



Characterization of sulfhydryl sites within bacterial cell envelopes using selective site-blocking and potentiometric titrations



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ABSTRACT

In this study, a novel approach was developed to estimate the concentration and acidity constants of sulfhydryl sites within bacterial cell envelopes, and we apply the approach to compare sulfhydryl site concentrations of *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus cereus*, *Shewanella oneidensis* and *Pseudomonas fluorescens*. The experiments involved the selective blocking of sulfhydryl sites using a thiol-specific molecule, coupled with total site concentration comparisons of blocked and un-blocked bacterial samples by potentiometric titration measurements to determine sulfhydryl concentrations. All five species studied contained measurable concentrations of sulfhydryl sites, ranging from $16.6 \pm 3.3 \mu\text{mol/g}$ for *B. cereus* to $33.1 \pm 7.6 \mu\text{mol/g}$ for *S. oneidensis*. No significant difference was found between sulfhydryl site concentrations on Gram-positive species relative to those on Gram-negative bacteria. However, the proportion of sulfhydryl sites relative to the total sites on each species was the highest for the thermophilic bacterium *B. licheniformis* with $14 \pm 3\%$, and the four mesophilic species exhibited an average of $8 \pm 2\%$. All species contained sulfhydryl sites with a pK_a of 9.2–9.4, but *B. subtilis* and *P. fluorescens* exhibited significant concentrations of sulfhydryl sites with much lower pK_a values as well. Our results suggest that sulfhydryl sites are present in relatively low concentrations over a wide range of bacterial diversity, but that their concentrations are high enough to control the binding of metals onto bacteria under low metal-loading conditions.

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1. Introduction

Due to the abundance of organic acid functional groups within bacterial cell envelopes (Beveridge and Murray, 1980; Fein et al., 1997), metal cations can readily adsorb onto bacterial surfaces, thereby potentially affecting the speciation, distribution, bioavailability and mobility of the metal in the environment (Beveridge and Murray, 1976; Templeton et al., 2001; Martinez et al., 2002; Borrok et al., 2005a; Li and Wong, 2010; Sheng et al., 2011). Previous research indicates that carboxyl, phosphoryl and amine groups are important binding site types within bacterial cell envelopes (Fein et al., 1997; Cox et al., 1999; Ngwenya et al., 2003; Jiang et al., 2004; Guiné et al., 2006). In addition to these site types, the importance of sulfhydryl groups in metal–bacteria interactions was identified and highlighted in recent studies (Guiné et al., 2006; Mishra et al., 2010, 2011; Kenney et al., 2012; Pokrovsky et al., 2012; Song et al., 2012; Hu et al., 2013). Mishra et al. (2010), using EXAFS spectroscopy to study the speciation of Cd adsorbed onto *Shewanella oneidensis* under a wide range of metal loading conditions, identified sulfhydryl groups as the dominant binding sites for Cd for systems in which the initial Cd:biomass ratio

was $300 \mu\text{g Cd/g}$ of bacteria in wet weight. Carboxyl and phosphoryl bindings become more important in the adsorbed Cd budget with increasing Cd loading on the bacteria, but sulfhydryl sites represent a detectable binding site in systems with an initial Cd:biomass ratio of less than 10 mg Cd/g of bacteria (Mishra et al., 2010). Au(I)–sulfhydryl (Kenney et al., 2012; Song et al., 2012) and Hg(II)–sulfhydryl (Mishra et al., 2011; Hu et al., 2013) bindings have also been observed as dominant mechanisms of Au and Hg adsorption onto bacteria when metal:biomass ratios are in the range of several hundred $\mu\text{g metal/g}$ of bacteria or less. The concentrations of metals such as Cd, Hg, Cu, Ni, Zn and Pb in uncontaminated surface and ground waters, and even in some contaminated systems, are low, typically ranging from ng/L to $\mu\text{g/L}$ (Klavins et al., 2000; Murano et al., 2007; Gupta et al., 2009; Lopez et al., 2010; Cui et al., 2011). Typical bacterial abundances in these systems can reach levels of approximately 10^9 to 10^{10} cell/L (Cole et al., 1993; Basu and Pick, 1997), or a bacterial concentration of approximately 0.1 to $1.0 \text{ g (wet mass)/L}$, using a conversion factor of 10^{10} cells/g (wet mass). Therefore, the metal loadings on bacterial cells in even contaminated geological systems are probably lower than concentrations that can saturate the sulfhydryl groups within bacterial cell envelopes, suggesting that metal–sulfhydryl binding may represent the dominant adsorption mechanism on bacteria. Metal–sulfhydryl binding, therefore, may control the transport, speciation, toxicity and bioavailability of

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metals in natural environments. For example, Hu et al. (2013) recently demonstrated that the reduction of Hg(II) to Hg(0) by *Geobacter sulfurreducens* was markedly inhibited by Hg(II) adsorption onto bacterial sulfhydryl sites, and that only the Hg(II) adsorbed onto the other, non-sulfhydryl sites on the bacteria is bioavailable. Despite the importance of sulfhydryl sites in controlling metal adsorption by bacteria under low metal loading conditions, the concentrations and acidity constant values of sulfhydryl sites within bacterial cell envelopes have not been well constrained, making it difficult to understand and predict the extent of metal-bacterial sulfhydryl binding that occurs in natural and engineered geologic systems.

Currently, the best available method to estimate the density and acidity constants of binding sites on bacterial cell envelopes is based on potentiometric titration and surface complexation modeling (SCM) (Xue et al., 1988; Fein et al., 1997; Cox et al., 1999; Pagnanelli et al., 2000; Ngwenya et al., 2003; Yee et al., 2004). Although potentiometric titrations provide a reliable means to derive total site concentrations, and have been used effectively to constrain the protonation behavior of cell envelope functional groups (Daughney and Fein, 1998; Ngwenya et al., 2003; Takahashi et al., 2005), the interpretation of potentiometric titration data to infer concentrations and acidity constant values for specific proton-active binding sites is model-dependent (Fein et al., 2005). EXAFS spectroscopy can be used to identify metal-sulfhydryl site binding (Sarret et al., 1998; Guiné et al., 2006; Mishra et al., 2009, 2010; Pokrovsky et al., 2012), but it is not capable of yielding values for the sulfhydryl site concentrations within cell envelopes or the acidity constants of those sites (Mishra et al., 2005). Commonly used methods for the analysis of small biological thiol molecules involve the selective derivatization of the thiols with thiol-specific labeling reagents, followed by separation of thiol derivatives from interferences and UV/vis or fluorescence measurements (Dalle-Donne and Rossi, 2009; Hansen and Winther, 2009). However, the separation technologies that are required in this approach, such as high-performance liquid chromatography (HPLC) (Fahey and Newton, 1987), are not suitable for micron-sized bacterial cells.

