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# Structure of the exopolysaccharide of *Vibrio diabolicus* isolated from a deep-sea hydrothermal vent

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**Abstract**

The structure of the exopolysaccharide produced under laboratory conditions by *Vibrio diabolicus*, a bacterium recovered from a deep-sea hydrothermal vent, has been investigated using sugar and methylation analysis and NMR spectroscopy. The polysaccharide consists of a linear tetrasaccharide repeating unit with the following structure.

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



*Keywords:* Bacterial polysaccharide; Hydrothermal vent; Uronic acid; Amino sugars

## 1. Introduction

Because of the extreme conditions of pressure, temperature and high concentrations of toxic elements observed near deep-sea hydrothermal vents, the presence of unusual microorganisms of biotechnological interest could be expected in terms of polysaccharide-producing bacteria. Since 1994 several polysaccharides have been obtained from laboratory-grown hydrothermal bacterial isolates and partially characterized. These exopolymers exhibited very different chemical and rheological properties compared with those of other bacterial polysaccharides [1,2]. Structural studies have been conducted on some interesting polymers of these, and to date the repeating units of two polysaccharides have been totally characterized [3,4]. The first species of *Vibrio* [5] to be isolated from such an extreme environment was a mesophile that secretes an exopolysaccharide of potential interest for its chemical resemblance to heparin. The present report describes the structural elucidation of this exopolysaccharide of *Vibrio diabolicus*, which is rich in aminodeoxy sugars and glucuronic acid.

## 2. Results and discussion

*Isolation and composition of the polysaccharide.*—A crude preparation of the polysaccharide was obtained by precipitation of the culture supernatant of *V. diabolicus* with 30% ethanol (v/v). The polymer was purified from compounds of low molecular weight by ultrafiltration. GLC analysis of the derived per-*O*-trimethylsilyl methyl glycosides revealed that this polymer was composed of glucuronic

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acid, 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose in a molar ratio of 2:1:1. Each glycosyl residue was determined to be in the D-configuration [6].

*Glycosyl linkage analysis.*—When the polysaccharide was methylated and then reduced with Super-Deuteride™ Aldrich Chemical Co., [$LiB(C_2H_5)_3D$], prior to hydrolysis, reduction with $NaBD_4$ and acetylation, subsequent analysis of the resulting partially methylated (partially deuterated) alditol acetates by GLC–MS revealed only three components (Table 1): 4,6-disubstituted Glc*p* containing two deuterium atoms at C-6; 4-substituted Gal*p*NAc and 3-substituted Glc*p*NAc. Since the composition results showed two glucuronyl residues in the repeating unit, and methylation data indicated that only one type of glucuronic acid residue was present, which was 4-linked, we could conclude that each repeating unit contained two 4-substituted Glc*p*A residues. These results are consistent with a linear tetrasaccharide repeating unit.

*NMR studies.*—The $^1H$ NMR spectrum of the exopolysaccharide at 325 K (Fig. 1) was consistent with a polysaccharide with a regular repeating structure. Signals were observed in the anomeric region at $\delta$ 5.40, 4.67, 4.61 and 4.53, and for Me protons of *N*-acyl groups at $\delta$ 2.10 (3 H) and 2.12 (3 H). The $^{13}C$ NMR spectrum showed signals for four anomeric carbons at 100.1, 102.9, 105.3 and 105.9 ppm, for two N-linked carbons at 52.9 and 56.7 ppm, for two Me carbons at 24.9 and 25.0 ppm, and for four carbonyl carbons at 173.3, 173.9, 177.5 and 177.6 ppm. These results are consistent with a tetrasaccharide repeating unit containing two uronic acids and two N-acetylated amino sugars. The residues were labeled **A**, **B**, **D** and **E** in order of decreasing chemical shift of their H-1 protons. The $^{13}C$ and $^1H$ resonances of the residues (Table 2) were assigned using COSY (Fig. 2), TOCSY and HMQC experiments with help from the intra-residue NOEs observed in the NOESY spectrum (Table 3) and from the long-range three-bond intra-residue $^1H-^{13}C$ correlations obtained in the HMBC experiment (Table 4). The following is a brief account of how the $^1H$ and $^{13}C$ chemical shifts were established for the individual residue of the repeating unit.

