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Novel bacterial exopolysaccharides from deep-sea hydrothermal vents

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Five bacterial strains recovered from deep-sea hydrothermal vents were studied for their ability to secrete extracellular polymers. A preliminary characterization displayed four different polysaccharides in terms of both chemical composition and rheological properties. One of them was secreted by *Alteromonas macleodii* subsp. *fijiensis* and exhibited similarities with xanthan, a commercial polysaccharide. Two of the three *Pseudoalteromonas* species were shown to produce the same polymer. The last polymer was secreted by a bacterium belonging to the *Vibrio* genus. They all contained glucose, galactose, mannose, glucuronic and galacturonic acids as the main sugars with the exception of the last one which was only constituted by uronic acids and hexosamines, in that similar to the structure of heparin, a glycosaminoglycan useful in pharmaceutical area. Applications for these polysaccharides could be expected in various biotechnological fields including the food industry, the wastewater treatment and pharmaceutical areas.

INTRODUCTION

Polysaccharides occur as important constituents of plants and microbial cells, as storage polymers and as biopolymers secreted outside the cell wall. Due to their many interesting physical–chemical and rheological properties, polysaccharides including microbial exopolysaccharides (EPS) have found many applications in many sectors of industry as detergents, textiles, adhesives, oil recovery (MEOR), wastewater treatments, cosmetology, pharmacology but primarily in the food industry.

Most of the polysaccharides from marine environment are obtained from seaweeds (agar, alginates and carrageenans) while microbial polysaccharides represent only a small fraction of the current polymer market. However, microbial polysaccharides are recently gaining more and more interest.

Deep-sea hydrothermal vents have been characterized by high pressures, high temperature gradient and high concentrations of toxic elements as sulfides and heavy metals. These extreme conditions raise interesting questions about survival and growth of micro-organisms in this particular environment. Because of the nature of the environment, the presence of unusual micro-organisms of biotechnological interest could be expected in terms of thermostable enzyme, polysaccharide and poly-β-hydroxyalcanoate (PHA) producing bacteria.

The study presented herein is a part of large research program on the discovery of biotechnologically important microorganisms originating from hydrothermal deep-sea environments, with potential applications in industry. A preliminary screening on isolates from hydrothermal vents has already shown that microbial polysaccharide producers exist under extreme conditions (Vincent *et al.*, 1994). These polymers were characterized and evaluated for potential applications (Guezennec *et al.*, 1994).

A subsequent screening was carried out on new samples recovered from different oceanographic cruises. The isolates were selected from their capability to produce exopolysaccharides (EPS) in lab conditions, and the polymers secreted were partially characterized. Five exopolysaccharides were analyzed for their carbohydrates, proteins and sulfate contents along with their monosaccharide compositions and rheological properties.

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MATERIALS AND METHODS

Strains origin

During different oceanographic cruises (Starmer 89, Hero 91 and Guaynaut 91) carried out near active hydrothermal vents located in the rift system of the North Fiji Basin, the 9°N East Pacific Rise and the Guaymas Basin respectively, different samples were collected. The bacterial strains ST 716, GY 788 were isolated from seawater and GY 768, GY 786, HE 800 from invertebrate tissues (*Alvinella* or *Riftia*).

Reference strains

Alteromonas macleodii (LMG 2843), *Pseudoalteromonas carrageenovora* (LMG 2154), *Pseudoalteromonas espejiana* (LMG 2866), *Pseudoalteromonas haloplanktis* (LMG 2852), *Pseudoalteromonas undina* (LMG 2880), *Vibrio alginolyticus* (LMG 4409), *Vibrio campbellii* (LMG 11216), *Vibrio harveyi* (LMG 4044), *Vibrio natriegens* (LMG 10935), *Vibrio parahaemolyticus* (LMG 2850), *Vibrio pelagius* (LMG 3897), *Shewennela putrefaciens* (LMG 2268) were obtained from the LMG collection, Ghent, Belgium.

