H. denitrificans* 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
→3)-α-D-Gal	**a**	5.170	3.862	3.862	4.097	3.903	3.703
		99.25	67.35	**75.91** [b]	66.66	71.11	61.54
→4)-α-D-GlcA2,3NAc	**b**	5.046	4.108	4.132	3.905	4.247	
		94.55	52.62	51.18	**78.53**	74.02	
→4)-β-D-GlcA2,3NAc	**c**	4.654	3.721	4.053	3.995	3.849	
		102.53	54.74	55.79	**75.07**	79.47	
→4)-β-D-GlcA2,3NAc	**d**	4.606	3.687	3.886	3.882	3.726	
		102.35	54.62	54.53	**78.88**	79.22	

[a] Chemical shifts in ppm relative to acetone at δ 2.230 for ^{1}H and 31.07 for ^{13}C, at 70 °C.
[b] Linkage carbons are indicated in bold.

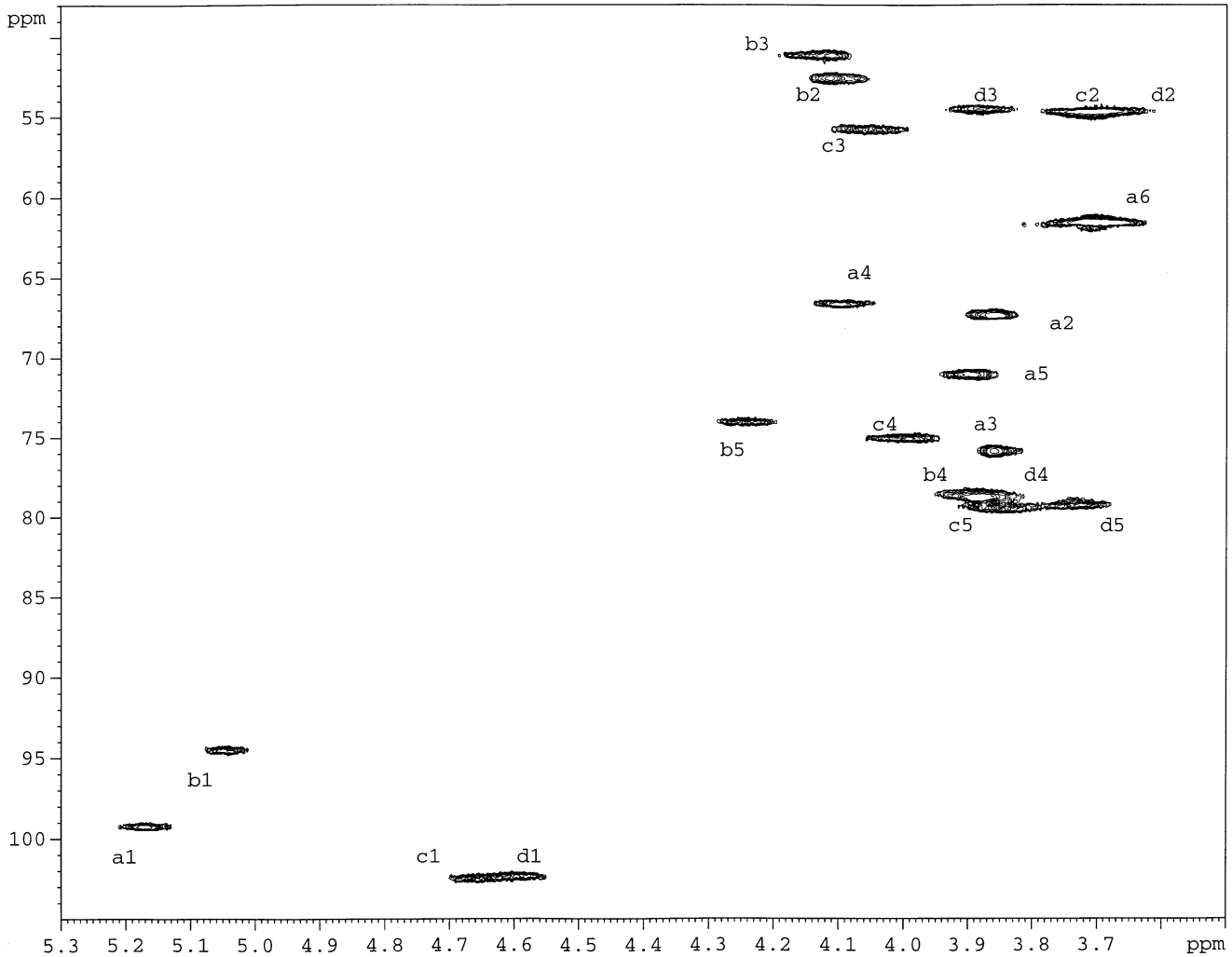

Fig. 3. Partial contour plot of the HMQC data for *H. denitrificans* EPS.

