



Importance of extracellular polysaccharides on proton and Cd binding to bacterial biomass: A comparative study

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ABSTRACT

The importance of extracellular polysaccharide (EPS) on proton and Cd binding was examined by comparing the adsorption behaviors of 4 bacterial species (*Pseudomonas putida*, *Shewanella oneidensis*, *Rhizobium tropici*, and *Agrobacterium* sp. [ATCC# 21680]) with intact capsular EPS to corresponding adsorption behaviors with the EPS enzymatically removed from the biomass. Potentiometric titrations were conducted to detect any differences in proton binding of the biomass with and without the presence of EPS. Enzymatic removal of the EPS from each of the bacterial species in this study resulted in no significant differences in biomass proton binding behavior. Batch Cd adsorption experiments also showed no significant differences in the adsorption capacities between the EPS and EPS-free systems for all 4 species of bacteria. Our results suggest that EPS contains proton-active functional groups that are similar to those on the cell wall, and that, on a mass-normalized basis, EPS and bacterial cell walls exhibit similar site concentrations and affinities for adsorbing protons and Cd from solution. Because EPS exhibits similar Cd and proton binding properties to bacterial cell walls, and because of the similarity in binding properties between species, it may be possible to model metal and proton binding to biofilms in general using a single set of stability constants. This general modeling approach would obviate the impossible task of determining binding constants for protons and each metal of interest with each EPS component and each bacterial cell wall of interest.

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1. Introduction

Metal adsorption onto bacterial cells has been studied extensively (e.g., Beveridge and Murray, 1976, 1980; Fein et al., 1997; Small et al., 1999; Haas et al., 2001; Ngwenya et al., 2003; Wu et al., 2006; Guiné et al., 2006; see Fein, 2006, for a recent review), and these studies have significantly improved our understanding of adsorption mechanisms and our ability to quantitatively model these reactions. The cell wall of a bacterium contains functional groups that include carboxyl, phosphoryl, hydroxyl, amino, and sulfhydryl groups (Beveridge and Murray, 1976, 1980; Kelly et al., 2001; Guiné et al., 2006; Mishra et al., 2009), which are binding sites for protons and aqueous metal cations. In addition to sequestering metals from solution, metal binding onto bacteria can change the environmental fate of a metal through reduction reactions (e.g., Fein et al., 2002; Loukidou et al., 2004; Cummings et al., 2007) or precipitation (Kappler et al., 2005), and metal adsorption is an important step in the bioavailability of metals (Di Toro et al., 2001; Santore et al., 2001; Paquin et al., 2002; Borrok et al., 2005; Slaveykova and Wilkinson, 2005).

A vast majority of laboratory studies of metal adsorption onto bacteria involve planktonic cells. A growing number of studies have

documented similar proton and metal binding behaviors by a range of planktonic cells, as well as by consortia of planktonic cells grown from natural environments (e.g., Daughney et al., 1998; Small et al., 1999; Yee and Fein, 2001; Borrok et al., 2004a; Borrok et al., 2005; Johnson et al., 2007; Ginn and Fein, 2008). However, it is likely that most bacterial cells in the environment exist within free-floating or attached biofilms (e.g., Christensen, 1989; Sutherland, 2001; Madigan and Martinko, 2006). Biofilms consist of an assemblage of bacterial cells held together by a network of extracellular polysaccharide (EPS), DNA, lysed cells, proteins, lipids, nucleic acids, and membrane vesicles (Wingender et al., 1999; Whitchurch et al., 2002; Branda et al., 2005; Schooling and Beveridge, 2006). EPS is produced by many bacterial species as a way to gather nutrients or to promote adhesion to surfaces (Duddridge et al., 1981; Characklis and Cooksey, 1983; Beveridge, 1989), and bacteria produce more EPS when nutrient is limited, or when exposed to toxic conditions (e.g., Decho, 1990; Wolfaardt et al., 1999; Decho, 2000; Hirst et al., 2003). The composition and structure of EPS vary greatly between species, but EPS generally contains polysaccharides such as mannose, glucose, and galactose, and can exist in dissolved or particulate form (Mort and Bauer, 1980; Christensen and Characklis, 1990; Decho, 1990; Hall and Meyer, 1998). EPS contains proton-active binding sites, and the proton and metal binding properties of EPS have been studied using EPS extracted from both microbial assemblages in activated sludge (Comte et al., 2006a, 2006b; Guibaud et al., 2006; Comte et al., 2008; Guibaud et al., 2008), and from

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pure monocultures (Guibaud et al., 2005; Bhaskar and Bhosle, 2006; Lamelas et al., 2006).

Although investigation of metal binding properties of EPS using extracted organic material from bacterial biofilms can isolate binding mechanisms, the approach may be misleading due to the difficulty in extracting all of the EPS material. The main methods of EPS extraction have been chemical extraction (using EDTA, formaldehyde + NaOH, glutaraldehyde, etc.) or physical extraction (using gravimetric approaches, cation exchange resins, sonication, and/or heating) (Liu and Fang, 2002; Guibaud et al., 2005; Comte et al., 2006a). Comte et al. (2006a) compared the effectiveness of extraction procedures and the effects that these extraction procedures have on the resulting extracted EPS material. Comte et al. (2006a) determined that the chemical extraction procedures were the most effective at separating EPS from bacterial cells, however these procedures also could contaminate and modify the EPS significantly. Modification of the EPS material could significantly affect the proton-active functional groups present on the EPS molecules. For example, Lamelas et al. (2006) used heating combined with the enzyme protease to remove EPS from cells, followed by chemical precipitation of the EPS from solution and lyophilization. They then performed potentiometric titrations on this dried material, inferring the presence of two types of functional groups within the EPS. However, according to the observations of Comte et al. (2006a), the fraction of EPS that Lamelas et al. (2006) collected was likely significantly modified by the chemical extraction procedure. Additionally, the EPS collected by Lamelas et al. (2006) likely represents that which is most soluble and therefore most easily lost to the environment around the biofilm, as even chemical extraction procedures are not completely effective at removing all EPS molecules from a biofilm (Comte et al., 2006a), and likely leave a significant amount of capsular EPS on the cell.