Preliminary strains characterization

The strains were isolated as described elsewhere (Raguénès *et al.*, 1997). Biolog GN microplates (Biolog Inc., Hayward, Calif.) were used to characterize the new strains. At this time, few marine bacteria appear in the Biolog GN database. Twelve marine reference strains selected for their phenotypic features were introduced in our database. A factor analysis in principal components connected with a hierarchical ascending classification was performed with the SPAD.N software package (CISIA, St Mandé, France).

Polymer production and isolation

Polymers were obtained from a 2-liter fermentor cultures using appropriate growth conditions, on medium supplemented with glucose. Samples were removed at regular intervals for viscosity measurements with a Brookfield viscosimeter model DV-II. Exopolysaccharides were isolated from the culture medium after 1 or 2 days when viscosity reached stable value. Cells were removed from the medium by high-speed centrifugation. Polymers were precipitated from the supernatant with pure ethanol, washed with mixture of ethanol and water as previously described (Talmont *et al.*, 1991; Vincent *et al.*, 1993), and stored at room temperature.

Chemical analysis

Protein content was determined by the method of Lowry *et al.* (1953) with bovine serum albumin as standard. Uronic acid contents were measured by the meta-hydroxydiphenyl method (Blumenkrantz *et al.*, 1973; Filisetti-Cozzi *et al.*, 1991) with D-glucuronolactone as the standard. Neutral sugars were analysed by the orcinol-sulfuric acid method (Tillmans *et al.*, 1929; Rimington, 1931), using the galactose:-mannose (1:1) ratio as standard. Hexosamines were determined by the Elson & Morgan, 1933 method modified by Belcher *et al.* (1954), with glucosamine as standard.

Alditol acetates were prepared as described by Blakeney *et al.* (1983) after hydrolysis of polysaccharides (10 mg) in 4 M trifluoroacetic acid (1 ml) at 100°C for 4 hours. Methanolysis was performed by adding 0.5 ml of 3 M HCl in dry methanol on freeze-dried samples (0.2 mg) and heating at 100°C for 4 hours. Neutralization of the acidic solution, re-*N*-acetylation and conversion of the methyl glycosides in the corresponding trimethylsilyl derivatives were performed as described by Montreuil *et al.* (1986). Gas chromatography (GC) was performed on a HRGC 5160 instrument equipped with a flame ionization detector. A fused silica CP-SIL-5CB capillary column was operated with temperature gradient (50°C to 120°C, 20°C/min, 120°C to 250°C, 2°C/min).

Sulfate contents were determined by Fourier transform infrared spectroscopy (Lijour *et al.*, 1993). Pellets were obtained by grinding a mixture of 2 mg of polysaccharide with 200 mg of dry KBr and then pressing in a 16 mm diameter mold. FTIR spectra were recorded on a BOMEM MB100 instrument with a resolution of 4 cm^{-1}. Spectra were obtained in the 4000–400 cm^{-1} region.

NMR analysis were performed on polysaccharides dissolved in D_2O (99.98% D). ^1H and natural abundance proton decoupled ^{13}C NMR spectra were recorded on a BRUKER DRX400 or a BRUKER AC300 at 55°C. ^1H and ^{13}C NMR chemical shifts are expressed in ppm relative to 2,2,3,3-tetradeutero-3-(trimethylsilyl) propionic acid sodium salt.

Physical analysis

Measurements of viscosity were carried out in 0.1 M NaCl at 20°C by using a Contraves Low Shear 40 viscosimeter in a range of shear rate from 10^{-2} to 15 s^{-1}. Intrinsic viscosity was determined with low polymer concentrations (0.02 to 0.15 g.$liter^{-1}$), by linear extrapolation, using the Huggins and the Kraemer relations. Flow curves were obtained for 3 g.$liter^{-1}$ samples solutions in 0.1 M NaCl.

Thermogravimetric measurements of samples (20 mg) were performed on a SETARAM TGA 92 instrument with temperature gradient (20°C to 100°C, 20°C/min, 100°C to 800°C, 50°C/min).

