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Article *in* Journal of Hazardous Materials · January 2020

DOI: 10.1016/j.jhazmat.2020.122209

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Role of bacterial cell surface sulfhydryl sites in cadmium detoxification by *Pseudomonas putida*

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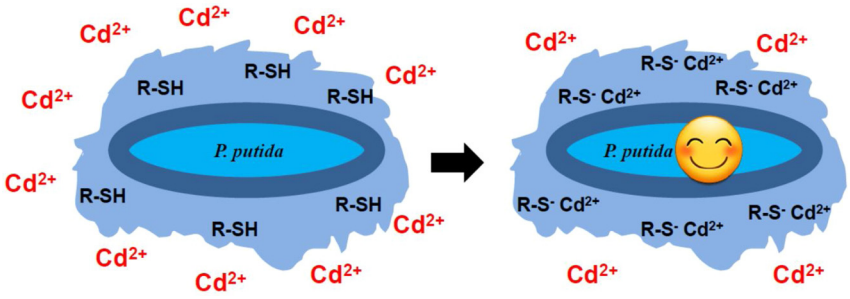
GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: G. Lyberatos

Keywords:
Sulfhydryl sites
P. putida
Toxicity
Cd

ABSTRACT

Understanding bacterial metal detoxification systems is crucial for determining the environmental impacts of metal pollution and for developing advanced bioremediation and water disinfection strategies. Here, we explore the role of cell surface sulfhydryl sites in bacterial detoxification of Cd, using *Pseudomonas putida* with surface sulfhydryl sites mostly on its EPS molecules as a model organism. Our results show that 5 and 20 ppm Cd in LB growth medium affects the lag phase of *P. putida*, but not the overall extent of cell growth at stationary phase, indicating that *P. putida* can detoxify Cd at these concentrations. EXAFS analysis of Cd bound to biomass from the different growth stages indicates that Cd binds to both sulfhydryl and non-sulfhydryl sites, but that the importance of Cd-sulfhydryl binding increases from early exponential to stationary phase. Cell growth is positively correlated to the measured sulfhydryl concentration on different biomass samples, but is independent of the measured non-sulfhydryl binding site concentration on the cell surfaces. Taken together, our results demonstrate that the sulfhydryl binding sites on EPS molecules can play an important role in binding and detoxifying toxic metals, significantly decreasing the bioavailability of the metal by sequestering it away from the bacterial cells.

1. Introduction

The presence of toxic metals in the environment, including both highly toxic metals (e.g., Hg and Cd) and excess essential metals (e.g., Zn and Cu), can pose a severe threat to ecosystems. Microorganisms, such as bacteria, can be sensitive to relatively low concentrations of toxic metals, and they have developed multiple defense strategies to protect cells from toxic metals, such as cell surface metal sequestration, metal efflux systems, intracellular metal sequestration, and metal redox transformation (Gadd and Griffiths, 1978; Nies, 1999; Chandrangsu et al., 2017; Shou et al., 2018). Understanding the metal detoxification systems of bacteria is crucial in order to determine the environmental

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https://doi.org/10.1016/j.jhazmat.2020.122209
Received 17 December 2019; Received in revised form 29 January 2020; Accepted 29 January 2020
Available online 30 January 2020
0304-3894/

impacts of metal pollution on ecosystems and to develop appropriate strategies for a variety of applications, such as bioremediation (Shamim, 2018; Liu et al., 2019), water disinfection (Li et al., 2008), antimicrobial design (Turner, 2017), and biosynthesis of nanomaterials (Wadhwani et al., 2016).

In most cases, metals must enter bacterial cells to cause toxic effects (Nies, 1999). Therefore, blocking toxic metals before they cross cell membranes, e.g., immobilizing metals within the cell envelope or on extracellular polymeric substances (EPS) (Gadd and Griffiths, 1978; Shou et al., 2018) becomes an effective approach for lowering the bioavailability of toxic metals. Bacterial cell envelopes and cell-bound EPS molecules (which together we refer to here as cell surfaces) can adsorb a wide range of metals due to the presence of abundant functional groups (metal binding sites) on molecules within cell surfaces, such as carboxyl, phosphoryl and sulfhydryl sites (Beveridge and Murray, 1976; Liu and Fang, 2002; Fein et al., 2019). However, the toxicity and bioavailability of the adsorbed metals on bacterial cell surfaces depends at least in part on the type and concentration of the bacterial surface complexes that form (Flynn et al., 2014; Sheng and Fein, 2014). These results suggest that the type and location of binding sites that interact with aqueous metals likely play an important role in regulating the toxicity of the adsorbed metals. To date, the role of specific metal binding sites (e.g., carboxyl, phosphoryl and sulfhydryl sites) in the detoxification of metals by bacteria has not been studied.

