**The Effect of Metal Loading on Cd Adsorption onto *Shewanella oneidensis* Bacterial
Cell Envelopes: The Role of Sulfhydryl Sites**

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Abstract

The adsorption and desorption of Cd onto *Shewanella oneidensis* bacterial cells with and without blocking of sulfhydryl sites was measured in order to determine the effect of metal loading and to understand the role of sulfhydryl sites in the adsorption reactions. The observed adsorption/desorption behaviors display strong dependence on metal loading. Under a high loading of 40 $\mu\text{mol Cd/g}$ bacterial cells, blocking the sulfhydryl sites within the cell envelope by exposure of the biomass to monobromo(trimethylammonio)bimane bromide (qBBr) does not significantly affect the extent of Cd adsorption, and we observed fully reversible adsorption under this condition. In contrast, under a low metal loading of 1.3 $\mu\text{mol Cd/g}$ bacterial cells, the extent of Cd adsorption onto sulfhydryl-blocked *S. oneidensis* cells was significantly lower than that onto untreated cells, and only approximately 50-60% of the adsorbed Cd desorbed from the cells upon acidification. In conjunction with previous EXAFS results, our findings demonstrate that Cd adsorption onto *S. oneidensis* under low metal loading conditions is dominated by sulfhydryl binding, and thus is controlled by a distinct adsorption mechanism from the non-sulfhydryl site binding which controls Cd adsorption under high metal loading conditions. We use the data to develop a surface complexation model that constrains the values of the stability constants for individual Cd-sulfhydryl and Cd-non-sulfhydryl bacterial complexes, and we use this approach to account for the Cd adsorption behavior as a function of both pH and metal loading. This approach is crucial in order to predict metal adsorption onto bacteria under environmentally relevant metal loading conditions where sulfhydryl binding sites can dominate the adsorption reaction.

Keywords: sulfhydryl; *Shewanella oneidensis*; metal loading; surface complexation modeling; biosorption

1. Introduction

Contamination of near-surface geologic systems by toxic metals is a major environmental concern (Jarup, 2003). Bacteria are ubiquitous in near-surface environments, and they can adsorb a wide range of metals through interactions with their abundant cell envelope binding sites (Beveridge and Murray, 1976, 1980; Plette et al., 1996; Fein et al., 1997), thereby affecting the speciation, distribution and mobility of metals in these systems (Beveridge and Murray, 1976; Templeton et al., 2001; Li and Wong, 2010). Bacterial adsorption of metal also can promote biomineralization reactions (Beveridge et al., 1983; Labrenz et al., 2000; Dunham-Cheatham et al., 2011) and can control metal bioavailability for a range of metabolic processes (Borrok et al., 2005; Hu et al., 2013; Flynn et al., 2014; Sheng and Fein, 2014). Determining the mechanisms responsible for metal adsorption onto bacterial cells, therefore, is critical in order to understand global cycling and transformation of many metals in the environment.

Metal adsorption onto bacteria has been studied using macroscopic bulk adsorption measurements (Beveridge and Murray, 1976, 1980; Plette et al., 1996; Fein et al., 1997; Yee and Fein, 2001), calorimetry (Gorman-Lewis et al., 2006; Gorman-Lewis, 2011) and molecular-scale tools such as Fourier transform infrared spectroscopy (FTIR) (Ha et al., 2010), X-ray photoelectron spectroscopy (XPS) (Boonaert and Rouxhet, 2000) and X-ray absorption spectroscopy (XAS) (Kelly et al., 2002; Boyanov et al., 2003; Guine et al., 2006; Ha et al., 2010). The earlier studies identified carboxyl, phosphoryl, and amino groups as the predominant metal binding sites within bacterial cell envelopes (Beveridge and Murray, 1980; Kelly et al., 2002; Boyanov et al., 2003; Jiang et al., 2004) and surface complexation modeling has been used to account for the observed metal adsorption behaviors (Xue et al., 1988; Plette et al., 1996; Fein et al., 1997; Cox et al., 1999; Pagnanelli et al., 2000; Ngwenya et al., 2003). However, most of

these studies were conducted under relatively high metal loading conditions, with values of higher than 10 μmol aqueous metal/gram of wet biomass common in the studied systems. Typical reported concentrations for heavy metal contaminants in surface and ground waters range from ng/L to low $\mu\text{g/L}$ (Klavins et al., 2000; Murano et al., 2007; Gupta et al., 2009; Lopez et al., 2010; Cui et al., 2011), where bacterial abundances can reach levels of approximately 10^9 to 10^{10} cell/L (Cole et al., 1993; Basu and Pick, 1997), suggesting that the metal:biomass ratios in these systems are significantly lower than 10 $\mu\text{mol/g}$ (calculated based on 10^{10} - 10^{11} cells/g, wet weight). As a result, extrapolating the established adsorption mechanisms using calculated stability constants of metal-bacteria complexes from previous studies to natural systems may lead to significant inaccuracies in predicted metal behaviors.

