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Structural data on a bacterial exopolysaccharide produced by a deep-sea *Alteromonas macleodii* strain

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ABSTRACT

Some marine bacteria collected around deep-sea hydrothermal vents are able to produce, in laboratory conditions, complex and innovative exopolysaccharides. In a previous study, the mesophilic strain *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane was collected on the East Pacific Rise at 2600 m depth. It was isolated from a polychaete annelid *Alvinella pompejana* and is able to synthesise and excrete the exopolysaccharide deepsane. Biological activities have been screened and some protective properties have been established. Deepsane is commercially available in cosmetics under the name of Abyssine® for soothing and reducing irritation of sensitive skin against chemical, mechanical and UVB aggression. This study presents structural data for this original and complex bacterial exopolysaccharide and highlights some structural similarities with other known EPS produced by marine *Alteromonas* strains.

1. Introduction

Exopolysaccharides (EPSs) are produced by various microorganisms, including proteobacteria as well as cyanobacteria and archaea. EPS-producing bacteria are widely present in marine ecosystems and can be isolated from the water column, sediments, animals, etc. Bacteria producing polymers with novel structures and innovative properties have been isolated in atypical environments, including extreme environments (Chi & Fang, 2005; Mancuso-Nichols, Guezennec, & Bowman, 2005). For example, deep-sea hydrothermal vents are areas of active tectonics with diverse physico-chemical characteristics. Thermal gradients allow the development of psychrophilic, mesophilic and thermophilic microorganisms. In addition to these temperature gradients, the hydrothermal environment exposes organisms to high hydrostatic pressure and high concentrations of toxic elements such as heavy metals and sulphides (Cambon-Bonavita, Raguenes, Jean, Vincent, & Guezennec, 2002; Grassle, 1987). Many bacteria living near hydrothermal vents are associated with other organisms (molluscs, worms, shrimp) and some can even produce exopolysaccharides under laboratory conditions. These bacteria primarily belong to genera *Vibrio*, *Alteromonas* or *Pseudoalteromonas* (Cambon-Bonavita et al., 2002; Raguenes, Christen, Guezennec, Pignet, & Barbier, 1997; Raguenes, Peres, et al., 1997; Raguenes et al., 1996; Rougeaux et al., 1999; Vincent et al., 1994).

Marine bacterial exopolysaccharides have various physiological roles: they are involved in responses to environmental stress, in recognition processes and cell-cell interactions, in adherence of biofilms to surfaces (Weiner, Langille, & Quintero, 1995). The relative importance of these roles depends on the environment in which the bacterium is located. In nature, most described functions have a protective role: the presence of a polysaccharide gel layer on the cell surface protects microorganisms from desiccation and predation by protozoa, or can have effects on diffusion properties (Dudman, 1977). For example, the polysaccharide gel layer can make the cell inaccessible to antibiotics. In addition, EPS can trap and concentrate nutrients, help locate and maintain exoenzymes and provide protection against heavy metals and other adsorbable toxins (Decho, 1990). In case of symbiosis, EPSs have several key functions for the bacterial cell and/or its host: they create microenvironments for cell function, metabolism and reproduction. Finally, EPS secretions play an important role in the formation of cellular aggregates and the initiation of flocculation processes (Sutherland, 2002).

Abbreviations: EPS, exopolysaccharide; GLC, gas–liquid chromatography; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple bond quantum coherence; amu, atomic mass units.

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The EPS HYD657, named “deepsane”, is produced and secreted by the strain *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane (Cambon-Bonavita et al., 2002). This strain was collected in 1987, close to a hydrothermal vent located on the East Pacific Rise at 2600 m depth. It was isolated from a polychaete annelid *Alvinella pompejana* (Desbruyeres & Laubier, 1980). Screening of its biological properties has shown that this EPS effectively protects keratinocytes from inflammatory agents, such as interferon gamma (INF-γ). Protective effects have also been demonstrated on Langerhans cells, which are sensitive to ultraviolet attacks and play a major role in the human cutaneous immune defence system (Thibodeau & Takeoka, 2006). Deepsane has already found applications in cosmetics and is commercially available under the name of Abyssine® (patent PCT 94907582-4) for soothing and reducing irritation of sensitive skin against chemical, mechanical and UVB aggression.

Nevertheless, there are currently no data available on the structure of deepsane. This study presents structural data for this original and complex bacterial exopolysaccharide and highlights some structural similarities with other known EPS.

2. Experimental

2.1. Production, isolation and purification of HYD657 exopolysaccharide

EPS HYD657 was extracted from the strain *A. macleodii* subsp. *fijiensis* biovar deepsane as described (Cambon-Bonavita et al., 2002). Briefly, EPS HYD657 was produced in a 20 L fermenter containing marine broth (30 g/L sea salts, 1 g/L yeast extracts, 5 g/L peptone) supplemented with glucose (30 g/L) at 28 °C. The culture medium was inoculated at 10% (v/v) with a bacterial suspension in the exponential growth phase. The pH was adjusted and maintained at 7.2 by automatic addition of NaOH. The medium was oxygenated at 30 L/h with an agitation rate of 200–1100 rpm. After 50 h of fermentation, bacterial cells were removed from the culture medium by centrifugation (15,000 × *g*, 80 min). The supernatant containing the excreted EPS was then purified by filtration through a cellulose membrane (0.7 μm) and then by ultrafiltration (100 kDa) before being freeze-dried and stored at room temperature away from light and moisture.

2.2. Molecular characteristics

2.2.1. Molecular weight

The molecular weight of EPS HYD657 was determined by high-performance size-exclusion chromatography (HPSEC) using a three-angle light scattering detector, coupled with refractive index and specific refractive index increment d*n*/d*c* (MiniDAWN^{TM} TREOS, Wyatt). Elution was performed on a PL-Aquagel-OH column (Varian, fractionation range < 10^7 g/mol) with 0.1 M NH_4OAc as the eluent. To calculate the molecular mass, the d*n*/d*c* value used was 0.145 mL/g. The polydispersity index was calculated from the Mw/Mn ratio.

2.2.2. Sulphate content

Elemental analysis was performed by the CNRS Microanalysis Facility (Gif-sur-Yvette, France). Sulphate content (sodium salt) was calculated from the sulphur analysis with the following equation: sulphate group = 3.2 × *S*%.

2.3. Monosaccharide analysis

The molar ratio of monosaccharides was determined according to Kamerling, Gerwig, Vliegenthart, and Clamp (1975) modified by Montreuil et al. (1986). The exopolysaccharide was hydrolysed by 3 M MeOH/HCl at 100 °C for 4 h, followed by re-N-acetylation with Ac_2O overnight at room temperature. The methyl glycosides were converted to their corresponding trimethylsilyl derivatives. Separation and quantification of the per-*O*-trimethylsilyl methyl glycosides were performed by gas–liquid chromatography (GLC) using a Thermo-Finnigan system equipped with a CP-SIL 5 CB capillary column (0.32 mm × 60 m). The trimethylsilyl derivatives were analysed using the following temperature program: 50 °C for 1 min, 50 °C → 120 °C at 20 °C/min, 120 °C → 250 °C at 2 °C/min, 250 °C for 5 min.

The absolute configuration of the sugar residues was established from native and carboxyl-reduced polysaccharides, by GLC analysis of the derived trimethylsilylated (S)-2-butylglycosides and comparison of their retention times with reference compounds (Gerwig, Kamerling, & Vliegenthart, 1978). The derived butylglycosides were separated using the following temperature program: 50 °C for 1 min, 50 °C → 135 °C at 20 °C/min, 135 °C → 200 °C at 1 °C/min.