RESULTS

Computer based analysis of the results of Biolog GN microplates clearly indicated that ST 716 was an *Alteromonas macleodii*-like bacterium, GY 768, GY 786 and GY 788 belonged to a new *Pseudoalteromonas* genus (Gauthier et al., 1995), and HE 800 to the *Vibrio* genus.

The gross chemical composition of the crude polysaccharides is listed in Table 1. The polymers were characterized by a great diversity in terms of neutral, acidic or even amino sugars. Protein concentrations were ranged from 1 to 6%, depending of the degree of purification obtained using the cleaning procedure above described. All the polysaccharides were polyanionic in nature due to the presence of uronic acids, ester sulfates or pyruvate ketals.

Infrared spectroscopy was performed on all polysaccharides (Fig. 1). Fourier Transform-infrared spectroscopy (FTIR) exhibited a broad O-H stretching band at 3420 cm^{-1}, a minor C-H stretching band at 2900 cm^{-1} and a large absorption band at 1630 cm^{-1}–1650 cm^{-1} with a shoulder at 1730 cm^{-1} for EPS 788, 786 and 768. Sulfate contents, as determined by the presence of a doublet at 1230–1250 cm^{-1} showed concentrations of 5.5%, 13%, 6.5%, 8.1% and 0% for the EPS 716, EPS 768, EPS 786, EPS 788 and EPS 800 respectively.

GC analysis of monosaccharides, following either acidic methanolysis or aqueous hydrolysis, is listed in Table 2. Glucose, galactose and mannose predominated as neutral sugars with concentrations ranging from 0.2 to 14.8%. Ribose and fucose were not found in any polysaccharide, with the exception of EPS 788 where concentrations as low as 3% were determined. It is well known that it is not very easy to fully hydrolyse acidic polysaccharides quantitatively into their respective monomer units and that data described above are not absolute but dependent principally on the hydrolysis conditions used. Glucuronic and galacturonic acids are the only acidic sugars found in these exopolymers with the first one predominating. *N*-Acetyl-glucosamine and *N*-acetyl-galactosamine were determined as amino sugars in low concentrations in most polysaccharides with the exception of EPS 800. The presence of pyruvate linked to the mannose was observed in exopolysaccharides EPS 716, EPS 768 and EPS 786, using xanthan as comparison.

Table 1. Colorimetric analysis of bacterial polysaccharides (grams per 100 g)

EPS	Neutral sugars	Hexuronic acids	Hexosamines	Total carbohydrates	Proteins
716	41	41	1	83	2
768	31	31	2	64	6
786	27	22	4	53	6
788	39	19	1	59	5
800	1	32	30	63	1

