Adsorption of copper on Pseudomonas aureofaciens: Protective role of surface exopolysaccharides

A.G. González a, L.S. Shirokova b,c, O.S. Pokrovsky b,* , E.E. Emnova d, R.E. Martínez e, J.M. Santana-Casiano a, M. González-Dávila a, G.S. Pokrovsky b

a Universidad de Las Palmas de Gran Canaria, Departamento de Química, Campus de Tafira, 35017 Las Palmas, Spain
b Géochimie et Biogéochimie Expérimentales, LMTG, Université de Toulouse, CNRS-IRD-OMP, 14 Avenue Edouard Belin, 31400 Toulouse, France
c Laboratoire de Aquatic Ecosystems, Institute of Ecological Problems of the North, Russian Academy of Science Ural Branch Russian Academy of Science, Arkhangels, Russia
d Institute of Plant Genetics and Physiology, Moldavian Academy of Science, Padurii St., 26/1, MS-2002, Moldavia
* GeoForschungsZentrum Potsdam, Section 4.1, Telegrafenberg, 14473 Potsdam, Germany

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A B S T R A C T

Adsorption of copper on exopolysaccharide (EPS)-rich and (EPS)-poor soil rhizospheric Pseudomonas aureofaciens cells was studied as a function of pH and copper concentration at different exposure time in order to assess the effect of cell exopolysaccharides on parameters of adsorption equilibria. The surface properties of bacteria were investigated as a function of pH and ionic strength using potentiometric acid–base titration and electrophoresis that permitted the assessment of the excess surface proton concentration and zeta-potential of the cells, respectively. For adsorption experiments, wide range of Cu concentration was investigated (0.1–375 μM) in order to probe both weak and strong binding sites at the surface. Experimental results were successively fitted using a Linear Programming Model approach. The groups with pK a of 4.2–4.8 and from 5.2 to 7.2, tentatively assigned as carboxylates and phosphoryl respectively, are the most abundant at the surface and thus essentially contribute to the metal binding. The presence of exopolysaccharides on the surface decreases the amount of copper adsorbed on the bacterial cell wall apparently via screening the underlying functional groups of the cell wall. At the same time, dissolved EPS substances do not contribute to Cu binding in aqueous solution. Results of this study allow quantification of the role played by the surface EPS matrix as a protective barrier for metal adsorption on bacterial cell walls.

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1. Introduction

Microorganisms have a high capacity of binding cationic metals onto their cell walls [1–8]. This process can control the mobility, speciation and bioavailability of metals in natural environments. Numerous studies have been conducted to address these issues, notably in the context of freshwater and saline environments [9,10], groundwaters [11,12], deep-sea hydrothermal systems [13,14], deep sedimentary basins [15,16] and sediments [17]. In addition, some physico-chemical research has been undertaken for marine and freshwater diatom species [18,19] spores of marine bacteria [20], freshwater algae [21], and whole bacteria and bacterial cell walls [6,22–28].

A common feature of many bacteria is the biosynthesis of extracellular polysaccharide (EPS) that can offer a protective barrier under environmental stress conditions [29]. In metal stress conditions, bacteria are highly stimulated to produce exopolysaccharides (EPS), which significantly increase the viability of cells exposed to metal-bearing solutions [30]. Commonly, exopolysaccharides are composed of glucose, fructose, manose, pyruvate and fucose, as well as mannuronic- and guluronic-acid complexes [31]. In addition, the EPS matrix comprises a mixture of heteropolysaccharides, proteins and nucleic acids [32,33]. Molecules of EPS from the majority of the bacterial species are negatively charged due to the presence of carboxyl and phosphoryl functional groups [34]. Carboxyl or hydroxyl groups are also known to be primarily involved in the metal-binding process by forming coordination bonds that facilitate the stability of ion polymer complexes [35].

Extracellular polysaccharides can bind toxic metal ions outside the cells, enabling organisms to ameliorate the presence of metal cations [36–39]. EPS may be considered as an efficient scavenger of heavy metals from water systems [40], thus reducing the concentration of free ionic metal activity [41]. EPS synthesis is affected by UV-radiation [42], dessication [43], and the presence of toxins [44]. However, the existing information contains certain contradictions/inconsistencies: on the one hand, EPS plays a role as a protective barrier of cell versus external metal load, and on the other
hand, it is reported to adsorb significant amount of metal in the form of rather labile carboxylate or hydroxide complexes which may become easily bioavailable due to their location in the vicinity of cell membrane. To better address these contradictions, specially designed experiments for the same bacteria species, of similar cell wall composition, but different proportion of exopolysaccharides, would be necessary.

