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Structural studies of the main exopolysaccharide produced by the deep-sea bacterium Alteromonas infernus

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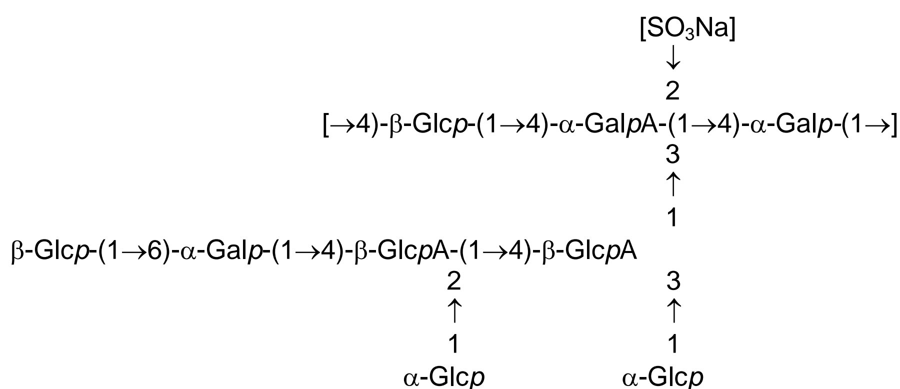
Abstract : The structure of the extracellular polysaccharide (EPS) produced by the mesophilic strain, *Alteromonas infernus*, found in deep-sea hydrothermal vents and grown under laboratory conditions, has been investigated using partial depolymerization, methylation analysis, mass spectrometry and NMR spectroscopy. This polysaccharide consists of a branched nonasaccharide repeating unit with the following structure:



***Keywords**: Alteromonas*; bacterial polysaccharide; structure; Hydrothermal vent.

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1. Introduction

Marine microorganisms, present near deep-sea hydrothermal vents, have been studied for several years because they grow in very exceptional conditions characterized by extreme pressures and temperature and high concentration of toxic elements. Several bacteria (e.g. *Alteromonas macleodii* subsp. *fijiensis* and *Vibrio diabolicus*) found in such environments produce extracellular polymers with original structures when they grow in an aerobic carbohydrate-based medium [1,2]. Such polysaccharides were first studied for their rheological properties or metal complexing abilities [3,4]. However, for several years, interest has developed in looking for the biological activities (especially heparin-like) of microbial polysaccharides. In this context, we have started to study the polysaccharide produced by *Alteromonas infernus*, a new species of bacterium [5] classified as a non-pathogenic microorganism by the Institut Pasteur. It secretes a water-soluble acidic heteropolysaccharide consisting mainly of glucose, galactose, glucuronic and galacturonic acids with variable quantities of rhamnose. This high-molecular-weight polysaccharide (10^6 g/mol) differs in both its monosaccharide and sulfate contents (10%) from other EPS produced by deep-sea hydrothermal bacteria and from polysaccharides of other origins. The native EPS secreted by *A. infernus* is without anticoagulant activity. Nevertheless, several oversulfated low-molecular-weight EPS fractions (sulfated LMW-EPS), with uronic acid and sulfate contents comparable to those of heparin, have been prepared by partial hydrolysis and chemical sulfation [6]; some of these fractions displayed interesting anticoagulant activity [7]. They were less efficient than low-molecular-weight heparin and unfractionated heparin in activated partial thromboplastin time (APTT) assays (2.5 and 6.5 times, respectively), but more efficient than LMW dermatan sulfate [8]. Affinity co-electrophoresis experiments performed in the presence of antithrombin or heparin cofactor II showed that all chains of sulfated LMW-EPS strongly bound antithrombin while only a subpopulation did the same with heparin cofactor II [7]. This latter result strongly suggests that the anticoagulant activity is modulated not only by the sulfate content, but also by the position of sulfate along the polysaccharide chain.

In a previous paper [7], only some structural data were described. They were obtained mainly by methylation analysis (with or without reduction) of the crude EPS. They indicated the presence of 11 different residues and of three side chains terminated by a Glc*p* or a Gal*p*, the branched residues being a 3,4-disubstituted GalA*p*, a 3,4-disubstituted

GlcA*p*, and a 2,4-disubstituted GlcA*p*. The present report provides the complete structural determination of the *Alteromonas infernus* EPS.

2. Results and discussion

2-1 Native EPS. A crude preparation of EPS was obtained by precipitation and ultrafiltration as previously described by [6]. Composition and methylation analyses (see Table 1) gave the same results as those previously reported. NMR spectra of the native EPS displayed very poor resolution owing to its too high MW. Moreover, NMR studies were carried out on two fractions obtained by partial hydrolysis: one prepared (LMW-EPS) in very mild conditions to avoid too extensive modification, and another one (OF) constituted by small oligosaccharides resistant to stronger hydrolysis conditions.

Table 1. Analysis of methylated and carboxyl reduced polysaccharide alditol acetates derived from alkylation of *Alteromonas infernus* strain exopolysaccharide (native EPS), oligosaccharide fraction (fraction OF) and very partially hydrolyzed EPS (LMW-EPS).

Alkylated sugars (as alditol acetates)	t_R [a]	Detector response (%)			
		Native EPS	Fraction OF	LMW-EPS	
2,4,6-Me_3Rha [c]	0.69	8.1	tr	tr	→3)-Rha*p*-(1→
2,3,4,6-Me_4Glc	0.71	19.3	22	21	Glc*p*-(1→
2,3,4,6-Me_4Gal	0.73	8.0	0	7	Gal*p*-(1→
2,3,6-Me_3Gal	0.79	14.3	8	7	→4)-Gal*p*-(1→
2,3,6-Me_3Glc	0.80	10.3	0	8	→4)-Glc*p*-(1→
2,3,4-Me_3Glc-*6*-d_2 [d]	0.82	4.0	29	0	Glc*pA*-(1→
2,3,4-Me_3Gal	0.85	6.7	0	2	→6)-Gal*p*-(1→
2,3-Me_2Glc-*6*-d_2	0.9	10.3	22	9	→4)-Glc*p*A-(1→
2,4-Me_2Glc-*6*-d_2	0.93	0	4	0	→3)-Gal*p*A-(1→
2-MeGlc-*6*-d_2	0.95	5.3	0	8	→3,4)-Glc*p*A-(1→
2-MeGal-*6*-d_2	0.965	4.0	2	9	→3,4)-Gal*p*A-(1→
3-MeGlc-*6*-d_2	0.97	4.1	0	4	→2,4)-Glc*p*A-(1→

[a] t_R = Retention time for the corresponding alditol acetate relative to that of *myo*-inositol hexaacetate (t_R=1.000).
[b] 2,4,6-Me_3Rha = 1,3,5-tri-*O*-acety-2,4,6-tri-*O*-methyl-rhamnitol, ect...
[c] 2,3,4-Me_3Glc-*6*-d_2 = 1,5,6-tri-*O*-acetyl-6,6-dideutero-2,3,4-di-*O*-methylglucitol.