2.4. Methylation analysis

Glycosyl-linkage positions were determined as described (Hakomori, 1964). Hydroxyl groups were methylated using the lithium dimethylsulphonyl as the anion and methyl iodide in Me_2SO (Blakeney & Stone, 1985; Kvernheim, 1987). The methylated compounds were recovered using SepPak C18 cartridges (Waeghe, Darvill, Mcneil, & Albersheim, 1983). The permethylated products were carboxyl-reduced by treatment with lithium triethylborodeuteride for 2 h at room temperature (York, Darvill, Mcneil, Stevenson, & Albersheim, 1985). The methylated products were then hydrolysed in 2 M TFA for 2 h at 120 °C, then reduced with $NaBD_4$ in a NH_4OH solution for 30 min at 80 °C, and finally acetylated with Ac_2O/pyr (1:1, v:v) for 30 min at 120 °C. GLC-mass spectrometry (MS) was performed on an Agilent instrument fitted with a CP-SIL 5 CB capillary column (0.25 mm × 60 m). The temperature program was 50 °C for 1 min, 50 °C → 170 °C at 20 °C/min, 170 °C → 240 °C at 4 °C/min, 240 °C for 10 min. Ionisation was carried out in electron impact mode (EI, 70 eV).

2.5. Smith degradation

Oligosaccharides were generated by Smith degradation (Goldstein, 1965). Briefly, 50 mg of exopolysaccharide was oxidised with 50 mL of 50 mM $NaIO_4$ at 4 °C. After 5 days in the dark, the excess of oxidant was destroyed by the addition of ethylene glycol. The oxidised polysaccharide solution was reduced with 10 mL of $NaBH_4$ (15 mg/mL in EtOH:H_2O 9:1, v:v), dialysed against distilled water for 48 h (6000–8000 Mw cut-off) and freeze-dried. 30.5 mg of the reduced product (reaction rate of 61% w/w) was hydrolysed with acetic acid (1.5%) for 2 h at 100 °C, neutralised by co-evaporation with methanol and freeze-dried again. 29.5 mg of the obtained products (reaction rate of 59%, w/w) was fractionated on a Bio-Gel P2 column (Bio-Rad, 1.6 cm × 100 cm) at a flow rate of 0.15 mL/min with 0.1 M NH_4OAc as the eluent.

2.6. Electrospray mass spectrometry

The samples were analysed by negative mode ESI-ITMSn using a LCQ Advantage ion trap mass spectrometer (Thermo Fisher, USA) to get structural information. They were diluted in MeOH/H_2O (50:50, v:v) to a final concentration of 100 μg/mL. Sample introduction was performed at a flow rate of 2.5 μL/min. Nitrogen was used as sheath gas (20 arbitrary units). The MS analyses were carried out using automatic gain control conditions, with a typical needle voltage of 4 kV and a heated capillary temperature of 200 °C. For MSn experiments, collision energies were adjusted between 25 and 35%

(normalised values) for each sample in order to optimise the signal and obtain the maximal amount of structural information from the selected ion. The activation time was set at 30 ms and 20 scans were accumulated for final analysis. The instrument was calibrated using tri-galacturonic acid at 20 ppm.

2.7. NMR spectroscopy

Samples were deuterium-exchanged three times by freeze-drying in 99.99% D_2O. Native EPS HYD657 was ball-milled for 15 min before the deuterium exchanges. NMR experiments were carried out on EPS HYD657 and oligosaccharides at 70 °C and 25 °C, respectively, using a Bruker DRX-500 spectrometer and a Bruker AVANCE 500 spectrometer equipped with an indirect 5 mm triple resonance cryoprobe TCI $^{1}H/^{13}C/^{15}N$ (SD-2 oligosaccharide only). Chemical shifts were expressed in ppm relative to TSP used as a chemical shift reference at 0 ppm. Assignments of signals were based on COSY, TOCSY, HMQC and HMBC experiments. A mixing time of 100 ms was used in the TOCSY experiment.

3. Results

3.1. Native exopolysaccharide

3.1.1. Compositional analysis

Deepsane was obtained by high-speed centrifugation and ultrafiltration of the culture supernatant of *A. macleodii* subsp. *fijiensis* biovar deepsane. HPSEC indicated that deepsane is a high-molecular-weight polymer of 1.1×10^6 g/mol with a polydispersity index of 1.3. Elemental analysis revealed a sulphate content of 7.5% (w/w). The protein amount present in the deepsane sample was found to be only 1% (w/w), indicating that the EPS was relatively pure.

The glycosyl residue composition, determined by GLC analysis of trimethylsilyl derivatives after methanolysis, was determined from five experiments carried out in triplicate and calculated in molar ratios relative to fucose. Deepsane was found to be composed of seven different types of monosaccharides: fucose, rhamnose, glucose, galactose, mannose, glucuronic acid and galacturonic acid at a molar ratio of 1.0:2.5:2.6:5.9:1.4:2.0:1.9. Two additional unidentified peaks were attributed to a substituted sugar with a non-osidic substituent (results not shown).

The absolute configuration of the monosaccharides in the polysaccharide was determined from the formation of trimethylsilylated (S)-2-butyl glycosides and from GLC analysis. Glucose, galactose, mannose, glucuronic acid and galacturonic acid were in the D configuration, whereas fucose and rhamnose residues were in the L configuration.

3.1.2. Methylation analysis

The results of the methylation analysis of deepsane are indicated in molar ratios relative to 3-linked galactopyranosyl (Table 1). Seven different residues were observed and were consistent with the GLC composition analysis. Methylation analysis indicated that there were many different types of terminal, mono- and disubstituted residues, suggesting that deepsane has a complex structure.

The most abundant residues were 3-linked and 4-linked galactopyranosyl, terminal and 3-linked rhamnopyranosyl and 4-linked glucopyranosyl. Uronic acid residues were also identified, such as terminal, 3-linked and especially 4-linked glucopyranosiduronic acid, but 3,4-disubstituted galactopyranosiduronic acid was also observed. The presence of disubstituted residues, e.g. 3,4-linked rhamnopyranosyl or 2,3-linked manopyranosyl, and terminal residues (fucose, rhamnose, glucose, galactose and glucuronic acid) revealed branch points and/or substitutions with non-osidic components. In particular, a 3-*O*-lactyl-hexopyranosiduronic acid was identified.

Table 1
Methylation analysis of the native exopolysaccharide HYD657 after carboxyl reduction.