A different approach to the direct study of extracted EPS from biofilms has been to examine the proton and metal binding behaviors of bacteria with and without EPS present and to infer the role of EPS by difference. Ha et al. (2010) tested the proton and metal adsorption capacities of cell-bound EPS by comparing an EPS-producing wild type of *Shewanella oneidensis* to a mutant that was genetically modified to inhibit the production of alginate, the polysaccharide found in EPS (Conti et al., 1994). Ha et al. (2010) found that there are 3 proton active functional groups in both strains of the bacteria, though the EPS-free mutant had higher concentrations of phosphoryl sites and lower concentrations of carboxyl and amino sites than the EPS-rich wild type. In addition, Ha et al. (2010) observed that the EPS-rich wild type adsorbed higher concentrations of metal than the mutant at a given pH and biomass concentration. However, prohibiting the production of alginate does not necessarily prohibit all capsular EPS around the cell, and the 'EPS-free' biomass created by Ha et al. (2010) likely contained other EPS components including extracellular DNA, proteins, and/or other cellular exudates.

Ueshima et al. (2008) used the enzyme glucoamylase to cut the α 1–4 glucosidic bonds in the EPS material from *Pseudomonas putida* cells, and then washed the EPS and extracellular material away from the cell, leaving only the bare cells behind. They compared the buffering and metal adsorption capacities of these "cleaned" cells to those of the EPS-rich cells. Ueshima et al. (2008) conducted potentiometric titrations and Cd adsorption experiments using the two types of biomass and found similar buffering capacity and Cd adsorption capacity for the biomass samples that contained *P. putida* with intact EPS and the biomass samples with the EPS removed enzymatically. Similarly, Tournay et al. (2008) found that *Bacillus licheniformis* S-86 cells, treated with a cation exchange resin to remove the EPS, exhibited similar proton buffering to the untreated, EPS-rich cells. Because the effect of EPS on proton and metal binding to biomass has only been studied for a limited number of species, and because some of these studies observed differences in the importance of EPS binding, the role of EPS remains poorly defined, and it is unclear

whether the EPS of different bacterial species exerts different roles in adsorption behavior.

In this study, we expand on the work conducted by Ueshima et al. (2008) to determine the proton and metal binding capacities of four EPS-producing bacteria, with and without the EPS enzymatically removed. The bacteria were chosen for their ability to produce significant amounts of EPS as well as for the variability in the composition of the EPS produced by each species. Our experiments with *P. putida* were conducted to overlap with the study by Ueshima et al. (2008). *P. putida* produces EPS that is rich in glycerol, acetylated amino sugars, and xylose (Celik et al., 2008); *S. oneidensis* yields EPS that has glycocalyx-like properties and contains some heme-containing proteins (Marshall et al., 2006); *Rhizobium tropici* produces a relatively large amount of soluble EPS consisting of monosaccharides such as glucose, galactose, mannose, and rhamnose (Zevenhuizen, 1971; Becker and Pühler, 1998); *Agrobacterium* sp. (ATCC# 21680) was chosen for its ability to yield high amounts of a gelatinous EPS, rich in curdlan (Hisamatsu et al., 1977; Harada and Harada, 1996; McIntosh et al., 2005). We conducted potentiometric titrations and Cd bulk adsorption experiments on the aforementioned bacterial species with EPS intact and with EPS enzymatically removed. The objectives of the study were to quantify the site concentrations, acidity constants, and Cd binding behaviors of biomass samples both with intact EPS and with EPS enzymatically removed for each of the four bacterial species, and to compare the results to determine whether proton and Cd binding behaviors of biofilms are significantly different from those exhibited by planktonic bacterial cells.

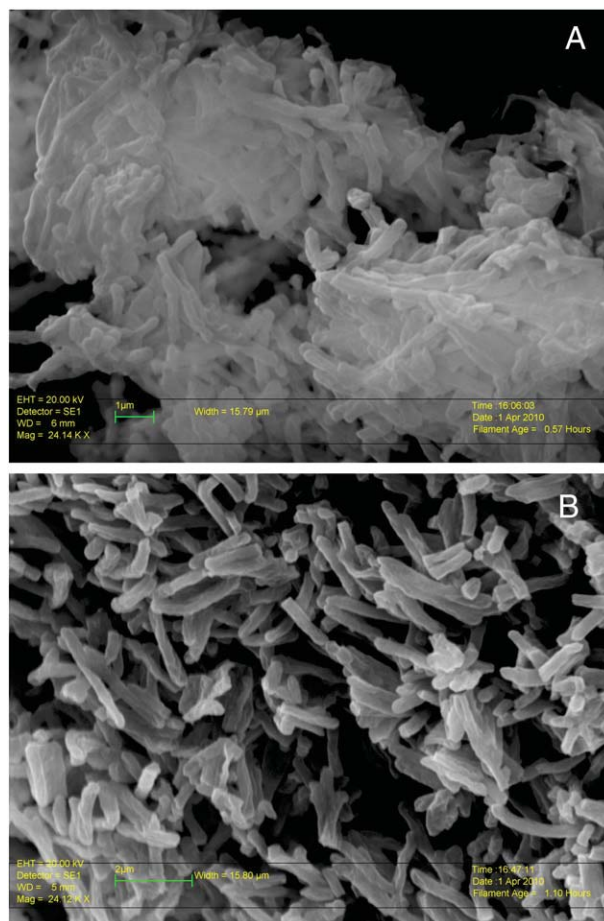


Fig. 1. Scanning electron microscope images of A) untreated and B) enzyme-treated *S. oneidensis* biomass.