*Residue* **A** *[→4)-α-*D*-Gal*p*NAc-(1→]*.—Assignments of chemical shifts of the $^1H$ resonances for this residue were difficult due to signal overlap within the spin system. However, the resonances for H-1 to H-4 were traced in the COSY spectrum, and the corresponding $^{13}C$ resonances were determined from the HMQC spectrum. These results were confirmed in the HMBC spectrum, in which an additional long-range correlation appeared between H-1 and C-5. The chemical shifts of the remaining $^1H$ resonances were assigned from the H-5/H-6,6′ cross-peaks in the NOESY spectrum. The $^1H$ and $^{13}C$ resonances of the NAc group could be attributed according to H-2/C=O and H($CH_3$)/C=O correlations observed in the HMBC experiment.

*Residue* **B** *[→4)-β-*D*-Glc*p*A-(1→]*.—All the $^1H$ resonances for this residue were traced in the COSY contour plots and the $^{13}C$ chemical shifts were assigned from the HMQC spectrum.

*Residue* **D** *[→3)-β-*D*-Glc*p*NAc-(1→]*.—The TOCSY spectrum allowed the determination of four $^1H$ resonances belonging to residue **D**. But due to severe signal overlaps within the spin system and with other spin systems, only the chemical shifts for H-1 and H-2 could be first assigned from the COSY spectrum. The chemical shifts for H-3 and H-4 were assigned from the C-2/H-3 and C-3/H-4 long-range correlations shown in the HMBC spectrum and from the $^1H$ and $^{13}C$ data. The chemical shifts of the remaining $^1H$ resonances were determined from the H-4/H-5 and H-5/

Table 1
Analysis of the alditol acetates derived from methylation of *V. diabolicus* exopolysaccharide

| Alkylated sugars (as alditol acetates) | $t_R$ [a] | Detector response (%) |
|---|---|---|
| 2,3-$Me_2$Glc-6-$d_2$ [b] | 0.84 | 31 |
| 3,6-$Me_2$GalNAc [c] | 1.09 | 29 |
| 4,6-$Me_2$GlcNAc | 1.13 | 40 |

[a] $t_R$ = retention time for the corresponding alditol acetate relative to that of *myo*-inositol hexaacetate ($t_R$ = 1.00).

[b] 2,3-$Me_2$Glc-6-$d_2$ = 1,4,5,6-tetra-*O*-acetyl-6,6-dideutero-2,3-di-*O*-methylglucitol.

[c] 3,6-$Me_2$GalNAc = 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol.