One of the most powerful tools able to characterize and predict metal interaction with bacterial cell walls and external envelopes is the thermodynamic approach. The thermodynamic description of interaction between microorganisms and aqueous solution often involves a surface complexation approach [6,20–23,26,45–49]. Surface speciation models allow rigorous description of reversible metal adsorption onto bacterial surface functional groups. This study represents a part of a concerted effort aimed at the expanding of the data base of adsorption parameters for aquatic and soil microorganisms with respect to metal pollutants. To achieve this goal, surface adsorption constants were measured for typical rhizospheric species Pseudomonas aureofaciens cultured in two different growth media that produced EPS-rich and EPS-poor cultures. This allowed quantifying the effect of EPS produced on the adsorption of copper by P. aureofaciens under rigorously constrained solution conditions.

The two specific questions addressed in this study are: (1) To what extent the abundant syntheses of EPS by bacteria modify metal adsorption parameters? (2) Are the charged (carboxyl-, phosphoryl-) groups in the cell wall screened by the EPS layers against aqueous Cu\(^{2+}\) or does soluble EPS complex copper ion in solution? Obtained results should allow better understanding of the effect of metal on bacterial growth through testing a link between the thermodynamic parameters of metal adsorption and the inhibition of culture growth.

2. Experimental

2.1. Culture characterization

The bacterial strain P. aureofaciens CNMN PsB-03 was obtained from the laboratory of Plant Mineral Nutrition and Hydric Regime (Institute of Genetics and Plant Physiology, Moldovan Academy of Sciences, Chisinau, Moldova). It was isolated from chernozem soil and selected due to its capacity to produce of exopolysaccharide (EPS) on a sucrose-containing medium [50].

The strain of P. aureofaciens was maintained at 4 °C in liquid succinic acid (SA) media [51], and cultured in two different media: (1) sucrose–peptone (SP) solution [52], thus promoting rich EPS synthesis which occurred in both Cu-free and Cu-rich media (up to 3 mM Cu\(^{2+}\)aq), or (2) SA media, yielding very poor EPS production. Cultivation was performed at 25 °C for 48–72 h with continuous shaking. The qualitative monosaccharide composition of the EPS produced in the indicated media was different: the SP-media yielded EPS composed of 76.1% fructose and 11.4% glucose, whereas the SA-media yielded EPS composed of 49.6% glucose, 22.3% fructose and 14% mannose [53]. Small amounts (<10% total sugars) of rhamnose, ribose, xylose and galactose were present in both EPS samples. Therefore, the most probable EPS for the SA-medium was the acidic polysaccharide levan (polyfructan). In contrast, glucon and some other heteropolymers were present in the SA-medium.

Experiments were undertaken for non-rinsed and rinsed bacterial biomass that produced two sub-samples for both EPS-rich and EPS-poor samples. In the first case, the bacteria were collected from the culture without rinsing, through centrifugation at 8000g for 20 mins. Therefore, for non-rinsed biomass, the EPS content of the cell wall was expected to remain intact. The rinsed biomass was produced by repeated centrifugation (three times) in an inert electrolyte solution (NaNO\(_3\) or NaCl). Repetitive rinsing of biomass in electrolyte solution brings about the removal of part of the EPS from the cell surface. For the EPS-rich (SP-media) and the EPS-poor (SA-media) cultures, 28% and 6% total biomass were removed, respectively. The biomass concentration is given in g humid/L (centrifuged 20 min at 8000g). The conversion factor of wet to dry biomass was 3.6 and 5.0 for EPS-rich and EPS-poor cultures, respectively.

2.2. Infrared characterization of soluble exopolymers

The exopolymers (EP) were isolated from cell-free culture supernatant of both SP and SA media by precipitation with acetone (1:3, v/v) and dried at 40 °C. The infrared (IR) spectra of these products confirmed the presence of several functional groups [54]. The IR spectra of non-purified EP were recorded on Specord-74 spectrometer for spectral range 400–4000 cm\(^{-1}\) and the IR band identification was performed according to Bellamy [55]. The aliphatic functional groups (–CH\(_2\)_n, –CH\(_3\)_m, –C=H) were represented by absorption bands at 2960, 2850, 1465, 1370 cm\(^{-1}\). The IR spectra showed absorption attributed to hydroxyl groups (O–H) at 3250 cm\(^{-1}\) (polymeric inter-molecular hydrogen bonds), 1070 and 1290 cm\(^{-1}\) (primary), 1100 cm\(^{-1}\) (secondary), 1140 cm\(^{-1}\) (tertiary). The IR absorption at 1086 cm\(^{-1}\) suggested the presence of ester bond C=O in EP molecules, which proved polysaccharide nature of tested exopolymers. The absorption bands at 715 and 525 cm\(^{-1}\) confirmed the presence in EP chains of sulfur-containing groups (C=S or S=O). Moreover, protein groups (at 1540 and 1640 cm\(^{-1}\)) were present in all tested samples. The characteristic absorption signals for carboxyl groups (\(>\text{C}=\text{O}\)) were not detected in IR spectra of investigated EP samples synthesized by strain PsB-03 on both type of nutrient media. Note that, since infrared characterization was performed on EPS recovered from supernatants, this constitutes soluble EPS which has been shown to display rather less carboxyl groups relative to the bound EPS [56].

