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Chelating properties of bacterial exopolysaccharides from deep-sea hydrothermal vents

M. Loaëc[a], R. Olier[b], J. Guezennec[a],*

[a]*IFREMER, Centre de Brest, DRV/VP/Laboratoire de Biotechnologie des Microorganismes Hydrothermaux, B.P.70 29280 Plouzané, France*

[b]*Université de Bretagne Occidentale, CR$_4$ Laboratoire de Spectroscopie Moléculaire, BP 809. 29285 Brest Cedex, France*

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The metal-binding properties of four bacterial exopolysaccharides originating from deep-sea hydrothermal vents were investigated in batch experiments. The effect of pH on ion uptake was also investigated. Removal of toxic elements such as lead, cadmium and zinc occurred according to a chemical, equilibrated and saturable mechanism, following the typical Langmuir model. The sorption isotherms were plotted to obtain the maximum of uptake capacities and apparent dissociation constants. Maximum uptake capacities reached 316 mg g^{-1}, 154 mg g^{-1} and 77 mg g^{-1} for lead, cadmium and zinc respectively. The strong chelating properties of all polymers for the three selected metals make their use as biosorbents in wastewater treatment as a possible alternative to other physical or chemical methods. © 1998 Published by Elsevier Science Limited. All rights reserved.

INTRODUCTION

Contamination of the environment by heavy metals is of growing concern because of the health risks posed by human and animal exposures. Physical and chemical techniques have been used in water treatment but are sometimes inefficient or costly. The use of biomass has been proposed and several groups have studied ion uptake by a range of bacterial materials, such as extracellular polysaccharides and cell wall components. Sorption and/or complexation of dissolved metals based on chemical activity of microbial biomass is an alternative and economically attractive treatment for a wide variety of polluted environments.

The accumulation of metals by microorganisms and microbial exopolysaccharides (EPS) has been recognized for a few decades (Bitton & Friehofer, 1978; Brown & Lester, 1979), but received more attention in recent years because of its potential application in environmental protection, recovery of precious or strategic metals and metal poisoning therapy.

Deep-sea hydrothermal vents are characterized by extreme conditions including high pressures, high temperature gradients and levels of toxic elements. These extreme environments are suggested to be a source of biotechnologically important microorganisms. Preliminary studies on mesophilic bacteria recovered from different deep-sea hydrothermal fields have shown the production of unusual eubacterial exopolysaccharides with innovative characteristics (Guezennec *et al.*, 1994). Four purified exopolysaccharides were selected with respect to their chemical composition and evaluated for their capability to bind toxic elements such as lead, cadmium and zinc.

MATERIALS AND METHODS

Polymer-producing strain

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). Four strains (ST 716, GY

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*Corresponding author: Dr. J. Guezennec. Tel: (33) 2 98 22 45 26; fax: (33) 2 98 22 47 57; e-mail:jguezenn@ifremer.fr

785, HE 800 and HYD 1574) were selected on the basis of the chemical composition of the polysaccharide production. On the basis of phenotypical and phylogenetic analyses and DNA/DNA relatedness, strain ST 716 was assigned to the species *Alteromonas macleodii* as a new sub-species (***Alteromonas macleodii*** subsp. ***fijiensis***) (Raguénès *et al.*, 1996) strain GY 785 as a new species of the genus *Alteromonas* (***Alteromonas infernus***) (Raguénès *et al.*, 1997a) while strain HE 800 was identified as a new species of the genus *Vibrio* for which the name of ***Vibrio diabolicus*** was proposed (Raguénès *et al.*, 1997b). The last strain (HYD 1574) belonged to the *Pseudoalteromonas* genus (Vincent, 1993) and its description is in progress.

Polysaccharide-producing bacteria were selected on the basis of the appearance of mucoid colonies when grown on solid agar medium supplemented with glucose. Polymers were obtained through fermentor cultures using appropriate conditions. Exopolysaccharides were isolated from the culture medium after 3 or 4 days, by high speed centrifugation and precipitation with ethanol. After sequential washes with mixtures of EtOH/H_2O, the polysaccharides were dried at 40°C (Talmont *et al.*, 1991; Vincent *et al.*, 1994).