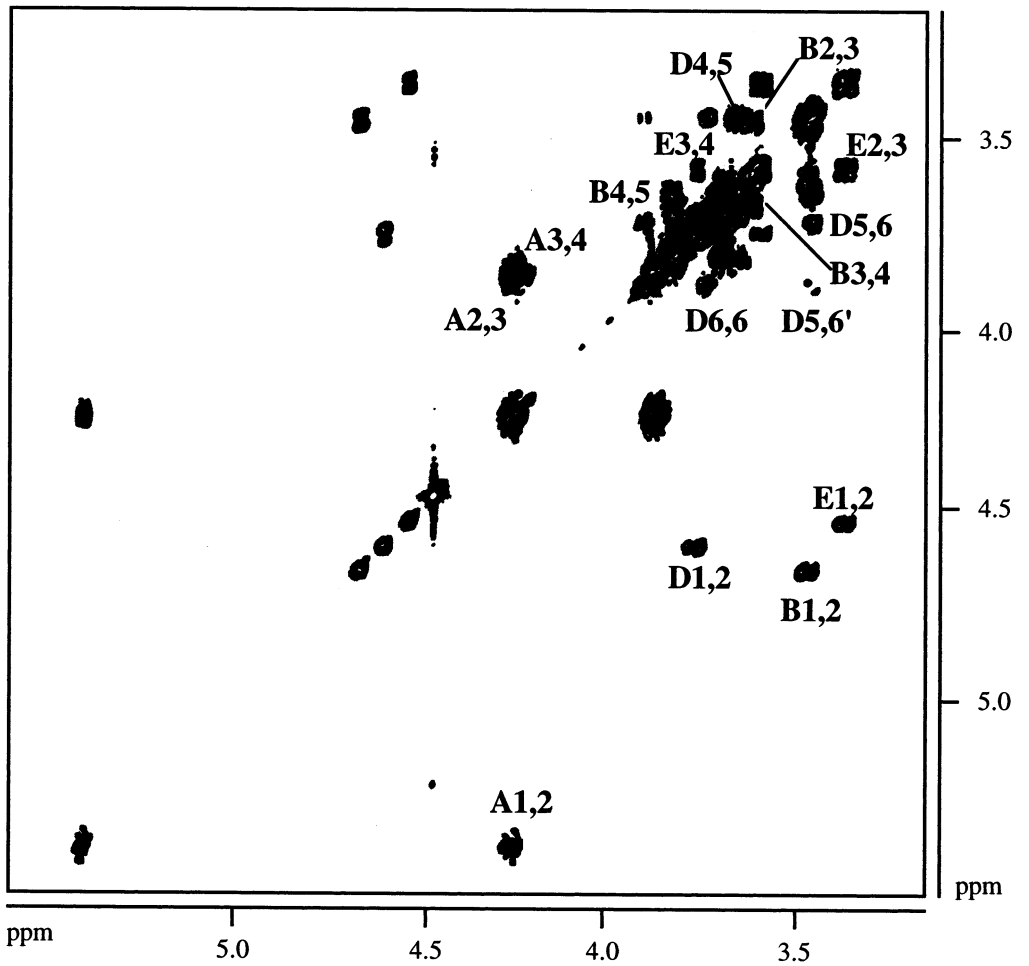


Fig. 1. 500 MHz $^{1}H$ NMR spectrum recorded at 325 K in deuterium oxide of *V. diabolicus* exopolysaccharide.

Table 2
Chemical shifts ($\delta$, ppm) of the signals in the $^{1}H$ and $^{13}C$ NMR spectra of the *V. diabolicus* exopolysaccharide

| Residue | $^{1}H/^{13}C$ [a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | C=O | $CH_3$ |
| →4)-α-D-Gal*p*NAc-(1→ | 5.40 | 4.26 | 3.88 | 4.20 | 3.84 | 3.84, 3.64 | | 2.12 |
| **A** | 100.1 | 52.9 | 70.7 | *79.1* | 73.2 | 62.6 | 177.5 | 25.0 |
| →4)-β-D-Glc*p*A-(1→ | 4.67 | 3.47 | 3.62 | 3.70 | 3.82 | | | |
| **B** | 105.9 | 75.8 | 73.7 | *83.8* | 77.9 | 173.3 [b] | | |
| →3)-β-D-Glc*p*NAc-(1→ | 4.61 | 3.76 | 3.71 | 3.65 | 3.46 | 3.76, 3.95 | | 2.10 |
| **D** | 102.9 | 56.7 | *81.3* | 70.3 | 78.6 | 63.1 | 177.6 | 24.9 |
| →4)-β-D-Glc*p*A-(1→ | 4.53 | 3.36 | 3.59 | 3.77 | 3.76 | | | |
| **E** | 105.3 | 75.8 | 76.3 | *82.5* | 79.2 | 173.9 [b] | | |

[a] Carbon protons involved in interglycosidic linkages are italicized.
[b] These assignments may be interchanged.