Subsequent investigations of EPS samples of P. aureofaciens PsB-03 using Bruker FTIR spectrometer with MST detector supplied with IR ATR (Attenuated Total Reflection), included ZnSe element for 4000–700 cm\(^{-1}\) have shown the same results. The hydroxyl, aliphatic, ester, protein, sulfur-containing groups were detected, while the carboxyl groups (\(>\text{C}=\text{O}\)) were not revealed.

2.3. Electrophoretic measurements

The microelectrophoresis is a powerful technique to characterize the electric double layer (EDL) of microbial cell surfaces and their surface electric potential [57–62]. The zeta-potential of EPS-rich P. aureofaciens cultures were measured using a CAD Instrumentation “Zetaphorometer IV” Z 4000, microelectrophorimeter. The electrophoretic measurements were carried out in a quartz cell connecting two Pd electrode chambers. The cell was illuminated by a 2 mW He/Ne laser. During each measurement an electric field of 80 V/cm was applied in each direction. Metabolically active bacteria were suspended in 0.1 and 0.01 M NaNO\(_3\) and pH was varied by adding 0.1 M HCl and NaOH in the range from 2.3 to 11 at 0.2 pH unit intervals. For each pH, three replicates of zeta-potential were performed.

2.4. Titration of bacterial surface

The acid–base titrations of bacteria cultured in SP and SA media were carried out in 0.1 M NaNO\(_3\) at 25 ± 0.5 °C. After harvesting, bacterial biomass was rinsed three times with 0.1 M NaNO\(_3\). The experiments were performed at constant biomass concentration of 10.0 g\(_{\text{wet}}\)/L. The suspension was pre-saturated with nitrogen
and first acidified using standardized 0.05 M HCl to initial pH of 3. The acid–base titration was done by adding small aliquots of NaOH (0.96 M) using an automatic titrator system Mettler® DL70 ES and continuous nitrogen bubbling. The addition interval was 0.1–0.4 pH units and the criteria of electrode potential stability between two subsequent additions was set as ±0.005 pH units/min. Note that this value is dramatically different from conventional 0.1 mV/s (~0.1 pH units/min) used in most surface titrations of bacterial biomass.

The excess surface proton concentration was calculated from the equation [23,63]:

$$[H^+]_s = (C_{aj} (\text{suspension}) - C_{aj} (\text{reference})) - (C_{bj} (\text{suspension}) - C_{bj} (\text{reference}))$$  \hspace{1cm} (1)

where $C_{aj}$ and $C_{bj}$ correspond to concentration of base and acid for the jth addition of titrant. As a reference system, the supernatant from the rinsed bacterial biomass was used and processed exactly in the same way as bacterial suspension. Note that this definition of excess surface proton concentration does not allow converting it directly to the cell surface charge, because the initial amount of surface protons is not known.

2.5. Metal Adsorption

The copper adsorption experiments were designed to provide a quantitative characterization of metal binding by bacterial cells in a wide range of pH and Cu(II) concentrations in solution. For this, two types of experiments were carried out: (i) adsorption at constant initial metal concentration in solution as a function of pH (pH-dependent adsorption edge) and (ii) adsorption at constant pH as a function of metal concentration in solution (adsorption isotherm). All experiments were performed in the solutions undersaturated with respect to any copper oxide, hydroxide or carbonate phase as verified by speciation calculations with the MINTEQA2 computer code and corresponding database [64,65].

The initial copper concentration was always $5.2 \times 10^{-5}$ M at variable pH and the copper concentration was varied between 0.44 and 375 $\mu$M at constant pH. The pH was adjusted by adding aliquots of NaOH (0.1 and 0.01 M) or HNO₃ (1, 0.1 and 0.01 M). pH was constant by adding MES (2- morpholinoethanesulfonic acid monohydrate) buffer, which was prepared at a concentration of 0.1 M and pH 5.5 and added to a final concentration of 0.005 M.

The adsorption of Cu²⁺ was followed as a function of time in series of kinetic experiments, performed at the initial concentration of 52 $\mu$M and pH 5.85. For this, EPS-rich bacteria were used as live (harvested at stationary stage), dead (autoclaved) and inactivated (0.01 M NaNO₃ media). In addition, adsorption on live EPS-rich bacteria was studied as a function of pH and [Cu²⁺]ₐₙ after 1 and 24 h of exposure time. All kinetic experiments were conducted in 0.1 M NaNO₃ under darkness with permanent stirring at a constant biomass concentration of 4 $\mu$gvet/L.