Compared to carboxyl and phosphoryl sites, sulfhydryl sites on bacterial cell surfaces are generally less abundant (Yu et al., 2014), but they form much stronger bonds with chalcophile metals such as Hg, Cd, Zn, Cu and Au (Yu and Fein, 2015; Nell and Fein, 2017; Yu and Fein, 2017b), resulting in the dominance of sulfhydryl sites in the adsorption of these metals onto bacterial cells under low metal loading conditions (Guine et al., 2006; Mishra et al., 2010; Pokrovsky et al., 2012; Yu and Fein, 2015). In addition, the concentration of sulfhydryl sites on bacterial cell surfaces can increase significantly as a function of growth conditions (Yu and Fein, 2017a), and hence sulfhydryl sites can contribute significantly to the adsorption of Cd, Hg and Au onto bacterial cells even under high metal loadings (Mishra et al., 2017; Yu and Fein, 2017b). Therefore, we focus our study on sulfhydryl sites, and hypothesize that they play a crucial role in sequestering toxic chalcophile metals on EPS molecules and away from the cell surface in order to detoxify the metals. *Pseudomonas putida*, a bacterial species that can be found in many toxic metal contaminated environments and exhibits excellent tolerance to chalcophile metals (Higham et al., 1986; Chen et al., 2006; Hu and Zhao, 2007), was used as a model organism in our experiments because under the growth conditions of our experiments it produces EPS molecules that contain abundant sulfhydryl binding sites with much lower sulfhydryl site concentrations on the cell walls. Therefore, *P. putida* is a prime candidate to exhibit EPS-dominated sulfhydryl binding of metals and hence for use as a probe of the bacterial strategy of sequestering toxic metals through EPS binding. Our results indicate that Cd adsorption onto EPS sulfhydryl sites represents a strategy adopted by *P. putida* and perhaps other similar bacterial species for binding and detoxifying Cd, and we demonstrate that the bioavailability, and hence the toxicity of Cd is inversely related to the concentration of sulfhydryl sites within the cell-produced EPS molecules.

2. Materials and methods

2.1. Cd toxicity tests

Two sets of toxicity tests were conducted using *Pseudomonas putida* ATCC#: 33015 as the model organism, and aqueous Cd as the toxic metal. The first set of experiments was used to test the responses and detoxification ability of *P. putida* cells to low concentrations of toxic Cd. Bacteria were first cultured aerobically in 1 mL of Cd-free Lysogeny Broth (LB10) medium at 32 °C for 24 h, and were then transferred to 50 mL of LB10 medium containing 0, 5, or 20 ppm Cd, and allowed to grow at the same temperature for 72 h. The LB10 medium consists of 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, and the Cd concentrations of the growth media were attained by adding appropriate volumes of a 2 g/L Cd stock solution which was prepared by dissolving $Cd(NO_3)_2$ in ultrapure water, and which was then sterilized by passing it through a 0.2 µm nylon filter membrane. Because of the high concentration of Cl^- in the medium, the Cd in the LB10 medium is present primarily as relatively non-toxic aqueous Cd-chloride complexes (Deheyn et al., 2004; Yu and Fein, 2015), and only a small percentage of the Cd is present in the toxic form as free Cd^{2+} (Sunda et al., 1978). The optical density of the cell suspensions at 600 nm (OD_{600}) was measured at different time intervals on a Cary 300 UV–vis spectrophotometer and the OD_{600} value of the LB10 medium was used as a background value to calculate the increase in OD_{600} caused by the growth of *P. putida*. Abiotic control experiments using LB10 medium only were also conducted under the same conditions in order to monitor if contamination occurs, and the measured OD_{600} values in these controls did not change within 72 h.

The second set of experiments was conducted to test the effects of cell surface sulfhydryl sites on Cd toxicity towards *P. putida* cells. In order to yield biomass with different concentrations of cell surface sulfhydryl sites, we used starter cells that were pre-cultured in Cd-free LB10 medium for 5, 6, 12, 24, or 72 h. The cell surface sulfhydryl site concentration for each biomass sample was quantified using a potentiometric titration approach as described in Section 2.3. In these toxicity experiments, we used a modified Lysogeny Broth medium (LB0.5) that consisted of a similar formula to the LB10 medium except the NaCl content was reduced to 0.5 g/L in order to decrease the prevalence of relatively non-toxic Cd-chloride aqueous complexes in the experimental solutions and to increase the importance of Cd^{2+}, which is toxic to the bacteria. The experiments involved the addition of 0, 2, 5, 10, or 20 ppm Cd to the LB0.5 medium. While the presence of tryptone and yeast extract makes the calculation of the Cd speciation in LB media difficult, we calculated the speciation of Cd in 10 g/L and 0.5 g/L NaCl solutions using FITEQL 2.0 (Westall, 1982). At any Cd concentration used in this study (2, 5, 10 or 20 ppm), we found that free Cd^{2+} accounts for 11 % of the total aqueous Cd in 10 g/L NaCl and 64 % of the total aqueous Cd in 0.5 g/L NaCl. These calculations suggest that the reduction of the NaCl concentration from 10 g/L to 0.5 g/L significantly increases the concentration of free Cd^{2+} in the LB medium. In order to compare the toxicity response of the cells with and without sulfhydryl sites blocked for Cd binding, additional experiments (referred to below as' 24 h-Q' experiments) were conducted in the presence of 20 ppm monobromo(trimethylammonio)-bimane bromide (qBBr; from Toronto Research Chemical) and 0 or 2 ppm Cd using starter cells that were pre-cultured for 24 h in LB10 medium. qBBr selectively and irreversibly reacts with sulfhydryl sites on bacterial cell surfaces, thus blocking them for Cd binding (Yu et al., 2014; Yu and Fein, 2015, 2017b). Besides these changes, the conditions for the second set of experiments were the same as those used in the first set of toxicity experiments previously described. Each of these toxicity experiments was conducted in triplicate and the Student's *t*-test was applied to the results to test for statistical significance.