H-6,6′ cross-peaks observed in the COSY spectrum. Corresponding $^{13}C$ resonances were established from the HMQC spectrum. The $^{1}H$ and $^{13}C$ resonances of the NAc group could be attributed according to H-2/C=O and $H(CH_3)$/C=O correlations observed in the HMBC experiment.

*Residue* **E** *[→4)-β-*D*-Glc*p*A-(1→]*.—Resonances for this residue were established as for the residue **B**.

Comparison of the $^{1}H$ and $^{13}C$ NMR data for the residues with literature values for methyl glycosides [7–9] and substituted sugars [3,4,10] permitted the residues of the repeating unit to be identified as indicated in Table 2. This identification was in accordance with the methylation data, as supported by the downfield positions of C-4 of **A**, **B** and **E**, and C-3 of **D**.

Glycosyl sequence information was obtained from the inter-residue NOEs (Table 3) and three-bond $^{1}H-^{13}C$ correlations (Table 4) obtained from the NOESY and HMBC experiments, respectively. An intense NOE correlation was observed between H-1 of **D** and H-4 of **E**, along with weaker contacts with H-2 and H-3 of **E**, demonstrating that **D** is linked to the 4-position of **E**. The NOESY spectrum showed a clear inter-residue contact between H-1 of **E** and H-4 of **B**, as well as a contact between H-1 of **B** with H-4 of **A**. The H-1 of **A** presented a clear NOE contact with H-3 of **D**, along with contacts of lower intensity with H-4 and H-5 of **D**, establishing the α-(1→3) linkage. The structure of the tetrasaccharide repeating unit is supported by the $^{1}H-^{13}C$

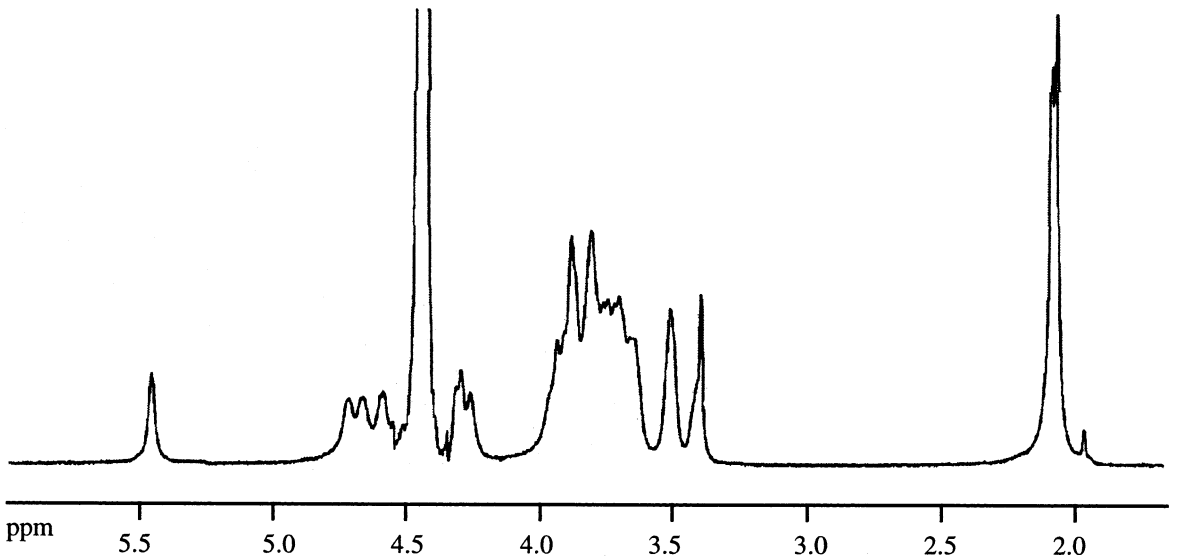


Fig. 2. 500 MHz COSY spectrum recorded at 325 K in deuterium oxide of *V. diabolicus* exopolysaccharide.