Most adsorption experiments were conducted during 1 h at 25 ± 0.2 °C in continuously agitated bacteria suspension with an ionic strength of 0.1 M NaNO₃ using 8 mL sterile polypropylene vials. Experimental conditions are listed in Table 1. The biomass concentration was kept constant at 4 $\mu$gvet/L and copper concentration in solution ([Cu²⁺]ₐₙ) ranged over four orders of magnitude from 0.13 $\mu$M to 4.72 mM (Table 1). This allowed proving not only abundant and weakly binding sites but also less abundant but much stronger binding sites. The pH was adjusted using aliquots of NaOH or HNO₃ and varied from 1.7 to 7.1 (Table 1). Live, freshly harvested (stationary stage) cells and dead (autoclaved) bacteria were used in the adsorption experiments.

To better account on the EPS presence on the cell surface, a number of experiments were carried out after rinsing the biomass to different degree. The biomass was centrifuged three times during 20 min at 5000g. The last supernatant was considered as a control and it was used for blank adsorption experiments in the absence of biomass. At the end of each experiment, the suspension was centrifuged at 4500g and the resulting supernatant was filtered through 0.45 $\mu$m acetate cellulose filter, acidified with ultrapure HNO₃ and stored in the refrigerator until the analysis. Adsorption of copper on cell walls for both EPS-rich and EPS-poor P. aureofaciens was investigated in the pH range between 1.7 and 6.8 (Fig. 1 and Table 1). The adsorption of copper on cell surface was quantified by subtracting, at each solution pH, the concentration of copper remaining in bacterial suspension from concentration of added copper in the supernatant (control experiments without biomass). The adsorption of copper on reactor walls at 2 $\leq$ pH $\leq$ 7 was negligible (<1%). It was verified by blank (supernatant) experiments.

Reversibility of Cu²⁺ adsorption on live bacterial biomass was verified via treating the cells with adsorbed Cu after 1.5 h of exposure by 0.01 M EDTA during 10 min and analyzing Cu in the supernatant. This conventional method is widely used to distinguish
Dissolved Organic carbon (DOC) concentration in solution was routinely monitored in all experiments of pH-dependent adsorption edge, at pH from 1.5 to 4.4, using a Carbon Total Analyzer (Shimadzu TOC-6000) with an uncertainty of 3% and a detection limit of 0.1 mg L⁻¹.

3. Results

3.1. Titration of bacterial surface and electrophoretic measurements

The surface titration experiments were carried out with rinsed EPS-rich and rinsed EPS-poor biomass in 0.1 M NaNO₃ for pH range from 3 to 10 (Fig. 1 and Table 2). Both types of cultures demonstrated high buffer capacity in the pH range from 5 to 10. The pH corresponding to zero excess proton adsorption was 4.0 ± 0.05 and 4.7 ± 0.11 for EPS-rich and EPS-poor bacteria, respectively (Fig. 1). This difference can be understood from the view point of EPS chemical composition: cells lacking in EPS should exhibit higher amount of positive charges, amine-like group of the cell surface envelopes, which are not protected by the exopolysaccharidic layer and having mostly neutral or negatively charged hydroxyl groups.

The electrophoretic experiments were carried out with EPS-rich and EPS-poor P. aureofaciens in 0.1 NaNO₃ (Fig. 2A). The value of pH isoelectric point (pH_{IEP}) is below 2 for both EPS-rich and EPS-poor cultures. The EPS-rich cells showed more negative mobility compared to EPS-poor cells in alkaline solutions (Fig. 2). This can be understood given a higher charge density in the EPS-rich cells, most likely linked to the high concentration of low pKₐ sites (see Table 2 and the modeling section below) since the decrease of electrophoretic mobility started almost exactly at pH = pKₐ. The chemical nature of these sites remains unknown. In addition to carboxylates/phosphodiester listed in Table 2, it could be also sulfate negatively charged groups. Another explanation could be the screening of positively-charged amine groups of the cell membrane by abundant hydroxylate moieties of the external EPS layers, as it was suggested for diatom cells (see for discussion Gélabert et al. [59]). The effect of ionic strength on bacterial zeta-potential was weakly pronounced (Fig. 2B), where the zeta-potential of EPS-rich cells, in 0.1 M and 0.01 M NaNO₃, was plotted as a function of pH. Note however, that further electrophoretic mobility measurements are necessary to assess the ionic strength dependence of the EPS-poor cells.

3.2. DOC and free copper concentration in the cell supernatant

The Dissolved Organic Carbon (DOC) was measured in the adsorption experiments as a function of pH at 1.5 < pH < 4.5 (Fig. 3). The DOC concentration was approximately 20 times higher