The sulfhydryl site (-SH) exhibits particularly high affinities for binding with chalcophilic metals such as Hg, As, Cd, Cu and Pb. Although the presence of sulfhydryl sites within bacterial cell envelopes has been demonstrated (Morris and Hansen, 1981; Morris et al., 1984), the role of these sites in metal adsorption onto bacteria has not been studied extensively, likely due to their presumed lower abundance relative to carboxyl and phosphoryl sites within cell envelopes. Recently, the importance of metal-sulfhydryl binding within bacterial cell envelopes was documented, and the nature of the bacterial surface complexes has been characterized by several research groups using Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy (Guine et al., 2006; Mishra et al., 2010; Mishra et al., 2011; Pokrovsky et al., 2012; Song et al., 2012). For example, Mishra et al. (2010) investigated Cd adsorption onto *Shewanella oneidensis* under a range of metal loading conditions using EXAFS spectroscopy, discovering that Cd-sulfhydryl binding dominates the adsorption in experiments with a Cd:biomass ratio of 2.7 $\mu\text{mol Cd/g}$ biomass (wet weight), and that Cd-sulfhydryl binding represents a detectable portion of the total

Cd binding until the Cd:biomass ratio increases to approximately 89 $\mu\text{mol/g}$. Carboxyl and phosphoryl binding become more important in the adsorbed Cd budget with increasing Cd loading on *Shewanella oneidensis* (Mishra et al., 2010). EXAFS spectroscopy has also revealed that sulfhydryl binding dominates the adsorption of Hg and Au onto bacteria when metal loadings are below several $\mu\text{mol/g}$ (wet weight) (Mishra et al., 2011; Song et al., 2012). These spectroscopic findings document the importance of metal-sulfhydryl binding under low metal loading conditions, but the results do not yield site concentrations or stability constants for these bacterial surface complexes.

Monobromo(trimethylammonio)bimane bromide (qBBBr), a molecule that binds strongly and specifically to sulfhydryl sites within cell envelopes, has been used in conjunction with fluorescence measurements (Joe-Wong et al., 2012) and with potentiometric titrations (Yu et al., 2014) to determine the concentration of sulfhydryl sites and associated acidity constants within bacterial cell envelopes for a range of bacterial species. The measured concentration of sulfhydryl sites is lower than those of the more abundant carboxyl and phosphoryl sites within cell envelopes, but models of the distribution of adsorbed metals on bacteria require not only site abundances, but also stability constants for the important metal-site complexes. The stability constants of metal-sulfhydryl aqueous complexes are typically several orders of magnitude higher than those of corresponding aqueous metal-carboxyl and metal-phosphoryl complexes (Haitzer et al., 2002), and a similar relationship for bacterial surface complexes likely explains the increased importance of metal-sulfhydryl binding with decreased metal loading on bacterial surfaces. However, currently there have been no direct measurements of metal-sulfhydryl stability constants for sites within bacterial cell envelopes, making it impossible to quantitatively model the effect of metal loading on the adsorption and surface speciation of metals onto bacteria.

We hypothesize that the stability constants for metal-sulfhydryl complexes on bacterial surfaces are significantly higher than those for metal-carboxyl and metal-phosphoryl complexes, causing the mechanism responsible for metal adsorption to vary as a function of metal loading. Under low metal loading conditions, the higher stability constants for metal-sulfhydryl complexes lead to dominance of sulfhydryl binding, despite the much lower abundance of sulfhydryl sites relative to carboxyl or phosphoryl site abundances. With increasing metal loading, however, the sulfhydryl sites become saturated and carboxyl and phosphoryl binding dominate. To test this hypothesis, we measured the adsorption of Cd onto *Shewanella oneidensis* bacterial cells under a wide range of metal loading conditions. Some experiments at selected low and high metal loadings were conducted using the sulfhydryl site blocking agent qBBBr in order to test the importance of sulfhydryl sites in Cd binding as a function of metal loading. We used the results of these tests to develop a surface complexation modeling approach which enables us to determine for the first time stability constants of Cd-sulfhydryl bacterial surface complexes. The resulting thermodynamic model can quantify and predict the adsorption of Cd onto *Shewanella oneidensis* under a wide range of metal loading conditions, accounting for the adsorption mechanism which changes as a function of both pH and metal loading.

2. Materials and Methods

2.1 Bacterial Cell Preparation

In this study, all the bacterial concentrations are reported in terms of wet weight of the biomass. The procedures for growth and washing of *Shewanella oneidensis* MR-1 cells were similar to those described previously (Yu et al., 2014). Briefly, bacteria were first cultured aerobically in 3 mL of trypticase soy broth with 0.5 % yeast extract at 32 °C for 24 h and then

transferred to 2 L of growth medium of the same composition at 32 °C for another 24 h. After incubation, bacterial cells in early stationary phase were harvested by centrifugation at 10,970 g for 5 min. The biomass pellets were rinsed with 0.1 M NaCl five times, with each rinse followed by centrifugation at 8,100 g for 5 min. Finally, the biomass pellets were transferred into pre-weighed test tubes and centrifuged for two 30-minute intervals at 8,100 g. After decanting the supernatant, the wet weight of the cells was used to calculate the bacterial concentrations in the subsequent experiments. To calculate the wet weight to dry weight conversion factor, approximately 2 gram (wet weight) of washed biomass was dried at 100 °C for 24 h until the weight did not change with time, with a resulting wet weight to dry weight ratio of 4.75.