2-2 Preparation, purification and structure determination of the oligosaccharide fraction (OF). A fraction containing only small oligosaccharides (called OF in the following) was prepared by acid hydrolysis followed by ultrafiltration through a 1000 D membrane. An aliquot of this fraction was also reduced by $NaBH_4$ (rOF). PMAA analysis of OF (see Table 1**Erreur ! Source du renvoi introuvable.**) showed the presence of six different residues, three being much more important corresponding to unsubstituted Glc*p*, unsubstituted Glc*p*A and 4-substituted Glc*p*A. The three others were due to 3,4-disubstituted Gal*p*A, 3-substituted Gal*p*A, and 4-substituted Gal*p*. OF did contain only traces of Rha residue.

Table 2.
Negative-ion mode ESI-MS analysis of the $NaBH_4$ reduced oligosaccharide fraction (rOF)

Oligosaccharide	m/z, assignment			
	$[M-H]^-$	$[M-2H+Na]^-$	$[M-2H]^{2-}/2$	$[M-3H+Na]^{2-}/2$
Sulfated pentasaccharide	951	973	475	486
Pentasaccharide	871	893	435	446
Sulfated tetrasaccharide	789	-	-	405
Tetrasaccharide	709	731	-	365

ESI-MS studies of the reduced oligosaccharide fraction (rOF). The rOF fraction was then analyzed by mass spectrometry with electrospray ionization (ESI-MS) in negative mode. The results (Table 2) established the presence of four different oligosaccharides. The peaks at m/z 951 and 973 (sodium form) corresponded to a mono-sulfated reduced pentasaccharide containing two neutral hexose residues and three uronic acids. Double charge ions were also detected (m/z 475 and 486). A second series of peaks with 80 (m/z 871 and 893) or 40 Da less (m/z 435, 446 for double charged ions) corresponded to an unsulfated pentasaccharide. The mass difference between the sulfated pentasaccharide and the ion m/z at 789 was 162. This represents a neutral dehydrated hexose (180-18) such as glucose or galactose. Consequently, this oligosaccharide corresponded to a reduced sulfated tetrasaccharide. The analogous unsulfated tetrasaccharide appeared at m/z 709. MS/MS

experiments were performed on the most abundant ion (m/z 871, unsulfated pentasaccharide). The MS/MS spectrum and the fragmentation pattern are shown in Fig. 1.

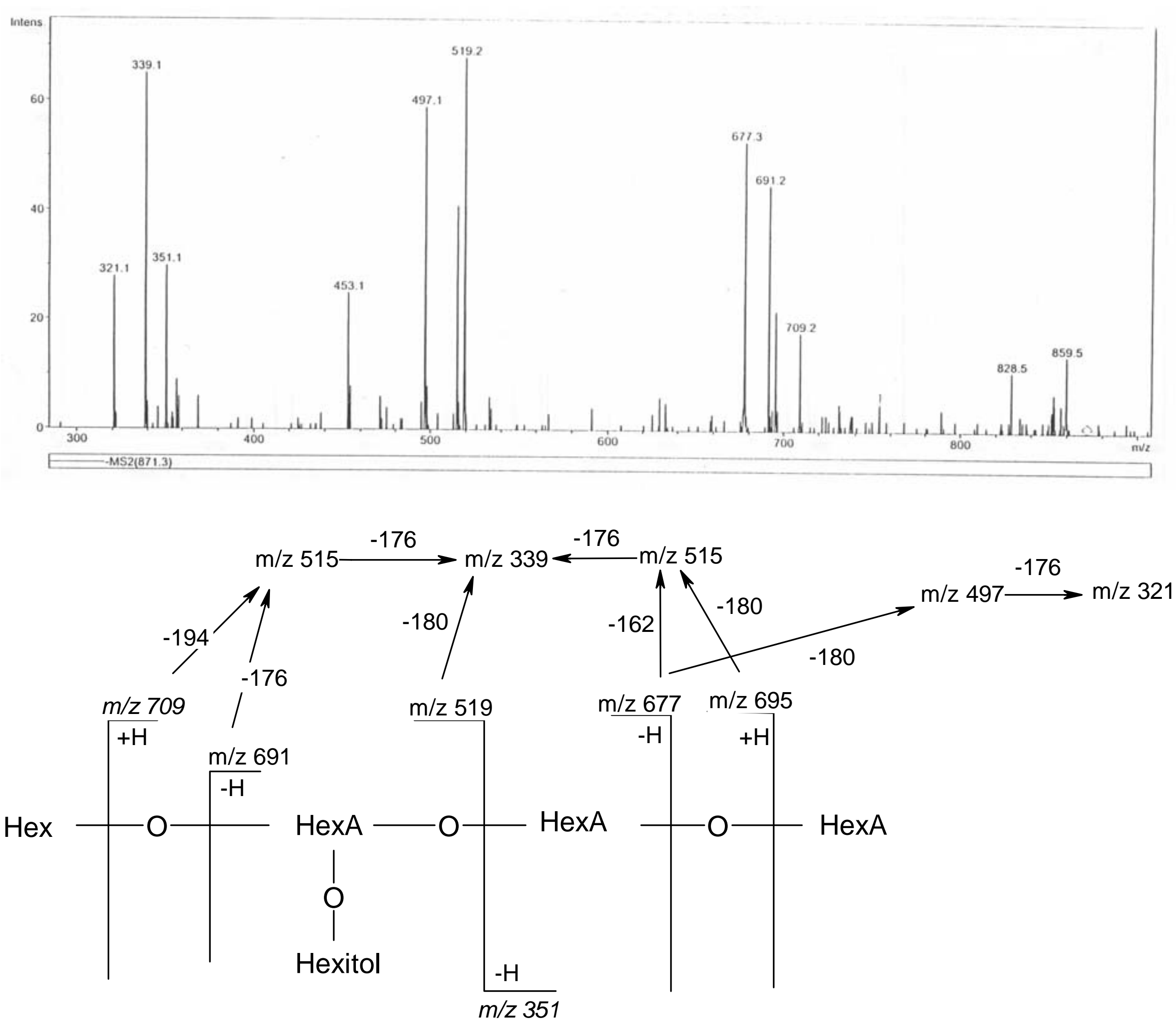


Fig. 1. MS/MS spectrum (above) and fragmentation pattern (below) of the precursor ion m/z 871 $[M-H]^-$. This ion was the most important observed in ESI-MS of the reduced fraction rOF in negative mode. Abbreviations are Hex for Glc*p* or Gal*p*, HexA for GlcA or GalA while hexitol is the reduced terminal residue.