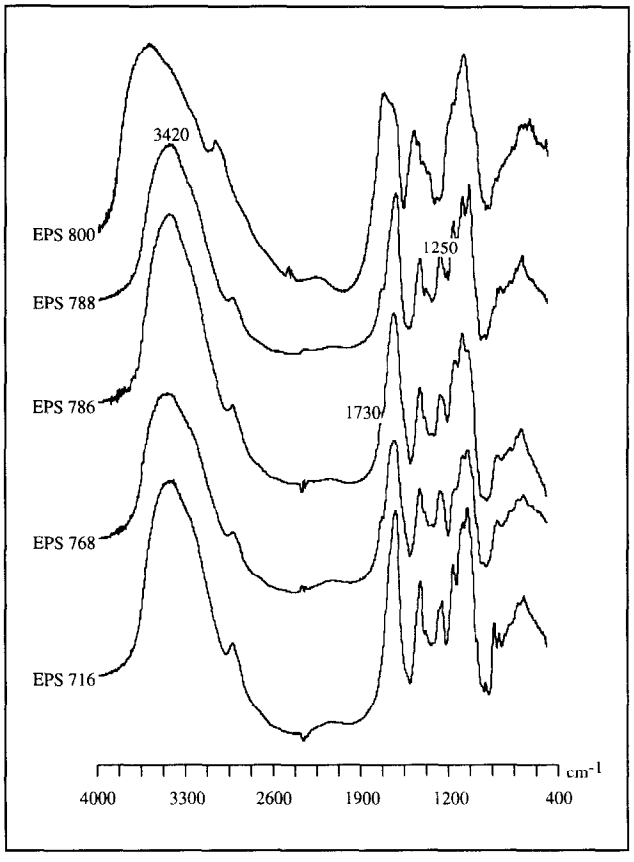

Fig. 1. FTIR spectra of exopolysaccharides

The ^{1}H and ^{13}C NMR spectra were poorly resolved primarily because of the viscosity of solutions. However, signals for both anomeric carbon and protons could be identified. The ^{13}C NMR spectrum of all polymers showed downfield resonances (170–180 ppm) indicating the presence of carboxyl carbons and upfield resonances due to methyl groups of 6-deoxy sugars or substituents like acetate or pyruvate. An example of a ^{13}C NMR spectrum is given in Fig. 2.

For rheological analysis, the ionic strength 0.1M was selected in order to screen the intramolecular electrostatic repulsions. Intrinsic viscosities of exopolymers reached values from 120 to 2600 ml/g, with Huggins constants ranging from 0.18 to 3.11 (Table 3). Flow curves of the five polysaccharides are shown on Fig. 3. A decrease of the viscosity was observed in the high shear rate range for most of the polymers (EPS 716, EPS 768 and EPS 786).

Thermogravimetric analysis of polymers showed water contents ranging from 13 to 16% and degradation temperatures ranging from 286 to 298°C.

DISCUSSION

Microbial polysaccharides possess a wide variety of properties that may not found in the polymers of tradi-

Table 2. Monosaccharide ratios (grams per 100 g) obtained after methanolysis and trimethylsilyl derivatization or hydrolysis and peracetylation

EPS	Method of release	Glc	Gal	Man	Fuc	Rib	Man Pyr	Glc UA	Gal UA	Glc N Ac	Gal N Ac	Total
716	Meth.	12.8	10.0	4.2			nq	16.1	8.0			51.1
	Hydr.	14.8	8.7	10.3			—	—	—			33.8
768	Meth.	12.7	9.0	2.3		tr	nq	6.0	5.2			35.2
	Hydr.	13.5	7.5	3.2		0	—	—	—			24.2
786	Meth.	10.2	7.3	1.9		tr	nq	4.8	4.2			28.4
	Hydr.	11.2	6.5	3.5		0	—	—	—			21.2
788	Meth.	10.4	12.8	1.7	2.6	1.9		6.0	3.9			39.3
	Hydr.	11.5	14.2	1.1	2.3	1.3		—	—			30.4
800	Meth.	0.2		0.3				11.2	0.5	18.0	7.9	38.1

Meth: Acidic methanolysis - Hydr: Acidic hydrolysis - tr: traces - nq: non quantified Glc: Glucose - Gal: Galactose - Man: Mannose - Fuc: Fucose - Rib: Ribose - Man Pyr: Pyruvated Mannose - Glc UA: Glucuronic Acid - Gal UA: Galacturonic Acid - Glc N Ac: *N*-acetyl Glucosamine - Gal N Ac: *N*-acetyl Galactosamine.

tional plant origin. Polysaccharides with novel and useful properties are expected to be discovered as the sources of microorganisms are continued to be trapped. Bacteria associated with deep-sea hydrothermal conditions have demonstrated their ability to produce in an aerobic carbohydrate-based medium, unusual extracellular polymers (Guezennec *et al.*, 1994). The present study displays very different polysaccharides in terms of chemical and rheological properties.