2.2. Extended X-ray absorption fine structure (EXAFS) measurements

We used EXAFS measurements of the binding environment of Cd as a function of growth stage in order to determine whether a relationship exists between the binding environment and the measured toxicity response. Cells for the EXAFS experiments were grown in LB10 medium in the presence of 20 ppm Cd, and were harvested at early exponential, early stationary and stationary phases by centrifugation at 10,970 $\times g$ for 5 min. In order to avoid Cd desorption from the biomass, EXAFS measurements were conducted without cell washing. Cd K edge (26,711 eV) EXAFS was measured in fluorescence mode using the third

harmonic of the undulator at sector 10-ID beamline of the Advanced Photon Source at Argonne National Laboratory (Segre et al., 2000). The energy of the incident X-rays was scanned using a Si(111) reflection plane of a cryogenically-cooled double-crystal monochromator. A Pt-coated mirror was used to remove X-rays of higher harmonic energies. The incident ionization chamber was filled with 100 % N_2 gas. The transmitted and reference ion chambers were filled with 100 % Ar gas. The fluorescence detector in the Stern–Heald geometry (Stern and Heald, 1983) was filled with Kr gas, and a Pd filter of six absorption lengths was used to reduce the background signal. Bacterial pellets were loaded into a slotted Plexiglas holder, covered with Kapton film, and transported immediately to the beamline for EXAFS measurements. All of the EXAFS measurements were performed within a day of the sample preparation, and the samples were refrigerated prior to the EXAFS measurements. Quick scans were used with signal sampling every 0.5 eV and with an integration time of 0.1 s per point.

The X-ray absorption near edge structure (XANES) for each sample was monitored for possible beam induced chemistry (and none was found), and the X-ray beam was moved to a fresh spot every 5 scans in order to further reduce the possibility of radiation-induced changes and to account for sample inhomogeneity. A total of 30–50 consecutive scans from each sample were collected and averaged, with Cd foil data collected simultaneously in the reference chamber. Data were analyzed using the UWXAFS package (Stern et al., 1995). Processing of the raw data, including alignment of datasets and background removal, was done using ATHENA (Ravel and Newville, 2005). The input parameter to ATHENA that determines the maximum frequency of the background, R_{bkg}, was set to 1.1 Å (Newville et al., 1993). The data range used for Fourier transforming the EXAFS data was 3.0–9.8 $Å^{-1}$ with a Hanning window function and a dk value of 1.0 $Å^{-1}$ (Newville et al., 1993). Simultaneous fitting of each of the three datasets with multiple k-weighting (k^1, k^2, k^3) of each spectrum was performed using the Fourier transformed spectra. The fitting range for all of the datasets was 1.2–2.8 Å. The simultaneous fitting approach reduces the possibility of obtaining erroneous parameters due to correlations at any single k-weighting (Mishra et al., 2010).

The EXAFS spectra from Cd-acetate, Cd-phosphate, and Cd-sulfide standards were used for qualitative comparison of the unknown samples, and these same spectra were also used to refine fitting parameters for the quantitative analysis of the Cd binding environment in the biomass samples. The best fit values for the O and S signal contributions to the EXAFS spectra from the standards were used as the initial guess parameters for simultaneous fitting of the three biomass samples. The relative contributions of Cd-S and Cd-O binding to the total Cd binding environment within the first shell of the biomass samples were determined by floating the O coordination number and constraining the S coordination number such that the total contribution of the two sites would sum to be 100 %. Because O coordinates octahedrally around a Cd atom, and S coordinates tetrahedrally, it was assumed that full coordination of the O-bearing and S-bearing sites was 6 O and 4 S atoms, respectively. In this analysis, we do not differentiate between carboxyl and phosphoryl binding sites because the Cd-O bond distances in the Cd-acetate and Cd-phosphate standards are the same (Mishra et al., 2010). An attempt to performed EXAFS fits using contribution from Cd-phosphate, Cd-acetate, and Cd-sulfide signals resulted in statistically inferior fits suggesting that inclusion of Cd-phosphate in addition to Cd-acetate and Cd-sulfide is not justified for a statistically meaningful fitting of biomass data. Therefore, we did not include the Cd-phosphate standard in our modeling.

2.3. Determination of sulfhydryl sites on bacterial cell surfaces

The preparation of the biomass samples that were used for sulfhydryl site measurements was as follows: after incubation in Cd-free LB10 medium at 32 °C for different periods of time (5, 6, 12, 24, or 72 h), the biomass was harvested by centrifugation at 10,970 ×*g* for 5 min. The biomass pellets were then washed three times with a 0.1 M NaCl solution, with centrifugation at 8,100 ×*g* for 5 min after each wash. The biomass pellets were then transferred into pre-weighed test tubes and centrifuged for two 30-minute intervals at 8100 ×*g*. After decanting the supernatant, the wet weight of the biomass was used to calculate the bacterial concentrations in the subsequent experiments, and the bacterial concentrations that are reported in this study are these wet weights.