In this study, we use a novel method that couples selective derivatization of sulfhydryl sites with potentiometric titrations and surface complexation modeling in order to analyze the sulfhydryl concentrations and acidity constant values within bacterial cell envelopes. Monobromo(trimethylammonio)bimane bromide (qBBr, Fig. 1), a cationic thiol-specific labeling molecule, was selected as the blocking molecule for bacterial sulfhydryl sites within cell envelopes due to its fast reaction rate specifically with thiol moieties within bacterial cell envelopes (Kosower et al., 1979; Kosower and Kosower, 1987). Previous studies demonstrate that qBBr can effectively block thiol sites on the surface of various biological cells such as human red blood cells (Kosower et al., 1979), guinea pig spermatozoa (Huang et al., 1984) and human leukocyte antigen (Whelan and Archer, 1993). Despite the differences in surface structures between various cells, the reaction mechanisms between qBBr and various biological sulfhydryl sites are the same (Fig. 1) (Kosower et al., 1979; Radkowsky and Kosower, 1986). During the interaction between qBBr and a sulfhydryl site, the bromine atom of qBBr is replaced by the sulfur atom from a sulfhydryl site. Therefore, exposed sulfhydryl sites should form strong covalent

bonds with qBBr. Since the reaction product is not proton active, the sulfhydryl sites blocked by qBBr on cell envelopes should be inert during potentiometric acid–base titrations. Thus, the change in total site concentrations on cell envelopes determined by potentiometric titrations of biomass with and without qBBr treatment should be a direct measure of the sulfhydryl site concentration. Recently, qBBr titrations were used by Joe-Wong et al. (2012) in order to probe the sulfhydryl site concentration within cell envelopes of *Bacillus subtilis* using fluorescence spectroscopy. This method yields total sulfhydryl site concentrations, but does not provide constraints on the acidity constants of the probed sulfhydryl sites. In this study, we test a related approach, using potentiometric titration experiments involving biomass with and without qBBr treatment to probe sulfhydryl site concentrations and acidity constant values for five bacterial species. We use the results to determine if differences in sulfhydryl site concentrations and acidity constants exist between bacterial species with different cell envelope compositions (Gram-negative vs Gram-positive and thermophilic vs mesophilic).

2. Materials and methods

2.1. Bacterial cell preparation and thiol blocking reaction

In this study, all the bacterial concentrations are reported in terms of wet weight of the biomass. Five bacterial species were used in this study, including three Gram-positive bacteria (*Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus*) and two Gram-negative bacteria (*Shewanella oneidensis* and *Pseudomonas fluorescens*). Among these bacteria, *B. licheniformis* is a thermophile, while the other four species are mesophiles, although they were cultured under the same temperature in this study. The procedures for growth and washing of the bacterial cells were similar to those described previously (Fein et al., 1997, 2005). Briefly, all of the bacterial species 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 and rinsed three times with 0.1 M NaCl to avoid any unwanted interactions between qBBr and components of the growth medium. After rinsing, *B. subtilis* cells were stained using a LIVE/DEAD BacLight bacterial viability kit, which contains SYTO9 stain and propidium iodide, and then observed under a fluorescence microscope in order to test the integrity of the bacterial cells. While SYTO9 alone labels both dead and live cells to make them fluoresce green, propidium iodide penetrates only those cells with damaged membranes and reduces SYTO9 fluorescence, causing dead cells to fluoresce red. Our test results on *B. subtilis* cells showed a uniform green fluorescent color with no visible red cells, indicating that virtually all of the bacterial cells were still live and intact after the rinsing procedure.

Monobromo(trimethylammonio)bimane bromide (qBBr) and *N*-Acetyl-L-cysteine (A-CYS) were purchased from Santa Cruz Biotechnology, Inc. and Sigma-Aldrich, Inc., respectively. To block sulfhydryl sites, freshly prepared qBBr solution in 0.1 M NaCl was added to the bacterial suspension or to a 1 mM A-CYS solution, and the mixture was allowed to react for 2 h at room temperature under continuous shaking on a rotating plate at 60 rpm. The A-CYS molecules have two proton-active functional groups within the pH range that we studied: a carboxyl group with a pK_a of ~3.2 and a sulfhydryl group with a pK_a of ~9.5 (HSDB, 2014). Therefore, the titrations of the A-CYS solutions with and without qBBr-treatment serve as controls to test the ability of qBBr to block sulfhydryl sites and the selectivity of qBBr for sulfhydryl sites by determining if any of the carboxyl sites on A-CYS are blocked by the qBBr treatment. Previous studies indicate that the reaction kinetics between bromobimanes and sulfhydryl sites are pH-dependent and relatively rapid at pH 6–8 in the presence of a phosphate salt buffer (Kosower et al., 1979; Radkowsky and Kosower, 1986). For example, although human red blood cells are several microns in diameter,

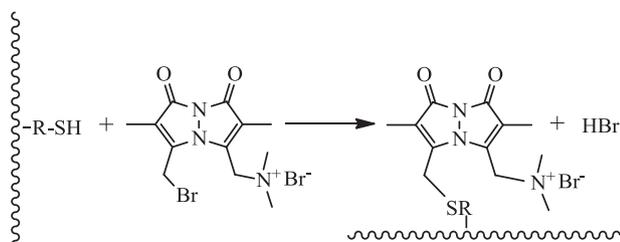


Fig. 1. Reaction of qBBr with sulfhydryl sites within bacterial cell envelopes.

monobromobimane (mBBr) and dibromobimane (bBBr), which have similar structures to qBBr, can diffuse through the whole cell and react with all intracellular sulfhydryl sites within 1 h (Kosower et al., 1979). Because the cell walls of bacteria are much thinner than whole red blood cells and because bacterial cell walls are porous with an estimated mean pore diameter of ~4 nm (Demchick and Koch, 1996), the diffusion of the <1 nm qBBr molecules throughout bacterial cell walls should be faster than 1 h. We extended the reaction time to 2 h to insure complete reaction between qBBr and all bacterial cell envelope sulfhydryl sites. In this study, typical suspension pH values after the qBBr reaction were 5.8–6.4 without pH buffers, so in order to simplify the experimental systems, pH buffers were not used. As the sulfhydryl concentrations of most species studied here were unknown, the sulfhydryl concentration of $23.7 \pm 2.1 \mu\text{mol}$ per gram for *B. subtilis* determined by Joe-Wong et al. (2012) was doubled and used to set the initially tested qBBr:bacteria ratio. We varied the qBBr concentration during the exposure step, and found that the calculated number of blocked sulfhydryl sites on *B. subtilis* did not change significantly using qBBr/sulfhydryl ratios of between 1.3 and 4.0 (based on $23.7 \mu\text{mol/g}$ of sulfhydryl sites on *B. subtilis* and a 1-to-1 qBBr to sulfhydryl stoichiometry), suggesting that a qBBr/sulfhydryl ratio of 2.0 was sufficient for blocking the bacterial sulfhydryl sites after 2 h of reaction. Longer exposure times than 2 h could not be used due to significant degradation of qBBr in aqueous solutions. In preliminary titrations for each species, bacteria were first exposed to a solution with a qBBr:bacteria ratio of about $47 \mu\text{mol}$ of qBBr/g of biomass. The qBBr concentrations in the exposure step for each bacterial species were adjusted to be approximately twice the concentration of sulfhydryl sites that was determined in the preliminary experiments in order to insure complete sulfhydryl site blocking. After reaction with qBBr, each cell suspension was centrifuged at 8100 g for 5 min and then rinsed three times with 0.1 M NaCl to remove the unreacted qBBr. Finally, the cell suspension was transferred into a pre-weighed test tube and centrifuged for two 30-minute intervals at 8100 g. After decanting the supernatant, the wet weight of cells was used to calculate the bacterial concentrations during the potentiometric titrations. The average conversion rates of wet weight to dry weight with 1σ uncertainties are as follows, *B. licheniformis*: 4.77 ± 0.05 , *B. subtilis*: 4.69 ± 0.23 , *B. cereus*: 4.50 ± 0.06 , *S. oneidensis*: 4.82 ± 0.00 , and *P. fluorescens*: 4.76 ± 0.02 . Because qBBr and A-CYS are dissolved molecules, separation could not be accomplished and the A-CYS titrations were conducted with qBBr present, and these controls were conducted with a 1:1 molal ratio of qBBr:A-CYS.