The results indicated the presence of a disaccharide composed of two uronic acid residues at one non-reductive position and a hexose at the other one. In conclusion, combining these results with those deduced from the PMAA experiments, the partial structure of the reduced unsulfated pentasaccharide is: [Glc*p*A-(1→4)-Glc*p*A-(1→3)[Glc-(1→4)]-Gal*p*A-(1→?)-Hexitol]. The loss of the terminal Glc-(1→4) residue gave the tetrasaccharides.

NMR spectral studies of OF. In the anomeric region of the ^{1}H NMR spectrum (Fig. 2), several peaks appeared between 4.5 and 5.32 ppm, but some were not due to anomeric proton, as shown by the HSQC spectrum (Fig. 3) which contained cross-peaks outside (for the ^{13}C) the region of anomeric signals.

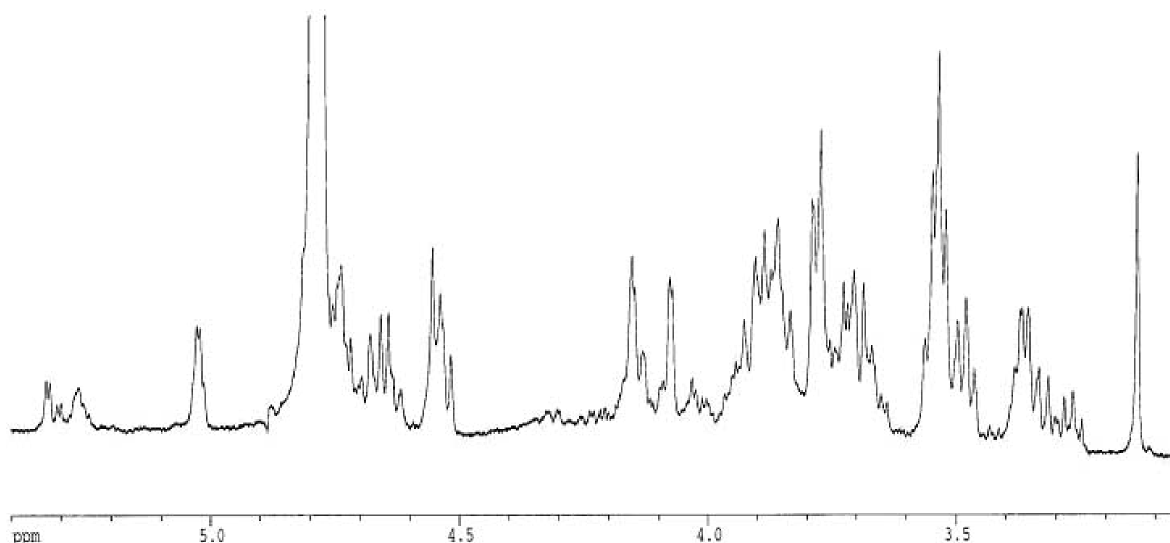


Fig. 2. 500 MHz ^{1}H NMR spectrum of fraction OF recorded at 325°K in deuterium oxide.

The HSQC spectrum displayed seven H-1/C-1 cross-peaks corresponding to nine different residues: five α-linked residues at δ 5.32/95.1; 5.26/101.0; 5.26/101.1; 5.03/102.4 (two residues); and four β-linked residues at δ ≈4.75/106.3; ≈4.73/106; 4.65/99.3 and 4.52/105.3. The residues are labeled **a-i** in this order. A combination of COSY, TOCSY and HSQC was performed to assign almost all ^{1}H and ^{13}C resonances of each sugar residue (Table 3). Residues **a** and **h** occupied the reducing end (in α and β form, respectively) as expected from the chemical shifts of the C-1 and H-1. The *galacto* series of both residues were in complete agreement with the downfield positions of the H-2 (at δ 3.87 and 3.56), H-3 (at δ 3.95 and 3.73) and H-4 (at δ 4.14 and 4.04) respectively. The chemical shift of C-4 (δ 81 and δ 79.2) indicated that **a** and **h** were 4-substituted. Both series of signals disappeared with $NaBH_4$ reduction, which confirmed these attributions.