Table 3
Correlations observed in the 2D NOESY spectrum recorded at 325 K in deuterium oxide of *V. diabolicus* polysaccharide

| Residue | Proton | Correlations [a] | Residue, atom |
|---|---|---|---|
| →4)-α-D-Gal*p*NAc-(1→ | H-1 (δ 5.40) | *3.46* | **D**, H-5 |
| **A** | | *3.65* | **D**, H-4 |
| | | *3.71* | **D**, H-3 |
| | | 4.26 | **A**, H-2 |
| | H-5 (δ 3.84) | 3.64 | **A**, H-6 |
| | | 3.84 | **A**, H-6′ |
| →4)-β-D-Glc*p*A-(1→ | H-1 (δ 4.67) | 3.47 | **B**, H-2 |
| **B** | | 3.62 | **B**, H-3 |
| | | 3.82 | **B**, H-5 |
| | | *4.20* | **A**, H-4 |
| →3)-β-D-Glc*p*NAc-(1→ | H-1 (δ 4.61) | *3.36* | **E**, H-2 |
| **D** | | *3.59* | **E**, H-3 |
| | | *3.77* | **E**, H-4 |
| →4)-β-D-Glc*p*A-(1→ | H-1 (δ 4.53) | 3.36 | **E**, H-2 |
| **E** | | 3.59 | **E**, H-3 |
| | | *3.70* | **B**, H-4 |

[a] Inter-residue NOEs are italicized.

Table 4
Correlations observed in the 500 MHz HMBC spectrum recorded at 325 K in $D_2O$ of *V. diabolicus* exopolysaccharide

| Residue | Proton | Correlations [a] | Residue, atom |
|---|---|---|---|
| →4)-α-D-Gal*p*NAc-(1→ | H-1 (δ 5.40) | 73.2 | **A**, C-5 |
| **A** | H-2 (δ 4.26) | 70.7 | **A**, C-3 |
| | | 177.5 | (C=O of NAc) |
| | H-3 (δ 3.88) | 52.9 | **A**, C-2 |
| | H-4 (δ 4.20) | *105.9* | **B**, C-1 |
| | $CH_3$ (δ 2.10) | 177.5 | (C=O of NAc) |
| →4)-β-D-Glc*p*A-(1→ | H-1 (δ 4.67) | *79.1* | **A**, C-4 |
| **B** | H-2 (δ 3.47) | 105.9 | **B**, C-1 |
| | H-3 (δ 3.62) | 75.8 | **B**, C-2 |
| | | 83.8 | **B**, C-4 |
| | H-4 (δ 3.70) | *105.3* | **E**, C-1 |
| | H-5 (δ 3.82) | 105.9 | **B**, C-1 |
| →3)-β-D-Glc*p*NAc-(1→ | H-1 (δ 4.61) | *82.5* | **E**, C-4 |
| **D** | H-2 (δ 3.76) | 81.3 | **D**, C-3 |
| | | 177.6 | (C=O of NAc) |
| | H-3 (δ 3.71) | 56.7 | **D**, C-2 |
| | H-4 (δ 3.65) | 81.3 | **D**, C-3 |
| | $CH_3$ (δ 2.12) | 177.6 | (C=O of NAc) |
| →4)-β-D-Glc*p*A-(1→ | H-1 (δ 4.53) | *83.8* | **B**, C-4 |
| **E** | H-2 (δ 3.36) | 105.3 | **E**, C-1 |
| | | 76.3 | **E**, C-3 |
| | H-3 (δ 3.59) | 75.8 | **E**, C-2 |
| | | 82.5 | **E**, C-4 |
| | H-4 (δ 3.77) | 76.3 | **E**, C-3 |
| | | *102.9* | **D**, C-1 |

[a] Interglycosidic correlations are italicized.

correlation data from the HMBC experiment: intermolecular correlations were observed between **D** and **E**, between **E** and **B**, and between **B** and **A**.