Type of sugar	PMAA[a]	Deduced linkage	Molar ratio[b]
Fucose	2,3,4-Me_3Fuc	Fuc*p*-(1→	0.09
Rhamnose	2,3,4-Me_3Rha	Rha*p*-(1→	0.38
	2,4-Me_2Rha	→3)-Rha*p*-(1→	0.87
	2-MeRha	→3,4)-Rha*p*-(1→	0.17
	4-MeRha	→2,3)-Rha*p*-(1→	0.08
Glucose	2,3,4,6-Me_4Glc	Glc*p*-(1→	0.08
	2,3,6-Me_3Glc	→4)-Glc*p*-(1→	0.83
Galactose	2,3,4,6-Me_4Gal	Gal*p*-(1→	0.15
	2,4,6-Me_3Gal	→3)-Gal*p*-(1→	1
	2,3,6-Me_3Gal	→4)-Gal*p*-(1→	0.56
	2,4-Me_2Gal	→3,6)-Gal*p*-(1→	0.04
Mannose	4,6-Me_2Man	→2,3)-Man*p*-(1→	0.16
	2,4-Me_2Man	→3,6)-Man*p*-(1→	0.03
Glucuronic acid	2,3,4-Me_3Glc-6-d_2	Glc*p*A-(1→	0.07
	2,4-Me_2Glc-6-d_2 [c]	→3)-Glc*p*A-(1→	0.09
	2,3-Me_2Glc-6-d_2	→4)-Glc*p*A-(1→	0.24
Galacturonic acid	2-MeGal-6-d_2	→3,4)-Gal*p*A-(1→	0.06
Lactate-uronic acid	Lac-2,4-Me_2Hex-6-d_2	Lac→3)-Hex*p*A-(1→	0.2

[a] Partially methylated alditol acetate.
[b] Results are expressed in molar ratios relative to the 3-linked-galactose residue.
[c] 2,4-Me_2Glc-6-d_2 = 1,3,5,6-tetra-*O*-acetyl-6,6-dideutero-2,4-di-*O*-methyl-glucitol.

3.1.3. NMR analysis

NMR analyses of native deepsane showed the presence of methyl signals in the 1–1.5 ppm region. Some were assigned to fucose and rhamnose residues, while others were identified as methyl signals of non-osidic substituents. The 1D proton (Fig. 1) and 2D COSY, HMQC, HMBC (not shown) NMR analyses of native deepsane, recorded at 70 °C after 15 min ball-milling, allowed to identify two different types of non-osidic substituents: lactyl and pyruvyl.

HMBC spectra showed that the single signal at 1.5 ppm was correlated with a carbon signal at 28 ppm, suggesting its methylic nature. No correlation of the 1.5 ppm signal with other protons

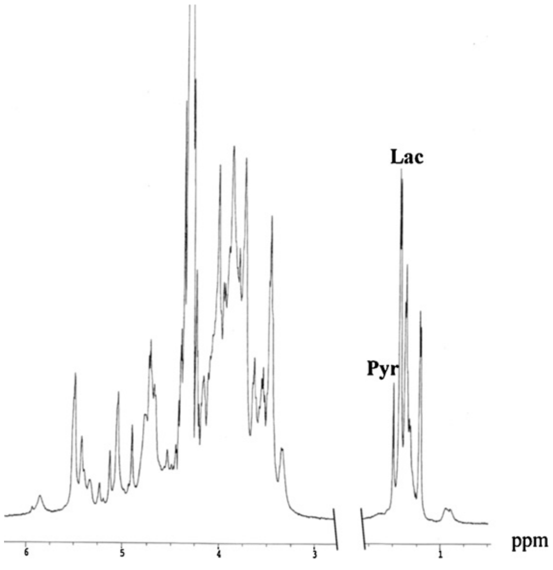


Fig. 1. ^{1}H NMR spectrum of native deepsane (70 °C, 500 MHz), after 15 min of ball milling. Lac, lactyl group; Pyr, pyruvyl group.

was observed in COSY, but HMBC analysis revealed correlations of the methyl proton with the carbon at 178.3 ppm of a carboxylic group and with the acetalic C-2 at 103.8 ppm. Taken together, these results suggest a pyruvyl group. The doublet signal at 1.43 ppm correlated with the carbon at 22 ppm was assigned to the methyl proton of a lactate moiety. The COSY and HMBC spectra showed correlations between methyl protons and H-2 at 4.35 ppm and between methyl protons and the carboxylic group at 184.8 ppm, respectively. The doublet at 1.43 ppm was also correlated with the downfield-shifted C-2 at 81.5 ppm, indicating the branch point of the ether-linked substituent to a glycosidic moiety. NMR analyses of native deepsane provided some information on the presence and nature of non-osidic substituents. However, insufficient resolution prevented the assignment of all spin systems and the elucidation of the complete structure. Different chemical methodologies have been tested to obtain low molecular weigh fractions i.e. acid hydrolysis, autohydrolysis, free-radical depolymerisation, lithium degradation and mild acid hydrolysis, as well as different fractionation conditions. Due to the complexity of such exopolysaccharide, response to those classical methods was either obtention of mono- and di-saccharides either too complex fractions with low-NMR resolution. Thus, the Smith degradation appears to be the most relevant method to start deeper structural analysis of deepsane. Consequently, further structural analyses were carried out with lower molecular weight fractions obtained by Smith degradation.

3.2. *Structural analysis of oligosaccharides*

After Smith degradation of 50 mg of the native exopolysaccharide, 29.5 mg of a mixture of oligosaccharides was obtained with a final reaction rate of 59% (w/w). These products were purified by SEC on a Bio-Gel P2 resulting in the recovery of nine fractions of oligosaccharides with a total mass of 16.8 mg with the hydrolysis rate of 33% (w/w). Some fractions containing oligosaccharides, named SD-1, SD-2 and SD-3, were characterised by GLC analysis of the trimethylsilyl derivatives, ion trap electrospray MS in negative mode and 1D and 2D NMR experiments (^{1}H, ^{13}C, COSY, TOCSY, HMQC, HMBC).

3.2.1. *Oligosaccharide SD-1*

The analysis of the monosaccharides from the SD-1 fraction was performed by GLC analysis of the trimethylsilyl derivatives after methanolysis. The analysis indicated the presence of galactose (Gal), erythronic acid (EryA) and two unidentified peaks previously attributed to a monosaccharide with a non-osidic substituent in the analysis of the native EPS (see Section 3.1.1).

The SD-1 fraction was analysed by MS with electrospray ionisation in negative mode and an ion trap for detection, allowing MSn analysis. Several fragments were generated when the major parent ion of the SD-1 oligosaccharide $[M-H]^-$ at m/z 545.2 was analysed by tandem MS (MS/MS) (Fig. 2A). The ions $[M-118-H]^-$ at m/z 427.2 and $[M-72-H]^-$ at m/z 473.2 corresponded to the loss of a uronic tetritol and a lactyl group, respectively. Other observed ions corresponded to the loss of uronic tetritol and hexose for m/z 265.1 and of a lactyl group and uronic hexose for m/z 297.2. These data helped elucidate the sequence proposed in Fig. 2A. Furthermore, in light of the GLC results, the uronic tetritol was assigned to erythronic acid and the hexose to galactose.

The SD-1 fraction was also submitted to 1D and 2D NMR analysis. Most of the proton chemical shifts for each spin system were assigned by inspecting the COSY and TOCSY contour plots and the ^{13}C resonances were assigned based on inspection of the HMQC plot. The chemical shifts for each spin system are reported in Table 2. The ^{13}C NMR spectrum revealed two signals in the anomeric region, at δ 106.4 for residue A and 102 for residue B; and, based on HMQC experiment, these two anomeric carbons were correlated with two anomeric protons at 4.72 for A and 5.22 for B, respectively (spectrum not shown).

3.2.1.1. *Residue A.*

Based on the observed $^{3}J_{H1,H2}$ value of 7.3 Hz and chemical shifts, the β anomeric form was allocated to this residue. The spin system of residue A was characterised by the absence of H-6 signals. An intra-residue correlation was observed in the HMBC spectrum between the H-5 and the carbon of a carboxyl group at 178.1 ppm (not shown). Based on the chemical shifts, signal multiplicity and the results of monosaccharide analysis by GLC, residue A was assigned to a glucuronic acid (GlcA). The downfield shift

Table 2
^{1}H and ^{13}C NMR data on the residues that occur in oligosaccharides SD-1 and SD-2 (δ, ppm).