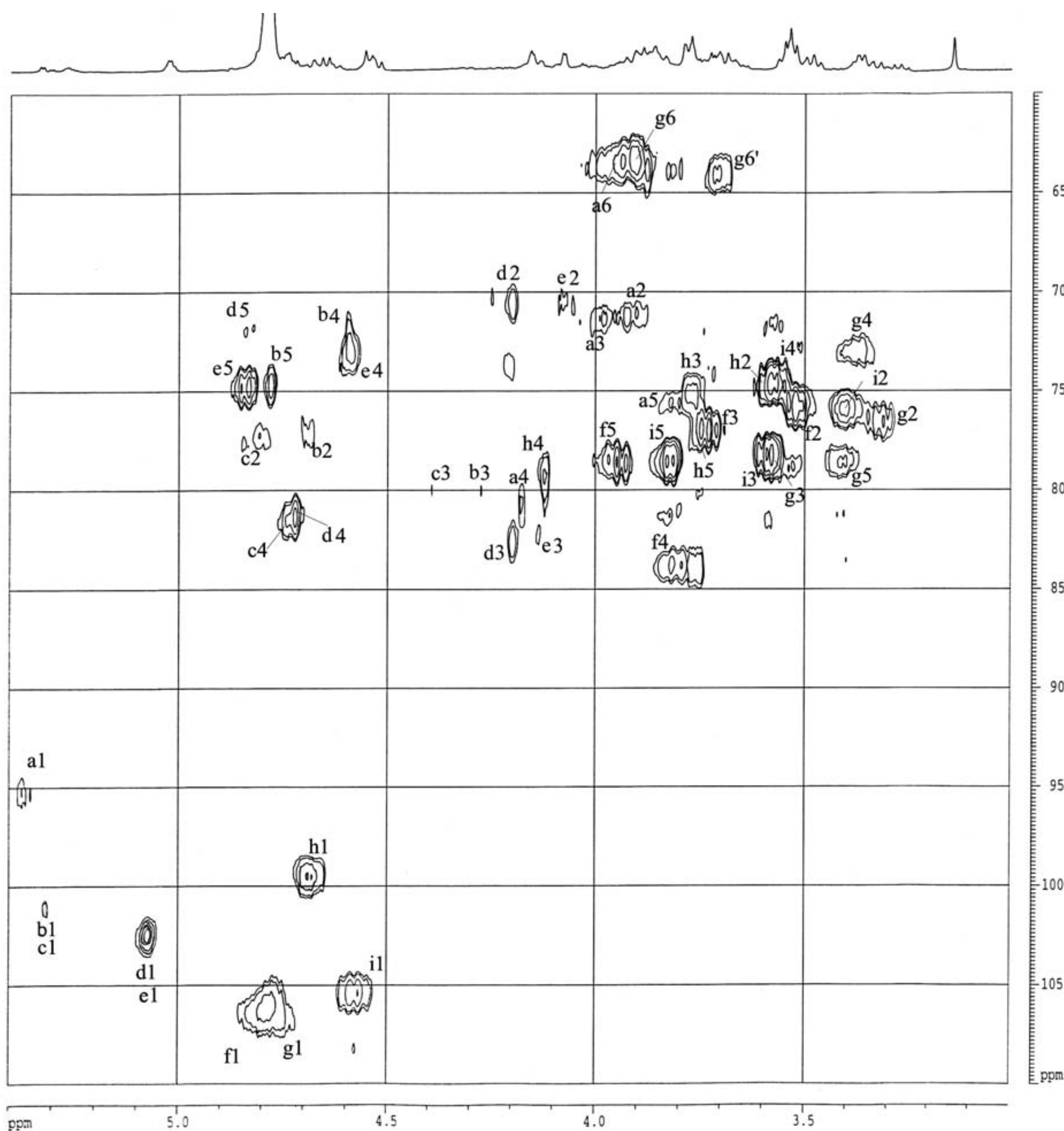


Fig. 3. 500 MHz HSQC spectrum of oligosaccharides (OF fraction) recorded at 325°K in deuterium oxide.

Residues **b** and **c** showed downfield chemical shifts of H-1 (δ 5.26) as expected for sugars of the α-configuration. The H-3/H-4 cross-peaks, in the COSY spectrum, displayed the characteristic shape of *galacto* residues (due to the low coupling constants of H-4 with H-3 and H-5). The H-4 chemical shifts at δ 4.56 and 4.69 respectively showed that **b** and **c** were galacturonic acid residues [1]. The C-3 at δ 80.0 for **b** and **c** and C-4 at δ 81.5 for **c** indicated that residue **b** was 3-substituted and residue **c** was 3,4-disubstituted. The particularly downfield chemical shifts of H-2 at δ 4.64 for **b** and δ 4.75 for **c** established that the positions-2 of these residues bore a sulfate group. Consequently, **b** and **c** were

[→3)-α-Gal*p*A-(2SO_3^-)-(1→ and [→3,4)-α-Gal*p*A-(2SO_3^-)-(1→], respectively. Residues **d** and **e** were very similar to residues **c** and **b** respectively except for the H-2. The H-2 resonances at δ 4.17 (**d**) and 4.03 (**e**) indicated that no sulfate group was present on those residues. Residue **d** was identified as a 3,4-disubstituted-α-D-Gal*p*A and residue **e** as a 3-monosubstituted-α-D-Gal*p*A. In fact, **b**, **d** and **e** were the various possible residues generated by partial hydrolysis from the same initial unit: **c**.

Table 3. Chemical shifts (δ, ppm) of ^{1}H and ^{13}C for the oligosaccharide fraction (OF).

Residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6	6'
a	5.32	3.87	3.95	4.14			
4→)-α-Gal*p*	95.1	71.3	73.8	81.0			
b	5.26	4.64	4.23	4.56	4.75		
→3)-α-Gal*p*A-(2SO_3^-)-(1→	101.0	77. 6	80.0	73.3	74.6		
c	5.26	4.75	4.30	4.69			
→3,4)-α-Gal*p*A-(2SO_3^-)-(1→	101.1	77.6	80.0	81.5			
d	5.03	4.17	4.18	4.68	4.83		
→3,4)-α-Gal*p*A-(1→	102.4	70.7	82.6	81.3	71		
e	5.03	4.03	4.13	4.52	4.84		
→3)-α-Gal*p*A-(1→	102.4	70.7	82.4	73.3	75		
f	~4.75	3.46	3.66	3.77	3.88		
→4)-β-Glc*p*A-(1→	106.3	76.0	77.3	84.0	78.8	178.1	
g	4.73	3.25	3.52	3.31	3.35	3.82	3.59
β-Glc*p*-(1→	106.0	76.8	78.5	73.0	78.5	64.0	64.0
h	4.65	3.56	3.73	4.11			
→4)-β-Gal*p*	99.3	74.8	75.7	79.2			
i	4.52	3.34	3.52	3.50	3.76		
β-Glc*p*A-(1→	105.3	75.9	78.5	74.7	78.8	178.6	

The *β-gluco* configuration attributed to the residues **f**, **g** and **i**, was in complete agreement with the upfield position of H-1 at δ 4.75, 4.73, 4.52 and H-2 signals δ 3.46,

3.25, 3.34, respectively. The chemical shift of the C-4 δ 84.0 for **f** indicated that this residue was 4-substituted, and consequently corresponded to the [→4)-β-Glc*p*A-(1→] residue identified by PMAA analysis. An intra-residue correlation observed in the HMBC spectrum between proton H-4 δ 3.77 of **f** and downfield carbon δ 178.1 confirmed that **f** was the 4-substituted-β-Glc*p*A. A similar correlation between H-4 (δ 3.5) of residue **i** and downfield carbon δ 178.6 showed it was the terminal β-Glc*p*A. The last residue **g** was necessarily identified as a terminal β-D-Glc*p*.

Glycosyl sequence information was obtained from ROESY and HMBC experiments. The HMBC spectrum showed an inter-residue correlation between H-1 of **g** (δ 4.73) and residue C-4 of **d** (δ 81.3) which demonstrated that **g** was linked to the 4-position of **d**. Correlation between H-1 of **f** (δ 4.75) and C-3 of **d** or **e** (δ 82.6; 82.4) confirmed the presence of the sequence [→4)-β-Glc*p*A-(1→3)-α-Gal*p*A-(1→]. The correlation between the anomeric proton and carbon of **i** and the C-4 and H-4 of **f,** respectively, indicated that **i** was linked to the 4-position of **f**. Correlations between H-1 (δ 5.03) of **d** and/or **e** and the C-4 of **h** confirmed the link between **d** or **e** and **h** [α-Gal*p*A-(1→4)-Gal*p*]. These results were confirmed by the ROESY correlations. Inter-residue correlations between H-3 of **d** and H-1 of **f** (δ 4.75) indicated that **f** was linked to the 3-position of the α-Gal*p*A ressidue. The correlations between H-1 of **i** and H-4 of **f** proved that **i** was linked to the 4-position of **f**. Therefore, the structures of the tetrasaccharides and the pentasaccharides were identified as:

```
                         R
                         ↓
g                        2  c, d           a/h
β-Glcp-(1→4)-α-GalpA-(1→4)-α/βGalp
                         3
                         ↑
                         1
                     β-GlcpA   f
                         4
                         ↑
                         1
                     β-GlcpA   i
```

Pentasaccharide

c) R=SO_3^-

d) R=H

```
          R
          ↓
          2  b, e        a/h
α-GalpA-(1→4)-α/βGalp
          3
          ↑
          1
      β-GlcpA   f
          4
          ↑
          1
      β-GlcpA   i
```

Tetrasaccharide

b) R= SO_3^-

e) R=H

The NMR data are in good agreement with those reported for a similar EPS produced by another deep-sea bacterium: *Alteromonas macleodii* [1]. It should be underlined that the

PMAAs corresponding to the [→3,4)-Gal*p*A] and [→3,4)-α-Gal*p*A-(2SO_3^-)-(1→] were very low or totally absent, respectively. These residues were probably destroyed (in part or totally), as well as the terminal reductive sugar, in the basic conditions used for the methylation.