2.2. Potentiometric titration experiments

Potentiometric titrations were conducted using an autotitrator assembly and using HCl or NaOH titrants with pre-determined concentrations of approximately 1 M. To prepare the bacterial suspension for titration, a 0.1 M NaCl solution was degassed by bubbling N_2 through the solution for at least 1 h in order to remove dissolved CO_2 . Bacteria with or without qBBr treatment were suspended in the 0.1 M NaCl solution to achieve a homogeneous bacteria suspension with a concentration of approximately 50 g/L, and 6–7 mL of suspension was titrated for each titration. To test the potential reactions between carboxyl sites and qBBr, potentiometric titrations were conducted with 1 mM acetic acid in a 0.1 M NaCl solution with 0 or 3 mM qBBr present in solution. Similarly, solutions of 1 mM disodium hydrogen phosphate with either 0 or 2 mM qBBr were titrated in order to evaluate the potential reactions between phosphoryl sites and qBBr. We also conducted control titrations of 0.1 M NaCl with 0, 1, or 2 mM qBBr present in solution to evaluate the acid–base response of qBBr alone.

All the titrations were conducted under a headspace of N_2 gas to exclude atmospheric CO_2 and each suspension was stirred continuously with a magnetic stir bar. Each titration consisted of three steps: 1) acidifying the bacterial suspension to pH 3.0 by adding aliquots of

the HCl standard; 2) a forward titration from pH 3.0 to pH 9.7 by adding aliquots of the NaOH standard. These data were the ones used for calculating the total sulfhydryl site concentrations; and 3) a reverse titration by acidifying the suspension back to pH 3.0 to test the reversibility of the protonation reactions. The titrator was set to operate using a method in which set criteria were used by the titrator to control the equilibration time for each step of the titration and to determine the volume of acid or base added at each step. New titrant was added after the signal drift reached a minimum stability, or after a maximum waiting time was achieved, with these factors optimized in preliminary experiments. The optimized minimum stability and maximum waiting time were 0.3 mV/min and 40 s, respectively. Titrations conducted with lower minimum stability values or with longer maximum waiting times were not significantly different from those conducted under our optimized conditions. For all the bacterial species measured, the forward titration curves matched well with their corresponding reverse titrations, indicating that no significant damage occurred to the cells during the forward titrations, and thus the forward titration data were used for the modeling to represent the buffering capacity of the bacterial cell envelopes. To compare titration results from different experiments, the results were plotted in terms of a mass normalized net concentration of protons added to the system:

$$[\text{H}^+]_{\text{net added}} = (C_a - C_b) / (m_b) \quad (1)$$

where C_a and C_b are the total concentrations of acid and base added to the solution at each step, respectively, with units of mmol/L; and m_b (g/L) is the concentration of bacteria in suspension during the titration. Each set of titrations consisted of two titrations for untreated bacteria and another two titrations for qBBr-treated bacteria, and at least three sets of titration data from separately grown batches inoculated from separately cultivated plate cultures were collected for each bacterial species for a total of at least 6 titrations of untreated biomass and 6 titrations of qBBr-treated biomass.

2.3. Modeling approach

The proton-active functional groups on bacterial cell envelopes consist of a number of discrete monoprotic acids, whose deprotonation reactions can be modeled using the following reaction (Borrok et al., 2005b; Fein et al., 2005):



where R denotes the bacterial envelope macromolecule to which the i th organic acid functional group, A_i , is attached. The acidity constant of the i th site, $K_{a,i}$, can be expressed as:

$$K_{a,i} = \frac{[R-A_i^-]a_{H^+}}{[R-A_iH^0]} \quad (3)$$

where $[R-A_i^-]$ and $[R-A_iH^0]$ represent the concentrations of the deprotonated and neutral i th organic acid functional group on the bacterial cell envelope, respectively, and a_{H^+} is the activity of H^+ in bulk solution.

A range of surface complexation models (SCMs) including the non-electrostatic model (NEM), the constant capacitance model, the diffuse layer model and the Donnan shell model have been used to describe titration data and to yield total and individual cell envelope site concentrations and acidity constants in previous studies (Plette et al., 1995; Fein et al., 1997; Martinez et al., 2002; Ngwenya et al., 2003; Yee et al., 2004; Borrok and Fein, 2005; Fein et al., 2005). Although different correction approaches can be applied to the acidity constant calculations to account for bacterial surface electric field effects, the fits to the experimental titration data by the different models are similar (Borrok and Fein, 2005; Fein et al., 2005). Since the available data do not show a

consistent ionic strength influence on the protonation behavior (Daughney and Fein, 1998; Martinez et al., 2002; Fein et al., 2005), it is impossible to rigorously calibrate any model of the bacterial surface electric field, and we use a non-electrostatic model in this study for determining the number and concentrations of bacterial envelope

sites along with their acidity constants. In this study, FITEQL (Westall, 1982) was used as a modeling tool for optimization of the titration data, solving for the initial proton concentration in the system at the beginning of the titrations, $T_{H^+}^0$, following the approach described by Fein et al. (2005).