Some experiments involved bacterial cells with EPS materials removed. In order to remove EPS from biomass samples, the freshly harvested and washed cell pellets were immediately re-suspended in 0.1 M NaCl with a cation exchange resin (Dowex® Marathon C sodium form, 20–50 mesh, 30 g of resin/g of biomass in wet weight) and allowed to react for 2 h at room temperature (~20 °C) with slow stirring in order to maintain homogeneous suspensions. The treated cells were then washed using the same procedure as described above. The biomass that is produced from this resin treatment procedure is virtually free of EPS materials, as previous studies have demonstrated with electron microscopy (Yu and Fein, 2016).

The approach used to determine the sulfhydryl site concentrations on bacterial cell surfaces was the same procedure that we developed and described in previous studies (Yu et al., 2014; Yu and Fein, 2017a). We used potentiometric titrations and surface complexation modeling to determine the total site concentrations within biomass samples. The concentration of sulfhydryl sites was determined by measuring the decrease in the total concentration of all binding sites after the sulfhydryl sites were selectively blocked using monobromo(trimethylammonio)-bimane bromide (qBBr), a molecule that itself does not protonate or deprotonate. In order to block sulfhydryl sites, cells were suspended for 2 h in a freshly prepared qBBr solution in 0.1 M NaCl with pH buffered to 7.0 ± 0.1 using a 1.8 mM Na_2HPO_4/18.2 mM NaH_2PO_4 buffer, with a qBBr:biomass ratio of approximately 200 µmol/g, followed by three biomass washes with a 0.1 M NaCl electrolyte solution. Potentiometric titrations of cells with and without qBBr treatment were conducted using an autotitrator assembly with ~10 g of a 0.1 M NaCl cell suspension containing 30 g (wet mass) of cell per liter. The cell suspensions were first adjusted to pH 3.0 using 1 M HCl, followed by a titration from pH 3.0–9.7 using 1 M NaOH. The 'up pH' titration was used for calculating the total bindings sites on each sample using a four-site non-electrostatic surface complexation model and FITEQL 2.0. All titrations were conducted in triplicate.

3. Results

The presence of 5 or 20 ppm Cd in LB10 medium caused strong negative effects on the growth of *P. putida* within the first few hours of the experiment, but did not affect the extent of overall growth in the long term (Fig. 1). When the Cd concentration in the growth medium increased from 0 to 20 ppm, the lag phase of *P. putida* extended markedly from about 5 h–12 h. However, in each case, once the lag phase was complete, cells in each of the three experiments multiplied rapidly. After 24 h, similar OD_{600} values were observed for cell suspensions in the presence of 0, 5 and 20 ppm Cd, indicating that *P. putida* cells completely detoxified the added Cd in these experiments. In LB10 medium, most of the Cd is present as aqueous Cd-chloride complexes (e.g., $CdCl^+$, $CdCl_2^\circ$), and only < 11 % of the Cd is present as relatively toxic Cd^{2+}. Therefore, our results show that *P. putida* cells are sensitive to very low concentrations of toxic Cd species, and that the cells can detoxify at least low concentrations of Cd^{2+}.

The EXAFS analysis of the *P. putida* biomass samples that grew in the presence of 20 ppm Cd in LB10 medium indicates that a significant amount of Cd adsorbed onto the biomass, and that the adsorbed Cd was partitioned between complexation with sulfur- and oxygen-bearing binding sites (Fig. 2). The Cd-sulfide spectrum exhibits a first shell (Cd-S) peak that is shifted to a significantly larger distance and amplitude relative to those associated with the first shell (Cd-O) peak from the

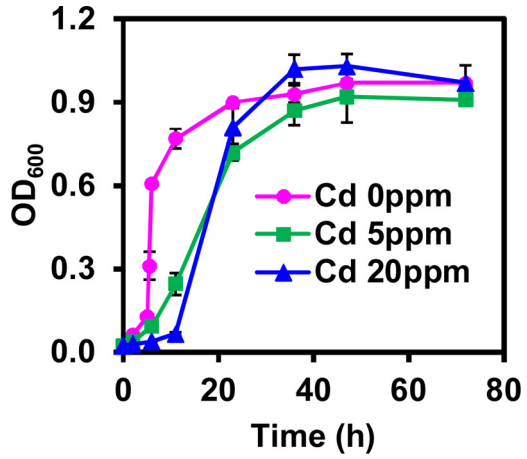

Fig. 1. Growth curves of *P. putida* in LB10 media containing 0, 5, or 20 ppm Cd.

spectrum for the Cd-acetate standard (Fig. 2a). In addition, the amplitude of the peak at 2.2 Å is larger in the real part of the Cd-sulfide EXAFS spectrum than it is in the Cd-acetate spectrum (Fig. 2b). These spectral features can be used to qualitatively determine the relative contributions of Cd-O and Cd-S bonds to the total adsorbed Cd on the biomass (Mishra et al., 2010).