2-*3 Preparation and structure determination of a low-molecular-weight EPS.*

Preparation, composition and methylation analysis. A low-molecular-weight EPS was obtained from the native EPS by very mild acid hydrolysis. The hydrolysate was first purified by ultrafiltration and then by size-exclusion chromatography of the retentate. A relatively homogeneous fraction of quite high mass (22 600 g/mol) with a polydispersity of 3.5, measured by high-performance size-exclusion chromatography (HPSEC) using pullulanes as standards, was selected for further study. It is called LMW-EPS in the following. GLC analysis of the per-*O*-trimethylsilylated methyl glycosides revealed that this fraction was composed of glucose, galactose, glucuronic acid and galacturonic acid with only some traces of rhamnose. 10% of sulfate (SO_3Na) was also present. Rhamnose was identified in several other fractions representing only a tiny quantity of the eluted material (results not shown). This confirmed that the crude EPS contained two polysaccharides, inseparable in the native form. Methylation analysis of LMW-EPS indicated the presence of ten different residues (Table 1) : glucose (accounting for at least two residues according to the peak intensity) and galactose in terminal position, four mono-substituted and three di-substituted residues [→2,4)-β-Glc*p*A-(1→]; [→3,4)-β-Glc*p*A-(1→] and [→3,4)-α-Gal*p*A-(1→]. This showed the existence of three ramifications in the repeating unit.

NMR studies of LMW-EPS. The ^{1}H NMR spectrum of fraction LMW-EPS (Fig. 4) showed clearly six different low-field signals between 5.1 and 5.6 ppm and an unresolved signal (4.5-4.9 ppm, accounting for 7 protons) corresponding to H1 of α- and β-linked sugars, respectively. The HSQC spectrum clearly allowed the identification of anomeric carbons corresponding to α-anomeric residues (δ 95-110). These residues were named **A** (δ ~5.53/100.8), **B** (δ 5.40/102.9), **C** (δ 5.32/102.1), **D** (δ 5.26/101.2), **E** (δ 5.24/101.6) and **F** (δ 5.20/102.8). However, in the same spectrum for the 4.5-4.9 cluster, only four H-1/C-1 cross-peaks corresponded to β-anomeric positions, which indicated that 3 protons in the region between 4.5 and 4.9 were not anomeric. The β-residues were named **K** (δ 4.73/105.4), **L** (δ 4.64/103.2), **M** (δ 4.66/102.9) and **N** (δ 4.67/107.8).

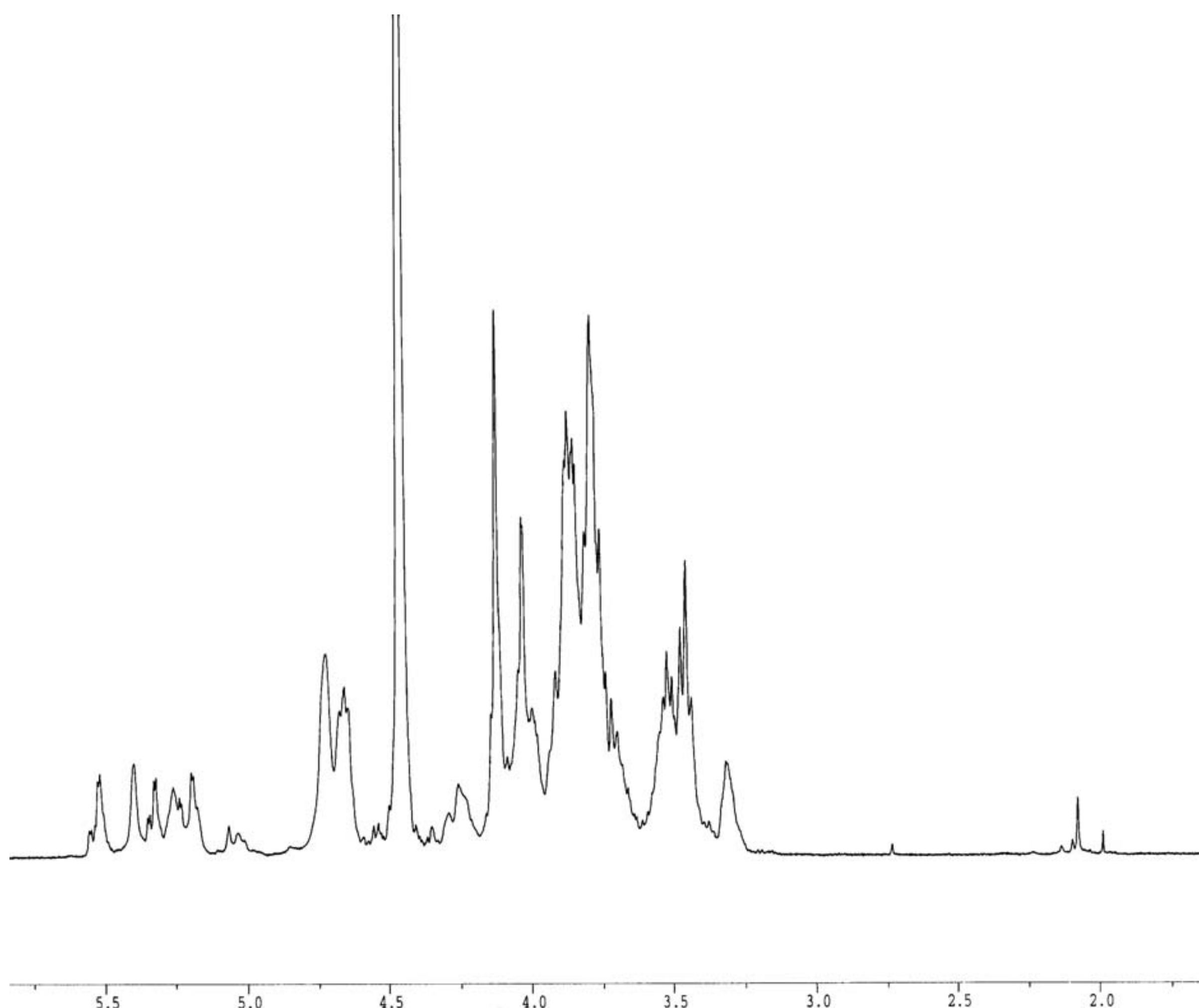


Fig. 4. 500 MHz ^{1}H NMR spectrum of fraction LMW-EPS recorded at 325°K in deuterium oxide.