Residue	Nucleus	Chemical shift (ppm)					
		1	2	3	4	5	6
Oligosaccharide SD-1							
→3)-β-Glc*p*A-(1→ (A)	^{1}H	4.72	3.49	3.47	3.63	3.78	–
	^{13}C	106.4	75.3	86.9	74.7	78.8	178.1
→3)-α-Gal*p*-(1→ (B)	^{1}H	5.22	4.00	4.02	4.25	4.10	3.82/3.74
	^{13}C	102	70.3	82.3	71.7	73.8	63.9
→2)-EryA (C)	^{1}H	3.83/3.74	4.07	4.42	–		
	^{13}C	62.9	83.4	75.2	179.7		
Lac	^{1}H	–	4.35	1.42			
	^{13}C	183.8	81.1	21.3			
Oligosaccharide SD-2							
Threitol form							
α-Gal*p*A-(1→ (D)	^{1}H	5.43	4.50	4.12	4.39	4.56	–
	^{13}C	100.7	78.1	70.3	73.8	74.5	177.4
→2)-Threitol (E)	^{1}H	3.84/3.75	3.81	3.94	3.78		
	^{13}C	65.2	83.1	74	63.8		
Hydroxyethylidene-threitol form							
α-Gal*p*A-(1→ (D)	^{1}H	5.55	4.50	4.09	4.38	4.55	–
	^{13}C	100.2	78	70.2	73.7	75.1	179
→2,3,4)-Threitol (E')	^{1}H	3.78/3.68	3.90	4.42	4.07		
	^{13}C	64.6	82	78.6	68.6		
2-Hydroxyethylidene (F)	^{1}H	5.05	3.71/3.69				
	^{13}C	106	63.9				

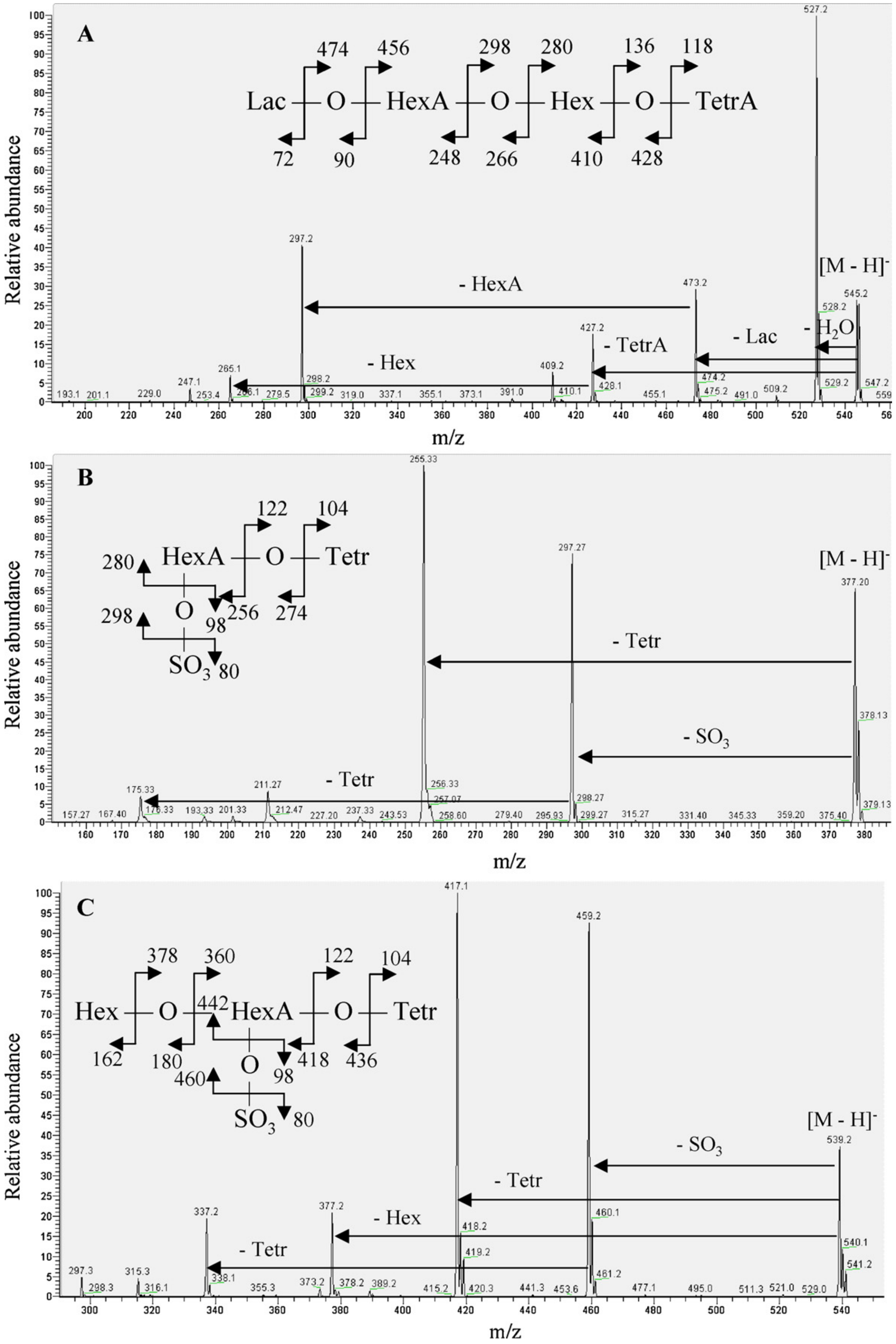


Fig. 2. Chemical structures and CID MS/MS spectra of the [M−H]⁻ precursor ions at (A): *m*/*z* 545.2 for the SD-1 oligosaccharide; (B): *m*/*z* 377.2 for the SD-2 oligosaccharide; (C): *m*/*z* 539.2 for the SD-3 oligosaccharide; with annotated fragments and losses. Lac, lactyl group; Hex, hexose; HexA, uronic hexose; TetrA, uronic tetritol; HexA, uronic hexose; Tetr, tetritol; SO_3, sulphate group.

of C-3 at 86.9 ppm (Table 2) indicated that this position had been substituted. Thus, the residue corresponded to →3)-β-GlcA(1→.

3.2.1.2. Residue B. Residue B was assigned to a galactose. Combining the observed $^3J_{H1,H2}$ value (3.5 Hz) and chemical shift values, an α anomeric form was allocated to this residue. The low-field shift of C-3 (82.3 ppm) indicated a linkage point, this residue being a unit of →3)-α-Gal(1→.

3.2.1.3. Residue C. This residue did not present any anomeric signals and was an aglycon. An intra-residue correlation was observed in the HMBC spectrum between the H at 4.42 ppm and the carbon of a carboxyl group at 179.7 ppm (not shown). Chemical shifts, signal multiplicity and results of monosaccharide analysis led to the identification of erythronic acid (EryA). This erythronic acid was also present in lactone form in another eluted fraction corresponding to a similar SD oligosaccharide, differing only by the aglycon moiety. According to the downfield shift of C-2 (83.4 ppm), the linkage was on position 2. This residue corresponded to →2)-EryA, derived from an oxidised 4-substituted glucuronic acid, followed by the hydrolysis of the acetal linkage of the sugar oxidised during the Smith degradation.