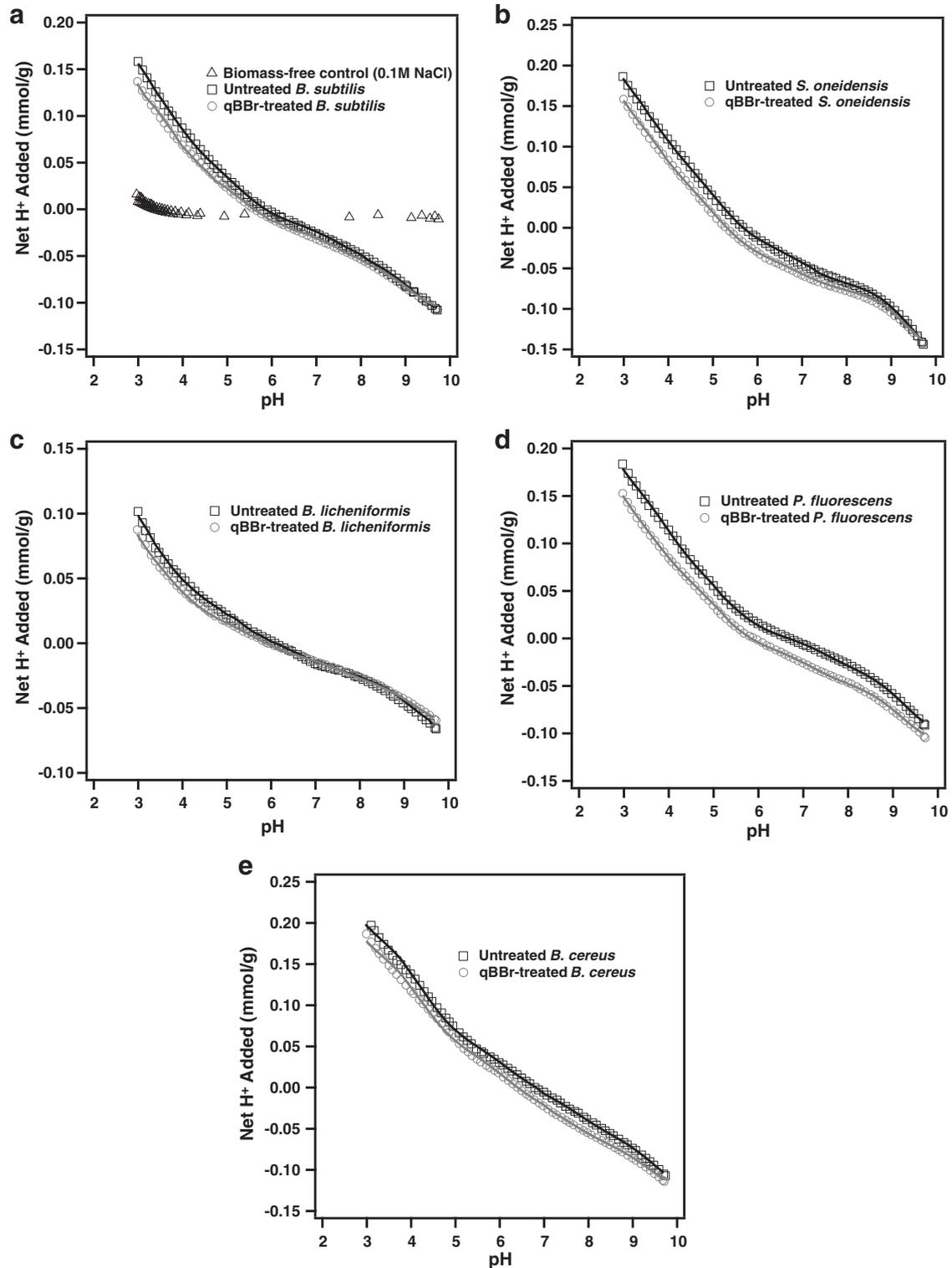


Fig. 2. Representative potentiometric titration curves of each species for 50 g/L of untreated and qBBR-treated bacterial suspensions: (a) *Bacillus subtilis* (the biomass-free control values were divided by 50 g/L to enable direct comparison with the mass normalized biomass data); (b) *Shewanella oneidensis*; (c) *Bacillus licheniformis*; (d) *Pseudomonas fluorescens*; (e) *Bacillus cereus*. The titration of each species with or without qBBR treatment was repeated for at least 6 times, and only one representative curve is shown here. Solid curves represent 4-site surface complexation modeling.

3. Results and discussions

3.1. Specificity of qBBr to bacterial sulfhydryl sites

The total concentration of sites that are accessible to protons over the pH range of these experiments is directly related to the integrated difference between the observed titration curve for the biomass sample and that of the biomass-free electrolyte control (Fig. 2a). For each bacterial species studied, the qBBr treated biomass exhibited a lower buffering capacity (Fig. 2), and hence a lower concentration of proton-active sites, than did the untreated biomass, demonstrating a decrease in the concentration of sites accessible to proton exchange after the biomass was exposed to qBBr. The qBBr molecule itself is inert to protonation reactions over the pH range investigated, as demonstrated by the similarity in titration curves between the electrolyte control (0.1 M NaCl solution) with 0, 1, or 2 mM qBBr present in solution (Fig. 3a). Therefore, we can conclude that the decrease in proton active sites that results from qBBr treatment of the biomass is due to qBBr blockage of certain sites and the inability of these blocked sites to respond to the acid or base added.

The observed decrease in buffering capacity that accompanies the qBBr treatment of the biomass is most likely due to qBBr blockage of cell envelope sulfhydryl sites. However, because the dominant sites on cell envelopes are carboxyl, phosphoryl and amino groups (Fein et al., 1997; Cox et al., 1999; Ngwenya et al., 2003; Jiang et al., 2004), they could also be responsible for the observed decrease in buffering capacity if any of those site types react with qBBr as well. Monobromobimane (mBBr), which is a neutral molecule with a similar structure to qBBr, can react slowly with amine, phosphoryl, carboxyl and other nucleophilic sites when the concentrations of those sites are sufficiently high in solution (Fahey and Newton, 1987). In this study, the reaction between qBBr and amine groups in our system can be excluded because: 1) it is unlikely that the positively charged qBBr molecule would interact with protonated and positively charged amine groups within the cell envelopes; 2) while a neutral amine is nucleophilic, it would lose its nucleophilicity after protonation (Soderberg, 2014). In addition, previous research shows that qBBr can selectively react with the thiol sites on a human leukocyte antigen (HLA) B27 molecule in the presence of a comparable concentration of positively charged amine groups (Whelan and Archer, 1993). Because previous studies widely used 10 mM of phosphate buffer to maintain solution pH for qBBr-thiol reactions and no significant interference between the qBBr and phosphoryl sites were detected (Kosower et al., 1979; Kosower and Kosower, 1987; Whelan and Archer, 1993), the reactions between qBBr and phosphoryl sites, which are present on bacterial cell envelopes at much lower concentrations than 10 mM, should also be negligible. The qBBr-treated A-CYS (Fig. 3b) displays a significantly lower buffering capacity than does the untreated A-CYS in the pH range close to the pK_a of the sulfhydryl site on A-CYS (~9.5), but no change was observed between the titrations of treated and untreated A-CYS in the pH range in which the carboxyl sites are proton active (~3.2), demonstrating that the presence of qBBr at a molality equal to that of A-CYS blocks virtually all proton activity of the sulfhydryl sites on A-CYS and does not react with carboxyl groups. Furthermore, our control experiments with acetic acid and with disodium hydrogen phosphate, which we use as analogs for carboxyl and phosphoryl sites within the cell envelope, respectively, indicate no significant change in buffering capacity of a 1 mM CH_3COOH or 1 mM Na_2HPO_4 solution with an excess of qBBr present (Fig. 3c). As a result, we conclude that blockage of carboxyl and phosphoryl sites by the qBBr does not occur and that all of the observed decrease in buffering capacity is due to blockage of sulfhydryl sites by the qBBr.