Most of the signals were attributed by a simultaneous interpretation of COSY, TOCSY, ROESY, HSQC and HMBC spectra. The chemical shifts are given in Table 4. In the ^{1}H NMR spectrum, the H-1 of **A** resulted from the superposition of 3 doublets at least. However, the TOCSY spectrum (Fig. 5) showed that all the H-2, H-3, H-4 and H-5 displayed exactly the same chemical shifts. The α-galacto configuration was deduced from the downfield resonance of H-2 and H-3. Carbon chemical shifts were consistent with no substitution at any of the 2,3,4 positions. Residue **B** was also identified as a α-Gal*p* residue, but the C-4 at δ 81.8 indicated that it was 4-substituted. Residue **D** was very similar to residue **c** previously described in oligosaccharides (see section 2,2) and characterized as a 3,4-disubstituted-α-D-Gal*p*A-[2-SO_3Na]. Chemical shifts of H-2, H-3, H-4 and H-5 of residues **C**, **E** and **F** were very close (see TOCSY spectrum) and in agreement with their assignment as residues with an α-*gluco* configuration. In addition, H-1 of **C** and **F** appeared

as two doublets, which meant that **C** and **F** were settled in two different, but very similar, environments. On the other hand, H-1 of **E** accounted for less than one proton. For all of these residues (**C**, **E** and **F**) no downfield location of carbon was detected so they were identified as terminal α-Glc*p*.

Table 4. Chemical shifts (δ, ppm) of ^{1}H and ^{13}C for the LMW-EPS fraction.

Residue	$^{1}H/^{13}C$					
	1	2	3	4	5	6
A 6→)-α-Gal*p*-(1→	5.49 to 5.57 100.8	3.79 71.3[1]	3.88 71.5	4.03 72.3	4.12 73.5	4.12 67.1
B →4)-α-Gal*p*-(1→	5.40 102.8	3.86 71.8	3.93 71.5	4.13 81.8	4.67?	- -
C α-Glc*p*-(1→	5.32 102.1	3.50 75.0[2]	3.74 75.6	3.46 72.2[2]	4.00 74.7	- -
D →3,4)-α-Gal*p*A-(2SO_3^-)-(1→	5.26 101.2	4.73 76.9	4.25 80.0	4.68 81.5	~4.7 74.6	177.6
E (C) α-Glc*p*-(1→	5.24 101.6	3.52 -	3.77 -	3.45 -	3.99 -	- -
F α- Glc*p*-(1→	5.20 102.8	3.47 74.8[2]	3.78 76.1[1]	3.47 72.2[2]	4.00 74.7	- -
K 4→)-β-Glc*p*-(1→	4.73 105.4	3.31 76.1	3.78 76.1	3.55 80.5	3.48 74.9[2]	-
L β-Glc*p*-(1→	4.64 103.2	3.33 76.1	3.78 -	3.86 -	- -	- -
M 2,4→)-β-Glc*p*A-(1→	4.66 102.9	3.44 82.8	3.76 ~79	3.86 ~79	- -	- 180.0
N 3,4→)-β-Glc*p*A-(1→	4.67 106.8	3.53 ~75	3.78 83.0	4.05 80.5	3.91 79.7	- 180.0

[1] These attributions could be interchanged.
[2] Idem.

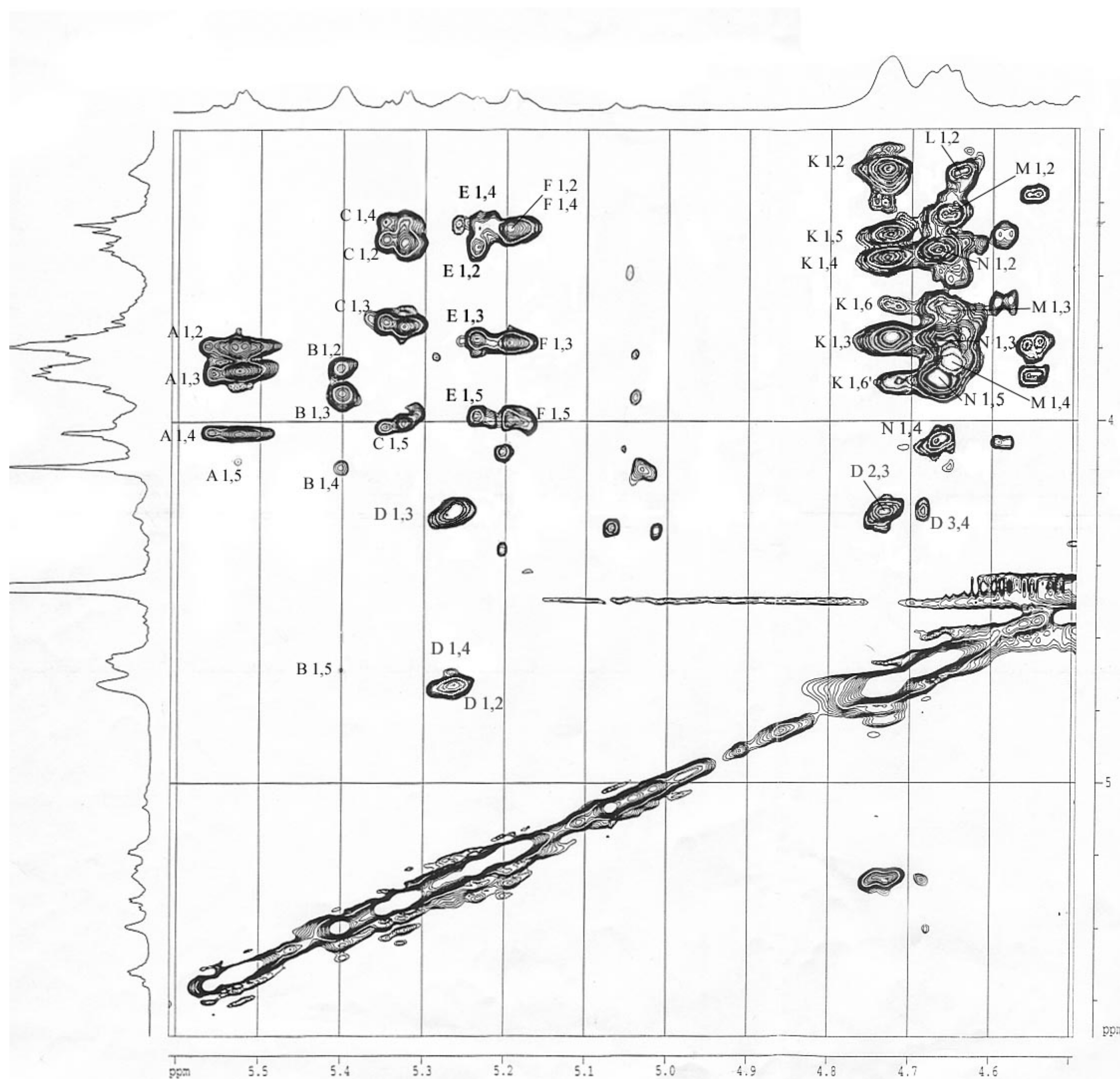


Fig. 5. 500 MHz TOCSY spectrum (anomeric region) of LMW-EPS recorded at 325°K in deuterium oxide.