3.2.1.4. Lactyl group (Lac). The oligosaccharide ^{1}H NMR spectrum showed a doublet signal at 1.42 ppm and the ^{13}C NMR spectrum showed a signal at 21.3 ppm which were both assigned to the methyl signal of a lactate moiety. The presence of a carboxyl group in this substituent was indicated by the ^{13}C signal at 183.8 ppm correlated with the H-2 at 4.35 ppm in the ^{1}H, ^{13}C HMBC spectrum (signals not shown).

3.2.1.5. Inter-residue correlations. The HMBC spectrum (Fig. 3) indicated a correlation between H-2 Lac and C-3 A, but also H-3 A and C-2 Lac, thus demonstrating the location of the lactyl group with an ether linkage at position 3 of glucuronic acid. This SD-1 oligosaccharide contained a unit of 3-*O*-lactyl-β-glucuronic acid.

Inspection of the HMBC plot indicated other interesting inter-residue connectivities (Fig. 3). In particular, the H-1 of residue A connected to C-3-B and H-3-B connected to C-1-A, thus establishing the following sequence: A-(1 → 3)-B. The HMBC spectrum showed another inter-residue connection between H-1-B and C-2-C and between H-2-C and C-1-B. The data confirmed and completed the MS results, showing that SD-1 has the following structure: Lac-(2 → 3)-β-GlcA-(1 → 3)-α-Gal-(1 → 2)-EryA, with 2-linked erythronic acid originating from a 4-linked glucuronic acid residue.

3.2.2. Oligosaccharides SD-2 and SD-3

Two additional eluted fractions were analysed and revealed the same oligosaccharide named SD-2 in two different forms. The GLC monosaccharide analysis of the SD-2 oligosaccharide indicated the presence of galacturonic acid (GalA) and threitol (Thre).

The negative mode MS/MS spectrum of the ion [M−H]⁻ *m*/*z* 377.2 from the first SD-2 fraction indicated losses corresponding

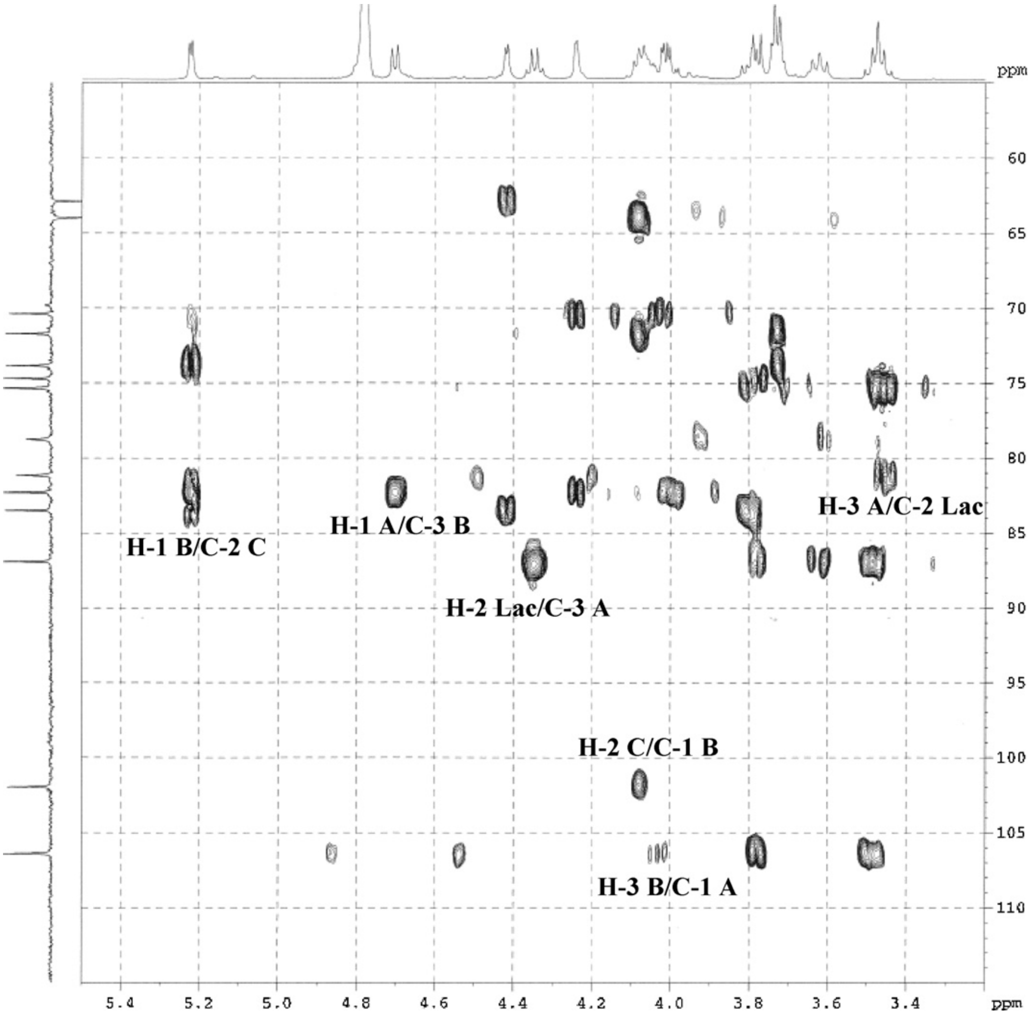


Fig. 3. Partial HMBC NMR spectrum for the SD-1 oligosaccharide (RT, 500 MHz).

to a sulphate group and a tetritol moiety. The fragmentation pattern showed the linkage of a sulphate group to a hexuronic acid (not shown). According to the GLC results, the uronic hexose was attributed to galacturonic acid and the tetritol to threitol. The MS/MS spectrum of the ion $[M-H]^-$ m/z 419.1 from the second SD-2 fraction showed the same fragmentation pattern, with a loss of 164 atomic mass units (amu) corresponding to an unknown compound instead of the loss of tetritol (results not shown). This 164 amu loss was assigned to deoxyhexose or to hydroxyethylidene-tetritol.

The two last eluted fractions that were analysed revealed an oligosaccharide named SD-3, also present in two different forms as was SD-2. The monosaccharide analysis of the SD-3 oligosaccharide indicated the presence of galacturonic acid (GalA), threitol (Thre) and glucose (Glc).

MS/MS analysis of ion $[M-H]^-$ m/z 539.2 from the first SD-3 (Fig. 2B) showed the ion m/z 377.2, already observed in the SD-2 fraction as $[M-H]^-$, but now resulting from a loss of hexose $[M-162-H]^-$. The SD-3 fragmentation pattern showed the same sequence as SD-2, but included an additional hexose at the non-reducing end of the hexuronic acid. In light of results from the GLC analysis, the hexose was attributed to glucose. Similar to the SD-2 oligosaccharide, the MS/MS analysis of the second SD-3 eluted fraction showed the presence of a compound with the loss of 164 amu (results not shown).

As in the characterisation of SD-1 oligosaccharides, most of the proton and carbon chemical shifts were assigned by inspecting 1D and 2D NMR plots (^{1}H, ^{13}C, COSY, TOCSY, HMQC, HMBC) and are presented in Tables 2 and 3 for the SD-2 and SD-3 oligosaccharides, respectively.