The EXAFS spectra of the three biomass samples indicate a significant increase in the importance of Cd-S binding as the growth phase progresses from early exponential to early stationary phase, followed by a small decrease in the importance of Cd-S binding from early stationary to stationary phase (Fig. 2). Quantitative modeling of the Cd EXAFS data with both Cd-O and Cd-S paths yields excellent fits to the data (Figs. S1 and S2, Tables 1 and S1). For the early exponential phase sample, O-bearing (non-sulfhydryl) binding sites dominate Cd adsorption onto the biomass, and only 18 % of the adsorbed Cd on the biomass is bound to S-bearing (sulfhydryl) binding sites (Table 1). In contrast, the calculated contribution of the S-bearing binding sites increases markedly to 51 % in the early stationary phase sample (Fig. S2 and Table 1), and then decreases slightly with extended growth time to 40 % in the stationary phase sample.

We used potentiometric titration experiments to measure the concentrations of sulfhydryl sites on cell surfaces of *P. putida* biomass that were cultured in Cd-free LB10 media, and were sampled at different points along its growth curve. The abundance of sulfhydryl sites is strongly affected by growth phase (Fig. 3 and Table S2). From early exponential phase (5 h) to early stationary phase (24 h), the measured concentration of sulfhydryl sites on the cell surfaces increases dramatically from 4 ± 12–118 ± 22 µmol/g, likely explaining the increase in Cd-sulfhydryl binding on biomass samples from exponential phase to stationary phase that was documented by our EXAFS measurements

Table 1
Relative contribution of Cd binding from O- and S-bearing sites on *P. putida* biomass samples that grew in the presence of 20 ppm Cd.

Growth Phase	Cd-O (%)	Cd-S (%)
Early Exponential	82 ± 5	18 ± 5
Early Stationary	49 ± 9	51 ± 8
Stationary	60 ± 7	40 ± 7

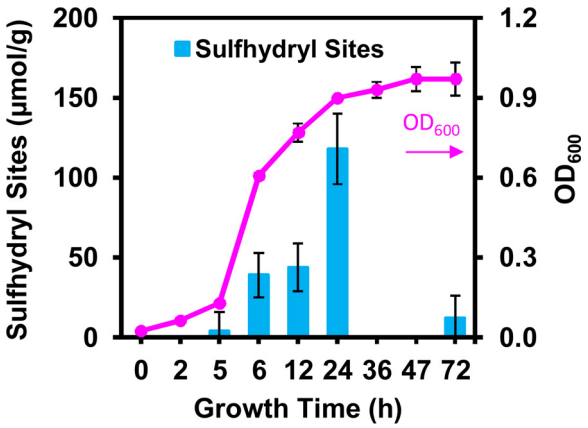

Fig. 3. Potentiometric titration measurement results of the concentrations of cell surface sulfhydryl sites on *P. putida* biomass at different growth stages that were cultured in Cd-free LB10 medium. Note the non-linear scale to the 'Growth Time' axis.

(Fig. 2 and Table 1). In contrast, the measured sulfhydryl site concentration for late stationary phase (72 h) cells is only 12 ± 14 µmol/g. However, it should be noted that the measured total concentrations of sulfhydryl sites from the potentiometric titration experiments are for samples grown in Cd-free media, and that the presence of Cd could potentially induce the synthesis of additional sulfhydryl sites by bacterial cells. Therefore a direct linkage of the measured sulfhydryl site concentrations to the relative importance of Cd-sulfhydryl binding at each growth phase as determined by EXAFS is not possible.

The above measurements were all conducted with intact biomass samples, which included both cells and bound EPS material. In order to identify the location of the sulfhydryl sites between the cell walls and cell-produced EPS material for the 24 h-biomass, we removed the EPS material through a cation exchange resin pre-treatment (Yu and Fein, 2016), and measured the surface sulfhydryl sites on the biomass without EPS. After the EPS was removed, the measured concentrations of sulfhydryl sites on the cell surfaces dropped dramatically from 118 ± 22 to 4 ± 18 µmol/g (Table S2). This result, similar to the findings in previous potentiometric titration and proteomic analyses for