2. Materials and methods

2.1. Bacterial growth

Cells and EPS of *P. putida* (ATCC# 33015), *S. oneidensis* MR-1 (ATCC# BAA-1096), *R. tropici* (ATCC# 49672), and *Agrobacterium* sp. (ATCC# 21680) were cultured aerobically at 32 °C in 3 mL of Luria-Bertani medium (or ATCC medium 3 for *Agrobacterium* sp.), and incubated for 24 h. The biomass was then transferred to 2 L of the same growth medium and incubated for another 24 h at 32 °C. After incubation, the biomass was harvested by centrifugation at 8100 g, and was rinsed 5 times with a 0.1 M NaClO₄ electrolyte solution. After each wash, the bacteria were suspended in clean electrolyte, a bacterial pellet was again formed by centrifugation for 5 min at 8100 g, and the supernatant was decanted. After the final wash, the wet mass of the bacterial pellet was determined by centrifuging the bacterial pellet twice at 8100 g for 30 min, removing as much solution as possible, and weighing the resulting pellet. The biomass pellet that was collected at this point consisted of a mix of cells, EPS, and other biofilm materials intact.

After the second of the five rinses of the bacteria described above, a portion of the biomass was separated for enzymatic removal of the EPS from the bacterial cells. The EPS was enzymatically removed using glucoamylase following the method used by Ueshima et al. (2008). 50 μL of glucoamylase was added aseptically for every 0.5 g of bacteria treated, and then 50 mL 0.1 M NaClO₄ was added, and the mixture was vortexed into a slurry. This slurry was placed in an incubator at 32 °C and agitated at 100 rpm for 1 h, after which the bacteria were separated from the supernatant through centrifugation, and were resuspended into a second identical batch of fresh enzyme-electrolyte solution for 1 h. The enzyme and extracted EPS material were rinsed and separated from the cells with 3 additional washes in 0.1 M

NaClO₄. In order to determine the extent of EPS removal by the glucoamylase, both the treated and untreated cells were imaged using scanning electron microscopy. The SEM samples were prepared by fixing the cells in 2% glutaraldehyde and then in 2% osmium tetroxide in a 0.1 M NaClO₄ electrolyte solution. The samples were then freeze dried, mounted onto adhesive carbon tape, attached to sample holders and sputter-coated with Au-Pd. Samples were imaged at an acceleration voltage of 20 kV under high vacuum with a spot size of 4.0 μm using a LEO EVO 50 environmental scanning electron microscope.

2.2. Potentiometric titrations

Potentiometric titrations were conducted using 30–50 g/L (wet mass) suspensions of biomass (cells with or without the EPS removed) suspended in 0.1 M NaClO₄ to buffer ionic strength, and were conducted under an N₂ atmosphere. Before use in the titrations, the electrolyte was bubbled with N₂ for 1 h in order to purge atmospheric CO₂. Each suspension began at circumneutral pH and was acidified to pH ~3 using 1.048 N HCl prior to the start of the titration. Aliquots of 1.030 N NaOH were used to adjust the pH of the suspension to 10, measuring pH and volume of base added at each step. After completion of the 'up pH' titration, a 'down pH' titration to pH 3 using the 1.048 M HCl was conducted to test the reversibility of proton binding. The titrations were conducted in triplicate, with 3 different cell suspensions, using an autotitrator and each individual suspension was stirred throughout the titration with a magnetic stir bar. The buffering capacity was calculated as: $(C_a - C_b - [H^+] + [OH^-])/m_b$, where C_a and C_b are the total concentrations (including initial concentration of acid added to the suspension prior to titration) of acid and base added at each step of the titration, respectively; the brackets indicate molar species concentrations, and m_b is the wet mass concentration (g/L) of the bacterial suspension.

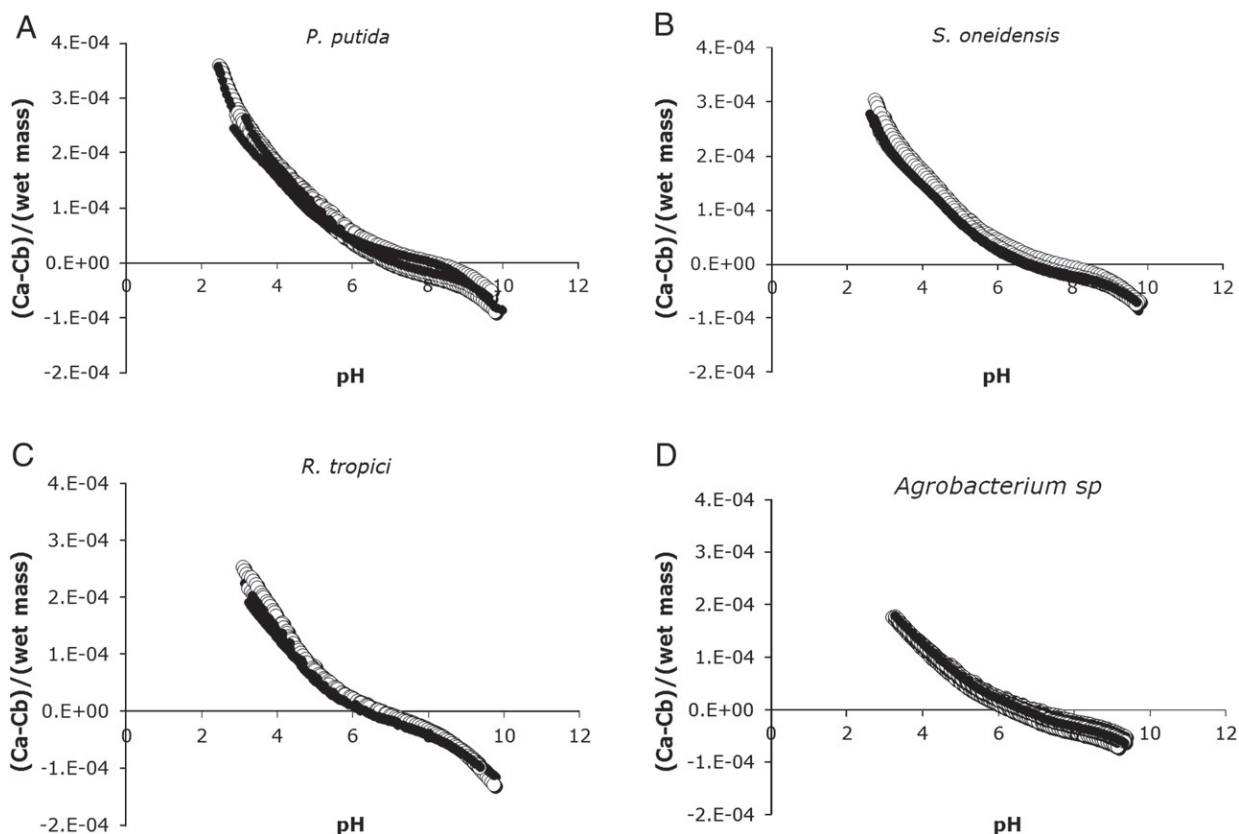


Fig. 2. Potentiometric titration data for A) *P. putida*, B) *S. oneidensis*, C) *R. tropici*, and D) *Agrobacterium* sp. (30–50 g/L wet mass) biomass in 0.1 M NaClO₄ electrolyte solution. Open circles represent data from experiments using biomass with intact EPS; black-filled circles represent data from experiments using enzyme-treated biomass.

