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ORIGINAL PAPER

Investigations into the uptake of copper, iron and selenium by a highly sulphated bacterial exopolysaccharide isolated from microbial mats

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Abstract A bacterium isolated from microbial mats located on a polynesian atoll produced a high molecular weight (3,000 kDa) and highly sulphated exopolysaccharide. Previous studies showed that the chemical structure of this EPS consisted of neutral sugars, uronic acids, and high proportions of acetate and sulphate groups. The copper- and iron-binding ability of the purified pre-treated native EPS was investigated. Results showed that this EPS had a very high affinity for both copper (9.84 mmol g^{-1} EPS) and ferrous iron (6.9 mmol g^{-1} EPS). Amazingly, this EPS did not show any affinity for either ferric ions or selenium salts. This finding is one of the first steps in assessing the biotechnological potential of this polysaccharide.

Keywords Binding capacity · Copper · Iron · Microbial mats · Sulphated exopolysaccharide

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Introduction

Polysaccharides occur as important constituents of plant and microbial cell walls, either as storage polysaccharides or as biopolymers secreted by microorganisms known as exopolysaccharides (EPS). Bacterial polysaccharides possess a wide variety of properties that may not be found in the more traditional polymers of plant origin. Although they compete with polysaccharides from other sources, e.g., from algae (alginates, carrageenans), crustacea (chitin) or plants, their production is less subject to variability due to marine pollution, crop failure or climatic impact [11, 15, 24].

Due to their many interesting physical and chemical properties, e.g., stabilizing, suspending, thickening, gelling, coagulating, film-forming and water retention capability, polysaccharides have found applications in many industrial sectors, e.g., those producing detergents, textiles, adhesives, paper and paint, the food and beverage industries, and those involved with pharmaceuticals and cancer therapy, drug delivery, oil recovery and metal recovery in the mining industry and from industrial wastes [3, 5, 12, 22, 25].

Heavy metals are known to be essential for almost all kinds of living organisms, but excessive concentrations can lead to severe health problems. Biosorption of heavy and/or radioactive metals can be considered as an alternative technology able to compete with other conventional technologies, such as chemical precipitation, electrolytic methods, adsorption on activated carbon, membrane processes and ion exchange using different chelating resins and fibers [26, 27]. Bacterial EPSs contain ionizable functional groups, such as carboxyl, amine, sulphate, acetate, and hydroxyl groups, which enable these polymers to bind heavy metals.

In the French Polynesian atolls, microbial mats develop in water ponds exposed to salinity fluctuation and high solar irradiation. These microbial mats, which are called “kopara” by the inhabitants of Tuamotu archipelago, are mainly composed of cyanobacteria and eubacteria, and contain large amounts of polysaccharides [16]. These exopolysaccharides preserve the integrity of the mat, prevent dessication and maintain cell hydrophobicity [2, 23]. They also are involved in the precipitation of carbonates [6, 7]. Since 2001, this ecosystem has been screened for innovative biomolecules, including novel microbial exopolysaccharides secreted under laboratory conditions by both bacteria and cyanobacteria [20, 21].

A marine bacterium (strain RA19), isolated from a microbial mat in Rangiroa atoll and belonging to the *Paracoccus* family, produced an EPS under laboratory conditions. This strain, *P. zeaxanthinifaciens* subsp. *payriae*, has been deposited in the Collection Nationale de Culture de Microorganismes (Institut Pasteur, Paris, France) as strain CNCM I-2926 [19]. The crude chemical composition included approximately 48% (w/w) neutral sugars, 8% (w/w) uronic acid, 8% (w/w) acetate and 27% (w/w) sulphate [19]. This study was conducted to investigate the ability of EPS produced by strain RA19 to bind heavy metals, such as copper and iron, as a first step in assessing the biotechnological potential of this EPS.

Materials and methods

In November 2001, samples of “kopara” were collected from the different microbial mats located on the atoll of Rangiroa. Enrichment cultures were purified on marine agar 2216E (MA, Difco Laboratories, Detroit, MI). Strain RA19 was selected because of its ability to exhibit a swarming mucoid phenotype on marine agar 2216E supplemented with 30 g l^{-1} of glucose along with a pigment further identified as pure all-*trans*-zeaxanthin [19].

