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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™ 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%