3.2. Model optimization for estimation of total site concentration

The potentiometric titration results indicate that each of the bacterial species studied contains proton-active sulfhydryl binding sites within

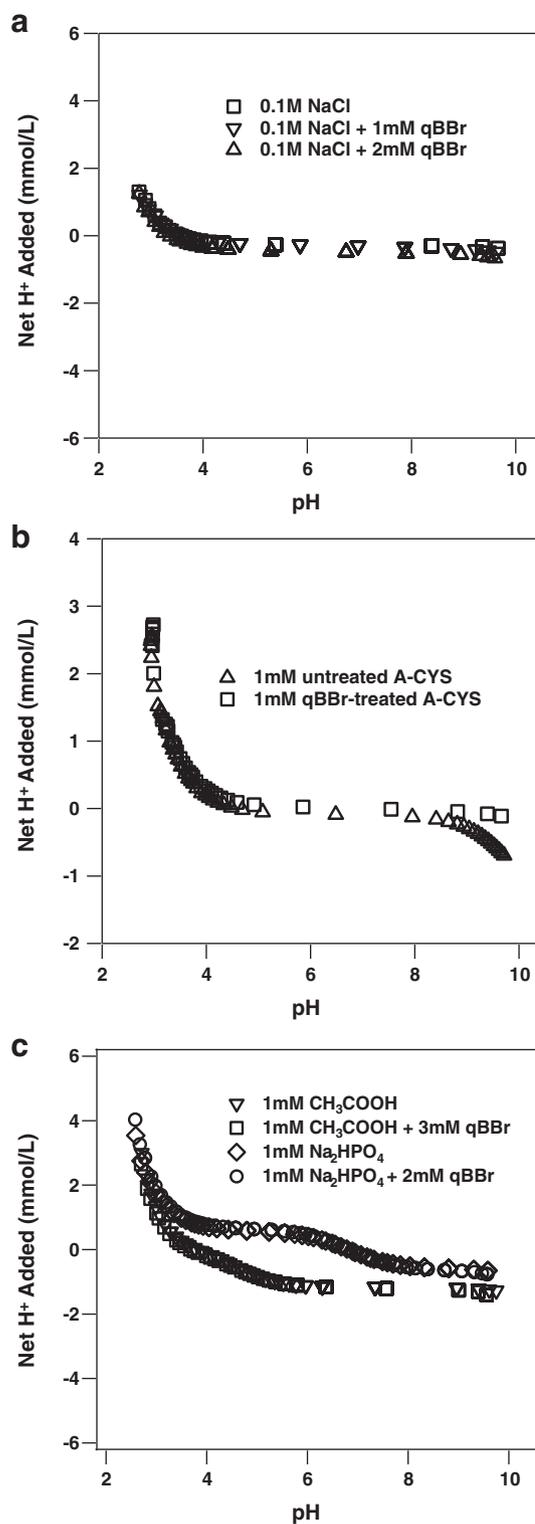


Fig. 3. Control experiments demonstrating that qBBr is not proton active over the pH range studied and that qBBr reacts with sulfhydryl sites but does not interact with carboxyl or phosphoryl sites: (a) 0.1 M NaCl with 0, 1, and 2 mM qBBr in solution; (b) 1 mM untreated and 1 mM qBBr-treated *N*-Acetyl-L-cysteine (A-CYS) in solution; and (c) 1 mM acetic acid with 0 and 3 mM qBBr in solution; 1 mM disodium hydrogen phosphate with 0 and 2 mM qBBr in solution.

their cell envelopes, and that the total sulfhydryl site concentrations on each species are small relative to the overall total site concentration of all of the sites together. We use a non-electrostatic surface complexation modeling approach to quantify the individual site concentrations and

acidity constants for biomass samples with and without qBBr treatment, and then ascribe the difference to the blockage of sulfhydryl sites. While modeling of the buffering capacity of bacterial cells yields specific site concentrations and acidity constants that are model-dependent (Fein et al., 2005), the determination of total site concentrations from these data is directly quantified by the titration measurements. Therefore, although the specific sulfhydryl site concentrations and their acidity constants calculated from our data are model-dependent, our approach yields precise and unequivocal determination of the total concentration of sulfhydryl sites on the bacteria that are proton-active over the pH range studied.

We use FITEQL 2.0 (Westall, 1982) modeling to determine the number of discrete functional group types that are required to account for the observed biomass buffering capacity, attempting to use one-, two-, three-, four-, and five-site models to fit the potentiometric titration data. In all cases, a five-site model does not converge, indicating that the system is under-constrained and that the data do not support a model with five discrete functional group types, while a four-site model matches the pH dependence of the buffering capacity significantly better than do models with fewer sites. This trend can be clearly seen in Fig. 4, which is a typical fitting result for one of *B. subtilis* titrations. FITEQL calculates a variance function, $V(Y)$, which quantifies the fit between the calculated and experimental data, and which decreases to an ideal value of 1 with increasing goodness of fit of the model to the data (Westall, 1982). For each titration in our study, the $V(Y)$ value improves significantly with each additional site considered in the model to a minimum for the four-site model, in good agreement with the visible difference between the fitting curves and experimental data. For example, the model fits to the *B. subtilis* biomass sample titration shown in Fig. 4 yield $V(Y)$ values for the one-, two-, three-, and four-site models of 781, 57, 7.2, and 1.3, respectively. This same modeling procedure was conducted for each of the titrations in this study, and both the $V(Y)$ values and the fitting curves suggest that a four-site model can best represent the titration data in each case.

The modeling results, including the four pK_a values and corresponding site concentrations for each site type are compiled in Table 1 for the set of untreated and qBBr-treated biomass samples for each bacterial species studied. The values shown in Table 1 represent the averages with 1σ uncertainties for all replicate experiments (>6) for each type of biomass sample tested. No significant difference was found for the average pK_a values of the four modeled sites on the five species studied, with the calculated values falling in the following four ranges: 3.7–4.1, 5.2–5.8, 7.0–7.7 and 9.2–9.4. Although the qBBr treatment decreased the total concentration of proton-active sites on the cell envelopes of

each species, the calculated pK_a distribution for the qBBr-treated biomass samples was not significantly different from the distribution of pK_a values calculated for the untreated biomass, probably because only a small proportion of sites were blocked in total. The total site concentrations of the untreated biomass samples vary markedly from one species to another, ranging from $167.7 \pm 5.8 \mu\text{mol/g}$ for *B. licheniformis* to $349.3 \pm 5.8 \mu\text{mol/g}$ for *S. oneidensis*. After qBBr exposure, the averages for total site concentrations of all the species significantly decreased and the Student's T test shows that the total site concentrations of qBBr treated bacteria are significantly different ($P < 0.05$, data not shown) from the untreated bacteria for all species, suggesting that the concentrations of sulfhydryls for the five bacteria studied here are detectable using the approach developed in this study.