Growth conditions

The optimal temperature for growth was between 30 and 35°C, the optimal pH was between 6.5 and 7.5, and the optimal ionic strength was between 20 and 40 g l^{-1} of NaCl. The doubling time, under optimal conditions, was 35 min [19].

Isolation and purification of EPS

Exopolysaccharide production was performed at 30°C in a 2-l fermenter (New Brunswick, Toulouse, France) containing 1 l of 2216E-glucose broth. A batch of culture medium was inoculated at 10% (v/v) with a suspension of cells in exponential phase. The pH was adjusted and maintained at 7.6 by automatic addition of NaOH 1 mol l^{-1}. Foaming was avoided by addition of Pluronic-PE6100 oil (BASF, Levallois/Perret, France) at 0.1% (v/v). The air flow was fixed at 30 l h^{-1} and the agitation rate from 200 to 400 rpm in order to maintain the level of dissolved O_2 at around 25%.

The water-soluble exopolysaccharide was recovered from the culture medium by high-speed centrifugation (20,000*g* for 2 h) after 4 days, then purified by ultracentrifugation against deionized water using a Pellicon-2 Mini Holder equipped with a Biomax 100 K filter (Millipore Corporation, Bedford, MA) and lyophilized prior to further analysis.

Metal-binding experiments

Purified exopolymer was dissolved in milliQ water. Preliminary experiments were conducted with EPS concentrations up to 1 mg ml^{-1} according to protocols described by Loaec et al. [13, 14]. Later, this concentration was reduced to 0.1 mg ml^{-1} when problems in the methodology were attributed to the high viscosity of the polysaccharide in solution. Metals tested were copper, iron and selenium. Solutions of copper were prepared from anhydrous $Cu(SO_4)_2$. Solutions of ferrous and ferric iron were prepared from $FeSO_4{\cdot}7H_2O$ and $Fe_2O_{12}S_3{\cdot}H_2O$, respectively, while the assays for selenium were performed with Na_2SeO_3, SeO_2, H_2SeO_3 and Na_2SeO_4.

Reaction solutions were prepared in duplicate by adding 40 ml of EPS solution to a 250-ml flask containing 10 ml of metal solution for a final concentration of 0.1 mg ml^{-1} EPS and metals in the range of 10–1,000 mg l^{-1}. The total sample volume was 50 ml. Samples were mixed thoroughly for 3 h at 200 rpm.

Metal uptake capacity was determined using the general equation [26]:

$$q = \frac{(C_i - C_{eq})V}{m}$$

where C_{eq} is the final metal concentration and C_i the initial metal concentration in solution of volume V, and m the mass of exopolysaccharide.

Appropriate blanks were examined throughout the sorption experiments to insure the absence of glassware sorption of metals and other potential side effects.

Metal analyses

After incubation, the pH of filtrates was reduced at 1.5 with concentrated nitric acid, and the metal concentration was determined by atomic absorption spectrometry or inductively coupled plasma spectrometry. All assays were run in triplicate.

FT-IR spectroscopy

Pellets for infrared analysis were obtained by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16-mm-diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the 4,000–400 cm^{-1} region.

Scanning electron microscopy

Samples for scanning electron microscopy (SEM) analysis were glued to aluminium stubs, gold-sputtered, and examined using a Philips XL 30 operating at 30 kV. EDAX analysis (EDAX Edx 4i) was carried out on non-gold-coated specimens.

Results and discussion

Chemical composition of EPS

The exopolysaccharide produced under laboratory conditions by strain RA19 is mainly composed of neutral sugars 48% (w/w), while hexuronic acids only accounted for 8% (w/w) [19]. Acetate groups accounted for 8% (w/w) of the total EPS. But the major feature of this bacterial EPS was its high amount of sulphate (27%) (w/w). Highly sulphated bacterial EPS are rare in nature, making this exopolymer different from others. Sulphate groups play an important role in the biological activities of microbial polysaccharides. For several years, there has been growing interest in the recognition of biological activities of microbial polysaccharides [30]. The anticoagulant activity of some bacterial polymers has to be linked to the high sulfate content associated with specific chemical composition [17, 28].