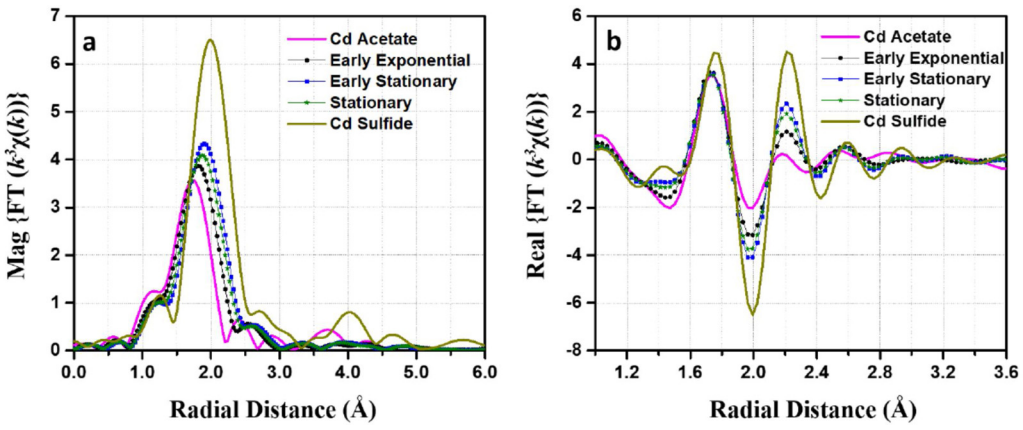

Fig. 2. (a) Magnitude and (b) real part of the Fourier transform of the measured Cd K-edge EXAFS spectra of three biomass samples compared to Cd-acetate and Cd-sulfide standards. The biomass samples were grown in LB10 medium in the presence of 20 ppm Cd and harvested at early exponential, early stationary and stationary phase.

P. putida (Yu and Fein, 2016; Fein et al., 2019), indicate that the sulfhydryl sites on cell surfaces of *P. putida* under our growth conditions are located mainly on the EPS molecules that are tightly bound to cells. Bacterial cells synthesize EPS primarily from mid-exponential phase to early stationary phase (Petry et al., 2000; Lbarburu et al., 2007). Therefore, the extremely low concentrations of sulfhydryl sites on the surfaces of the early exponential phase (5 h) cells likely arise because the cells did not yet synthesize EPS molecules at this growth stage. Although abundant sulfhydryl sites were detected on the 24 h biomass, the concentration of sulfhydryl sites on the 72 h biomass decreased dramatically (Fig. 3). We interpret these results to indicate that sulfhydryl-bearing EPS molecules likely detach from the cell surfaces upon the death and degradation of the 24 h-cells, and that newly synthesized cells which contain much lower concentrations of bound sulfhydryl-rich EPS molecules become the dominant cells at late stationary phase (72 h), resulting in the low sulfhydryl site concentration of the 72 h-cells sample.

The second set of toxicity experiments used an LB0.5 medium containing only 0.5 g/L NaCl, and starter biomass that was extracted from growth in an LB10 medium at different stages along the growth curve. Hence, each starter biomass contained different concentrations of cell surface sulfhydryl sites (Fig. 3). Because starter cells with different pre-culturing times exhibit different lag phases and growth rates when placed in the Cd-free LB0.5 medium, in order to compare the effects of Cd toxicity on each type of biomass, we report cell growth in terms of growth factor values that are calculated as the OD_{600} of a cell suspension in the presence of Cd divided by the OD_{600} in corresponding Cd-free controls. A growth factor of 1 indicates that the added Cd has no effect on the growth of bacterial cells, and lower growth factor values indicate stronger Cd toxicity.

In contrast to the minor influence of 20 ppm Cd on the growth of *P. putida* in the LB10 medium (Fig. 1), the presence of 2 – 20 ppm Cd strongly inhibits the growth of *P. putida* in the LB0.5 medium due to the dramatically increased concentration of free Cd^{2+}, with growth factors in most experiments smaller than 0.6 (Fig. S3). The pre-culturing time of the starter cells exerts a strong influence on the toxicity of Cd toward the cells, and the influence decreases with duration of the toxicity experiment. For example, at 2 h in the toxicity experiments in the presence of 2 ppm Cd, the growth factors in the different experiments vary markedly from 0.3 to 0.9, but these differences become negligible at 24 h (Fig. S3). Similar trends are also observed in experiments in the presence of elevated Cd concentrations (Fig. S3). The decreasing difference in growth factors for the different starter cells with increasing time likely occurs because the proportion of newly produced *P. putida* cells keeps increasing with time in each experiment, and the properties of these newly created cells are independent of the pre-culturing time of the starter cells. That is, as the toxicity experiment proceeds, the starter cells become a smaller proportion of the total number of cells in each experiment, and hence the differences in growth disappear with increasing time. Therefore, because the objective of this set of experiments is to study the influence of the surface sulfhydryl site concentration on Cd toxicity to *P. putida*, we focus on the growth factors of the *P. putida* cells at 2 h because at that time the experimental systems have the largest proportion of starter cells present and hence best represent the toxic response of the starter cells (with different sulfhydryl site concentrations) to Cd.

Among the different batches of starter cells, the 24 h-cells (the cells that were pre-cultured for 24 h in LB10 medium), which have the highest concentration of cell surface sulfhydryl sites (Fig. S3), always showed the lowest toxicity responses to Cd, with 2 h growth factors significantly higher than other experiments at any studied Cd concentration (Fig. S3). In contrast, the 5h-cells that contain the lowest concentration of sulfhydryl sites exhibited the smallest 2 h growth factors (Fig. S3), with their growth completely inhibited at 2 h in the presence of 5, 10 or 20 ppm Cd (2 h growth factors = 0). Based on the Student's *t*-test results for the 2 h growth factors of different starter