3.3. Cell envelope sulfhydryl site concentrations

Among the five bacterial species in this study, *S. oneidensis* displays the highest sulfhydryl site concentration of $33.1 \pm 7.6 \mu\text{mol/g}$, and *B. cereus* has the lowest concentration of sulfhydryl sites with only $16.6 \pm 3.3 \mu\text{mol/g}$ (Fig. 5a). For most of the species, the determined sulfhydryl concentrations have relatively high experimental uncertainties that account for 20–35% of the average values, suggesting that the sulfhydryl concentrations of the same bacterial species vary from one batch to another. As a result, although there are differences between the measured concentrations of sulfhydryl sites between the bacterial species studied here, the difference is not large. Despite the structural differences between Gram-positive and Gram-negative bacteria, there is no clear distinction in sulfhydryl concentrations between them based on the results in Fig. 5a. Some researchers suggest that sulfhydryl groups are mainly present in certain proteins within cell walls (Norrod et al., 1993; Michelon et al., 2010; Mishra et al., 2010). If that is true, then our results indicate that these sulfhydryl-containing proteins not only are present within the outer membrane and periplasmic layer of the selected Gram-negative bacteria in this study (Herrmann et al., 2009), but also are present within the peptidoglycan layer of the selected Gram-positive bacteria in our study. The only thermophilic bacterial species in this study, *B. licheniformis*, did not exhibit a significantly different sulfhydryl site concentration than any of the four mesophilic bacterial species studied. In a previous study, Culha et al. (2008) identified sulfhydryl-related peaks in the Raman spectra of three thermophilic bacterial species, including *B. licheniformis*, but these peaks were not present in the spectra of two mesophilic bacteria, leading Culha et al. to conclude that thermophilic bacteria contain higher concentrations of thiol residues in their cell envelope structures than do mesophilic species. This result, coupled with our observations, may indicate that the bulk of the thiol residues within thermophilic bacteria are present mainly as structural units and not as proton-active binding sites within the cell envelope. However, our experiments show that the proportion of sulfhydryl sites to total sites within the cell envelopes of *B. licheniformis* is $14 \pm 3\%$, higher than that of the four mesophilic species studied which range from $5 \pm 1\%$ to $9 \pm 2\%$ (Fig. 5b). The higher proportion of sulfhydryl sites on *B. licheniformis* may explain the results of Culha et al. in that their Raman approach may be more sensitive to site proportion than to the absolute site concentration due to potential Raman signal interferences between sulfhydryl and other site types (Culha et al., 2008).

To the best of our knowledge, *B. subtilis* is the only species for which sulfhydryl site concentrations on cell envelopes have been reported in previous studies (Joe-Wong et al., 2012; Kenney et al., 2012). Kenney et al. (2012) estimated a sulfhydryl site concentration of $13.9 \pm 4.8 \mu\text{mol/g}$ on *B. subtilis* based on EXAFS data and the extent of Au desorption from bacteria due to exposure of the biomass to a cysteine wash solution. A number of assumptions were involved in this estimation, and hence their calculated concentration probably underestimated the true concentration of sulfhydryl sites. As we describe above, Joe-Wong et al. (2012) determined the sulfhydryl site concentration of

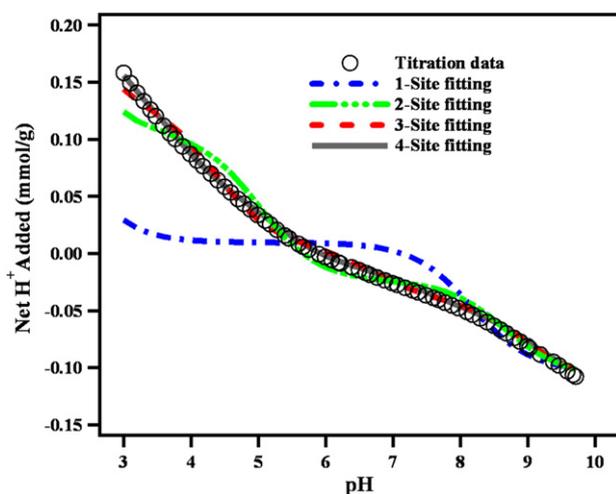


Fig. 4. Representative potentiometric titration data for a 50 g/L suspension of untreated *Bacillus subtilis*. Curves represent the best-fitting 1-, 2-, 3-, and 4-site non-electrostatic surface complexation models.

Table 1
Comparison of acidity constants and site concentrations for the five bacterial species studied, with and without qBBr treatment.

Bacteria		N ^a	pK _{a1}	C ₁ μmol/g	pK _{a2}	C ₂ μmol/g	pK _{a3}	C ₃ μmol/g	pK _{a4}	C ₄ μmol/g	C _{total} μmol/g
SO ^b	Untreated	10	3.9 ± 0.2	105.0 ± 9.5	5.2 ± 0.2	86.3 ± 15.1	7.0 ± 0.1	44.0 ± 2.9	9.4 ± 0.1	113.7 ± 8.7	349.3 ± 14.1
	Treated	9	3.9 ± 0.2	102.5 ± 9.1	5.3 ± 0.3	82.8 ± 16.6	7.1 ± 0.2	40.6 ± 4.1	9.4 ± 0.1	90.4 ± 8.8	316.2 ± 18.9
BS ^b	Untreated	7	3.9 ± 0.1	100.1 ± 7.6	5.6 ± 0.2	54.6 ± 8.0	7.7 ± 0.2	40.2 ± 2.9	9.2 ± 0.1	62.4 ± 4.0	257.3 ± 17.3
	Treated	7	3.8 ± 0.1	88.6 ± 6.2	5.6 ± 0.1	50.6 ± 5.4	7.6 ± 0.1	38.7 ± 2.9	9.2 ± 0.1	57.1 ± 2.2	234.9 ± 8.6
BL ^b	Untreated	10	3.7 ± 0.2	59.4 ± 5.0	5.5 ± 0.3	34.0 ± 4.9	7.5 ± 0.5	24.6 ± 5.1	9.4 ± 0.3	49.8 ± 11.0	167.7 ± 15.8
	Treated	9	3.8 ± 0.2	59.0 ± 1.7	5.7 ± 0.2	33.0 ± 4.6	7.7 ± 0.2	21.0 ± 1.6	9.4 ± 0.1	35.1 ± 1.3	144.1 ± 7.0
BC ^b	Untreated	10	4.0 ± 0.1	118.1 ± 8.1	5.7 ± 0.2	66.6 ± 6.5	7.5 ± 0.2	53.3 ± 3.6	9.3 ± 0.1	75.7 ± 2.2	313.7 ± 7.2
	Treated	7	4.1 ± 0.1	113.3 ± 4.8	5.8 ± 0.2	65.1 ± 3.5	7.5 ± 0.2	52.4 ± 5.9	9.3 ± 0.1	67.0 ± 2.8	297.1 ± 6.0
PF ^b	Untreated	10	4.0 ± 0.2	101.2 ± 7.5	5.6 ± 0.3	60.0 ± 6.1	7.7 ± 0.2	39.7 ± 3.6	9.4 ± 0.1	78.5 ± 3.6	279.5 ± 12.5
	Treated	6	3.8 ± 0.2	86.4 ± 9.3	5.4 ± 0.3	63.0 ± 7.6	7.4 ± 0.2	38.0 ± 3.0	9.2 ± 0.1	71.8 ± 2.7	259.2 ± 13.9

All the values shown represent the averages with 1σ.