Heavy metal binding capacity

Light cations, including K, Na, Mg and Ca, are usually present in the crude exopolysaccharide due to both the medium culture and the extraction procedure. These metals were first eliminated by treatment with a cationic exchange column (Dowex 50×8) followed by an ultrafiltration. The biopolymer was then saturated with a single counter ion (Li^+) to ensure homogeneity of the cationic composition for all experiments. Complete elimination was achieved following this pre-treatment (Table 1).

The initial pH for copper sorption ranged from 4.5 to 5 and remained stable throughout the experiments. Initial pH for ferrous ion sorption was in the same range, but a slight deposit of iron oxyhydroxyde (FeOOH) was observed in the medium at the end of preliminary experiments. The pH was then adjusted to pH 3, and all experiments were conducted under nitrogen to ensure the formation of such deposits.

Table 1 Percentage of light cations in EPS produced by strain RA19 (*P. zeaxanthinifaciens* subsp. *payriae*) (%, w/w)

EPS RA19	Na^+	K^+	Ca^{2+}	Mg^{2+}
Native state	3.73	0.48	1.88	1.27
Pre-treatment	<0.01	<0.01	<0.01	<0.01

Equilibrium sorption isotherms for copper and iron (Fe^{+II}) by EPS of *P. zeaxanthinifaciens*. subsp. *payriae* (RA19 EPS) are shown in Fig. 1. Sorption isotherms represent the equilibrium distribution of metal between the aqueous and the gel phase versus metal concentration. The sorption increases with the initial metal concentration as long as binding sites are not saturated. Thus, it can be hypothesized that metal accumulation by RA19 EPS is a chemical, equilibrated and saturable mechanism for both copper and iron (Fe^{+II}).

The results are presented on both the basis of mass (mg) uptake per gram EPS and on a molar basis (mmol g^{-1} EPS). Copper uptake reached 625 mg g^{-1} EPS (9.84 mmol g^{-1} EPS), while iron (Fe^{+II}) uptake was lower with an uptake of 385 mg g^{-1} EPS (6.9 mmol g^{-1} EPS). Thus, the dissociation constant K_d was 54.37 mg l^{-1} (0.85 mmol l^{-1}) for copper and 32.6 mg l^{-1} (0.58 mmol l^{-1}) for iron (Fe^{+II}).

Experiments were also conducted with ferric ions using $Fe_2O_{12}S_3{\cdot}H_2O$ salts. Due to the formation of a significant amount of FeOOH, the pH of the solutions was lowered to 1.8. Under such conditions, no significant and reproducible metal uptake was observed for this polymer. The pH of the solution strongly affects the sorption capacity of this

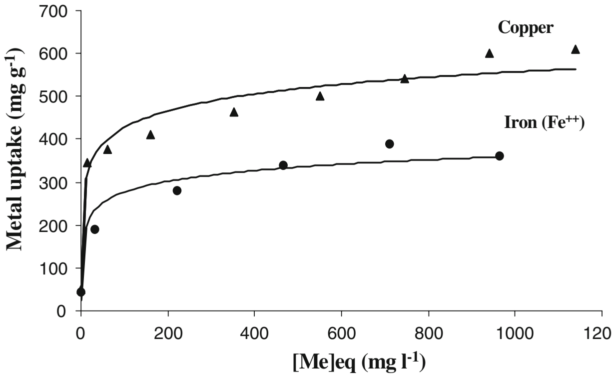

Fig. 1 Equilibrium sorption isotherms of copper and iron (Fe^{+II}) by RA19 EPS (3 h, room temperature). Standard deviations of triplicate measurements are smaller than symbol diameters

polymer. In strongly acidic solutions, protons compete with metal ions and are more available to protonate carboxyl groups then reducing the number of binding sites for ferric ions. In addition, degradation of the polymer occurred after 3 h at such low pH.

Experiments conducted with different selenium salts and under different pH conditions did not lead to any uptake of these ions by this EPS in its native state. Additional experiments will be conducted with partially depolymerized or deacetylated RA19 EPS in order to explain the absence of sorption by this biopolymer [8, 9].

These results have to be compared to other natural biosorbents. The value of Q derived from the Langmuir isotherm equation was up to 625 mg g^{-1} EPS, and this value was much higher than the observed capacities or the estimated Q values by other natural bacterial or modified exopolysaccharides and biomasses as well reported so far in the literature. Maximum copper uptake up to 323 mg g^{-1} EPS was reported for zooglane, a polysaccharide produced by *Zoogloea ramigera* [18].