$$\rightarrow 4)\text{-}\beta\text{-D-}\underset{\mathbf{c}}{\text{Glc}}p\text{A2,3NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-}$$

$$\underset{\mathbf{d}}{\text{Glc}}p\text{A2,3NAc-}(1\rightarrow 4)\text{-}\alpha\text{-D-}\underset{\mathbf{b}}{\text{Glc}}p\text{A-}$$

$$2,3\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-}\underset{\mathbf{a}}{\text{Gal}}p\text{-}(1\rightarrow$$

where D-Glc*p*A2,3NAc represents 2,3-diacetamido-2,3-dideoxy-D-glucopyranosiduronic acid.

Partial acid hydrolysis of the EPS.—In order to confirm the structure of the polysaccharide deduced from the experiments described above, partial acid hydrolysis studies were carried out on the EPS. Advantage was taken of the extreme resistance to acid hydrolysis of the diaminouronic acid glycosidic bond and the relative lability of the Gal linkage. A portion of the EPS was treated with 2 M CF_3CO_2H at 100 °C for 4 h, the acid was removed by evaporation under vacuum, and the products were then N-acetylated according to the method of Altman et al. [15]. Gel permeation chromatography on a column of ToyoPearl TSK-40 revealed one main product, **A**, eluting with a V_e corresponding to a hexasaccharide, and three lower-molecular-weight products in much smaller amounts. Fraction **A** was desalted on Bio-Gel P-2 and after analysis by NMR spectroscopy the purified **A** was reduced with $NaBD_4$ and the resulting alditol, **A-ol**, was also examined by NMR spectroscopy.

NMR spectroscopy of **A** *and* **A-ol**.—The 1D ^{1}H NMR spectrum of **A** at 30 °C (Fig. 1) showed five signals in the anomeric region, two for α-linked sugars at δ 5.304 (0.4 H, $J_{1,2}$

2.9 Hz) and 5.088/5.104 (1 H, twinned, $J_{1,2}$ 3.2 Hz), and three for β-linked sugars at δ 4.632 (2 H, $J_{1,2}$ 7.7 Hz), and 4.679 ($\sim$0.5 H, $J_{1,2}$ 8.4 Hz). On reduction to the alditol, the signals for the reducing sugar at δ 5.304 and 4.679 disappeared, the signal at 5.088/5.104 shifted downfield slightly to δ 5.159 and the twinning effect due to mutarotation of the reducing group was removed. The twinning effect on the signal at δ 4.496/4.479 (H-5 of **b**) was also removed by the reduction, indicating that this is the sugar immediately interior to the reducing end. The three signals integrating for one H each at δ 4.635 ($J_{1,2}$ 8.5 Hz), 4.647 ($J_{1,2}$ 8.3 Hz) and 5.159 ($J_{1,2}$ 2.8 Hz) in the anomeric region of the spectrum of **A-ol** (Fig. 1) confirmed that **A-ol** was the tetrasaccharide alditol derived from **A**. The ^{13}C spectrum of **A** (Fig. 2) showed five signals in the anomeric region while that for **A-ol**, as expected, showed only three. In the spectrum of **A-ol** five signals for C=O, six signals for methyl groups and six for carbons linked to N indicated that the tetrasaccharide contained the three diacetamidouronic acids and an alditol.

As the spectra of **A-ol** were less complicated than those for **A** these were assigned first, thus allowing for easier interpretation of the data for **A**. The ^{1}H signals for **A-ol** were assigned using the COSY spectrum and the ^{13}C signals were then assigned from the HMQC plot. All the spin systems were confirmed by the HMQC-TOCSY experiment and the data are collected in Table 3. The sequence of the sugars was determined from the HMBC [16] experiment, which also showed the connectivities between the carboxylic acid carbonyl signals and H-4 and H-5 of the diacetamidouronic acids, and between the carbonyl signals of the NAc groups and H-2 and H-3 of these sugars. The assignment of the signals for oligosaccharide **A** was made in a similar way and the data are also collected in Table 3. The *gluco* configuration for the acidic sugars was confirmed from the values for the $J_{2,3}$, $J_{3,4}$ and $J_{4,5}$ coupling constants. The following structures can thus be written for **A** and **A-ol**:

A-ol

β-D-Glc*p*A2,3NAc-(1→4)-β-D-Glc*p*A2,3NAc-(1→4)-α-D-Glc*p*A2,3NAc-(1→3)-galactitol

A

β-D-Glc*p*A2,3NAc-(1→4)-β-D-Glc*p*A2,3NAc-(1→4)-α-D-Glc*p*A2,3NAc-(1→3)-D-Gal

c **d** **b** **a**

3. Conclusions

The structure of the exopolysaccharide from *H. denitrificans* is thus as shown in the Abstract. Although the structure is novel, the unusual 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid has been found before in the O-specific polysaccharide from *Pseudomonas aeruginosa* type O:6 LPS [13]. This is only the third exopolysaccharide to be described from the genus *Haloferax* with that of *H. gibbonsii*

Table 2
HMQC-NOESY data for *H. denitrificans* polysaccharide

H or C			NOE to H or C							
5.170	**a**	H-1	67.35	**a**	C-2	**75.07**	**c**	**C-4** [a]		
4.097	**a**	H-4	71.11	**a**	C-5					
3.903	**a**	H-5	66.66	**a**	C-4	61.54	**a**	C-6		
3.703	**a**	H-6	71.11	**a**	C-5					
99.25	**a**	C-1	3.862	**a**	H-2					
5.046	**b**	H-1	52.63	**b**	C-2					
94.55	**b**	C-1	4.108	**b**	H-2	**3.862**	**a**	**H-2,3**		
4.654	**c**	H-1	55.79	**c**	C-3	**78.88**	**d**	**C-4**		
102.53	**c**	C-1	4.053	**c**	H-3	**3.882**	**d**	**H-4**	3.849	**c** H-5
4.606	**d**	H-1	79.22	**d**	C-5	**78.53**	**b**	**C-4**		
102.35	**d**	C-1	**3.905**	**b**	**H-4**	3.726	**d**	H-5		

[a] Interresidue NOEs are in bold. **a** H-1 denotes H-1 of residue **a**, etc.

Table 3
^{1}H and ^{13}C NMR data [a] for *H. denitrificans* tetrasaccharide **A** and alditol **A-ol**

Residue	A-ol	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
β-D-GlcA2,3NAc	**c**	4.635 (8.5) [b]	3.687 (11.4)	3.998(11.0)	3.672(11.4)	4.002	
		102.81	54.33	55.07	70.27	76.91	172.65 [c]
→4)-β-D-GlcA2,3NAc	**d**	4.647 (8.3)	3.717 (11.2)	4.033 (8.9)	3.923 (10.1)	3.987	
		102.92	54.49	53.62	**78.31**	76.33	171.79
→4)-α-D-GlcA2,3NAc	**b**	5.159 (2.8)	4.162	4.162	3.964 (10.1)	4.316	
		97.51	52.10	51.23	**78.37**	72.31	172.63 [c]
→3)-galactitol	**a**	3.74 (4.4)	4.090 (2.4)	3.859 (6.3)	3.907 (2.5)	3.812 (4.8,6.8)	3.66 (9.2)
		63.46	72.36	**78.55**	70.65	71.26	63.73
	A						
β-D-GlcA2,3NAc	**c**	4.632 (7.7)	3.708	3.986	3.663	3.954	
		102.91 [c]	54.47 [c]	55.13	70.31	76.68	
→4)-β-D-GlcA2,3NAc	**d**	4.632 (7.7)	3.684	4.014	3.910	3.982	
		102.83 [c]	54.34 [c]	53.61	**78.55**	77.08	
→4)-α-D-GlcA2,3NAc	**b**	5.088/5.104 (3.2)	4.225(11.6)	4.168(9.9)	3.953	4.496/4.479 (9.8)	
		94.18	51.16	51.92	**78.48**	71.76	
→3)-β-D-Gal∼OH	**a**	4.679 (8.4)	3.802	3.762	4.092	3.654	3.75
		97.17	70.91	**78.39**	65.83	75.80	61.80
→3)-α-D-Gal∼OH	**a′**	5.304 (2.9)	3.974	3.976	4.146	4.046	3.73
		93.15	67.33	**75.24**	66.53	71.12	61.97

[a] Chemical shifts in ppm relative to acetone at δ 2.230 for ^{1}H and 31.07 for ^{13}C, at 30 °C.
[b] Coupling constants in Hz.
[c] Values may have to be interchanged. Linkage carbons are in bold.