Chemical analysis

The total neutral and acid carbohydrate contents were determined by the orcinol-sulfuric method (Rimington, 1931) and meta-hydroxydiphenyl method (Filisetti-Cozzi & Carpita, 1991), respectively. *N*-acetyl-hexosamines were analysed by the modified Elson–Morgan method (Elson & Morgan, 1933; Belcher *et al.*, 1954). Sulfate and protein contents were determined by FTIR (Lijour *et al.*, 1994) and the method of Lowry *et al.* (1951), respectively.

Monosaccharide determinations were performed by hydrolysis followed by methanolysis. 500 μl of 2 M HCl were added to 2 mg of exopolysaccharides along with 50 μg of myo-inositol as internal standard and heated at 100°C for 4 h. The samples were dried for 24 h in a vacuum desiccator over P_2O_5. Methanolysis was performed by adding 500 μl of methanolic HCl 2 M to samples and heated at 100°C for 4 h. Neutralization, re-*N*-acetylation and conversion of the methylglycosides to the corresponding trimethylsilyl ethers were performed as described by Montreuil *et al.* (1986). The retention times, number of the peaks and relative peak heights for each monosaccharides were determined separately using a standard mixture. The gas chromatograph (HRGC 5160, Carlo Erba, Milano, Italy) was equipped with a polar fused silica capillary column (CP-Sil-5CB, Chrompack, Middelburg, Netherlands) and a flame-ionisation detection system. A temperature gradient was programmed from 50°C to 140°C at 20°C min^{-1}, from 140°C to 220°C at 1.8°C min^{-1} and from 220°C to 250°C at 20°C min^{-1}.

Metal uptake analysis

Selected metals were lead, cadmium and zinc as nitrate salts. The initial metal concentrations ranged from 50 to 1000 mg l^{-1}. After dissolution of EPS in water (MilliQ quality), metallic ion solutions of different concentrations were added leading to a final concentration of polymer at 0.1% (w/v). Flasks were shake at room temperature in a orbital shaker at 200 rpm for 3 h (equilibrium time). At the end of the experiment, the solution was sampled through a 30.000 Da porosity Centrifree filter (Amicon, Grasse, France) and acidified with 1% nitric acid solution for analysis. The initial $[Me]_i$ and final metal $[Me]_{eq}$ concentrations were determined by atomic absorption spectrophotometry (SP250, Varian, Les Ulis, France), leading to calculated metal uptake values (q) in each sorption system using the general equation (Volesky, 1990):

$$q = \frac{([\mathrm{Me}]_i - [\mathrm{Me}]_{eq})}{m} \times V \tag{1}$$

where V and m were the volume of solution and the mass of exopolysaccharide, respectively.

pH was maintained with a 682 Titroprocessor connected to a 665 Dosimat and 719S Titrino (Metrohm, Herisan, Switzerland). The pH of solutions was adjusted and maintained constant throughout the sorption experiments by addition of micro-quantities of HNO_3 or NaOH (0.1 M), respectively.

RESULTS AND DISCUSSION

Selected exopolysaccharides from hydrothermal origin are characterized by different proportion of hexoses, hexuronic acids and *N*-acetyl-hexosamines (Tables 1, 2). Because of the large number of possible binding sites, a model that can be used to interpret and compare experimental data is based on some simplifying assumptions (Cabaniss *et al.*, 1984), assuming a 1:1 metal to binding site stoichiometry. This

Table 1. Raw neutral sugar, uronic acid, hexosamine and protein content of bacterial exopolysaccharides [% w,w]

EPS	Neutral sugars [a]	Hexuronic acid [b]	*N*-Ac-Hexosamines [c]	Total carbohydrate	Protein
ST 716	40	31	–	71	4
GY 785	47	25	–	72	3.5
HYD 1574	61	10	–	71	3
HE 800	2.5	32	33	67.5	2.5

[a] Galactose:mannose (1:1) ratio as standard.

[b] Glucuronolactone as standard.

[c] Glucosamine as standard.