2.3. Cadmium adsorption experiments

Batch Cd adsorption experiments were conducted using the treated and untreated cells. A parent solution of aqueous 10 ppm Cd(II) in 0.1 M NaClO₄ was prepared from a 1000 ppm Cd standard reference solution. The 10 ppm Cd-bearing parent electrolyte solution was adjusted to pH 7 and a weighed pellet of cells was suspended into it such that the final bacterial concentration was 1.2 g/L of bacteria (wet mass). The suspensions were then divided into 10 mL volumes in 15 mL polypropylene test tubes. The pH of each 10 mL suspension was adjusted to a desired starting pH, ranging from pH values of 2 to 10, using small aliquots of 0.1 to 1 M HCl or NaOH, and the systems were allowed to equilibrate via end-over-end rotation at 24 rpm for 2 h. The final pH was measured, the suspension was centrifuged at 8100 g and the supernatant was filtered through a 0.45 μm disposable nylon filter to remove biomass from solution. The concentration of dissolved Cd was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) with matrix-matched standards. The concentration of Cd that was adsorbed onto the biomass in each system was calculated by subtracting the concentration of Cd remaining in solution at the end of the experiment from the original Cd concentration. The Cd adsorption experiments were conducted in duplicate.

3. Results and discussion

3.1. Scanning electron microscopy

The effectiveness of the enzyme treatment in removing EPS material from the bacterial cells was determined using SEM. Fig. 1A and B shows *S. oneidensis* cells with the biomass with EPS intact and with EPS enzymatically removed, respectively. The untreated biomass contains a thick coating of EPS and biofilm material surrounding all of the cells, indicating that normal sample washing procedure did not remove this thick layer of extracellular material. In the image of the biomass that was treated with glucoamylase, there is no evidence of a coating over the biomass, and whole cells are distinct and clearly visible, strongly suggesting that the enzyme treatment was able to remove at least a large proportion of the extracellular material from the biomass. Imaging of treated and untreated biomass was conducted for all of the bacterial species in the study, and the results shown are representative of the removal efficiency in each case.

3.2. Potentiometric titrations

For each species in this study, the removal of EPS did not significantly affect the buffering capacity of the biomass over the pH range studied. Fig. 2 shows the 'up-pH' potentiometric titration data for each bacterial species with and without the presence of EPS. The shapes and positions of the titration curves for the 4 bacterial species are similar whether the EPS was present or removed. Although some slight differences can be seen between the treated and untreated biomass, in general the differences are smaller than the differences observed between replicate titrations. The titrations for the four different bacterial species studied here are also similar to each other. Fig. 3 depicts a representative example for *Agrobacterium* sp. that demonstrates the reversibility of the proton adsorption reactions. Although differences exist between the 'up pH' and the 'down pH' titration data, the differences are smaller than those observed from one replicate to another, and we conclude that within the time scale of these experiments the protonation reactions are fully reversible and that proton adsorption equilibrium was attained. Similar results were obtained for each of the other species studied here.

The buffering capacity of each bacterial species did not change significantly with the removal of EPS from the biomass. The average buffering capacity for the bacteria in this study, with and without

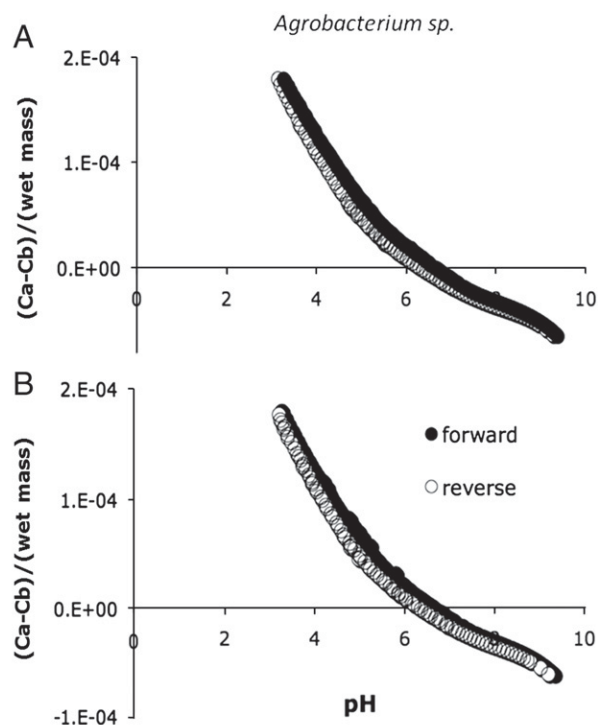


Fig. 3. Representative potentiometric titration data showing the reversibility of proton adsorption for *Agrobacterium* sp. biomass A) with and B) without EPS.

EPS, over the pH range of 3.0 to 9.5 was $4.1 \pm 0.8 \times 10^{-4}$ mol/g, which is close to the value observed for bacterial consortia grown from a range of near-surface environments (5.5×10^{-4} mol/g; Johnson et al., 2007), and is also similar to the buffering capacity of a number of single bacterial species studied previously (e.g., *Bacillus subtilis*: 2.9×10^{-4} mol/g, Fein et al., 2005). Ueshima et al. (2008) also observed no significant change in the proton binding behavior accompanying EPS removal. Our study confirms this result for *P. putida* and documents a similar result for the 3 new species studied. The similarities between the proton binding behaviors between the 4 species studied are consistent with observations made by a number of researchers who have directly compared protonation behavior of a wide range of planktonic bacteria and planktonic bacterial consortia (Yee and Fein, 2001; Borrok et al., 2004a, 2004b; Johnson et al., 2007).