The above data permit the structure of the repeating unit of the *V. diabolicus* polysaccharide to be written as

→3)-β-D-G$\overset{\mathbf{D}}{\text{l}}$c*p*NAc-(1→4)-β-D-G$\overset{\mathbf{E}}{\text{l}}$c*p*A-(1→
4)-β-D-G$\overset{\mathbf{B}}{\text{l}}$c*p*A-(1→4)-α-D-G$\overset{\mathbf{A}}{\text{a}}$l*p*NAc-(1→

## 3. Experimental

*Bacterial strain.*—*V. diabolicus* was isolated from the dorsal integument of the polychaete annelid, *Alvinella pompejana*. This annelid was collected during the 'Hero' oceanographic cruise in 1991, near the active hydrothermal vent ELSA located in a rift system of the East Pacific Rise (12°48.13′ N, 103°56.30′ W).

*Production, isolation and purification of the polysaccharide.*—The polysaccharide was produced as previously described [11,12] using a 2-L fermenter containing 1 L of marine 2216 broth medium supplemented with glucose, at atmospheric pressure, a temperature of 25 °C and at pH 7.2. After 48 h, the viscosity of the medium reached a stable value of 35 Pa s. Cells were removed from the medium by high-speed centrifugation at 20,000*g* for 2 h. The exopolysaccharide was precipitated from the supernatant by adding EtOH to a final concentration of 30% alcohol (v/v) and washed with 70–100% EtOH–water (v/v). The polysaccharide was then dissolved in water and subjected to ultrafiltration using a Millipore system with PM-10 membrane (10,000 Da cut-off), washed with water, concentrated and finally lyophilized. The yield was 2 g of polysaccharide, which was stored at room temperature (rt).

*General methods.*—GLC–MS was carried out on partially methylated alditol acetates on an HP-5890 system using a DB-1 fused-silica column (0.25 mm × 30 m) and a temperature program of 140–220 °C at 2 °C/min. GLC on the per-*O*-trimethylsilyl methyl glycosides was performed on a GC-8000 system using a CP-Sil-5CB fused-silica column (0.25 mm × 50 m) and a temperature program of 120–240 °C at 2 °C/min.

*Constituent analysis.*—Methanolysis was performed in 2 M MeOH–HCl at 100 °C for 4 h, and the resulting methylglycosides were N-acetylated and converted to the corresponding trimethylsilyl derivatives as described by Montreuil et al. [13]. The absolute configuration of the sugars was determined as devised by Gerwig et al. [6].

*Methylation analysis.*—Glycosyl-linkage positions were determined using a modification of the Hakomori procedure [14] using the lithium dimethylsulfinyl anion [15,16] and MeI in $Me_2SO$. The methylated compounds were recovered by use of SepPak $C_{18}$ cartridges (Millipore) [17]. Reduction of ester groups with Super-Deuteride™ [$LiB(C_2H_5)_3D$] was carried out according to the procedure of York et al. [18]. The methylated product was then hydrolyzed in 2 M TFA (2 h, 120 °C), reduced at rt with 1 M $NH_4OH$ soln containing 10 mg/mL of $NaBD_4$, and acetylated with $Ac_2O$ and 1-methylimidazole [18].

*NMR studies.*—NMR spectra were recorded at 325 K on solution of polysaccharide in $D_2O$ on Bruker DRX-400 and AMX 500 spectrometers using UXNMR software. $^1H-^1H$ correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) with a mixing time of 80 ms, and heteronuclear multiple quantum coherence (HMQC) were employed to assign signals and were performed according to standard pulse sequences. For inter-residue correlation, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment, with a mixing time of 350 ms, and a heteronuclear multiple bond correlation (HMBC), with a delay of 60 ms, were used. $^1H$ and $^{13}C$ NMR chemical shifts were expressed in ppm relative to sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-$d_4$.

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