Table 2. Monosaccharide ratios obtained after hydrolysis, methanolysis and trimethylsilyl derivatization [% w,w]

EPS	Glc	Gal	Man	Rha	Fuc	GlcA	GalA	GlcNAc	GalNAc
ST 716	11.8	10.7	11.3 [a]	–	–	19.7	8.5	–	–
GY 785	17.2	15.3	2.6	0.9	0.8	13.0	6.4	–	–
HYD 1574	13	13.4	12.2	6.3	–	7.6	–	–	–
HE 800	0.9	0.7	1.3	–	–	20.8	–	13.9	12.0

Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; Fuc, fucose; GlcA, glucuronic acid; GalA, galacturonic acid; GlcNAc, *N*-acetyl-glucosamine; GalNAc, *N*-acetyl-galactosamine.

[b] Mannose + pyruvated mannose.

model lead to the following equation:

$$q = \frac{Q_{\max} \times [\mathrm{Me}]_{\mathrm{eq}}}{K_{\mathrm{d}} + [\mathrm{Me}]_{\mathrm{eq}}} \tag{2}$$

The parameters $Q_{\max}$ and K_{d} are the saturation capacity and the apparent dissociation constant respectively.

The measure of the strength of complex formation between a metallic ion (Me) and a polysaccharide (L) is the stability constant K_{a}, defined as

$$K_{\mathrm{a}} = \frac{[\mathrm{Me{\cdot}L}]}{[\mathrm{Me}][\mathrm{L}]} = 1/K_{\mathrm{d}} \tag{3}$$

The determination of the stability constants has not been wholly satisfactory. These constants are calculated by using the concentrations rather than activities of ions or complexes. However, approximate stability constants or apparent stability constants, calculated for 1:1 complexes, allow comparison of the complexing ability of various cations and various polysaccharides.

During the addition of metallic cation to polysaccharide solutions, a sudden decrease in pH immediately occurred due to the acidic character of metallic ions. The pH of the solution then remained stable with values ranging from 4.5 to 6 depending on the initial concentration of the added cation (data not shown). The influence of pH with values ranging from 3 to 7 was studied in order to obtain the optimal experimental conditions.

Experiments under pH control conducted with lead, cadmium and zinc solutions at initial concentrations of 500 mg l^{-1} (Fig. 1) demonstrated that, below pH 4, the binding capacities of the ST 716 were reduced by the competition between metal and proton on the carboxylic site. The pK_{a} of ST 716 was measured at 4.1 and the alduronic acids in their undissociated forms, did not form complexes (Angyal, 1989). At pH 7, the concentrations of free metal decreased as the consequence of the simultaneous formation of the metallic hydroxides. Conversely between pH 4.5 and 6, the metal uptakes were stable with maximum values similar to those calculated by the Langmuir model with no pH control (Table 3). Consequently, within this range of pH, additional uptake experiments were conducted without pH control.

Equilibrium sorption isotherms of lead, cadmium and zinc by ST 716, GY 785, HYD 1574 and HE 800 have been plotted. The ST 716 isotherms are shown in Fig. 2 and represent the equilibrium distribution between the free and the complexed metal versus initial cation concentration. These results show that metal accumulation by exopolysaccharides