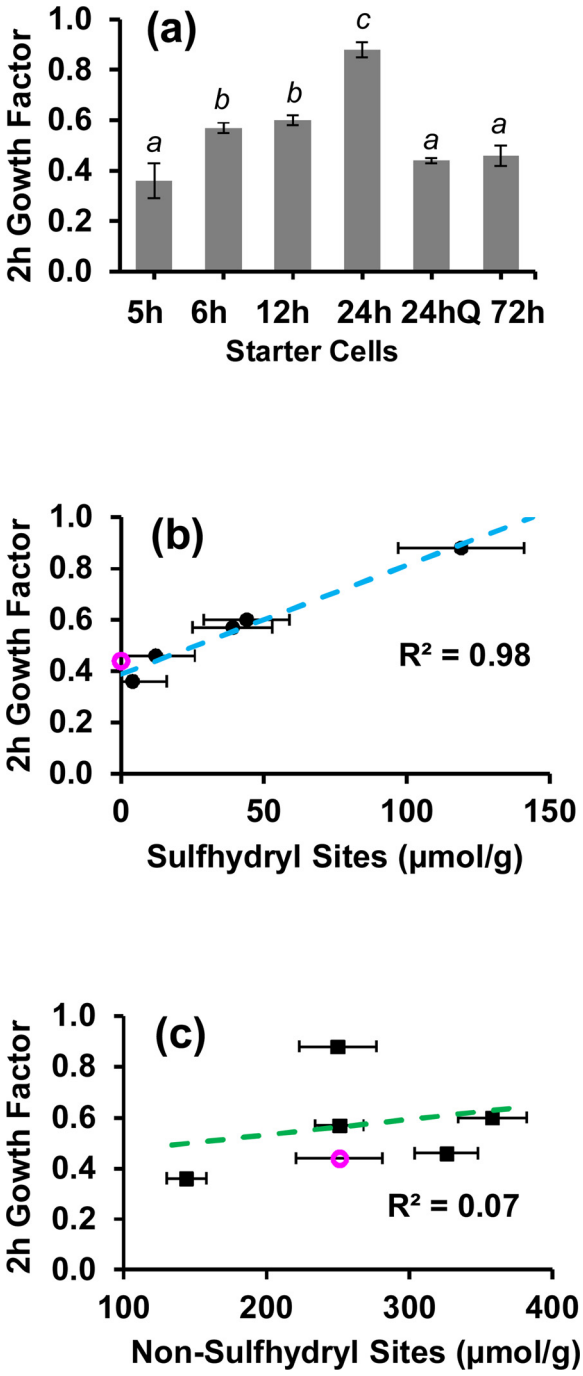

Fig. 4. Toxicity test results in LB0.5 medium that contains 2 ppm Cd using *P. putida* starter cells that were pre-cultured in LB10 medium for 5 h, 6 h, 12 h, 24 h or 72 h, reported in terms of the 2 h growth factor, which is calculated by normalizing the OD_{600} of a cell suspension at 2 h in a Cd-bearing experiment by the OD_{600} in the Cd-free control at 2 h. The 24 h-Q experiment was conducted using the 24 h-cells as starter cells in the presence of qBBr that blocks cell surface sulfhydryl sites to Cd binding. (a) The 2 h growth factor values using starter cells from different extraction times from the parent growth medium; (b) Relationship between measured 2 h growth factor values and measured cell surface sulfhydryl site concentrations on the starter biomass; (c) Relationship between measured 2 h growth factor values and measured concentrations of the non-sulfhydryl sites on the starter biomass. The letters on top of each column in (a) represent the Student's *t*-test results: $p < 0.05$ for any two samples with different letters and $p > 0.05$ for any two samples with the same letters. The pink hollow circles in (b) and (c) represent the results of the 24 hQ experiment.

cells, the starter cells can be divided into three groups, with 2 h growth factors for the 5h- and 72 h-cells lower than the growth factors for the 6h- and 12 h-cells, which in turn are lower than the growth factors of the 24 h-cells (Fig. 4a). Here the 2 h growth factors of any two starter cells from different groups are significantly different ($p < 0.05$), and those from the same group show no statistical difference ($p > 0.05$). For the 24 h-cells, we also conducted a comparison experiment in the presence of qBBr, a molecule that selectively and irreversibly blocks cell surface and EPS sulfhydryl sites to interactions with Cd in the experiment. In this experiment (24 hQ), with sulfhydryl binding sites blocked, the toxicity of 2 ppm Cd to the cells was significantly higher than was observed in the qBBr-free experiments, with the 2 h growth factor dropping from 0.88 ± 0.03 for the qBBr-free experiment to 0.44 ± 0.01 for the qBBr experiment ($p < 0.05$, Fig. 4a).