3.2.2.1. Residue D. The $^3J_{H1,H2}$ value (<4 Hz) and chemical shifts allocated an α anomeric form to this residue. This spin system did not show any H-6 signals and an intra-residue correlation was observed in the HMBC spectrum between the H-5 and the carbon of a carboxylic group (not shown), indicating the uronic acid nature of this residue. Results from the GLC analysis and H-4 signal multiplicity led to the attribution of a galacturonic acid. No carbon downfield shifts were observed, identifying a terminal non-reductive position for this residue: α-GalA-(1→.

The chemical shifts of SD-3 showed the same residue as in the SD-2 oligosaccharide with the C-4 at 82.9 ppm in the SD-3 oligosaccharide, instead of the C-4 at 73.8 ppm in SD-2 (Table 3), indicating a substitution point in the C-4 position of the α-galacturonic acid residue in SD-3. Thus, residue D corresponded to →4)-α-GalA-(1→.

3.2.2.2. Residue E. For the first analysed SD-2 and SD-3 fractions, an erythritol or threitol spin system was identified (Tables 2 and 3). Results from the monosaccharide composition analysis assigned a threitol to this residue. The low-field shift of C-2 indicated that this carbon was involved in glycosidic linkage, this residue being a 2)-threitol generated from a 4-linked galactose by Smith degradation.

3.2.2.3. Residue E′F. The residue named E seemed to exist in another form (E′). Furthermore, residue F showed a triplet signal at 5.05 ppm which was correlated with an acetalic carbon at 106 ppm according to the HMQC spectrum. The hypothesised E′F structure was formulated combining SD-2 and SD-3 mass spectrometry and NMR spectra. Chemical shifts (Tables 2 and 3 for SD-2 and SD-3, respectively), *J*-mod ^{13}C, HMQC (not shown), TOCSY (Fig. 4) and inspection of HMBC results (not shown) all suggest the presence of a hydroxyethylidene-threitol. Threitol or 3,4-*O*-(2′-hydroxyethylidene)-threitol at the reducing end were consistent with the hypothesis of a hydroxyethylidene-tetritol residue to explain the 164 amu loss in the MS analysis. Similar residues have been described (Gorin & Eveleigh, 1970; Gorin & Spencer, 1965; Richards, Perry, & Kniskern, 1989), where both threitol (E) and hydroxyethylidene-threitol (E′F) residues originate from a polyalcool formed during the Smith degradation of a 4-linked galactose residue, the formation of hydroxyethylidene is sometimes observed. In particular, 3,4-*O*-(2′-hydroxyethylidene)-tetritol formation, as observed in the SD-2 and SD-3 oligosaccharides, has been already observed in residues with an α configuration (Gorin & Spencer, 1965). Given these data, we suggest that the initial residue is →4)-α-Gal-(1→.

3.2.2.4. Residue G. Residue G was assigned to a β-Glc(1→ in light of the results from the GLC monosaccharide analysis, signal multiplicity, the $^3J_{H1,H2}$ value (>7 Hz) and chemical shifts (Table 3 and Fig. 4).

Residue D might be linked to E and E′ in each of the fractions as shown in the HMBC spectrum where inter-residue correlations were observed between the H-1 D and the C-2 E or E′ and vice versa (not shown). According to the SD-3 HMBC spectrum, an additional correlation was observed between H-1 G and C-4 D and between

Table 3
^{1}H and ^{13}C NMR data on the residues that occur in SD-3 oligosaccharides (δ, ppm).

Residue	Nucleus	Chemical shift (ppm)					
		1	2	3	4	5	6
Oligosaccharide SD-3							
Threitol form							
β-Glc*p*-(1→ (G)	^{1}H	4.61	3.35	3.48	3.37	3.39	3.87/3.69
	^{13}C	106.3	76.4	78.4	72.5	78.8	63.9
→4)-α-Gal*p*A-(1→ (D)	^{1}H	5.41	4.57	4.19	4.56	4.51	–
	^{13}C	100.5	78.3	71	82.9	74.1	177.4
→2)-Threitol (E)	^{1}H	3.84/3.75	3.82	3.95	3.74		
	^{13}C	65.1	83.3	73.9	63.7		
Hydroxyethylidene-threitol form							
β-Glc*p*-(1→ (G)	^{1}H	4.60	3.33	3.48	3.34	3.37	3.85/3.65
	^{13}C	106.9	76.8	78.7	73.2	78.9	64.3
→4)-α-Gal*p*A-(1→ (D)	^{1}H	5.48	4.56	4.15	4.54	4.48	–
	^{13}C	100.8	78.9	71.5	83.2	74.9	178.5
→2,3,4)-Threitol (E')	^{1}H	3.77/3.72	3.88	4.41	4.05		
	^{13}C	64.1	82.2	79.5	69.1		
2-Hydroxyethylidene (F)	^{1}H	5.05	3.72/3.70				
	^{13}C	106.6	64.5				

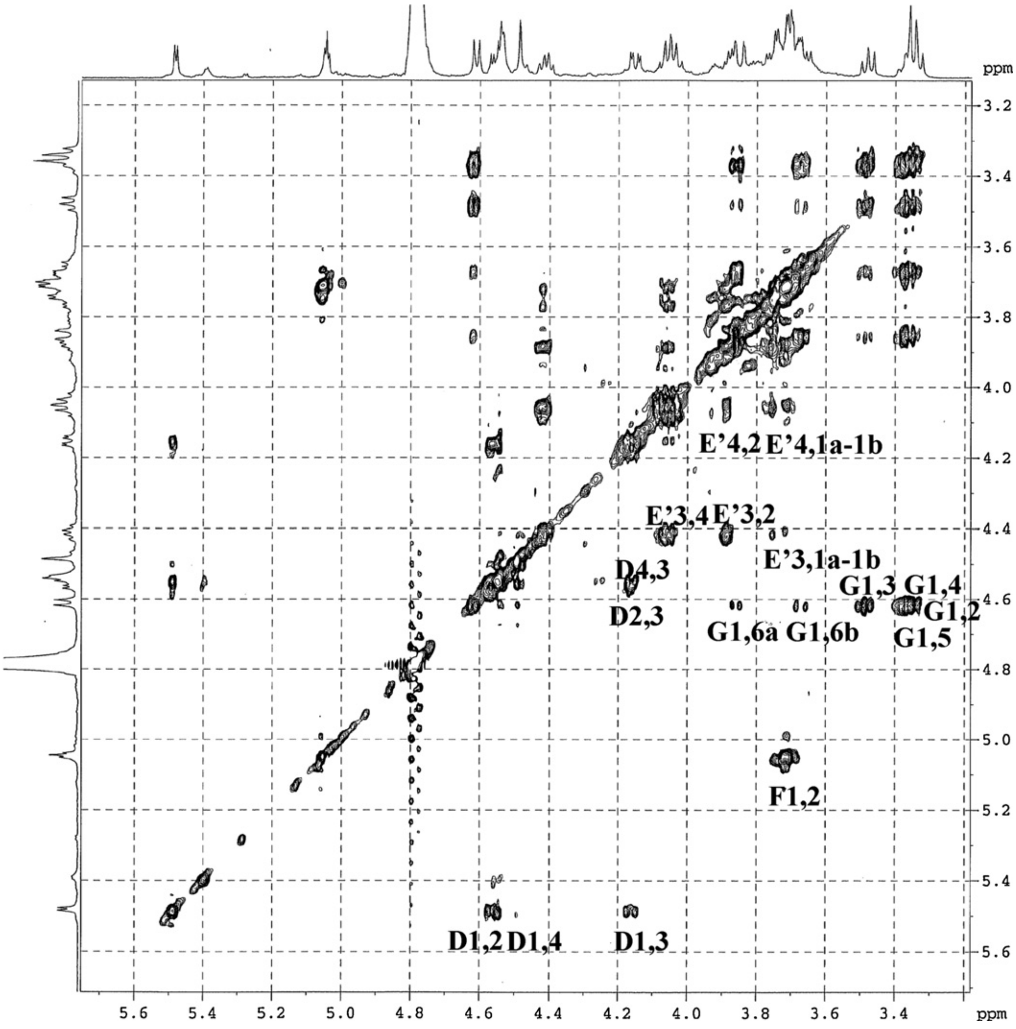


Fig. 4. Partial TOCSY NMR spectrum for the SD-3 oligosaccharide in the hydroxyethylidene form (RT, 500 MHz).