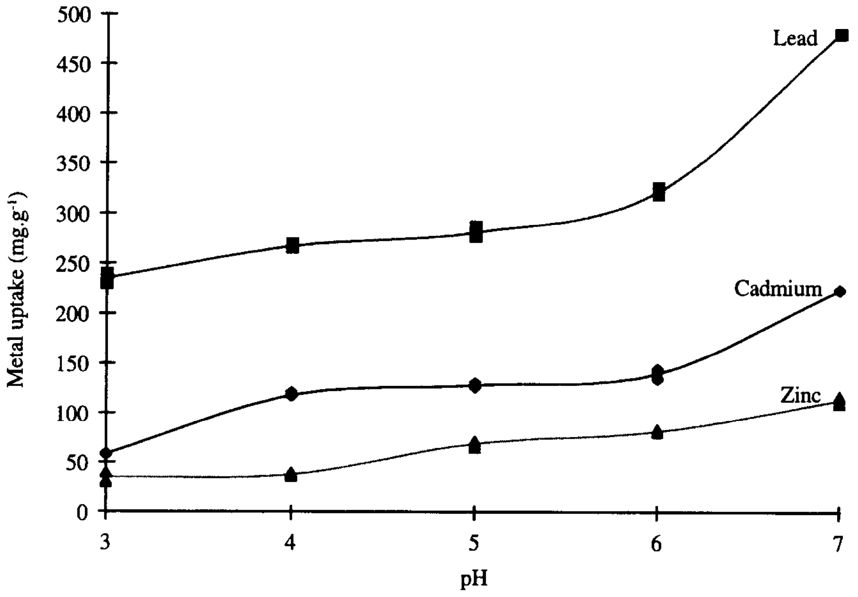

Fig. 1. Influence of pH control upon metal removal ($[Me]_{\mathrm{i}}$ = 500 mg l^{-1}) by ST 716. Standard deviations of six replicate measurements are smaller than symbol diameters.

Table 3. Uptake capacities Q_{max} and dissociation constants K_d of various heavy metals by exopolysaccharides

EPS	Lead		Cadmium		Zinc	
	Q_{max}	K_d	Q_{max}	K_d	Q_{max}	K_d
ST 716	1.52	0.007	1.12	0.044	1.15	0.038
GY 785	1.19	0.037	1.37	0.225	1.18	0.240
HYD 1574	0.74	0.032	0.69	0.051	0.79	0.143
HE 800	1.0	0.054	0.86	0.238	0.88	0.394

Q_{max}, mmol g^{-1}; K_d, mmol l^{-1}.

was a chemical, equilibrated and saturable mechanism, i.e. uptake capacities increase with the initial metal concentration as long as binding sites were not saturated. A linear transformation of Eq. (2) allows to calculate the parameters Q_{max} and K_d (Table 3).

Independent of the polysaccharide type and on the basis of mass (mg) metal uptake per gram EPS, lead uptake was always higher than cadmium and zinc. However, a more effective approach was to compare metal uptake capacities on a molar basis (mmol g^{-1}). Using this approach, differences appeared with the nature of exopolysaccharide and metal (Table 3). For both exopolysaccharides ST 716 and HE 800, lead uptake was higher than cadmium and zinc while cadmium and zinc uptake was strongest for GY 785 and HY 1574 respectively. ST 716 was the most efficient for lead removal with a binding capacity as high as 1.52 mmol g^{-1} while the maximum for cadmium was obtained by GY 785 (1.37 mmol g^{-1}). These two polymers exhibited the greatest zinc binding capacity (1.15 mmol g^{-1}).

In terms of net values, these purified bacterial biopolymers showed interesting chelating properties which can be compared to other values found in the literature. According to Volesky & Holan (1995), biosorbents of microbial origin have good performance in batch system with uptake values as high as 601 mg g^{-1} (2.90 mmol g^{-1}) for lead and 137 mg g^{-1} (2.09 mmol g^{-1}) for zinc with EPS secreted by *Bacillus subtilis*. However, in this case the biosorbent was not in its natural state (Brierley *et al.*, 1986). Moreover the capacity of hydrothermal EPS could certainly be increased by chemical modification such as sulfation, phosphatation or oxidation of the primary alcoholic group at C_6 to carboxylic acid.

Bonding between cation and ligand arises from the attraction that a metal (acceptor) ion has for one or more lone pairs