Titration data for the 'up pH' titrations for the four bacterial species studied here were modeled using a discrete proton-active site surface complexation model, following the approach of Fein et al. (1997, 2005), where the functional groups on the biomass are represented by distinct sites that deprotonate according to the following reaction:



where R represents the biomass molecule (either cell wall macromolecule or EPS molecule) to which each type of functional group, A_x, is attached. The equilibrium constant (K) for this reaction is expressed as:

$$K_a = \frac{[R-A_x^-] \cdot \alpha_{H^+}}{[R-A_xH^+]} \quad (2)$$

where [R-A_x⁻] and [R-A_xH⁺] represent the concentrations of the deprotonated and protonated form of site type A_x, respectively, and α_{H⁺} represents the activity of protons in solution. Although a range of other models could be used to account for the observed buffering capacities, including ones that account for electric field effects (e.g.,

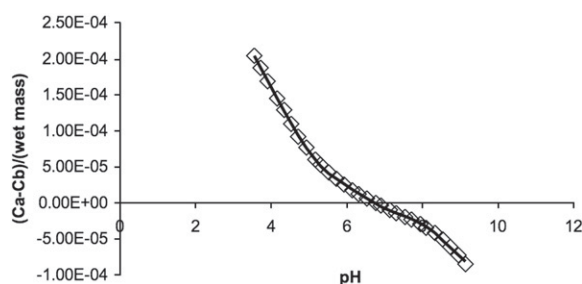


Fig. 4. A representative example of the goodness of fit of the non-electrostatic model (solid curve) to the potentiometric titration data (open diamonds) for *R. tropici* biomass.

Plette et al., 1996; Cox et al., 1999; Martinez et al., 2002), we chose to use a non-electrostatic model as the simplest approach to fit the data and to facilitate comparison with previous studies of the buffering capacities of bacterial cells (Yee and Fein, 2001; Borrok and Fein, 2005; Johnson et al., 2007; Ueshima et al., 2008). Using FITEQL 2.0 (Westall, 1982), we determined the total number of discrete sites necessary to account for the observed buffering capacity by sequentially testing models with 1 through 5 proton-active site types until the best fit to the data was determined. The goodness of fit for each model was quantified using the residual function in FITEQL 2.0, $V(Y)$. The proximity of the $V(Y)$ term to the ideal value of 1 characterizes the fit of the model to the data (Westall, 1982).

For each titration modeled here, a four site model provides the best fit to the data. The average $V(Y)$ values for 1-, 2-, 3- and 4-site models of all of the titration data are 132.6, 54.8, 3.1, and 0.4, respectively. In all cases, five-site models do not converge, indicating that the data are not sufficient to adequately constrain the properties of 5 proton-active sites. The 4-site models consistently yield the $V(Y)$ values closest to 1, as well as the best visual fits to the data for each titration, and we depict a representative model fit to the data in Fig. 4. The resulting calculated site concentrations and acidity constants are compiled in Table 1 and the values are compared graphically in Fig. 5. In every case, and for both the pK_a values and site concentrations, the values calculated for the biomass with EPS intact agree within experimental uncertainty with the values calculated for the EPS-free biomass. Furthermore, there is good agreement between the calculated pK_a and site concentration values and the generalized model parameters (also shown in Table 1) calculated by Borrok and Fein (2005) from titrations of 36 bacterial species and consortia. Therefore, the general model proposed by Borrok and Fein (2005) yields a reasonable fit to the protonation behavior of the species studied here. Because the presence of EPS does not significantly alter the protonation behavior of the biomass, these results suggest that the

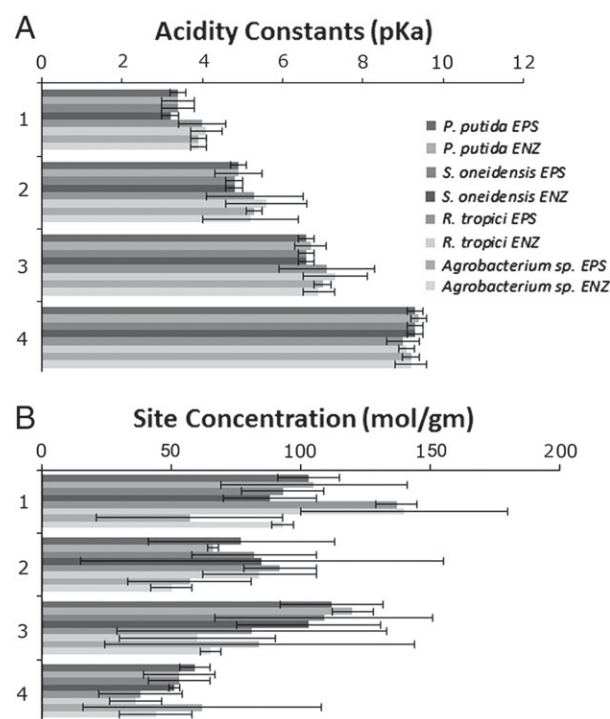


Fig. 5. Histogram for the average A) acidity constants (pK_a) and B) site concentrations (mol/g) for each bacterial species, with (labeled 'EPS') and without (labeled 'ENZ') the presence of EPS.

Borrok and Fein (2005) model can account for proton binding of a complex biofilm that contains not only multiple bacterial species, but EPS molecules as well.

3.3. Cd adsorption experiments

Cd adsorption experiments were conducted as a function of pH for each of the 4 bacterial species studied here, with and without the presence of EPS. Each of the bacterial species adsorbed similar concentrations of Cd from solution at a given pH, and the presence of EPS did not affect the extent of Cd adsorption significantly for any of the species studied (Fig. 6). In general, the extent of Cd adsorption increases with increasing pH for each species studied. The low pH *Agrobacterium* sp. experiments do not follow this general trend, as below pH 4 the extent of Cd adsorption increases with decreasing pH for biomass both with and without EPS. This anomalous enhancement

Table 1

Proton binding constants (K_a) and site concentrations with 2σ uncertainties, resulting from triplicate titrations.