### Table 2

<table>
<thead>
<tr>
<th>Cultures</th>
<th>pKₐ</th>
<th>Binding sites (mmol/g dry biomass)</th>
<th>Possible functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPS-rich</td>
<td>3.45</td>
<td>14.036</td>
<td>Carboxyl/phosphodiester</td>
</tr>
<tr>
<td>4.75</td>
<td>1.4396</td>
<td></td>
<td>Carboxyl</td>
</tr>
<tr>
<td>6.27</td>
<td>0.626</td>
<td></td>
<td>Phosphoryl</td>
</tr>
<tr>
<td>7.15</td>
<td>1.231</td>
<td></td>
<td>Phosphoryl</td>
</tr>
<tr>
<td>7.95</td>
<td>1.478</td>
<td></td>
<td>Amine</td>
</tr>
<tr>
<td>9.10</td>
<td>1.126</td>
<td></td>
<td>Amine</td>
</tr>
<tr>
<td>EPS-poor</td>
<td>3.55</td>
<td>0.755</td>
<td>Carboxyl/phosphodiester</td>
</tr>
<tr>
<td>4.15</td>
<td>4.324</td>
<td></td>
<td>Carboxyl</td>
</tr>
<tr>
<td>5.20</td>
<td>3.713</td>
<td></td>
<td>Phosphoryl</td>
</tr>
<tr>
<td>6.25</td>
<td>0.475</td>
<td></td>
<td>Phosphoryl</td>
</tr>
<tr>
<td>7.60</td>
<td>0.361</td>
<td></td>
<td>Amine</td>
</tr>
<tr>
<td>8.95</td>
<td>3.235</td>
<td></td>
<td>Amine</td>
</tr>
</tbody>
</table>
in EPS-rich cultures compared to the EPS-poor ones, consisting with much higher amount of EPS in the former. Some amount of this EPS can be released in solution in the course of the adsorption experiment. Several [DOC] measurements at 6 < pH < 8 yielded average values of 25 ± 5 and 5 ± 2 mg/L for EPS-rich and EPS-poor cultures, respectively. These values followed the trend shown in Fig. 3. The increase of [DOC] with decrease of pH is most likely to due to increase the EPS detachment/cells coagulation and surface layer degradation with increase of solution acidity. At the present time, we do not have a straightforward explanation for the higher [DOC] values in NaCl compared to NaNO3.

The Dissolved Organic Carbon concentration was significantly higher in the experiments with EPS-rich P. aureofaciens. In order to study the role of DOC concentration in the solution and its capacity to complex copper, several experiments were carried out to measure free Cu\(^{2+}\) in cell supernatant solutions and in 0.1 M NaNO3 using Cu\(^{2+}\)-selective electrode. Measuring free Cu\(^{2+}\) in EPS-rich and EPS-poor supernatants should help to distinguish the effect of Cu\(^{2+}\) complexation in solution and on cell surface. Plotted in Fig. 4 are the electrode potentials in the presence of various Cu\(^{2+}\) concentrations measured in 0.1 M NaNO3 and in the EPS-rich and the EPS-poor media. There was approximately 2.1 ± 0.24 mV difference between the inert electrolyte (0.1 M NaNO3) and the cell supernatant which signified that only 16 ± 2% of Cu\(^{2+}\) is bound in aqueous EPS complexes. However, free Cu\(^{2+}\) concentration was the same within ±5% in EPS-rich and EPS-poor supernatants. Thus no difference was detected for Cu\(^{2+}\) speciation in solution between two types of cultures. Therefore, the adsorption of Cu onto bacterial biomass was most likely controlled by the nature and abundance of cell surface groups, and not by soluble exopolymeric substances.

### 3.3. Adsorption of copper as a function of pH and copper concentration in solution

The pH-dependent adsorption edge for P. aureofaciens is illustrated in Fig. 5. Adsorption yield was always higher in bacteria subjected to rinse. The copper adsorption started at pH 2 and the maximum of adsorption was reached at pH 6.0 for both EPS-rich and EPS-poor cultures. However, copper adsorption as a function of Cu concentration in solution was undertaken at constant pH of 5.5 with copper concentration varying between 0.13 \(\mu\)M and 4.72 mM (Fig. 6 and Table 1). These experiments were carried out in both EPS-rich and EPS-poor P. aureofaciens. Similar to pH-dependent adsorption edge, the adsorption of copper at constant pH was higher for the EPS-poor culture compared to the EPS-rich culture. The slopes of dependence between \([\text{Cu}^{2+}]_{\text{adsorbed}}\) and \([\text{Cu}^{2+}]_{\text{solution}}\) in 0.01 M NaNO3 were 1.06 ± 0.01 \((r^2 = 0.99)\) and 0.94 ± 0.01 \((r^2 = 0.99)\) for EPS-rich and EPS-poor cultures, respectively. These slopes were valid in the full range of metal concentration investigated. The slope was 0.75 in
the adsorption of copper in 0.1 M NaCl electrolyte solution and EPS-rich culture. It reflected some copper complexation with Cl/C0(aq) which was explicitly taken into account during thermodynamic modeling.

Reversibility of Cu adsorption on live EPS-rich cultures was assessed at pH from 2.3 to 5.8. We found that, after 1.5 h of metal exposure to viable cells, between 2% and 13% of Cu was non-EDTA extractable, and the majority of the metal was reversibly adsorbed on the surface. These observations were consistent with results of the kinetic experiments demonstrating that the percentage of adsorbed Cu was practically constant (73 ± 4%) from 1 h to 24 h of exposure time (Fig. 7).