2.2 Sulfhydryl Blocking Treatment

Monobromo(trimethylammonio)bimane bromide (qBBBr), purchased from Santa Cruz Biotechnology, Inc, was used in a pretreatment solution and exposed to the suspended biomass in order to block cell envelope sulfhydryl sites from further binding of metals. qBBBr effectively blocks cell envelope sulfhydryl sites (Joe-Wong et al., 2012; Yu et al., 2014), but does not react with bacterial carboxyl or phosphoryl sites and is not proton-active itself (Yu et al., 2014). The bacterial pellets were suspended in a freshly prepared qBBBr solution in 0.1 M NaCl with pH buffered to 7.0 ± 0.1 using $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, with a qBBBr:biomass ratio of approximately 70 $\mu\text{mol/g}$, and the suspension was allowed to react for 2 h at room temperature under continuous shaking on a rotating plate at 60 rpm. Our previous study demonstrated that the reaction between qBBBr and the cell envelope sulfhydryl sites is complete after 2 h of reaction (Yu et al., 2014). After reaction, bacterial cells were separated from the qBBBr solution by centrifugation at 8,100 g for 5 min. The qBBBr-treated biomass pellets were rinsed with 0.1 M NaCl three times, with each rinse followed by centrifugation at 8,100 g for 5 min. Finally, the biomass pellets were

transferred into pre-weighed test tubes and centrifuged for two 30-minute intervals at 8,100 g. After decanting the supernatant, the wet weight of the cells was used to calculate the bacterial concentrations in the subsequent experiments.

2.3 Adsorption Experiments

Sets of batch Cd adsorption experiments were conducted as a function of pH and metal:biomass ratio, both with and without the qBBR pretreatment procedure on the biomass, so our approach yields biomass samples for the experiments that either have sulfhydryl sites blocked or not blocked by qBBR. Prior to the adsorption experiments, a 1000 mg/L Cd(NO₃)₂ stock solution was diluted to the desired Cd concentrations using 0.1 M NaCl, and the pH values of the resulting solutions were adjusted to 6.5 ± 0.2 using 1 M NaOH. The pre-weighed bacterial cell pellet was then suspended into the dilute Cd solution with a vortex mixer to achieve a biomass concentration of 10 g/L in each experimental system. Different metal:biomass ratios were achieved by varying the initial Cd concentrations from 5-400 $\mu\text{mol/L}$, yielding Cd:biomass ratios from 0.5 - 40.0 $\mu\text{mol Cd/g}$ bacteria. The well mixed Cd-biomass suspensions were then transferred into test tubes with 10 mL of suspension in each, and their pH values were adjusted using 0.1 M NaOH or 0.1 M HNO₃ in order to achieve initial pH values within the range of 3.0-7.0. The tubes were slowly rotated for 2 h at room temperature in order to insure that adsorption equilibrium was achieved (Borrok and Fein, 2005), after which the final pH of the solution was measured. In this study, all the reported pH values are final pH readings. Finally, the bacterial suspensions were filtered using 0.22 μm nylon membranes, and the concentrations of Cd in the aqueous phase were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). To test for the potential release of metal cations from the cell envelope due to exposure to and exchange with protons under acidic conditions (Borrok et al., 2004; Sheng et al.,

2005), 10 g/L suspensions of *S. oneidensis* under the same ionic strength and pH conditions as the adsorption experiments with or without Cd added, were created. After 2 h of rotation at room temperature, the bacterial suspensions were filtered using 0.22 µm nylon membranes, and the concentrations of Mg, Ca, Fe, Cu, Zn and Ni in the aqueous phase were measured using ICP-OES. The detailed procedures for the metal analyses are provided in Section 2.5.

2.4 Desorption Experiments

In order to test the reversibility of Cd adsorption onto *S. oneidensis*, desorption experiments were conducted for two selected metal:biomass ratios: 1.3 and 40 µmol Cd/g (wet weight) of bacteria. The first step in these experiments was to conduct duplicate sets of adsorption experiments as a function of pH, following the same procedure as described above. After the 2 h adsorption equilibration step, the solution pH in each experimental system was measured. The suspensions in one of the two duplicate sets of test tubes were then filtered using 0.22 µm nylon membranes, and the Cd concentration in the filtered solution was analyzed using ICP-OES to determine the extent of Cd adsorption during this adsorption step. Meanwhile, the suspension pH in the other of the two duplicate sets of test tubes was adjusted to $\text{pH } 3.4 \pm 0.1$ in order to promote Cd desorption, and the remaining test tubes were slowly rotated for an additional 2 h at room temperature. The pH in each system was checked and if needed was adjusted back to pH 3.4 every 30 min until no further change was observed. Finally, the bacterial suspensions were filtered using 0.22 µm nylon membranes, and the concentration of Cd present in each solution was analyzed using the ICP-OES.