Iron uptake was also higher than for any bacterial polysaccharide reported in the literature. Maximum uptake was observed with the cell walls of *Bacillus subtillis* (201 mg g^{-1} compared to 385 mg g^{-1} for RA19 EPS). The capacity of chitosan and cross-linked chitosan to adsorb ferrous ions did not exceed 64 mg g^{-1} [29], while Brierley and Brierley [4] related a Fe^{+II} retention capacity of 107 mg g^{-1} by bacterial biomass.

Interestingly, up to initial concentrations of 500 and 300 mg l^{-1} of copper and ferrous iron, respectively, the removal efficiency was near 100% (w/w). For higher concentrations, a significant decrease in the metal uptakes can be observed.

Scanning electron microscopy

Biosorption of both copper and iron salts by RA19 EPS induces change in the conformation of the polymer with the formation of microspheres up to 3 µm diameter (Fig 2). Microanalysis performed on these microspheres indicated large concentrations of metal ions up to 70% (w/w).

FTIR study

Infrared spectroscopy has proven to be a powerful tool for studying biological molecules and for obtaining information about metal-EPS binding. Figure 3 shows the FTIR spectra of Li-treated RA19 EPS before and after binding experiments with either copper or iron (II). Analysis of the FTIR spectrum of the pre-treated polysaccharide showed intensive bands in the range of 3,700–3,200 cm^{-1}, corresponding to the stretching band of v OH of polysaccharides. The FTIR spectrum also exhibited an intense band at 1,640 cm^{-1} along with an absorption peak at 1,740 cm^{-1}, corresponding to the stretching band of the free carboxyl double bond from the functional carboxylic groups present in this biopolymer. The doublet at 1,230–1,250 cm^{-1} corresponded to the presence of ester sulphate groups present in a large proportion in this polymer.

Complexation with either copper or iron induced different shifts in the wave numbers corresponding to specific vibrational modes of native exopolysaccharide. The most

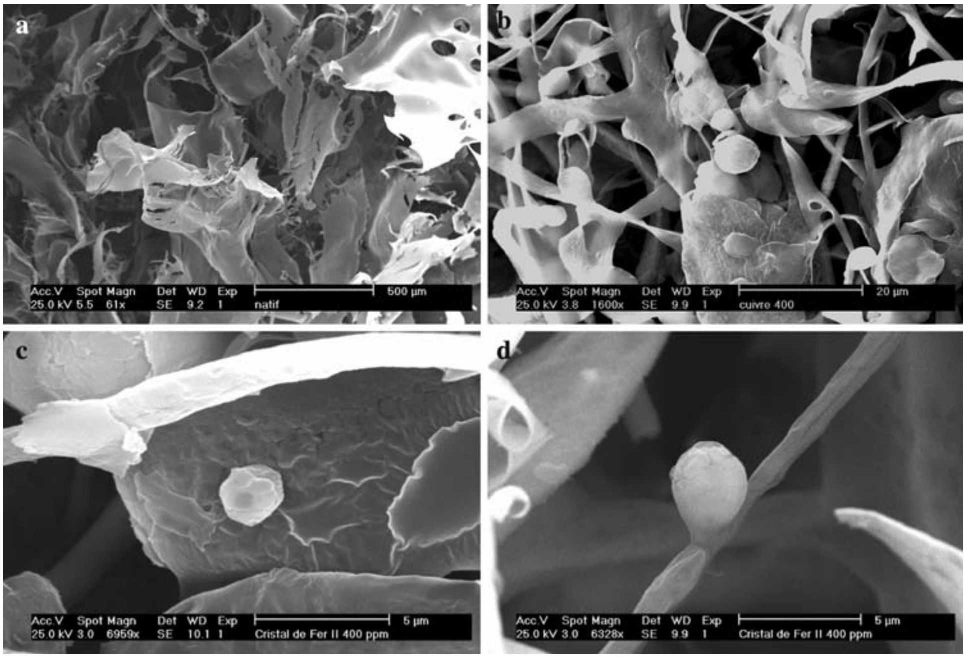

Fig. 2 Scanning electronic microscopy photography of **a** native RA19 EPS; **b** RA19 EPS–Cu; **c**, **d** RA19 EPS–Fe