EPS 716:

Preliminary rheological analysis of EPS 716, produced by *Alteromonas macleodii* subsp. *fijiensis* (Raguénès *et al.*, 1996), indicated a high intrinsic viscosity (2600 ml/g) and a shear-thinning flow behaviour with a Newtonian region in the low shear rate range (Fig. 3). Gelification was observed when calcium was added to the polymer solution. This polymer contains glucose and galactose and mannose as the main hexoses, and glucuronic and galacturonic acids in molar ratio 1.3:1:1.2:1.5:0.7. Gas chromatography analysis following methanolysis showed the presence of pyruvate linked to mannose. The ^{13}C NMR spectrum indicated a signal at 27.5 ppm assigned to carbon C1 of the pyruvate group. Pyruvate and acetate levels are thought to affect the viscosity of polymers (Sandford *et al.*, 1977; Cheetham *et al.*, 1989). The influence of the pyruvylation on the rheological behaviour of EPS 716 will be further studied. Regarding both its chemical composition and rheological properties, this polymer show very interesting similarities with xanthan. In that, applications as thickening agent in the food-processing industries could be expected. More structural and rheological studies of this polysaccharide are in progress.

EPS 768–786 :

Both strain GY 768 and strain GY 786 were assigned to the genus *Pseudoalteromonas*. The former one was close to *P. carrageenovora*, while the latter belonged to a group of new species close to *P. undina*. However, regarding the colorimetric analysis, their exopolysaccharides exhibited a similar carbohydrate composition. Neutral sugars and uronic acids were found in equal amounts in both polymers. Monosaccharides identified by GC analysis confirmed the similarities between the two polymers EPS 768 and EPS 786. They both contained glucose, galactose and mannose as the main hexoses, glucuronic and galacturonic acids as

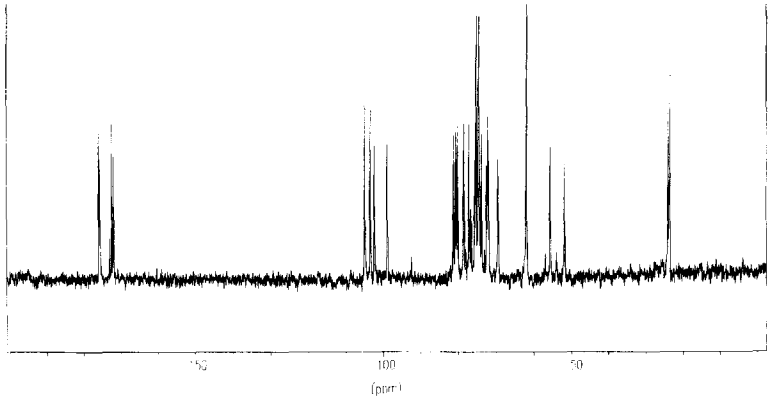

Fig. 2. ^{13}C NMR spectrum of EPS 800

Table 3. Viscosimetric analysis of bacterial exopolysaccharides

EPS	Intrinsic viscosity ml/g	Huggins constant
716	2600	0.18
768	1080	0.28
786	880	0.26
788	120	3.11
800	570	2.76

hexuronic acids in the same ratio (1.4:1:0.4:0.6:0.5). GC analysis also indicated the presence of pyruvated mannose for both polymers. A shoulder at 1730 cm^{-1} on FTIR spectra suggested the presence of acetate groups in the polymers. ^{13}C NMR spectra confirmed the presence of the pyruvyl and acetyl groups with signals at 27.5 ppm and 24.9 ppm respectively. The exact sites of acetylation remain yet unknown. The only difference between the two polymers remains their sulfate contents, 13% and 6.5% respectively. Their rheological behaviours were similar regarding the flow curves determined in a 0.1 M NaCl solution. Intrinsic viscosity was higher for EPS 768 than for EPS 786, 1080 ml/g and 880 ml/g respectively.