^a N: number of trials.

^b BL: *Bacillus licheniformis*; BC: *Bacillus cereus*; SO: *Shewanella oneidensis*; BS: *Bacillus subtilis*; PF: *Pseudomonas fluorescens*.

B. subtilis to be 23.7 ± 2.1 μmol/g, a value which agrees well with the value of 22.5 ± 7.3 μmol/g that we obtained. The higher uncertainty associated with our value is partially attributable to the different measurement techniques, but may also reflect the real variation that occurs between growth cultures as our study used more separately grown batches inoculated from separately cultivated plate cultures than did that of Joe-Wong et al. (2012).

3.4. Estimation of sulfhydryl site pK_a values

The acidity constant is another critical parameter for modeling and predicting the adsorption behaviors of metals on sulfhydryl sites within bacterial cell envelopes. Potentiometric titrations of untreated bacterial

biomass alone cannot be used to unequivocally estimate the pK_a values of bacterial sulfhydryl sites. However, by tracking the concentration loss of each site type due to the qBBr treatment in our study, it becomes possible to probe the pK_a values of the major sulfhydryl sites within bacterial cell envelopes. The qBBr treatment approach enables us for the first time to determine the contribution of a particular site type to the buffering behavior ascribed to a particular acidity constant value.

Fig. 6 depicts the change in site concentration of the qBBr-treated biomass samples relative to the untreated samples for each pK_a value or site type. As we observed with the sulfhydryl site concentrations, the distribution of sulfhydryl pK_a values also varies significantly from one species to another. In general, the site concentrations for Site 2 and Site 3 (pK_a values 5.2–5.8 and 7.0–7.7) do not vary markedly between the qBBr-treated and untreated samples, indicating that either these sites are not sulfhydryl sites or the sulfhydryl concentrations with these pK_a values are too low compared with the uncertainties of Site 2 and Site 3 measured by our method. All of the bacterial species studied exhibited significant changes in concentration of Site 4 (pK_a 9.2–9.4), which is consistent with typical pK_a values for small thiol-bearing molecules such as cysteine, glutathione and homocysteine with pK_a values also in the range of 8.0–10.0 (Noguchi et al., 1981; Stark et al., 1989). One species, *S. oneidensis*, exhibited a significant change in Site 3 (pK_a ~7.0) concentration, and two species, *B. subtilis* and *P. fluorescens*, exhibited changes in Site 1 (pK_a 3.7–4.1) site concentrations. In fact, for these two species, Site 1 sulfhydryl site concentrations were in excess of the Site 4 sulfhydryl site concentrations.

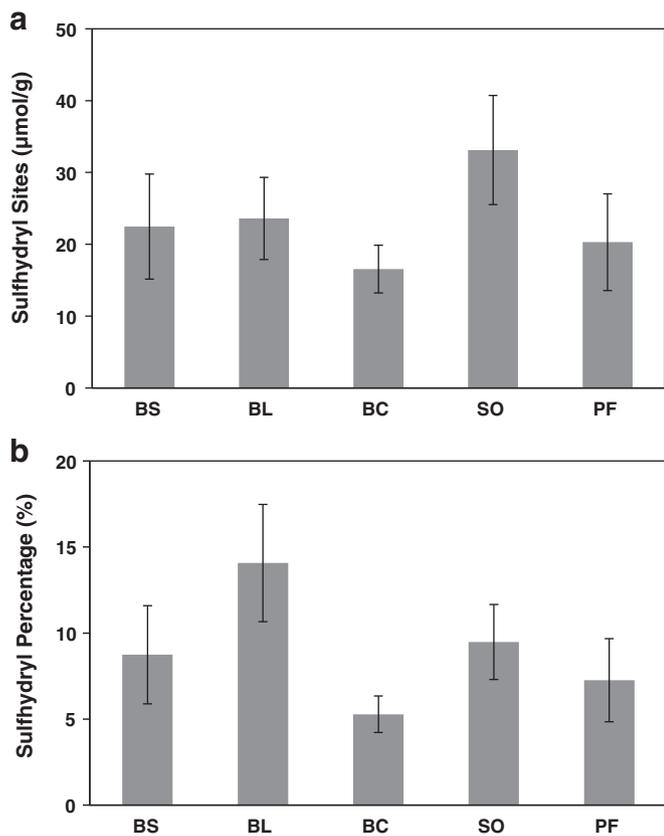


Fig. 5. (a) Sulfhydryl site concentrations and (b) percentage of sulfhydryl sites relative to total sites within bacterial cell envelopes for the five bacteria studied here (error bars represent 1σ values, BL: *Bacillus licheniformis*; BC: *Bacillus cereus*; SO: *Shewanella oneidensis*; BS: *Bacillus subtilis*; PF: *Pseudomonas fluorescens*).

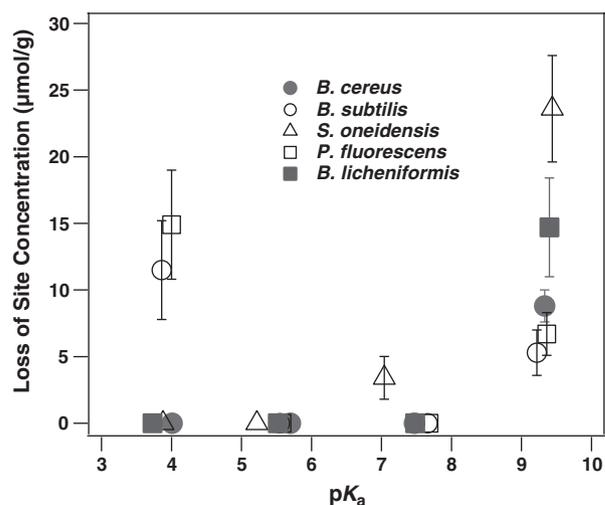


Fig. 6. Concentration loss for individual sites within the cell envelopes of the five bacterial species studied after qBBr treatment (error bars represent 1σ values).