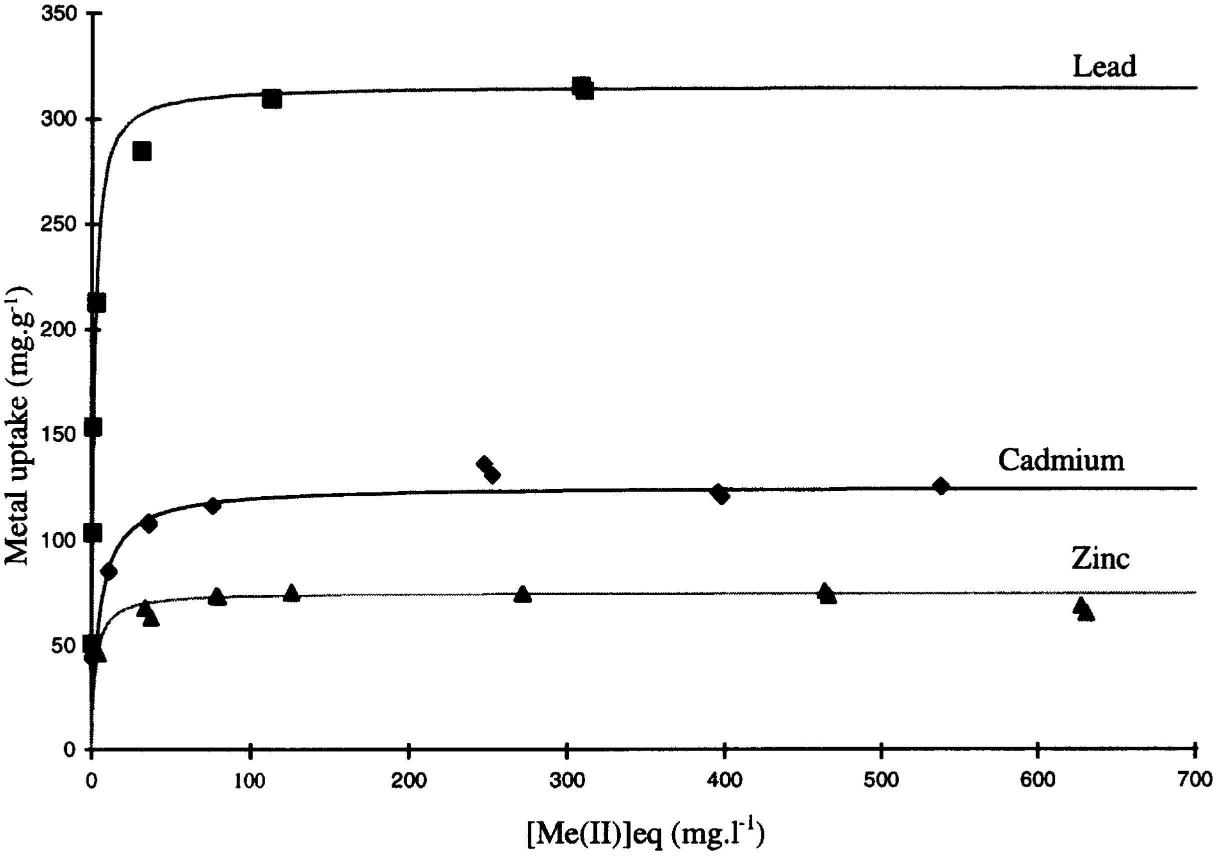

Fig. 2. Equilibrium sorption isotherms of lead, cadmium and zinc by ST 716 (room temperature, 3 h). Standard deviations of triplicate measurements are smaller than symbol diameters.

of electrons of the donor groups of a ligand. EPS possess a number and different kinds of chelating groups. The exopolysaccharides considered in this study differ in the chemical composition including the nature and the proportion of monosaccharides. ST 716 and GY 785 consisted of glucose, galactose and mannose as main hexoses, and glucuronic and galacturonic acids in molar ratios 1.6:1.4:1.1:2.4:1.0 and 3.2:2.9:0.5:2.0:1.0 respectively. In addition, ST 716 was shown to contain pyruvated mannose (Rougeaux *et al.*, 1996) which increases its acidic character. HYD 1574 was more neutral with glucose, galactose, mannose, rhamnose and glucuronic acid (2.0:2.0:1.8:1.0:1.0). HE 800 differed from others by the presence of *N*-acetyl hexosamines, GlcNAc and GalNAc, and GlcA (1.1:1.0:1.9). Neutral sugars present in very low proportion in this exopolysaccharide were contaminants. It can be noted the presence of sulfate ester groups on ST 716 (4%), GY 785 (8%) and HYD 1574 (12%).