2.5 Analysis of Metals

A PerkinElmer Optima 8000 ICP-OES system was used to analyze the concentrations of metals in solution. Matrix-matched standards were prepared by diluting 1000 ppm commercial

standards with 0.1 M NaCl to cover a range of 50 to 1000 ppb. Prior to analysis, all the samples were diluted to the concentration ranges covered by the standards using 0.1 M NaCl, and then all the samples and standards were acidified using 20 μ L of 15.8 M HNO₃ per 10 mL sample. The blanks and standards were analyzed every 10-15 samples in order to monitor instrument drift. The determined analytical uncertainty was approximately 2-4% for the metals used in this study. The wavelengths used for the analysis of Cd, Mg, Ca, Zn, Fe, Cu and Ni were 226.5, 285.2, 317.9, 206.2, 238.2, 327.4 and 231.6 nm, respectively.

3. Results

The measured extents of Cd adsorption onto *S. oneidensis* under metal loadings ranging from 0.5 to 40 μ mol Cd/g biomass are presented in Figure 1. For ease of discussion, we refer to the six metal loadings considered in the present study as low (0.5 and 1.3 μ mol/g), medium (2.5 and 5.0 μ mol/g) and high (13.4 and 40 μ mol/g). Under low metal loadings (Figure 1A-B), the extent of Cd adsorption is nearly independent of pH under the high pH conditions studied, where all or nearly all of the aqueous Cd is adsorbed onto the bacterial cells. Below approximately pH 5-6, Cd adsorption gradually decreases with decreasing pH. However, under medium and high metal loading conditions (Figure 1C-F), although Cd adsorption generally decreases with decreasing pH in the ranges of pH 5.5-7.0 and 3.0-5.0, the extent of adsorption increases significantly as pH decreases from approximately pH 5.5 to 5.0, which is significantly different than the behavior that was observed for this pH range under low metal loading conditions.

Adsorption experiments were also conducted using qBBR-treated biomass (sulfhydryl sites blocked) under selected low and high metal loading conditions in order to estimate Cd adsorption onto sulfhydryl and non-sulfhydryl sites. The blocking of sulfhydryl sites causes

distinctly different effects on Cd adsorption onto *S. oneidensis* under the two metal loadings studied. Under a low metal loading of 1.3 $\mu\text{mol/g}$, Cd adsorption onto qBBR-treated *S. oneidensis* cells was significantly inhibited relative to that observed using untreated *S. oneidensis* cells across the pH range studied (Figure 1B). In addition, while a decreased adsorption with decreasing pH was observed in the experiments using untreated *S. oneidensis*, the experiments with sulfhydryl-blocked biomass exhibited a significant increase in adsorption from pH 5.5 to 5.0 (Figure 1B), similar with that observed for the experiments with untreated biomass under higher metal loading conditions (Figure 1C-F). In contrast, under a high metal loading of 40 $\mu\text{mol/g}$, the qBBR treatment affects the *S. oneidensis* Cd adsorption edge only slightly and only at approximately pH 5.0 (Figure 1F).

In addition to the adsorption experiments, we also measured the concentrations of a range of metals including Mg, Ca, Fe, Cu, Zn and Ni that are released by *S. oneidensis* cells into solution. Although the concentrations of most of the metals were either below the detection limits (Cu and Ni) or were present at concentrations of less than 0.5 $\mu\text{mol/g}$ of biomass (Ca, Fe, Zn), Mg was present at concentrations up to 18 $\mu\text{mol/g}$ below a pH of approximately 5.0 (Figure 2). The concentration of Mg released from the bacteria to the aqueous phase decreases sharply at pH 5.0-5.5, with only approximately 2-3 $\mu\text{mol/g}$ Mg released above pH 5.5. In addition, the systems with and without aqueous Cd present exhibited very similar Mg release trends as a function of pH (Figure 2), suggesting no significant exchange between Mg^{2+} within bacterial envelopes and aqueous Cd, and that it is only protons under acidic conditions that lead to Mg release.

The results of the desorption experiments (Figure 3) suggest that the adsorption mechanism changes as a function of metal loading. Under a high metal loading of 40 $\mu\text{mol/g}$,

adsorption is almost completely reversible, with nearly all of the adsorbed Cd desorbing from the bacterial cells after the solution pH was lowered to 3.4. Conversely, under a low metal loading of 1.3 $\mu\text{mol/g}$, only 50-60% of the adsorbed Cd desorbs upon acidification.