We did not find significant differences in adsorption yield between live and inactivated EPS-rich bacteria: the adsorption of copper(I) started at 45% and 37%, respectively after 1 min of exposure time, reaching the maximum at 20 h (77%) for both cultures. Bearing in mind that there were not significant differences in the adsorption yield for the EPS-rich cultures between 1 h and 24 h of exposure time (6% at pH 6, Fig. 5), our observations support neither significant efflux of copper from the interior of cells to the external solution nor the irreversible assimilation in the cell compartments. Only for EPS-poor cultures at high Cu loading, we observed some increase in the adsorption yield between 1 and 24 h exposure time (Fig. 6A). Although high-resolution kinetic data for EPS-poor culture were not available, this observation was consistent with a 10% increase in relative amount of adsorbed Cu for EPS-rich cultures within the same time interval (Fig. 7).

Noteworthy that there was a clear difference in the rate of Cu adsorption on dead bacteria, showing much faster achievement of a constant adsorption yield compared to the live biomass (Fig. 7). Most probably, this is linked to modified physical structure of heat-killed cells surface and complete removal of diffuse highly hydrated EPS layers after biomass autoclaving and extensive rinsing.

3.4. Linear programming modeling

Linear Programming Model approach (LPM) developed by Martinez et al. [23,48,49] was used to rationalize experimental results in order to obtain the main parameters of surface equilibria such as pHk and pHm of metal adsorption and pHa of acid dissociation of P. aureofaciens cell surface functional groups. In contrast to simplified treatment of adsorption data using Langmuirian formalism used in previous study at high bacterial biomass/solution ratio (i.e., Pokrovsky et al. [28]), the present approach allows rigorous and much more realistic representation of continuum surface sites of cell surface polymers responsible for metal binding. The original code was modified in order to account for copper speciation in solution via calculating the percentage of free ion copper as a function of Cl/C0 concentration and pH taking into account Cl/C0 and OH/C0 complexation with Cu2+ using the stability constant from Ref. [65]. Linear
programming regression techniques automatically minimize the number of binding sites and the absolute error, rather than the least squares. This approach finds one global minimum for the error function, which emphasizes zero as a possible solution and avoids convergence problems such as those found in FITEQ, where the solution could be a local minimum [48]. Note that the LPM should be considered as a mathematical construction with no basis in true chemical nature of surface sites, as most surface complexation models. Indeed, the LPM uses a grid of fixed pHK values and optimizes parameters such as total binding site concentrations. Each site density, $B_j$, is assigned a positive value where zero is a possible result. This generates a pHK spectrum where discrete metal-binding sites are determined by the number of pHKj values.

In order to describe the interaction of metal cations ($Me^{2+}$) and protons with bacterial surface in pH-dependent adsorption edge experiments, a $Me^{2+}/H^+$ competition reaction, in Eq. (2) was assumed:

$$Me^{2+} + B_jH^+ \rightleftharpoons MeB_j^+ + H^+$$

where $B_j$ represents a surface reactive site and $K_{sj}$ the concentration apparent equilibrium constant conditional on ionic strength. For a jth deprotonated binding site at the ith pH value, $K_{sj}$ can be defined as:

$$K_{sj} = \frac{[MeB_j^+] \cdot [H^+]_{\text{meas},i}}{[Me^{2+}]_{\text{meas},i} \cdot [B_j]_{\text{meas},i}}$$

(3)

In the above expression, $K_{sj}$ is a function of experimentally determined proton and metal concentrations, $[H^+]_{\text{meas},i}$ and $[Me^{2+}]_{\text{meas},i}$, and of the amount of $Me^{2+}$ bound to the jth site at the ith pH value. $K_{sj}$ can be defined as:

$$K_{mj} = \frac{[MeB_j^+]_{\text{meas},i}}{[Me^{2+}]_{\text{meas},i} \cdot [B_j]_{\text{meas},i}}$$

(5)

In the above expression, $K_{mj}$ is a function of experimentally metal concentrations, $([Me^{2+}]_{\text{meas},i}$ and of the amount of $Me^{2+}$ bound to the jth site as a function of increasing biomass and a fixed pH value, $[MeB_j^+]_{\text{meas},i}$. Binding site concentrations will be calculated and assigned to a fixed pHKm grid as explained previously for the pH-dependent adsorption experiments. The values of $K_{sj}$ and $K_{mj}$ found in this study for pH-edge and fixed pH metal complexation experiments are not directly comparable because $K_j$ is a function of $K_m$, $K_j = K_m \times K_0$, where $K_0$ is the acidity constant of a specific functional group on the bacterial surface. Therefore, the main shortcoming of this model is that the same proton adsorption sites constrained with the titrations are not used to constrain metal adsorption. However, we think that this may partially reflect the physical reality of the complex multilayer structure of the bacterial surface: the gelatinous EPS layer might slow down the diffusion rate of the copper ion into the cell wall chelating matrix where as this diffusion barrier for protons should be much smaller.