Biomass type	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	[Site 1] ^a	[Site 2]	[Site 3]	[Site 4]
Untreated <i>P. putida</i>	3.4 ± 0.2	4.9 ± 0.2	6.6 ± 0.2	9.3 ± 0.2	103 ± 12	77 ± 36	112 ± 20	59 ± 6
Enzyme-treated <i>P. putida</i>	3.4 ± 0.4	4.9 ± 0.6	6.7 ± 0.4	9.4 ± 0.2	105 ± 36	66 ± 2	120 ± 8	53 ± 14
Untreated <i>S. oneidensis</i>	3.4 ± 0.4	4.8 ± 0.2	6.6 ± 0.2	9.3 ± 0.2	93 ± 16	82 ± 24	109 ± 42	53 ± 12
Enzyme-treated <i>S. oneidensis</i>	3.2 ± 0.2	4.8 ± 0.2	6.6 ± 0.2	9.3 ± 0.2	88 ± 18	85 ± 70	103 ± 28	51 ± 2
Untreated <i>R. tropici</i>	4.0 ± 0.6	5.3 ± 1.2	7.1 ± 1.2	9.0 ± 0.2	164 ± 8	100 ± 14	97 ± 52	46 ± 16
Enzyme-treated <i>R. tropici</i>	4.1 ± 0.4	5.6 ± 1.0	7.3 ± 0.8	9.1 ± 0.2	168 ± 40	84 ± 22	60 ± 30	36 ± 10
Untreated <i>Agrobacterium</i> sp.	3.9 ± 0.2	5.3 ± 0.2	7.0 ± 0.2	9.2 ± 0.2	57 ± 36	57 ± 24	84 ± 60	62 ± 46
Enzyme-treated <i>Agrobacterium</i> sp.	3.9 ± 0.2	5.2 ± 1.2	6.9 ± 0.4	9.2 ± 0.4	93 ± 4	50 ± 8	65 ± 4	44 ± 14
Untreated <i>P. putida</i> ^b	3.5 ± 0.4	5.0 ± 0.4	6.9 ± 0.8	10.1 ± 0.2	82 ± 36	105 ± 24	42 ± 14	128 ± 66
Enzyme-treated <i>P. putida</i> ^b	3.2 ± 1.0	4.7 ± 0.8	6.4 ± 1.2	9.8 ± 1.0	86 ± 22	99 ± 38	56 ± 46	119 ± 80
Borrok et al. (2005) ^{c,d}	3.1	4.7	6.6	9.0	110 ± 70	91 ± 38	53 ± 21	66 ± 30

^a Site concentrations in $\mu\text{mol/g}$ wet mass.

^b Ueshima et al. (2008) determined the pK_a values and site concentrations of treated and untreated *P. putida*.

^c Borrok et al. (2005) determined the site concentrations of 36 bacterial species and consortia using fixed pK_a values.

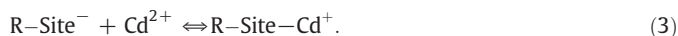
^d Data for site concentration shows 1σ uncertainty.

of Cd removal at low pH may be due to precipitation or internalization of Cd, but it is most likely due to decreased integrity of the cell wall of this species under these extremely low pH conditions (Borrok et al., 2004c). Some minor differences were observed between extents of Cd removal by biomass with EPS compared to biomass without EPS. However, the variability between replicate experiments (approximately $\pm 3\%$) was at least as large as the differences between experiments involving biomass with and without EPS present. Given these uncertainties, we conclude that the presence of the EPS does not significantly affect the extent of Cd removal under any of the conditions of these experiments.

Because the presence of EPS does not significantly affect the adsorption of Cd, we consider all of the data together for each species and compare the extent of Cd adsorption that we observed between the species studied here. Fig. 6 demonstrates that all of the species studied here adsorb similar extents of Cd from solution over the pH range studied, with the exception of the anomalous behavior reported for *Agrobacterium* sp. The variability that we observed between species is similar to the inter-species variability observed by Yee and Fein (2001), Borrok et al. (2004a), and Johnson et al. (2007). The dashed black curve shown in Fig. 7 was calculated from the proton- and Cd-biomass stability constants calculated by Johnson et al. (2007), determined by averaging the adsorption behaviors of the bacterial consortia studied. The adsorption behavior that is predicted from the Johnson et al. (2007) model agrees well with the adsorption of each of the bacterial species studied here. Therefore, the stability constants from this generalized model can be used to yield reasonable estimates for the extent of adsorption that occurs in EPS-bearing biofilms that contain a range of bacterial species.

A non-electrostatic surface complexation approach was used to calculate the stability constants for the important Cd-bacterial

biomass complexes from the Cd adsorption data. In order to compare calculated stability constants determined from experiments with and without EPS present, we considered only the adsorption data from pH 4 to 8.5. We modeled the adsorption of Cd as the formation of a bacterial surface complex with a 1:1 Cd:site molal ratio (Fein et al., 1997):



The mass action equation for Reaction (3) can be expressed as:

$$K_{\text{ads}} = \frac{[\text{R-Site-Cd}^+]}{[\text{R-Site}^-] \cdot \alpha_{\text{Cd}^{2+}}} \quad (4)$$

where the brackets represent molal concentrations, K_{ads} is the equilibrium constant for the Cd adsorption reaction, and $\alpha_{\text{Cd}^{2+}}$ is the activity of Cd^{2+} in solution. The average site concentrations and pKa values for each species, with and without EPS that we calculated from the titration modeling were used to describe the protonation of each site type. For each species, we modeled the data from experiments conducted with and without EPS separately, and determined goodness of fit using $V(Y)$ values from FITEQL to distinguish between models. In all cases, two-site models involving Cd^{2+} adsorption onto Sites 2 and 3 yielded significantly better fits to the data than any of the one-site models, and three-site models did not converge, suggesting over-constraint of the system. Fig. 8 depicts a representative example of how the model fits the data. Calculated K_{ads} values are located in Table 2, and a representative example for *R. tropici* is compared visually in Fig. 9. As can be seen for each bacterial species, the K values for both treated and untreated biomass are within experimental uncertainty of each other. The Cd binding constants for Sites 2 and 3