4. Discussion

4.1 Controls on Cd adsorption onto *S. oneidensis* under different metal loading conditions

In this study, the most significant effect of metal loading occurs at approximately pH 5.0-5.5, where increased Cd adsorption with decreasing pH was observed only under medium and high metal loadings (Figure 1C-F). An increase in adsorption with decreasing pH may be explained as either a change in the aqueous speciation of the solute over the pH range of interest (e.g., from a neutral aqueous complex species to cationic species) or an increase in the available binding sites within the bacterial cell envelopes under lower pH values. The first possibility can be excluded for our experiments because the calculated speciation of aqueous Cd does not change significantly across the pH range studied, with CdCl^+ and Cd^{2+} being the dominant forms of aqueous Cd under all experimental conditions. Because the protonation of functional groups within the cell envelope reduces the number of negatively-charged binding sites with decreasing pH, the concentrations of available binding sites should decrease with decreasing pH across the pH range studied. Hence, neither aqueous Cd speciation changes nor protonation/deprotonation of the cell envelope functional groups can explain the observed adsorption behavior. However, the metal release measurements (Figure 2) suggest that the release of Mg^{2+} from the cell envelope due to exposure to and exchange with protons under low pH conditions (Borrok et al., 2004; Sheng et al., 2005) actually leads to the creation of additional sites that originally were not reactive, and that these newly created sites are responsible for the enhanced adsorption that we

observed below approximately pH 5.5. In contrast, above pH 5.5 there is insufficient driving force for proton-attack, and most of these sites remain bound with Mg^{2+} and are not available for Cd adsorption.

Under low metal loading conditions, Cd adsorption does not appear to be affected by the Mg release as much as it is under higher metal loading conditions, likely because Cd-sulfhydryl binding dominates under low metal loading conditions (Mishra et al., 2010). Exposure of bacteria to acid treatment is used for the extraction of carbohydrates from bacterial cell envelopes, but this approach is less effective for extraction of proteins (Sheng et al., 2005), where most of the sulfhydryl sites within bacterial cell envelopes are likely present (Herrmann et al., 2009). Moreover, only $<0.1 \mu\text{mol/g}$ of iron, a prevalent metal in bacterial proteins (Waldron and Robinson, 2009), was released from bacterial cells in our systems even under acidic exposure (data not shown). These findings suggest that the newly created binding sites caused by proton- Mg^{2+} exchange are likely non-sulfhydryl sites, and thus the proton attack does not affect the concentration of sulfhydryl sites, the dominant binding sites for Cd adsorption under low metal loading conditions (Mishra et al., 2010). However, after the sulfhydryl sites are blocked by qBBr, the mechanism of Cd adsorption under low loadings switches to binding with non-sulfhydryl sites within the cell envelopes, and the appearance of the increased adsorption with decreasing pH at pH 5.0–5.5 (Figure 1B) is evidence for this change in adsorption mechanism. Under high metal loadings, the small difference between Cd adsorption onto untreated and qBBr-treated *S. oneidensis* (Figure 1F) suggests that non-sulfhydryl sites dominate the adsorption, consistent with the EXAFS results of Mishra et al. (2010).

The results from our desorption experiments (Figure 3) are consistent with a significantly changing contribution of Cd-sulfhydryl binding to the overall adsorption of Cd as a function of

metal loading. Previous studies of metal adsorption onto bacteria have noted full reversibility of the metal adsorption reaction under high metal loading conditions as well (Fowle and Fein, 2000; Yee and Fein, 2001; Lo et al., 2003), and adsorption under these conditions is dominated by carboxyl and phosphoryl binding (Toner et al., 2005; Ha et al., 2010; Mishra et al., 2010). Hence, our results suggest that Cd binding onto carboxyl and phosphoryl sites is fully and rapidly reversible, and relatively weak compared to Cd binding onto sulfhydryl sites. The desorption results shown here indicate that all of the Cd that does not desorb under the low metal loading condition is tightly bound to cell envelope functional groups which are likely sulfhydryl sites, and which account for 40-50% of the adsorbed Cd under these conditions. Similarly, Ledin et al. (1997) exposed Cd-loaded *P. putida* cells under a Cd loading of approximately 0.03 $\mu\text{mol/g(wet)}$ to a high concentration of EDTA solution for 24 h, and found that about 25% of the originally adsorbed Cd was still bound to the bacterial cells. Under the same adsorption and desorption conditions, the percentage of Hg that did not desorb was even higher, up to approximately 80%, likely because Hg-sulfhydryl binding is stronger than Cd-sulfhydryl binding (Ledin et al., 1997). However, our data do not constrain the concentration of the desorbed Cd ions that were originally adsorbed onto sulfhydryl sites. It is possible that partial desorption of the sulfhydryl-bound Cd occurred during the desorption experiments described here.