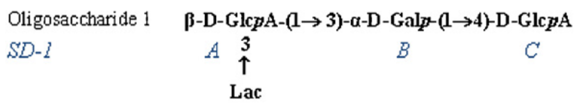
H-4 D and C-1 G, indicating a substitution of the α-galacturonic acid on position 4 with β-glucose (not shown).

These results led us to propose the following initial structure: α-GalA-(1 → 4)-α-Gal-(1→ and β-Glc-(1 → 4)-α-GalA-(1 → 4)-α-Gal-(1→ for SD-2 and SD-3, respectively. MS data also indicated the presence of a sulphate group carried by the galacturonic acid which suggest the following sequences: SD-2, [SO_3^-]-α-GalA-(1→)-α-Gal-(1→ and SD-3, β-Glc-(1 → 4)-[SO_3^-]-α-GalA-(1→)-α-Gal-(1→.

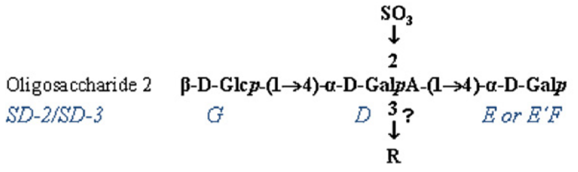
The downfield chemical shift of the galacturonic acid C-2 at 78 ppm in the SD-2 and SD-3 oligosaccharides indicated the position of the sulphate group. An acetylation experiment confirmed the linkage of the sulphate group with the C-2 of galacturonic acid and supported the hypothesis of a 3,4-(2′-hydroxyethylidene)-threitol moiety. No significant shifts (+0.15 ppm) were observed for the H-2 of galacturonic acid (residue D) or for H-3 E′ (+0.07 ppm), H-4 E′ (+0.02 ppm) and H-1 F (+0.17 ppm) of the hydroxyethylidene-threitol. In contrast, higher shifts were observed for other protons not involved in any linkage, such as H-3-D (+1.29 ppm), H-4-D (+1.38 ppm), H-1a/1b-E′ (+0.45/0.51 ppm) and H-2a/2b (+0.60/0.59 ppm) (results not shown).

Therefore, a sulphate group may be bound to the C-2 of a 4-linked galacturonic acid. However, methylation analysis did not reveal any 4- or 2,4-linked galacturonic acid, but only 3,4-linked galacturonic acid. We suggest the presence of a 2,3,4-linked galacturonic acid, with a loss of the sulphate group during the methylation experiment. Our results indicate the presence of an additional unknown substitution on the C-3 of the initial galacturonic acid moiety that was lost during the Smith degradation.

Based on the combined analysis of all the structural data of the oligosaccharides SD-1, SD-2 and SD-3, we propose the chemical structures shown below for the two oligosaccharides belonging to EPS HYD657. Oligosaccharide 1 structural hypothesis derived from SD-1 data and the structure of oligosaccharide 2 was inferred from SD-2/SD-3 data as well as from methylation analysis results. The residues indicated at the reducing ends were deduced from the combination of the composition of the native EPS and of the identified tetritols:



$$\underset{\substack{\uparrow\\ \text{Lac}}}{\overset{}{\beta\text{-D-Glc}p\text{A}}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-D-Glc}p\text{A}$$



$$\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\overset{\substack{SO_3\\ \downarrow\\ 2}}{\underset{\substack{3\,?\\ \downarrow\\ R}}{\alpha\text{-D-Gal}p\text{A}}}\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p$$

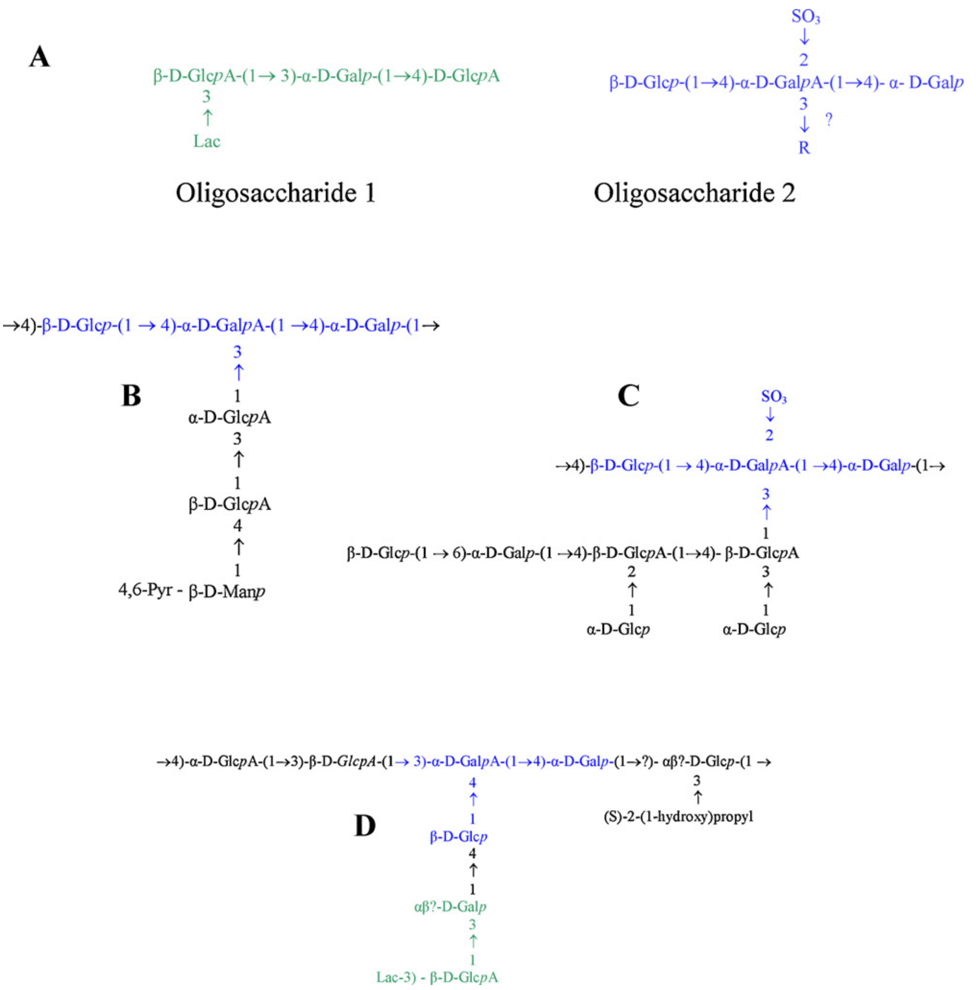


Fig. 5. Repeating units of EPSs with similar structures and produced by *Alteromonas* sp. strains isolated from deep-sea hydrothermal vents. (A) Part of EPS HYD657 (deepsane) from *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane; (B) EPS ST716 from *Alteromonas macleodii* subsp. *fijensis* (Rougeaux et al., 1998); (C) EPS GY785 from *Alteromonas infernus* (Roger et al., 2004); (D) EPS HYD1644 from *Alteromonas* sp. (Dubreucq et al., 1996).