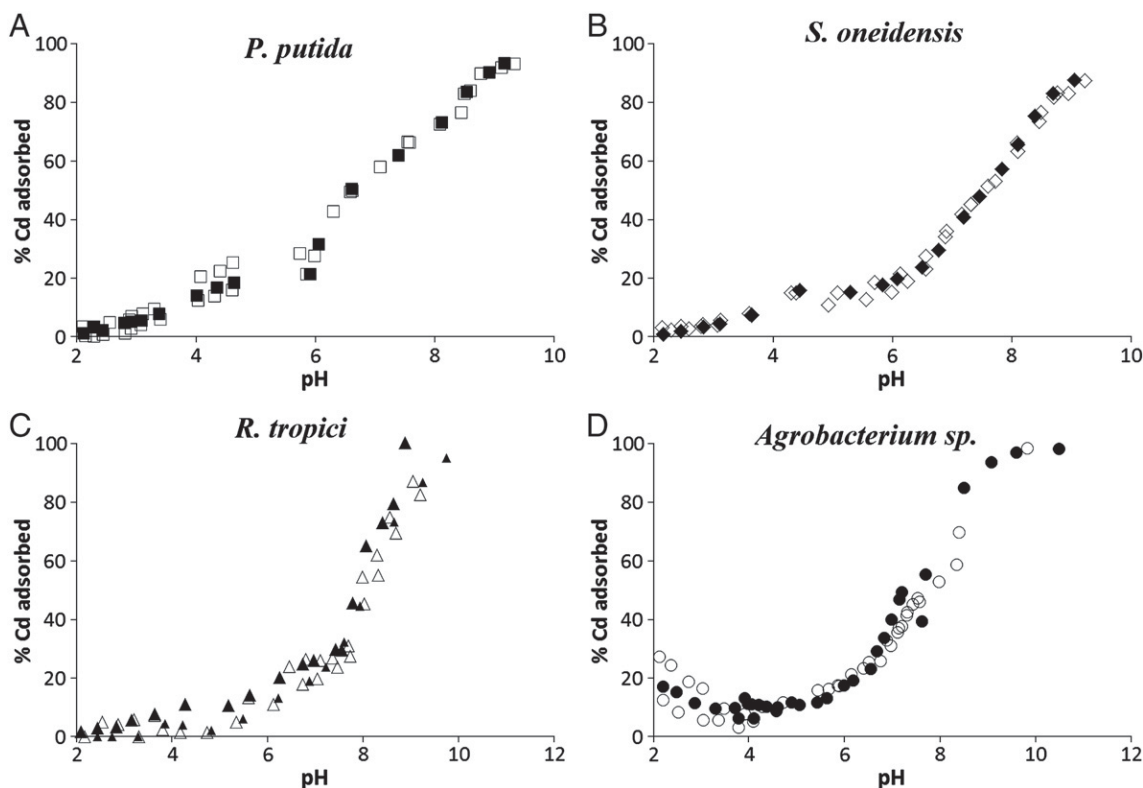


Fig. 6. Cd adsorption data for untreated, EPS-intact biomass (black symbols) and enzyme-treated biomass (open symbols) for A) *P. putida* (squares), B) *S. oneidensis* (diamonds), C) *R. tropici* (triangles), and D) *Agrobacterium* sp. (circles) showing the percentage of adsorbed Cd as a function of pH. All experiments contained 1.2 g/L (wet mass) of biomass and 10 ppm Cd in a 0.1 M NaClO_4 electrolyte solution.

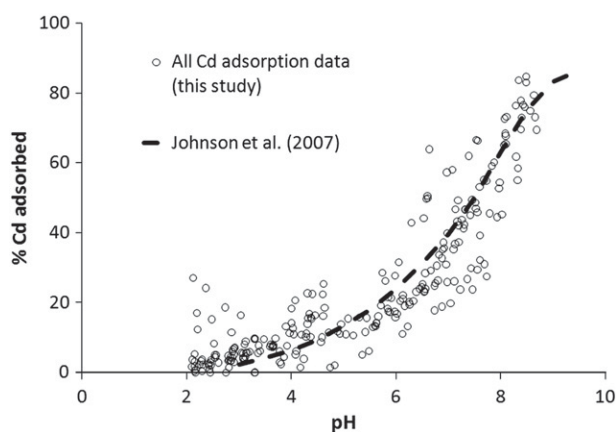


Fig. 7. Cd adsorption data for treated and untreated biomass for all the bacteria in this study (open circles). All experiments contained 1.2 g/L (wet mass) of biomass and 10 ppm Cd in a 0.1 M NaClO₄ electrolyte solution. The dashed black curve depicts a model for 10 ppm Cd adsorbed onto 1.2 g/L of natural consortia calculated with FITEQL using data from Johnson et al. (2007).

from Johnson et al. (2007) are within error of all of the species in this study, with the exception of Site 2 for *P. putida* and Site 2 for the enzyme-treated *Agrobacterium* biomass. In general, these models can account for Cd adsorption onto biofilms that contain a range of EPS molecules and containing a range of bacterial species.