Although cell envelope sulfhydryl sites often exhibit pK_a values similar to small thiol-bearing molecules, clearly a large number of exceptions were found and the pK_a of sulfhydryl sites must also depend on the micro-environment and neighboring groups of the site in question. For example, despite of the similar structure of cysteine and cysteinylglycine, the sulfhydryl sites of cysteinylglycine have a significantly lower pK_a value than that of cysteine (7.0 vs 8.9) (Noguchi et al., 1981). In another study, it was reported that positive charge on neighboring groups near sulfhydryls could stabilize the thiolates and thus lead to a shift of pK_a from 9.5 to 5.7 for sulfhydryl sites of cysteine-51 in methionine sulfoxide reductases (Antoine et al., 2006). Similarly, sulfhydryl sites with pK_a values lower than 5.8 were also documented by Whelan and Archer (1993), with the low value possibly due to interactions between sulfhydryl and protonated amino groups. Furthermore, significant contribution of Cu(I)-sulfhydryl binding was found by a recent study of EXAFS spectroscopy for Cu adsorption onto *Pseudomonas aureofaciens* cells under pH 2.2 (Pokrovsky et al., 2012), suggesting that sulfhydryl sites with very acidic pK_a values may be present within bacterial cell envelopes. We postulate that the low pK_a values of sulfhydryl sites within *B. subtilis* and *P. fluorescens* cell envelopes are similarly a result of the micro-environment of the sites. It is noteworthy to emphasize that these sulfhydryl sites with low pK_a values account for about 50% and 73% of the total sulfhydryl sites for *B. subtilis* and *P. fluorescens*, respectively. Since deprotonated sulfhydryl sites typically exhibit a higher capacity to bind with positively charged metals than the neutral state (Radkowsky and Kosower, 1986; Whelan and Archer, 1993), *B. subtilis* and *P. fluorescens* are expected to display higher reactivity to metals at neutral pH and low metal loadings compared to the other three bacteria studied here which exhibit a majority of their sulfhydryl sites with higher pK_a values of ~9.3.

3.5. Role of sulfhydryl sites in metal adsorption onto bacterial cells

Bacterial cell envelopes exhibit up to several hundred μmol of total sites per gram of wet biomass, as shown in Table 1. This study demonstrates that sulfhydryl sites for most of the bacterial species considered here account for only about 10% of the total sites within bacterial cell envelopes, and most of the sulfhydryl sites have pK_a values of 9.2–9.4. When metal concentrations greatly exceed sulfhydryl site concentrations, other sites such as carboxyl and phosphoryl will dominate metal binding onto bacteria. Under these conditions, metal adsorption exhibits strong pH dependence (Pagnanelli et al., 2000; Ngwenya et al., 2003) and is typically fully reversible (Fowle and Fein, 2000; Yee and Fein, 2001; Lo et al., 2003). However, when metal loadings on bacterial cells are much lower than the total site concentration, the adsorption of metal cations onto bacterial cells may be dominated by sulfhydryl binding and the adsorption behaviors could be quite different than is observed under higher loading conditions. Several studies (KrantzRulcker et al., 1996; Ledin et al., 1997) have reported a negligible pH dependence of metal adsorption onto bacterial cells for metal loadings of less than 1 μmol of metal/g of bacteria. Furthermore, Ledin et al. (1997) found that only 20% of Hg^{2+} desorbed from *Pseudomonas putida* when exposed to 5 mM of EDTA in desorption experiments after exposing the biomass to a metal loading of about 0.8–1.0 μmol of Hg/g of bacteria. Hu et al. (2013) adsorbed Hg onto *G. sulfurreducens* with an initial ratio of 40 nmol of Hg/g of bacteria, but did not observe significant Hg desorption after exposing the Hg-adsorbed biomass to 80 μmol of EDTA/g of bacteria. This extent of non-reversible sorption suggests strong bonds between the adsorbed Hg and the binding sites on the bacteria, and may be due to Hg-sulfhydryl binding as the Hg loading in these experiments was in the range where sulfhydryl binding can dominate. Recent EXAFS studies (Mishra et al., 2010, 2011; Song et al., 2012) document the dominance of sulfhydryl site binding of metals under conditions of low metal loading. For example, Mishra et al. (2010) found that Cd^{2+} exclusively adsorbs onto sulfhydryl sites of *S. oneidensis* at pH 5.9 when the initial Cd:biomass ratio is about

2.7 μmol of Cd/g of bacteria. In another study, Mishra et al. (2011) demonstrate that sulfhydryl sites dominate the adsorption of Hg^{2+} onto *B. subtilis* at pH 5 when the initial Hg:biomass ratio is 2.5 μmol Hg/g of bacteria. The sulfhydryl site concentrations within the cell envelopes determined in this study provide a quantitative basis to support these findings, as the sulfhydryl sites within cell envelopes of *S. oneidensis* and *B. subtilis* are approximately 20–30 μmol of sulfhydryl sites/g of bacteria (Fig. 5), much higher than the metal loadings used in the studies that are described above. In these cases, the high affinity sulfhydryl sites are about 10 times more abundant than the concentration of metal in solution. Even though carboxyl and phosphoryl sites are much more abundant than sulfhydryl sites within cell envelopes, the lower affinity of the carboxyl and phosphoryl sites for the metal allows sulfhydryl sites to dominate the budget of the adsorbed metal. Although EXAFS studies of metal adsorption under high loading conditions have identified carboxyl and phosphoryl sites as the dominant metal binding sites, metal binding onto sulfhydryl sites likely still occurs under high metal loading conditions (Mishra et al., 2010; Pokrovsky et al., 2012). Because of the low abundance of sulfhydryl sites relative to other sites within the cell envelope, the role of sulfhydryl binding in metal adsorption onto bacteria has been underestimated in most previous studies.

In summary, this study successfully developed a method to characterize the sulfhydryl sites within bacterial cell envelopes. Because of their importance in metal binding, especially for chalcophilic metals such as Hg, Zn, Cd and Cu under low metal loading conditions, the determined concentration and acidity constants for cell envelope sulfhydryl sites could improve our understanding and ability to predict the fate and transport of these metals in natural and engineered bacteria-bearing environments. It is noteworthy that the qBBR molecule is bigger than most metal cations, making it possible that some metal-accessible sulfhydryl sites are actually inaccessible to the qBBR molecule due to diffusion and/or steric effects. Probes of metal-sulfhydryl binding after exposure of the cells to qBBR would indicate the presence of these qBBR-inaccessible sulfhydryl sites. The approach described in this study of comparing potentiometric titrations of biomass that has had sulfhydryl sites blocked by treatment with qBBR to titrations of untreated biomass is the first to be capable of simultaneously determining the concentration and pK_a values of a specific site type within bacterial cell envelopes. Besides sulfhydryl sites, other site types could also be characterized using this approach if appropriate site-specific blocking molecules that are not proton-active over the pH range of interest can be identified.

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