4.2 Development of Predictive Approach for the Effect of Metal Loading

We use the adsorption measurements as a function of pH and metal loading to constrain the value of the stability constant for the Cd-sulfhydryl complex formed within the cell envelopes. Because Cd desorption from the sulfhydryl sites was not complete under the tested desorption conditions, it is possible that our adsorption experiments were not at equilibrium and

that our adsorption measurements represent a minimum concentration of adsorbed Cd at equilibrium. Therefore, the following equilibrium model should be considered conditional and yields a conservative estimate of the thermodynamic stability of the Cd-sulfhydryl complex. Yu et al. (2014) determined that the functional groups within *S. oneidensis* cell envelopes can be described with 4 proton-active sites with the following pK_a values: 3.9 ± 0.2 , 5.2 ± 0.2 , 7.0 ± 0.1 , and 9.4 ± 0.1 (referred to as Sites 1 – 4), with site concentrations of 105.0 ± 9.5 , 86.3 ± 15.1 , 44.0 ± 2.9 and 113.7 ± 8.7 $\mu\text{mol/g}$ (wet weight), respectively, and sulfhydryl sites comprise at least a portion of Sites 3 and 4. Because of the effects of Mg^{2+} release and the poor constraints on the concentration of sites liberated as a result of Mg^{2+} release under acidic conditions, only the adsorption data between pH 5.3-7.0 were used for the modeling exercise described here. Given the pH range of our adsorption data, we only considered Sites 2 and 3 as the most likely binding sites responsible for the observed Cd adsorption in this study, and we attempted to use either Site 2 or Site 3 to fit the experimental data based on a 1:1 Cd:site stoichiometry, as determined previously (Mishra et al., 2010). Correspondingly, Cd adsorption onto bacteria can be described using the following reactions:

where $R - A_i^-$ represents the deprotonated form of Site i ($i = 2$ or 3) within the *S. oneidensis* envelope and $R - A_i - \text{Cd}^+$ represents the bacterial complex formed between Cd^{2+} and Site i . The stability constant of the $R - A_i - \text{Cd}^+$ complex, $K_{\text{Cd-Site } i}$, can be expressed as:

$$K_{\text{Cd-Site } i} = \frac{[R - A_i - \text{Cd}^+]}{[R - A_i^-] a_{\text{Cd}^{2+}}} \quad (3)$$

where $a_{\text{Cd}^{2+}}$ is the activity of Cd^{2+} in bulk solution, and the brackets represent the concentrations of the species shown. Because most previous studies demonstrate only a weak ionic strength

influence on proton and metal adsorption onto bacteria (Martinez et al., 2002; Borrok and Fein, 2005; Gonzalez et al., 2010), we use a non-electrostatic surface complexation model to fit our Cd adsorption data. FITEQL 2.0 (Westall, 1982) was used as a modeling tool for optimization of the stability constants of the Cd-bacterial complexes.

The one-site model that invokes Cd binding onto the deprotonated form of Site 2 (Reaction 1), fails to adequately account for the pH dependence under most of the metal loading conditions studied, but models that invoke Cd adsorption onto Site 3 (Reaction 2) generally fit the experimental data well as a function of both pH and metal loading (Figure 4). In this initial modeling step, we used the data for each metal loading condition separately to solve for the $K_{Cd-Site\ 3}$ value that yielded the best fit to each set of data for each metal loading condition. However, as metal loading increases from 0.5 to 40 $\mu\text{mol/g}$, these calculated Log $K_{Cd-Site\ 3}$ values decrease from 7.0 to 5.2 (Figure 5A). $K_{Cd-Site\ 3}$ is a thermodynamic equilibrium constant for Reaction (2), and therefore if Reaction (2) is the only reaction responsible for adsorption, then the calculated $K_{Cd-Site\ 3}$ values should be independent of metal loading. The consistent decrease in calculated $K_{Cd-Site\ 3}$ values with increasing metal loading, therefore, strongly suggests that Reaction (2) is not the only adsorption mechanism. We attempted to model the data using a two-site model involving Cd adsorption onto both Site 2 and Site 3, and also using a one-site model involving a 1:2 Cd:Site 3 stoichiometry, but in both cases the calculated $K_{Cd-Site\ 3}$ values were still dependent on metal loading (data not shown). Therefore, we conclude that neither Reaction (1) nor binuclear binding can be the other adsorption mechanism involved here.

Based on the findings above, we re-modeled the Cd adsorption data using a two-site model in which both sites have the same $\text{p}K_a$ value of 7.0, but one is a high affinity sulfhydryl

site, $R - A_{3,Sulf}^-$, and the other is a lower affinity 'non-sulphydryl' site, $R - A_{3,non-Sulf}^-$, so that the following two reactions control the Cd adsorption:

where $R - A_{3,Sulf} - Cd^+$ and $R - A_{3,non-Sulf} - Cd^+$ represent the Cd complexes with the high- and low-affinity sites, respectively. The stability constants for these Cd-bacterial complexes can be expressed as:

$$K_{Cd-3,Sulf} = \frac{[R - A_{3,Sulf} - Cd^+]}{[R - A_{3,Sulf}^-]a_{Cd^{2+}}} \quad (6)$$

$$K_{Cd-3,non-Sulf} = \frac{[R - A_{3,non-Sulf} - Cd^+]}{[R - A_{3,non-Sulf}^-]a_{Cd^{2+}}} \quad (7)$$