EPS 788:

This polysaccharide was secreted by a bacterium close to *P. haloplanktis* and contained more neutral sugars than uronic acids. Glucose, galactose, mannose along with glucuronic and galacturonic acids were found in this exopolymer, as in most of microbial exopolysaccharides. Fucose and ribose were also determined with concentrations of 2.6% and 1.9% respectively. The ^{13}C NMR spectrum indeed showed a signal at 18.5 ppm assigned to the methyl group of the fucose. Pyruvate and acetate were found as substituents with signal at 27.4 ppm and 24.8 ppm on the ^{13}C NMR spectrum, but non additional information allowed to identify the location of these groups. The sulfate content was estimated to be in the same order of magnitude than other polymers (8.1%). The intrinsic viscosity in a 0.1 M NaCl solution reached 120 ml/g, a low value compared to the other bacterial exopolysaccharides, with a high Huggins constant, probably due to a poor solubilization of the polymer.

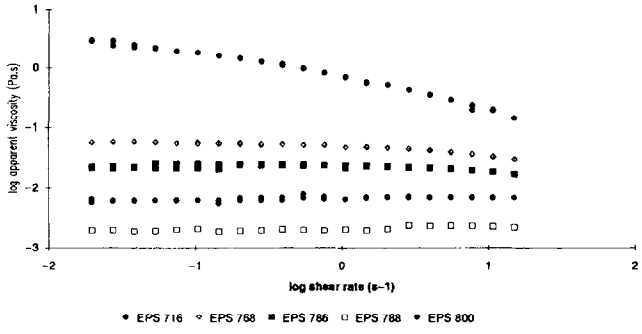

Fig. 3. Flow curves of 0.3% exopolysaccharides solutions at 0.1 M NaCl ionic strength

EPS 800:

Conversely to other polymers secreted by *Alteromonas* and *Pseudoalteromonas* species, this polymer was produced by a *Vibrio* species. Some marine vibrios are known to produce exopolysaccharides (Okutani, 1985). The viscosity was moderate compared to the other ones, with an intrinsic viscosity of 570 ml/g and a high Huggins constant. Interestingly this polymer was characterized by a specific composition. The colorimetric analysis showed large proportions of uronic acids and hexosamines. Glucuronic and galacturonic acids with the first predominating were identified by GC analysis along with glucosamine and galactosamine as amino sugars (1:0.04:1.4:0.6). Only traces of glucose and mannose were also found at very low concentrations and suggested to be contaminants. The ^{13}C NMR spectrum of EPS 800 is shown on Fig. 2. The occurence of four sugars was supported by four anomeric carbons (100.3, 103.7, 104.9 and 106.3 ppm). The ^{13}C NMR spectrum contained signals for four carboxyl carbons at 173.6, 174.2, 177.0 and 177.3 ppm. Signals at 25.0 ppm and 25.5 ppm were assigned to methyl of acetyl groups indicating the *N*-acetylation of the hexosamines. To a certain extent, EPS 800 can be compared to heparin, a well-known natural polysaccharide consisting of a repeated basic disaccharide sequence made of a uronic acid (glucuronic acid) and a glucosamine which are 1–4 linked, with high concentration of sulfate. Heparin shows anticoagulant activity, anti-HIV activity, and still more, several other physiological or pharmaceutical activities (Petitou *et al.*, 1983). The similarity in terms of chemical composition makes the EPS 800 polymer very attractive. However, regarding the absence of sulfate for this polymer, experiments have to be done to increase the sulfate content of EPS 800 before any evaluation of its biological activity.

CONCLUSION

Extremophiles living near deep-sea hydrothermal vents are potential sources of polysaccharide producers. Two different bacterial species (GY 768 and GY 786) belonging to the genus *Pseudoalteromonas* produce the same polymer. Regarding their chemical composition, along with additional rheological properties, several EPS could be used in various biotechnological fields. In particular, EPS of *Alteromonas macleodii* subsp. *fijiensis* (ST 716) presents rheological properties similar to those of xanthan, and applications for this polymer can be expected in food-processing industry. The polyelectrolytic character of EPS 768 and 786 polymers

could be used in biodetoxification or wastewater treatment. The specific chemical composition of the EPS 800, secreted by a *Vibrio* species, similar to heparin could make this polymer useful for applications in the pharmaceutical industry. More structural and rheological studies will bring additional informations for more specific applications of these polymers in many sectors of the industry.

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