Results of the model fit to adsorption experiments are shown as solid lines in Figs. 1, 5 and 6 and the model parameters are illustrated in Table 1 and 2. The pHK values were calculated from the pH-dependent adsorption edges (Table 1). The amount of available sites capable of binding copper varied from 0.01 to 0.05 mmol/g dry biomass. The higher negative magnitude of the pHK, the stronger the binding capacity of the each functional group on bacterial surface [49]. Thus, the most important and negative pHK value (~1.75) corresponded to EPS-poor culture grown on SA media (Exp # 5), for which the concentration of available sites was also high, 0.052 mmol/g dry biomass. Consequently, the concentration of available sites was lower for the EPS-rich culture (Exp # 1, 0.046 mmol/g dry biomass) and the stability of surface binding groups was lower (pHK = −1.25) than the EPS-poor strain (pHK = −1.75).

The pHK values were obtained from the adsorption experiments as a function of metal aqueous concentration at constant pH (Fig. 6). Results were presented in Table 1. The amount of total metal sites available for metal adsorption ranges from 0.01 to 0.96 mmol/g dry biomass. As for pH-dependent adsorption edge, the highest number of surface binding sites occurred in the EPS-poor culture (0.96 mmol/g dry biomass and pHK = 2.5), which was about one order of magnitude higher than for the EPS-rich culture at similar conditions. Dead bacteria yielded total amount of copper binding sites (0.080 and 0.085 mmol/g dry biomass) and of the amount of metal bound to the jth site at the ith pH value $[Me^{2+}]_{\text{meas},i}$.

For constant-pH adsorption isotherm data, the LPM model described earlier was modified to account for $Me^{2+}$ binding as a function of increasing metal concentration. Therefore, reaction 2 is replaced by:

$$Me^{2+} + B_jK_{mj}MeB_j^+$$

where $B_j$ represents a specific surface functional group and $K_{mj}$ the apparent metal–ligand binding constant conditional on ionic strength. For a jth deprotonated functional group at a fixed pH value, $K_{mj}$ can be defined as:

4. Discussion

Bacteria cell walls play a dominant role in the metal scavenging by microorganisms because various functional groups of the cell wall display a strong affinity for metal cations. With pH increase, these groups are progressively deprotonated forming anionic ligands capable of binding metals [47]. Results of this work demonstrated that adsorption of copper was higher by bacteria subjected to rinsing compared to those having their EPS envelopes intact. For example, non-rinsed bacteria exhibited the maximal adsorption yield of 70% versus 80% for 0.1 M NaNO3-rinsed cultures (Fig. 5). The repetitive rinsing of bacteria removed external exopolysaccharide layer and allowed to reactive functional groups of cell wall to interact with aqueous solution. The effect of rinsing was much
more pronounced for EPS-rich cultures where the loss of biomass was more important (28% and 6% for EPS-rich and EPS-poor media, respectively). Therefore, irrespective of the difference in chemical composition of cell walls for two subcultures that may stem from cell growing in SP and SA media, removing the EPS clearly increased the adsorption capacity of the total biomass *P. aureofaciens* with respect to Cu\(^{2+}\). At the same time, the relative change in adsorption yield after cell rinsing (e.g., ~1/3 at pH 4, see Fig. 5) had similar magnitude for both EPS-rich and EPS-poor cultures. Apparently, the rinsing of bacteria brought about removal of similar relative proportion of the cell surface EPS layers because the physical nature of the EPS and the degree of the EPS attachment to the cell surface were similar among two subcultures.

Note that although the bulk of existing information on the cells-EPS-metal interaction demonstrates increasing capacity of metal adsorption on bacteria in the presence of EPS (see Refs. [71,72] and references therein), some previous studies indicated weak or even negative effect of bacterial EPS on metal adsorption. In particularly, Tourney et al. [73] demonstrated that the EPS removal does not affect the metal adsorption capacity of *Bacillus licheniformis* as the concentration of main binding sites, carboxyl and phosphate functional groups, was only slightly affected by EPS extraction. Moreover, macroscopic adsorption and FTIR analysis indicated that the EPS layer did not significantly enhance Cd binding to *Pseudomonas putida* [30] and in some cases, the EPS layer demonstrated a lower affinity for metal binding than the isolated cell walls [74]. Tourney et al. [73] proposed two possible explanation for these findings of weak effect of EPS on metal binding. First, the EPS production is known to induce a considerable degree of cell aggregation in suspension, which is likely to reduce the surface area available for metal adsorption. Second, the release of large quantities of DOC from EPS layer may complex metal in solution, in competition to cell walls. Our potentiometric measurements (Fig. 4) unequivocally demonstrated the absence of Cu\(^{2+}\) complexation with external EPS of *P. aureofaciens* and as such we submit that the EPS for this bacterium can be considered simply as an outer envelope shielding the cell surface from metal exposure, a hypothesis firstly suggested by Tourney et al. [73].