Chemical shifts of H-1, H-2 and H-3 of residues **K** and **L** were very similar and consistent with a *β-gluc*o configuration. The C-4 of **K** (δ 80.5) indicated that this residue was a 4-substituted-Glc*p*. The signal of the H-1 of **L** (δ 4.64) was close to H-1 of **M** (δ 4.66) and **N** (δ 4.67) and consequently impossible to estimate, but the integration of H-2 of **L** and **K** together represented 1.5 protons, consequently **L** was a residue partially eliminated during the hydrolysis step. Cross-peaks identified in the HMBC spectrum, between H-1 of residues **M** and **N** and high-field signals (δ 180.0), indicated that these residues were β-glucuronic acids. **M** and **N** could, therefore, be assigned respectively to the [→2,4)-Glc*p*A] and the [→3,4)-Glc*p*A] as supported by the downfield positions of C-2 (δ 82.8) and C-4 (δ 79) of **M** and C-3 (δ 83.0) and C-4 (δ 80.5) of **N**.

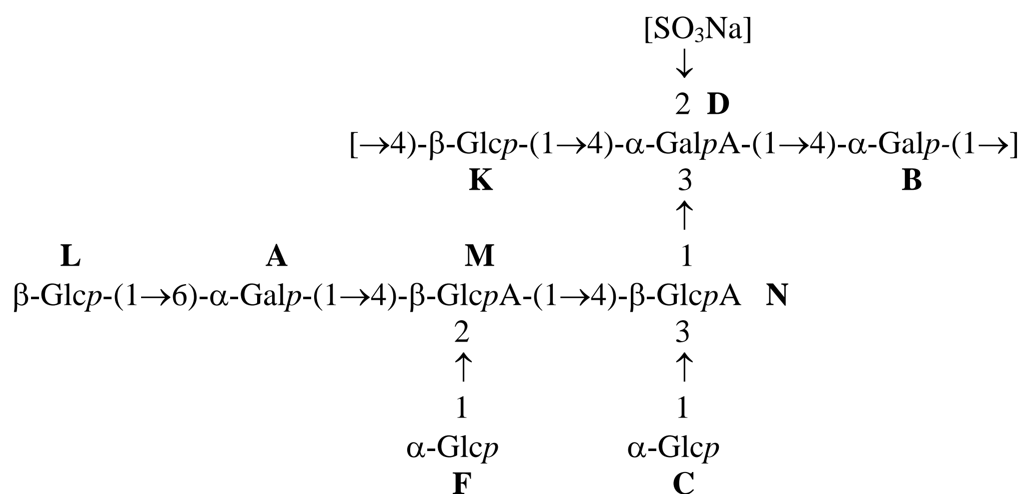
Table 5. Correlations observed in the ROESY and HMBC spectra for the LMW-EPS fraction, recorded at 325°K in deuterium oxide

Residue (anomeric proton δ_H)	**linkage**	**Correlations ^{1}H/^{1}H** (ROESY) δ_H ppm		**Long range Correlations ^{1}H/^{13}C** (HMBC) δ_C ppm	
A (δ 5.53)	**A**(1→4)**M**	3.79	**A**, H-2 and **M**, H-3	71.3	**A**, C-2
		3.86	**M**, H-4		
				78.9	**M**, C-4
B (δ 5.40)	**B**(1→4)**K**	3.86	**B**, H-2	71.8	**B**, C-3
		3.93	**B**, H-3		
		3.48	**K**, H-5	80.5	**K**, C-4
		3.55	**K**, H-4		
		3.78	**K**, H-3		
C (δ 5.32)	**C**(1→3)**N**	3.50	**C**, H-2 and **N**, H-2	75.6	**C**, C-3
		3.78	**N**, H-3	74.7	**C**, C-5
		3.91	**N**, H-5		
		4.05	**N**, H-4	83.0	**N**, C-3
D (δ 5.26)	**D**(1→4)**B**	4.73	**D**, H-2	81.8	**B**, C-4
		3.93	**B**, H-3		
		4.13	**B**, H-4		
F (δ 5.20)	**F**(1→2)**M**	3.44	**M**, H-2	76.1	**F**, C-3
		3.77	**F**, H-3 and **M**, H-3	74.8	**F**, C-2 or C-5
		3.86	**M**, H-4		
				82.8	**M**, C-2
K (δ 4.73)	**K**(1→4)**D**	3.31	**K**, H-2	81.5	**D**, C-4
		3.48	**K**, H-5		
		3.78	**K**, H-3		
		4.25	**D**, H-3		
		4.68	**D**, H-4		
M (δ 4.66)	**M**(1→4)**N**	3.44	**M**, H-2	80.5	**N**, C-4
		3.78	**N**, H-3		
		4.05	**N**, H-4		
N (δ 4.67)	**N**(1→3)**D**	3.53	**N**, H-2	80.0	**D**, C-3
		3.91	**N**, H-5		
		4.25	**D**, H-3		

The glycosyl sequence was obtained from HMBC and ROESY (Table 5) experiments and by asserting that the sulfated pentasaccharide is also constitutive of the LMW-EPS. In the HMBC spectrum, a correlation was observed between C-4 of **B** (δ 81.8) and the proton H-1 of **D** (δ 5.26) as well as between C-1 of **D** and H-4 of **B** (δ 4.12) which showed a linkage between **B** and **D** according to the [→3,4)-α-Gal*p*A-[2-SO_3^-]-(1→4)-α-Gal*p*-(1→] sequence previously identified in the pentasaccharide. The HMBC spectrum also showed