Surface complexation modeling can simultaneously determine two (or more) stability constants for binding sites responsible for a measured total extent of metal adsorption only when there is also an independent measurement of the distribution of the metal between the two sites. Because our measurements yield total adsorbed Cd concentrations only and not the concentration of Cd specifically bound to each of the sulphydryl and non-sulphydryl portions of Site 3, the Cd adsorption measurements alone cannot be used to simultaneously solve for unique values of $K_{Cd-3,non-Sulf}$ and $K_{Cd-3,Sulf}$. However, if we fix one K value, we can use the Cd adsorption measurements to solve for the other K value at each metal loading condition studied. The calculated $K_{Cd-Site\ 3}$ values shown in Figure 5A decrease from 7.0 to 5.7 over the metal loading range of 0.5-5.0 $\mu\text{mol/g}$, but only decrease from 5.7 to 5.2 over the metal loading range of 5.0-40.0 $\mu\text{mol/g}$. The relative constancy of the calculated $K_{Cd-Site\ 3}$ values under high metal loading

conditions suggests that one binding mechanism controls Cd adsorption under these conditions. Our results, as well as the EXAFS evidence from [Mishra et al. \(2010\)](#), indicate that a non-sulfhydryl site dominates the adsorption under high metal loading conditions. Therefore, the calculated $K_{Cd-Site\ 3}$ value from the highest metal loading condition studied (a value of 5.2 for the $\log K_{Cd-Site\ 3}$ value obtained at 40.0 $\mu\text{mol/g}$) represents a reasonable estimation of the value of $K_{Cd-3,non-Sulf}$.

We then use this estimated $K_{Cd-3,non-Sulf}$ value as a basis for calculating the stability constant for the complex between Cd^{2+} and the sulfhydryl sites of Site 3, $K_{Cd-3,Sulf}$. Because the contribution of sulfhydryl sites to the overall Cd adsorption is small under the high metal loading conditions studied, only the measured adsorption behaviors under low and medium metal loadings could be used to constrain the $K_{Cd-3,Sulf}$ value. Previously, [Yu et al. \(2014\)](#) determined that the total (sulfhydryl plus non-sulfhydryl) site concentration for Site 3 is 44.0 ± 2.9 (1σ) $\mu\text{mol/g}$, and that the sulfhydryl portion of this total is 3.4 ± 1.6 (1σ) $\mu\text{mol/g}$. However, using 3.4 $\mu\text{mol/g}$ as the sulfhydryl site concentration yields calculated $\log K_{Cd-3,Sulf}$ values that systematically decrease with increasing Cd loading, changing from a value of 8.1 to 7.0 as Cd loading increases from 0.5 to 5.0 $\mu\text{mol/g}$. The data of [Yu et al. \(2014\)](#) do not yield tight constraints on the Site 3 sulfhydryl concentration, and the uncertainty in the site concentration is high relative to its absolute value. Therefore, we re-modeled the Cd adsorption data using 5.0 $\mu\text{mol/g}$ (the average value plus the 1σ uncertainty) and 1.8 $\mu\text{mol/g}$ (the average value minus the 1σ uncertainty) as Site 3 sulfhydryl site concentrations, and the remaining portion of Site 3 as non-sulfhydryl sites to calculate $K_{Cd-3,Sulf}$. Using 5.0 $\mu\text{mol/g}$ as the sulfhydryl concentration yields a similar dependence of the calculated $K_{Cd-3,Sulf}$ values on metal loading relative to the dependence observed using 3.4 $\mu\text{mol/g}$. However, as shown in Figure 5B, the calculated $\log$

$K_{Cd-3,Sulf}$ values using 1.8 $\mu\text{mol/g}$ as the Site 3 sulfhydryl site concentration are independent of metal loading, suggesting that a value of 1.8 $\mu\text{mol/g}$ is closer to the actual concentration of Site 3 sulfhydryl sites within *S. oneidensis* cell envelopes.

The average value of the calculated $\text{Log } K_{Cd-3,Sulf}$ using 1.8 $\mu\text{mol/g}$ as the concentration of Site 3 sulfhydryl sites is 8.3 ± 0.3 (1σ), which is similar to the reported stability constant values for the aqueous Cd-glutathione complex (Perrin and Watt, 1971; Walsh and Ahner, 2013), but is lower than that for the aqueous Cd-cysteine complex (Cole et al., 1985; Walsh and Ahner, 2013). Our results indicate that the observed adsorption behavior can be explained with two adsorption mechanisms: Cd-sulfhydryl binding, involving relatively few sites, but with a log stability constant value of 8.3 that dominates the adsorbed Cd budget under low metal loading conditions, and binding between Cd and a non-sulfhydryl binding site with a similar $\text{p}K_a$ value to that of the sulfhydryl site, but with a log stability constant value of approximately 5.2. The non-sulfhydryl site is more plentiful, but has a lower affinity for Cd, and hence it becomes the dominant form of adsorbed Cd only under high metal loading conditions.