[4] producing a neutral polymer made up of repeating heptasaccharide units containing Glc, Gal, Man and Rha, and that of *H. mediterranei* [3] producing a highly charged sulphated polymer with a trisaccharide repeating unit containing GlcANAc and Man.

4. Experimental

General methods.—Analytical GLC and GLC-MS were performed as previously described [17]. 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 [18]. Gel permeation chromatography was performed on columns (1.6 × 68 cm) of Sephacryl S-500 and ToyoPearl TSK-40 in 0.1 M NaOAc buffer, pH 5.0, and of Bio-Gel P-2 in water. Fractions were monitored by refractive index. Polysaccharide samples were hydrolysed with 2 M HCl for 4 h at 120 °C and after neutralisation alditol acetates were prepared as described previously [17]. Acetylated (−)-2-octyl glycosides were prepared from a hydrolysate of the EPS as described by Leontein et al. [8].

Production of polysaccharide.—*Haloferax denitrificans* ATCC 35960 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%) [5] 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 [6], 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 (77 mg) was suspended in 2% aq HOAc and heated at 100 °C for 1.5 h. 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 (19 mg).

Preparation of oligosaccharides.—A portion of EPS (15 mg) was treated with 2 M CF_3CO_2H at 100 °C for 4 h followed by removal of the acid under vacuum. The products were N-acetylated in 5:0.3 water–MeOH by the addition of Ac_2O (0.1 mL) [15]. After 2 h the reagents were removed by evaporation and NH_3 (0.1 mL) added and the products lyophilised. Fractionation on ToyoPearl TSK-40 gel gave a major fraction **A** (10 mg, ($[\alpha]_D$ 18.3°, *c* 0.47, water), which was then desalted using Bio-Gel P-2. After recording the NMR spectra, **A** was reduced with $NaBD_4$ in water for 1 h to produce **A-ol**.

NMR spectroscopy.—Samples were deuterium exchanged by lyophilising several times from 99.6% D_2O and were then examined as solutions 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 30 and 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) 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) HMQC-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) HMBC with presaturation during relaxation delay and a mixing delay of 60 ms.

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References

[1] C.R. Woese, O. Kandler, M.L. Wheelis, *Proc. Natl. Acad. Sci.*, 87 (1990) 4576–4579.
[2] G.A. Tomlinson, L.L. Jahnke, L.I. Hochstein, *Int. J. Syst. Bacteriol.*, 36 (1986) 66–70.
[3] H. Parolis, L.A.S. Parolis, I.F. Boán, F. Rodríguez-Valera, G. Widmalm, M.C. Manca, P.E. Jansson, I.W. Sutherland, *Carbohydr. Res.*, 295 (1996) 147–156.
[4] N.A. Paramonov, L.A.S. Parolis, H. Parolis, I.F. Boán, J. Antón, F. Rodríguez-Valera, *Carbohydr. Res.*, 309 (1998) 89–94.
[5] F. Rodríguez-Valera, G. Juez, D.G. Kushner, *Syst. Appl. Microbiol.*, 4 (1983) 369–381.
[6] F.L.A. Buckmire, *Microbios.*, 41 (1984) 49–63.
[7] K. Bock, H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
[8] K. Leontein, B. Lindberg, J. Lönngren, *Carbohydr. Res.*, 62 (1978) 358–362.
[9] W.P. Aue, E. Bartholdi, R.R. Ernst, *J. Chem. Phys.*, 64 (1976) 2229–2246.
[10] D.G. Davis, A. Bax, *J. Am. Chem. Soc.*, 103 (1985) 2820–2821.
[11] A. Bax, S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–567.
[12] L. Lerner, A. Bax, *J. Magn. Reson.*, 69 (1986) 365–380.
[13] B.A. Dmitriev, N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.K. Kochetkov, E.S. Stanislavski, G.M. Mashilova, *Eur. J. Biochem.*, 125 (1982) 229–237.
[14] G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, N.K. Kochetkov, *Carbohydr. Res.*, 175 (1988) 59–75.
[15] E. Altman, M.B. Perry, J.-R. Brisson, *Carbohydr. Res.*, 191 (1989) 295–303.
[16] A. Bax, M.F. Summers, *J. Amer. Chem. Soc.*, 108 (1986) 2093–2094.
[17] Y.M. Choy, G.G.S. Dutton, M.R. Leslie, H. Parolis, L.A.S. Parolis, *Carbohydr. Res.*, 269 (1995) 295–302.
[18] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Anal. Chem.*, 28 (1956) 350–356.