one correlation between the H-1 of **K** (δ 4.73) and C-4 of **D** (δ 81.5) and between C-1 of **K** and H-4 of **D,** establishing the α-(1→4) linkage between **K** and **D**. Two others between C-4 of **K** and H-1 of **B** and between H-4 of **K** and C-1 of **B** were also observed. Consequently, the main chain of this EPS was constituted by the trisaccharide **K-D-B** (**g-c-a** in the pentasaccharide). From the structure of the pentasaccharide, the positions of β-Glc*p*A were easily deduced. In addition, a weak but conspicuous cross-peak (**N**, C-1 to **D**, H-3) indicated that **N** was the first residue of the side chain (**f** in the pentasaccharide), while **M** was the second one, because of the connectivity: **M**, C-1 to **N**, H-4. The reversed connectivities were also observed (**N**, H-1 to **D**, C-3 and **M**, H-1 to **N**, C-4) but were not discriminant because H-1 chemical shifts of both **M** and **N** were too close (δ 4.66 and 4.67). The positions of the last four residues (**C**, **F**, **A** and **L**) had to be determined. Correlations between H-1 of **C** (δ 5.32) and C-3 of **N** (δ 83.0) as well as between H-1 of **F** (δ 5.20) and C-2 of **M** (δ 82.8) indicated linkages between **N** and **C** (terminal α-Glc*p*) on the one hand and between **M** and **F** (terminal α-Glc*p*) on the other hand. The following connectivities (**C**, C-1 (δ 101.6) to **N**, H-3 (δ 3.78) and **F**, C-1 (δ 102.8) to **M**, H-2 (δ 3.44) corroborated this result, which was in agreement with the presence of [→3,4)-Glc*p*A-(1→] and [→2,4)-Glc*p*A-(→] as deduced from the methylation analysis. The chemical shift of the C-4 of **M** (δ 79) and the correlation with the H-1 of **A** (δ 5.53) indicated a α-(1→4) linkage between **A** and residue **M**. However, the ^{13}C signal at 69.3 ppm was due to a substituted methylene group according to the DEPT ^{13}C spectrum while the corresponding ^{1}H signal (observed in HSQC) was at 4.12 ppm. This proton signal corresponded to H-5 of **A** and H-4 of **B** but also to H-6 of **A**, therefore **A** was 6-substituted. In addition, in the HMBC spectrum, 4.12 ppm protons were connected to C-1 of **D** (see above) and to C-1 of **L** (although weakly). Consequently, the last residue **L**, corresponding to less than one residue (see above), was bound to the position-6 of **A**. **L** was probably easily removed from **A**. Methylation analysis sustained this hypothesis because the [→6)-Gal*p*-(1→] residue was detected in low quantities in LMW-EPS but in higher quantities in the native EPS. Terminal residues (**L**, **C** and **F**) were indeed partially removed, which explained the presence of relatively large quantities of terminal Gal and [→4)-Glc*p*A-(1→] in PMAA analysis and which also generated the already noticed heterogeneity for H-1 of residues **A, C** and **F**. The presence of **E** could be explained in a similar way. Obviously some desulfation also occurred, H-1 of **C** was probably close to the sulfate group of **D** (according to Dreiding model) and consequently appeared at low field (δ 5.32), when the sulfate was removed that generated a

new residue **E** differing from **C** only by its H-1 chemical shift (δ 5.24, see Table 4). An additional evidence showing that **E** was generated from **C** by desulfation of **D** is that **E** was undetectable in LMW-EPS produced by a free radical depolymerization process [7] producing no desulfation. Thus, the structure of the repeating unit of the *Alteromonas infernus* polysaccharide can be written as:



As already noticed, the backbone of this polysaccharide displays some similarities to that described by [1] for the EPS of *A. macleodii*. This is not surprising since both strains belong to the *Alteromonas* genus and were collected in similar environments. At the same time, *A. infernus* EPS differs *inter alia* by its exceptionally crowded GalA and by its very uncommon disubstituted side-branch. The knowledge of its structure will allow this molecule to be modified more rationally in order to prepare well-characterized oligosaccharides as required for medicinal use.

3. Experimental

*3.1. Production, purification and characterization of native exopolysaccharides.*_ The isolation procedure and characteristics of the GY785 strain have previously been reported [5]. EPS was produced and purified as previously described [6]. Monosaccharide content and methylation analyses were performed as described below.

*3.2. Preparation of partially hydrolyzed exopolysaccharides (LMW-EPS).*_ Crude EPS was dissolved in 1 M H_2SO_4 at 5 g/L and heated at 60°C for 90 min. The preparation was then neutralized with a solution of 1 M NaOH, ultrafiltrated (1000 Da cut-off

membrane) to eliminate salts and small oligosaccharides and finally freeze-dried. The polysaccharide (40 mg) was then dissolved in 0.1 M ammonium bicarbonate and applied to a size-exclusion chromatographic column (40 × 4.8 cm) of Sephacryl HR (Pharmacia) and eluted with the same buffer. Detection was performed using a refractometer (Gilson), eluted material was collected in x (nombre) fractions of y (volume) mL. The fraction z was then applied to a cation exchange chromatographic column (45 × 1.5 cm) of Dowex HCR-S/Na^+ form. Elution was performed using water and the polysaccharide fractions were freeze-dried. The molecular weight of this LMW-EPS was determined as previously described in [7].

3.3. Preparation of the oligosaccharide fraction (OF). An oligosaccharide preparation was obtained from the crude EPS dissolved in 1 M H_2SO_4 at 2 g/L and heated at 85°C for 3h30. After cooling at room temperature, calcium carbonate (25.8 g) was added to obtain a final pH of 7 and the solution was filtered. It was then ultrafiltrated (1000 Da cut-off membrane), converted to sodium salt using cation exchange chromatography as described above and finally freeze-dried. An aliquot was reduced by $NaBH_4$

Sulfate content. Elemental analysis was performed by the Central Microanalysis Department of the CNRS (Gif/Yvette, France). Sulfate content (sodium salt) was calculated from sulfur analysis according to the following relation: sulfate group = 3.22×S%.

Constituent analysis. Monosaccharide content was determined after methanolysis by gas chromatography [2]. Methanolysis was performed in 2 M MeOH–HCl at 100°C for 4 h, and the resulting methylglycosides were converted to the corresponding trimethylsilyl derivatives as described by Montreuil *et al.* [9]. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides was performed on a 5890 series II (Hewlett Packard) system with FID detection, using a Chrompack CPSil-5CB fused-silica column (0.25 mm×50 m) and a temperature program of 50-120°C at 20°C/min then 120–240°C at 2°C/min and finally 240-280°C at 10°C/min.

Methylation analysis. Methylation analysis was performed using a modification of the Hakomori procedure [10]. Hydroxyl groups were methylated by methyl iodide in DMSO using lithium dimethylsulfonyl as anion. Methyl esters of uronic acids were reduced by lithium triethylborodeuteride, as previously described [11]. Methylated EPS was then hydrolyzed with 2 M trifluoroacetic acid for 2 h at 120°C. The derivatives were reduced by $NaBD_4$ and acetylated with Ac_2O and 1-methylimidazole prior to analysis by gas

chromatography-mass spectrometry (GC/MS) [1] carried out 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.

Electrospray ionization-mass spectrometry (ESI-MS). ESI-MS was run in negative mode using a high resolution MS/MS spectrometer (Micromass ZABSpecTOF). Analyses were carried out in electron impact (Ei, 70 eV) using aq MeCN and NH_3 as the mobile phase.

NMR studies. NMR spectra were recorded at 325°K on a 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 to 100 ms, and heteronuclear single quantum coherence (HSQC) were employed to assign signals and were performed according to standard pulse sequences. For inter-residue correlation, a two-dimensional Rotating-frame Overhauser Enhancement Spectroscopy (ROESY) 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 2,2,3,3,-tetradeuterio-4,4-dimethyl-4-silapentanoate.

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