The growth conditions in our study were controlled, and kept the same for all 4 bacterial species. It has been shown that different culture media can change the composition of EPS (Cérantola and Montrozier, 2001), which in turn may lead to differences in proton binding properties. However, these effects on binding behaviors still need to be investigated, and were not the subject of this study. The similarity in proton- and Cd-adsorption behaviors exhibited by the treated and untreated biomass in this study suggests that the EPS molecules from the species studied here either contain no significant proton active sites, or that the reactivity is similar to that of the cell wall in general for all four bacterial species studied. Previous studies provide unequivocal evidence that EPS molecules bind protons and metals (e.g., Guibaud et al., 2006; Lamelas et al., 2006; Ueshima et al., 2008). Therefore, our data strongly suggest that the EPS from the four bacterial species studied here exhibit virtually identical proton- and Cd-binding capacities per gram of biomass. If EPS had no adsorption capacity, then we would expect to see less adsorption per gram with EPS present than without, because the EPS would dilute the binding sites on the cell wall. Generalized models of proton- and Cd-binding, determined by averaging the modeling parameters for a wide range of individual bacterial species and bacterial consortia (e.g., Johnson et al., 2007) yield reasonable agreement with the extent of proton and Cd adsorption observed for the four bacterial species studied here. This observation, coupled with the conclusion that EPS binding can also be modeled using these same modeling parameters, suggests that these same parameters can yield reasonable estimates of proton and metal adsorption onto EPS-bearing biofilms that contain a range of bacterial species and types of EPS molecules.

4. Conclusions

This study expands on the results of Ueshima et al. (2008) by determining the effect of EPS removal on the proton and Cd binding behaviors of four bacterial species. Our experimental results indicate that enzymatic removal of EPS from each of the bacterial species studied does not affect either the proton reactivity or the Cd adsorption behavior of the biomass. Coupled with previous evidence that shows that EPS molecules contain metal- and proton-binding functional groups, our observations suggest that the proton-active

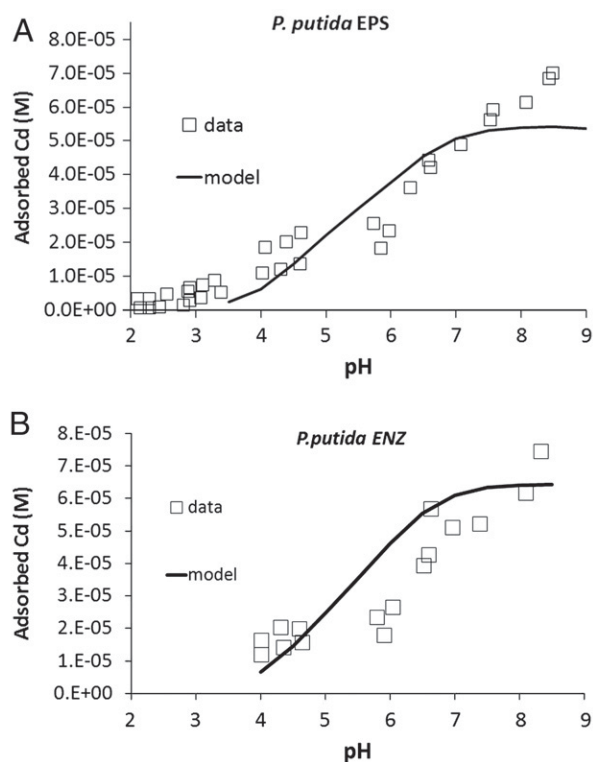


Fig. 8. A representative example best-fitting model (curve) of the measured extent of Cd adsorption (open squares) onto *P. putida* A) with EPS intact and B) with EPS enzymatically removed, as a function of pH. These experiments were conducted with 1.2 g/L of bacteria and 10 ppm Cd in 0.1 M NaClO₄.

binding sites within these four different types of EPS molecules are similar to those present on the cell walls of these species. In addition, the proton- and Cd-binding behaviors of the four species studied here are similar to each other. Most of the calculated site concentrations (normalized to mass), acidity constants, and Cd binding stability constants of both the EPS and cell wall sites for the four species studied here are within experimental uncertainty of those of a wide range of bacterial cell walls, suggesting that separate modeling parameters would not be needed when modeling biomass that contains EPS and a range of bacterial species. Our results suggest that reasonable

Table 2
Cd metal binding constants (K_{ads}) for the best-fit adsorption models.

Biomass type	Cd binding constants (log K_{ads})			
	Site 1	Site 2	Site 3	Site 4
Untreated		4.42	4.56	
<i>P. putida</i>		+0.34/−0.40	+0.10/−0.10	
Enzyme-treated		4.56	4.50	
<i>P. putida</i>		+0.42/−0.43	+0.34/−0.03	
Untreated		3.64	4.49	
<i>S. oneidensis</i>		+0.40/−0.49	+0.11/−0.08	
Enzyme-treated		3.54	4.53	
<i>S. oneidensis</i>		+0.19/−0.29	+0.23/−0.15	
Untreated		3.31	4.56	
<i>R. tropici</i>		+0.19/−0.21	+0.42/−0.16	
Enzyme-treated		3.04	4.52	
<i>R. tropici</i>		+0.24/−0.12	+0.32/−0.24	
Untreated		4.07	4.73	
<i>Agrobacterium</i> sp.		+0.31/−0.29	+0.22/−0.09	
Enzyme-treated		4.08	4.63	
<i>Agrobacterium</i> sp.		+0.18/−0.19	+0.32/−0.11	
Johnson et al. (2007)	3.1	3.4	4.3	5.8
	+0.20/−0.38	+0.18/−0.29	+0.23/−0.50	+0.28/−0.94

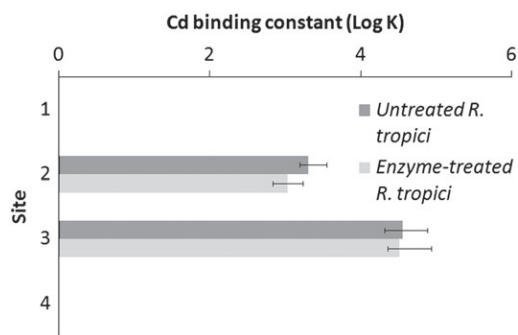


Fig. 9. Histogram comparing the Cd binding constants ($\log K_{ads}$) for Sites 2 and 3 of *R. tropici* with (labeled “untreated”) and without (labeled “enzyme-treated”) the presence of EPS.

approximations of proton and metal adsorptions onto EPS-bearing biofilms require knowledge of the average stability constants for the important proton- and metal-site complexes, as well as total site concentrations, but that individual behavior of the different species present and differentiation between EPS binding and cell wall binding may not be important to quantify.

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