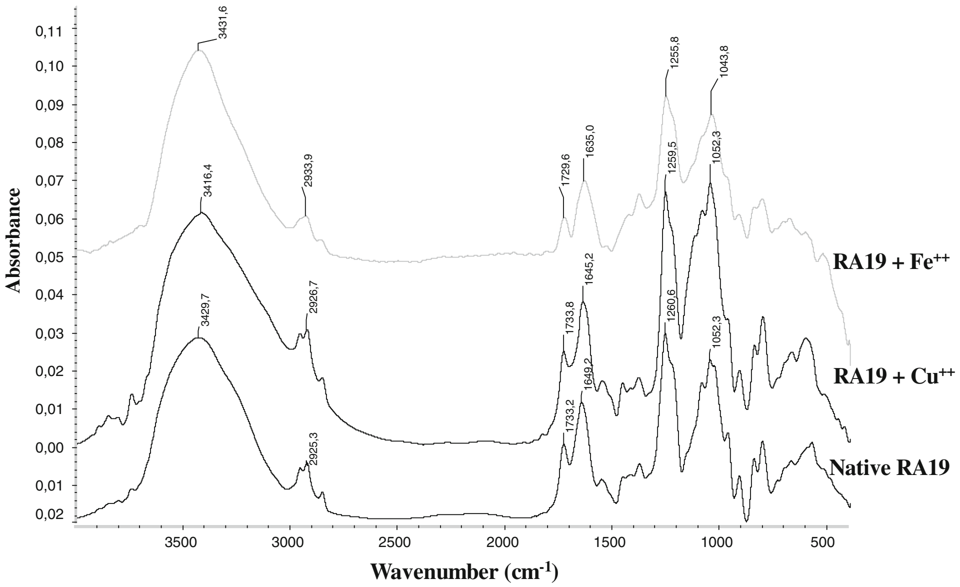

Fig. 3 FT-IR spectra of RA19 EPS before and after copper or iron (Fe^{+II}) biosorption

important differences in the vibrational spectra of the copper complexes seem to occur for the broad absorption peaks in the region of 3,200–3,700 cm^{-1} assigned to the existence of hydroxyl groups involved in the H-bond network. Neither the copper nor the iron ions induced significant shifts in the vibration bands associated with carboxylic and sulphate groups as well.

The heavy metal-binding capacity of polysaccharides is usually attributed to the high hydrophilicity of the polymer due to the presence of hydroxyl groups, the presence of functional and reactional groups (sulphate, acetamino, primary amino and ester groups) and the flexible structure of the polymer chains. RA19 EPS is characterized by high proportions of sulphate (27% w/w of the total sugar) and significant amounts of acetate groups (up to 8% of the total sugar). It is widely accepted that carboxylic groups from either uronic acids or non-sugar substituents are responsible for the metal-binding capacity of EPS specifically in pH solutions ranging from 4 to 5. However, comparison between different bacterial EPSs has clearly demonstrated that metal uptake capacities are not proportional to the concentration of uronic acids and carboxylic groups [13]. From our data, it can be hypothesized that hydroxyl groups are probably involved in the chelation of copper and iron by RA19 EPS as oxygen atoms from these groups could be weak donors [1].

The role of sulphate groups in the metal-binding capacity of polysaccharides is controversial [10, 14]. Despite the very high content in sulphate of EPS RA19, neither copper ions nor ferrous ions seemed to affect the vibrational bands of sulphonate groups. Conversely, sulphate groups are known to be involved in the overall uptake of trivalent ions, such as ferric ions. In our experiments, low Fe^{+III} sorption was observed with this highly sulphated polysaccharide.

Conclusion

Human activities have released large amounts of toxic elements that can be harmful to human beings into the environment. Biosorbents and primarily exopolysaccharides can act as substitutes to other technologies. The EPSs produced under laboratory conditions by the strain isolated from a microbial mat show a very high binding capacity for both copper and iron salts. This finding could be the first step in the development of a low cost biosorbent used in its native state or as modified polysaccharide-based materials.

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