It is widely accepted that carboxylic acids and amide groups are mainly responsible for the metal binding capacity of EPS. But comparison between tested exopolysaccharides show that the metal uptake capacities are not proportional to the concentration of hexuronic acids. Hydroxyl groups are probably involved in the chelation of metallic ions as oxygen atoms from these groups could be weak electron donors and complexes of metal ions with neutral carbohydrates are well-documented (Angyal, 1989). The presence of an a,e,a (axial–equatorial–axial) sequence or 1,3,5-triaxial arrangement of hydroxyl groups in the ring chain are most favourable to tridentate complex formation. Alduronate ions form much stronger complexes with cations than neutral sugars. The presence of a hydroxyl group in the β-position can contribute to the stability of such complexes. Furthermore, D-galacturonate complexes are stronger than D-glucuronate ones. This can be explained by a complex formation at the axial position of O-4 in the former. In addition, metal–carbohydrate complexes can be formed by two or more oxygens belonging to adjacent sugars in the polysaccharide chain. Chandrasekaran *et al.* (1988) have shown that in the potassium salt of gellan, ions are coordinated to both carboxylate oxygen atoms of β-D-glucuronate and O-6 of an adjacent β-D-glucose in one polymer strand, to O-2 of a β-D-glucuronate and O-6 of β-D-glucose in another strand, and to a molecule of water as well. In the case of alginate–calcium complex, a quinquedentate structure (O-4, O-5, and O-6 of a guluronate residue and O-2 and O-3 of a contiguous guluronate) can explain the mechanism of gelling of alginate. Finally, sulfate ester function can be also responsible for the observed metal uptakes, although binding is thought to be due mainly to the electrostatic interactions between sulfate ions and metals. According to Kaplan *et al.* (1987), the sulfate ester group plays a minor role compared with uronic acid.

The gross chemical composition and monosaccharide ratios of the four exopolysaccharides are not sufficient, alone, to totally explain their different metal uptake capacities. On the sole basis of the presence of amide and carboxylate groups, a greater binding capacity should have been observed for HE 800. The relative good metal uptake capacity of HYD 1574 such as zinc ions can be explained by its high sulfate content. Other parameters such as molecular weight or structural rearrangements and conformation are undoubtedly involved in the binding process. In order to understand the $Q_{\max}$ values obtained between the different exopolysaccharides, structural studies of EPS with and without metallic ions are in progress.

Complexation and the strength of the bond also depend on the nature of cations. Although of the same charge, the Pb^{2+} ion is, in aqueous solution, much larger (112 pm) than Cd^{2+} (97 pm) and Zn^{2+} (74 pm). The ionic radius is crucial, and the best radius for complex formation is suggested to be in the range of 100–110 pm. As a general point of view, anionic polysaccharide prefers to bind cations of large ionic radii (Angyal, 1989). For all polymers, lead exhibits greater affinity than cadmium and zinc. Except for ST 716, strength of complexation for cadmium ions are higher than for zinc (Table 3). From these data, it can be concluded that there is a relationship between stability constant and the ionic radius of cations.

Previous studies of the competition between two metallic ions on ST 716 had concluded that cadmium and zinc compete for the same binding site (Loaëc *et al.*, 1997). It can be noted that $Q_{\max}$ and K_d of cadmium and zinc ions are very similar. This result can be explained considering that Cd(II) and Zn(II) belong to the same chemical group whereas Pb(II) have a d^{12} electronic configuration, and thus the same type of coordination chemistry.

CONCLUSION

Bacterial exopolysaccharides from deep-sea hydrothermal vents have been shown to possess good metal binding properties. Maximum uptake capacities as high as 316 mg g^{-1} for lead, 154 mg g^{-1} for cadmium and 77 mg g^{-1} for zinc were obtained with ST 716 and GY 785. These values have to be related to the specific chemical nature of EPS and the number of possible binding sites. Strong electron donors such as carboxylate (alduronate, pyruvate), and *N*-acetyl hexosamines are involved in the binding process but weaker donors as hydroxyl groups can form tridentate complexes or contribute to the stability of carboxylate complexes. Determination of the repeating unit of these four EPS are in progress and will lead to a better understanding of polyoside–metal complexes. The different chemical nature of metallic ions (ionic radius and electronic configuration) has an effect upon the number and types of possible complexes. Finally and closely associated with these factors are the multitude of three-dimensional structures available to the biopolymers.

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