4. Discussion

Although trisubstituted uronic acid residues can be found in nature, the presence of a trisubstituted galacturonic acid as observed in oligosaccharide 2 is not frequently reported. In contrast, as observed in the oligosaccharide 1, a lactate group at position 3 of a glucuronic acid, has been observed in another EPS from *Alteromonas* sp. (Dubreucq, Domon, & Fournet, 1996). Different types of residues carrying lactate groups have been described in polysaccharides produced by microorganisms and they are usually located in position 3, position 4 or, more rarely, in position 6. In addition to this lactate group, pyruvate and sulphate were detected in EPS HYD657. Different types of simultaneous organic substituents have been described on several structures of bacterial EPS. For example, succinoglycan produced by *Rhizobium meliloti*, contains succinate, acetate and pyruvate groups (Chouly, Colquhoun, Jodelet, York, & Walker, 1995; Jansson et al., 1977); EPSs from *Butyrivibrio fibrisolvens* (Andersson, Cotta, & Kenne, 2003) and *Pseudomonas flavescens* (Cescutti et al., 1998) contain lactate and acetate groups, and the EPS from *Pseudomonas marginalis* strains carry pyruvate and succinate (Osman & Fett, 1989) or pyruvate and acetate (Osman & Fett, 1990). Similarly, a capsular polysaccharide of *Rhodococcus equi* contains both lactate and pyruvate groups in its repeating unit (Severn & Richards, 1992). Some studies indicate the presence of pyruvate and sulphate for EPS produced by *Bacillus thermantarticus* (Nicolaus, Schiano Moriello, Lama, Poli, & Gambacorta, 2004), or phosphate and sulphate for EPS from *Halomonas maura* (Arias et al., 2003). To date, few bacterial EPS with three types of substituents have been described.

Nevertheless, the two different oligosaccharides identified in EPS HYD657 are partially present in the structures of four EPS produced by other *Alteromonas* sp. strains isolated from a deep-sea hydrothermal vent environment, i.e. EPS ST716 (Rougeaux, Talaga, Carlson, & Guezennec, 1998), EPS GY785 (Roger, Kervarec, Ratiskol, Colliec-Jouault, & Chevolot, 2004) and EPS HYD1644 (Dubreucq et al., 1996) (Fig. 5). Structures of these EPS were elucidated using the same methodologies than for the EPS HYD657, with chemical and specific degradation experiments. These results may therefore reflect similar biosynthetic pathways for EPSs of this species. However, few structural data are available for EPS produced by *Alteromonas* and these structural similarities are only found in EPS synthesised by *Alteromonas* from hydrothermal vents. Of the three *Alteromonas* EPS, the structure of EPS HYD1644 shows the highest similarity with the oligosaccharides identified for EPS HYD657 in this study. Both *Alteromonas* strains 1644 and 657 were isolated from very similar environments: from *Alvinellidae* annelids at the same hydrothermal site. Other bacterial species (*Pseudoalteromonas*, *Vibrio diabolicus*) were collected during the same cruise in the same type of environment, particularly in the tegument of *A. pompejana*, but their EPSs have different structures. Thus, producing polysaccharides of similar nature may depend on two parameters: species identity and the type of environment.

If oligosaccharides 1 and 2 potentially belong to a repeating unit comprising seven types of monosaccharides and three types of substituents, some residues identified from the GLC analysis of native deepsane do not appear in the fractions described. This is the case for mannose, fucose and rhamnose, and also for the pyruvate group. Other more complex fractions obtained by the various tested cleavage methods (not shown) indicated the presence of deoxy-carbohydrates and/or pyruvate. However, we were not able to characterise all of the oligosaccharides from these fractions due to their complexity. Although deoxy-carbohydrates were observed in a small minority of the NMR spectra for the SD-3 threitol form fraction, they were not clearly identified. Fucose, which was identified in a non-reducing terminal position from methylation analysis, had probably been eliminated during the Smith degradation, as one of the rhamnose residues, also identified as a terminal residue. Finally, all the results on EPS HYD657 structure indicate a complex polysaccharide involving seven types of monosaccharides, ramifications and substituents and a sugar ratio suggesting some variability in terms of the repeating unit. The most complex structures reported for bacterial EPS include succinoglycan produced by *Rhizobium meliloti* (Chouly et al., 1995; Jansson et al., 1977) with a repeating unit of eight monosaccharides with three types of organic substituents and EPS GY785 from *Alteromonas infernus* with a repeating unit of nine residues and a sulphate group (Roger et al., 2004).

Complex structures have also been identified in polysaccharides originating from cyanobacteria (Sutherland, 2002). For example, the capsular polysaccharide produced by *Mastigocladus laminosus* has a repeating unit of 15 monosaccharides, including eight different types of sugars and ramifications (Gloaguen et al., 1999).

Structures of other EPS have not been completely elucidated, but their composition suggests complex repeating units. For example, EPSs produced by *Pseudomonas* strains contain six and nine different types of monosaccharides and 5% of sulphate (Mancuso-Nichols, Garon, Bowman, Raguenes, & Guezennec, 2004). Eight types of neutral monosaccharides and uronic acids have been described in an EPS produced by another *Pseudomonas* strain (Saravanan & Jayachandran, 2008).

5. Conclusion

We partially determined the structure of high molecular weight (10^6 g/mol) EPS HYD657. The main structural unit is branched and appears to contain from 16 to 18 monosaccharides, including seven types of monosaccharide (fucose, rhamnose, glucose, galactose, mannose, galacturonic acid and glucuronic acid) and three types of substituent (sulphate, lactate and pyruvate). The number of sulphate groups was estimated at three per repeating unit. Although all residues and groups have not yet been clearly identified, the carbohydrate sequences were proposed for two oligosaccharides as well as the position of two substituents: lactate and sulphate groups. The first trisaccharide (called oligosaccharide 1) consists of an α-galactose bound to α glucuronic acid and 3-substituted by a β-glucuronic acid carrying a lactate group in position 3. The second trisaccharide (oligosaccharide 2) was an α-galactose 4-substituted by an α-galacturonic acid. The galacturonic acid is substituted in position 4 by a β-glucose, in position 2 by a sulphate group and may have another substitution in position 3. The composition and structure of EPS HYD657 were partially elucidated and similarities with other known EPS from *Alteromonas* strains of hydrothermal environments were identified. Comparison with other exopolysaccharides illustrates the diversity of polysaccharide structures produced by microorganisms. According to a statistical study comparing the carbohydrate structures of mammals and bacteria from multiple databases, bacterial structures (excluding cyanobacteria) show diversity that is 10 times higher for monosaccharides and 9 times higher for glycosidic bonds compared to those reported from mammals (Herget et al., 2008).

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