The experimental results for pH-dependent adsorption edge were properly fitted using Linear Programming Model approach. This model is convenient for studied complex 3-D multiple layer system having both EPS component and rigid bacterial cell walls [23,63,75]. Note that, because the binding to EPS was weak and the effect of background electrolyte on surface potential was not pronounced, the Donnan shell-like model was not necessary. Other work used electrostatic approaches such as the constant capacitance model converged to three sites [25,76] whereas non-electrostatic approach used electrostatic interactions such as the constant capacitance model converged to two sites [25,76] whereas non-electrostatic approach used electrostatic interactions such as the constant capacitance model converged to two sites [25,76].

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Our results also demonstrate that the effect of ionic strength on zeta-potential of EPS-rich culture is rather small and poorly pronounced (Fig. 2B). This observation can be accounted for the presence of diffuse 3-D highly porous EPS layer of rhizospheric bacteria. Due to this spatial organization, protonation/deprotonation of functional groups at the cell envelopes occurs at the distance from the shear plane that is much larger than that for individual cells or inorganic particles. As a result, large ions of background electrolyte do not sufficiently approach the charged surface and thus weakly contribute to potential at the shear plane. A weak effect of ionic strength on electrophoretic mobility of EPS-rich *B. licheniformis* observed in the range of 0.1–0.001 M has been explained by the contraction of the EPS layer with ionic strength [78]. Therefore, it is possible that the change in charge density associated with swelling of the EPS envelope at low ionic strength, which would compensate for charge-shielding [82] can be responsible for independence of electrophoretic mobilities of *P. aureofaciens* on ionic strength, observed in the present study.

Results of potentiometric surface titration allowed constraining stability constants of the acid–base protonation reactions controlling the buffering capacity of bacterial surfaces (Table 2). Resulted pK\(_a\) values can be separated in four groups: \(\approx 3.5, 4–5, 5–7\) and 8–10. In accordance with previous studies [23,26,73,76,83,84], they can be identified as carboxyl/phosphodiester, carboxylate, phosphoryl, and amine, respectively. Note that the chemical nature of surface sites listed in Table 2 cannot be assessed solely from surface
Adsorption isotherm and pH-dependent adsorption edge (Tables 1). This reflects the possibility of large-size cations such as Cu(II) being more abundant in the EPS-poor bacterial (4.2–4.8, 5.2–7.2, and 7.6–9.1 which may tentatively correspond to the EPS-poor bacterial species (0.89–1.62 mmol/g dry). These points out the similarity of proton- and metal-binding properties between soil rhizospheric and aquatic microorganisms stemming from similar molecular mechanisms involved in passive surface reaction centers of the cell wall and this, presumably, may decrease the intracellular metal assimilation. Compared to most other studied bacteria, the EPS production by P. aureofaciens does not contribute to complexation of aqueous ions Cu(II) while prevents the metal adsorption on the cell surface. In the wide range of metal concentration studied in this work (0.1–375 µM), the strong binding sites such as sulfhydryl moieties are not evidenced from macroscopic physico-chemical treatment of adsorption equilibrium. Further in situ spectroscopic techniques are necessary to reveal the true heterogeneity of surface sites and the change of Cu speciation upon its translocation from the surface layer to the interior of the cell.

5. Conclusions

The presence of exopolysaccharides on the cell walls of P. aureofaciens decreased the adsorption of copper via screening the cell surface reactive centers by relatively chemically inert polysaccharide. This was observed during adsorption experiments performed on EPS-poor and EPS-rich cultures of the same species as a function of pH and copper concentration in solution. The external EPS do not possess significant amount of metal-binding centers being composed mainly of hydroxyl, ester, and aliphatic components. As such, they present a physical (diffusion) barrier for metal ion to approach reactive surface sites of the cell wall. Acid–base titration and electrophoretic measurements of P. aureofaciens revealed the presence of groups having pKₐ in the range of 3.5, 4.2–4.8, 5.2–7.2, and 7.6–9.1 which may tentatively correspond to carboxyl, phosphodiester, phosphoryl, and amine moieties as the main surface functional groups capable of proton- and metal-binding. Concentration of Dissolved Organic Carbon was significantly higher in experiments with EPS-rich culture compared to those with EPS-poor culture. The complexation of Cu(II) with exopolymeric substances in aqueous solution was very weak and similar between two types of cultures as confirmed by Cu(II) activity measurement via Cu(II)-selective electrode. Therefore, the main mechanism of P. aureofaciens cell protection from toxic metal stems from screening of strongly-binding functional groups of the cell wall such as phosphoryl (pKₐ = 5.2–7.2) by abundant exopolysaccharides of the most surface layers.

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