We used the two stability constants determined in this study ($K_{Cd-3,Sulf}$ and $K_{Cd-3,non-Sulf}$) to calculate the distribution of adsorbed Cd under the experimental conditions, and the results are presented in Figure 6. Although the data from this study cover approximately 2 orders of magnitude in metal loading conditions (0.5-40 $\mu\text{mol Cd/g}$ bacteria), the predicted total extents of Cd adsorption are in excellent agreement with the experimental data, demonstrating that the two stability constants that were determined in this study can account for Cd adsorption as a function of both pH and metal loading condition over a range in which the Cd adsorption mechanism changes markedly. Under the lowest metal loading conditions studied (Figure 6A and B), Cd-sulfhydryl binding dominates, representing virtually all of the adsorbed Cd budget. With

increasing metal loading, consistent with EXAFS results (Mishra et al., 2010), the relative importance of Cd-sulfhydryl binding decreases, and under the highest metal loading conditions studied (Figure 6F), the non-sulfhydryl sites are responsible for binding virtually all of the adsorbed Cd. The strength of the surface complexation modeling approach is demonstrated by the range of behaviors for which the model can account, based solely on the stability constants for the important Cd-bacterial surface complexes.

5. Conclusions

In the present study, we demonstrate that the mechanism of Cd adsorption onto *S. oneidensis* cell envelopes depends strongly on metal loading, due to the presence of sulfhydryl and non-sulfhydryl Cd binding sites that have high and low affinities for Cd, respectively. This study is the first to deconvolve metal binding onto sites having similar pK_a values and is the first to yield quantitative estimates for the value of the stability constant for a metal-sulfhydryl bacterial complex. We developed a surface complexation model based on the observed adsorption mechanisms to account for the effect of metal loading. This model successfully accounts for adsorption as a function of metal loading over a range of approximately 2 orders of magnitude, and covers adsorption in regimes in which sulfhydryl binding dominates and in which non-sulfhydryl sites dominate. Despite the low abundance of sulfhydryl sites relative to the total concentration of other binding sites within bacterial cell envelopes, their high affinities for chalcophilic metals such as Hg, Cd, As, Cu, Zn, Pb and Au likely enable them to out-compete the non-sulfhydryl sites such as carboxyl and phosphoryl groups in the adsorption of these metals, especially under low metal loading conditions. Furthermore, at least a portion of the Cd-sulfhydryl complexes are more resistant to desorption than are the Cd-non-sulfhydryl complexes.

Because the ratios of metal:bacteria in most natural environments are lower than $\mu\text{mol/g}$ (wet weight) (Cole et al., 1993; Basu and Pick, 1997; Klavins et al., 2000; Gupta et al., 2009), metal-sulfhydryl binding likely represents a major mechanism for metal adsorption onto bacteria in these systems. Thus, it is crucial to determine stability constants for the range of metal-sulfhydryl complexes that can form within cell envelopes. Our study demonstrates that surface complexation models are able to account for the extent of metal adsorption onto bacteria and for the speciation of the cell-bound metal, both in natural environments where the metal loadings are usually low, and in systems with much higher metal loadings, such as metal-contaminated soils and groundwater.

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601 **Figure 1. Measured extent of Cd adsorption onto untreated (●) and qBBr-treated (○) *S.***
 602 ***oneidensis* cells under metal loadings of: (A) 0.5, (B) 1.3, (C) 2.5, (D) 5.0, (E) 13.4, and (F)**
 603 **40.0 $\mu\text{mol Cd/g}$ (wet mass) bacteria.**

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Figure 2. Concentration of Mg released from *S. oneidensis* cells under different metal loadings (Cd:biomass ratios) as a function of pH.

Figure 3. Extent of Cd desorbed from *S. oneidensis* cells after an adsorption step under metal loadings of 1.3 and 40.0 µmol/g followed by 2 h of re-equilibration at pH 3.4±0.1.

Figure 4. Surface complexation modeling results using non-electrostatic one-site models (dashed curve for a model involving Site 2; solid curve for a model involving Site 3) of Cd adsorption onto *S. oneidensis* cells under metal loadings of: A) 0.5 , B) 1.3 , C) 2.5 , D) 5.0 , E) 13.4, and F) 40.0 $\mu\text{mol Cd/g}$ (wet mass) bacteria.

Figure 5. Calculated stability constant values for: A) $K_{\text{Cd-Site } 3}$ as a function of metal loading using a one-site model based on Reaction (2); B) $K_{\text{Cd-3,Sulf}}$ as a function of metal loading using a two-site model based on Reactions (4)-(5) and with $\text{Log } K_{\text{Cd-3,non-Sulf}}$ fixed at a value of 5.2.

Figure 6. Calculated speciation of adsorbed Cd using the two-site model based on $\text{Log } K_{\text{Cd-3,Sulf}}$ and $\text{Log } K_{\text{Cd-3,non-Sulf}}$ values of 8.3 and 5.2, respectively, under metal loadings of: A) 0.5 , B) 1.3 , C) 2.5 , D) 5.0 , E) 13.4, and F) 40.0 $\mu\text{mol Cd/g}$ (wet mass) bacteria. Open circles represent the experimental data, and solid curves, dashed curves, and dotted curves represent the predicted total adsorbed Cd concentration, the concentration of Cd adsorbed onto non-sulfhydryl sites, and the concentration of Cd adsorbed onto sulfhydryl sites, respectively.