By plotting the sulfhydryl site concentrations of the starter cells with their 2 h growth factors in the presence of 2 ppm Cd, we find a strong positive correlation between the growth factors and the sulfhydryl site concentrations of the starter cells (Fig. 4b, $R^2 = 0.98$). It is noteworthy that the growth factor in the 24 hQ qBBr experiment with the sulfhydryl sites on the 24 h-cells blocked matches well with the predicted growth factor for starter cells with no cell surface sulfhydryl sites (pink circle in Fig. 4b). In contrast, the 2 h growth factors exhibit a poor correlation to the concentrations of the non-sulfhydryl sites on the biomass samples, which include all of the other binding sites on the cell surfaces (Fig. 4c, $R^2 = 0.07$). The observed correlation is not limited to the 2 ppm Cd dataset, as the measured 2 h growth factors in the 5, 10, and 20 ppm Cd experiments all exhibit strong correlations with cell surface sulfhydryl site concentrations (Fig. S4). The goodness of the correlations decreases in general with increasing Cd concentrations, likely because for the experiments with higher total Cd concentrations, the concentration of adsorbed Cd exceeds the concentration of sulfhydryl binding sites, and Cd in these systems also binds to some extent to non-sulfhydryl sites under these elevated metal loadings (Mishra et al., 2010; Yu and Fein, 2015; Nell and Fein, 2017). This result is consistent with sulfhydryl sites playing a crucial role in diminishing Cd toxicity, and for the high Cd systems in which significant non-sulfhydryl binding occurs, Cd toxicity increases and the relationship between sulfhydryl site concentration and Cd toxicity breaks down to some extent.

4. Discussion

An important finding of this study is that the *P. putida* cells cultured in the LB10 medium contain relatively high concentrations of sulfhydryl sites on their cell surfaces compared to sulfhydryl site concentrations that have been measured for other bacterial species (Joe-Wong et al., 2012; Yu et al., 2014; Yu and Fein, 2016, 2017a). For example, the measured concentration of surface sulfhydryl sites on the *P. putida* cells that were cultured in LB10 medium to early stationary phase (the 24 h sample in Fig. 3) is 118 ± 22 μmol/g, which is at least three-fold higher than the reported values for most other bacterial species that have been studied (Joe-Wong et al., 2012; Yu et al., 2014; Yu and Fein, 2016, 2017a), including *P. putida* cells that were grown in a nutrient-rich TSB medium (Yu et al., 2014; Yu and Fein, 2017a) and a M9 minimal medium (Yu and Fein, 2017a). Although it is not clear why the *P. putida* cells contain such high concentrations of cell surface sulfhydryl sites when they are cultured in the LB10 medium, Choi et al. (2014) found that *P. putida* cells cultured in the LB10 medium have more than a three-fold higher protein content within their EPS matrix compared to *P. putida* cells grown in two other minimal media. Proteins are likely the primary hosts of sulfhydryl sites both within cell envelopes and on EPS material (Norrod et al., 1993; Fein et al., 2019). Therefore, our findings suggest that the energy source and perhaps other environmental factors during cell growth play important roles in the production of surface proteins and hence cell surface sulfhydryl binding sites by *P. putida* (Choi et al., 2014). Further studies aimed at identifying the factors that control sulfhydryl-bearing protein production and distribution, and their influence on heavy metal toxicity to other bacterial species are crucial.

The high concentration of sulfhydryl sites on the cell surface of *P. putida* leads to Cd-sulfhydryl binding representing a large proportion of the total adsorbed Cd budget under high Cd loading conditions (Table 1). Typically, because of the limited abundance of high affinity sulfhydryl binding sites on bacteria, metal-sulfhydryl binding decreases in importance when metal loadings exceed approximately 10 μmol/g (Guine et al., 2006; Mishra et al., 2010; Pokrovsky et al., 2012; Yu and Fein, 2015; Nell and Fein, 2017). However, our EXAFS analysis indicates that Cd-sulfhydryl binding represents over 50 % of the total adsorbed Cd budget in the early stationary phase biomass sample (Fig. S2, Table 1) which was exposed to a metal loading of approximately 130 μmol/g. Most bacterial species that have been studied exhibit cell surface sulfhydryl site concentrations of 20–40 μmol/g (Joe-Wong et al., 2012; Yu et al., 2014; Mishra et al., 2017; Yu and Fein, 2017a). In two cases, much higher concentrations have been reported: 93 ± 8 μmol/g for *Bacillus subtilis* cells that were grown in a TSB medium containing 50 g/L of glucose (Yu and Fein, 2017a), and 68 ± 23 μmol/g for *Geobacter sulfurreducens* cells that were grown in a fresh basal medium (Mishra et al., 2017). Similar to the finding of this study, sulfhydryl sites were shown to play an important role in the adsorption of metals onto these biomass samples even under relatively high metal loadings, ranging from 20 − 50 μmol/g (Mishra et al., 2017; Yu and Fein, 2017b).

In addition to the high abundance of sulfhydryl sites, the location of the sulfhydryl sites primarily on EPS molecules also contributes to the dominance of Cd-sulfhydryl complexes on cell surfaces of the *P. putida* cells. Aqueous metals interact with biomass first through interactions with EPS molecules due to their location on the outermost layers of the cell surface. If EPS molecules contain a higher ratio of sulfhydryl sites relative to other possible metal binding sites than does the bacterial cell envelope, then Cd-sulfhydryl binding will be favored even under higher metal loadings than would be the case if the sulfhydryl sites were located within the cell envelope with the plentiful other non-sulfhydryl binding sites.

The sulfhydryl sites on *P. putida* EPS molecules dominate Cd adsorption onto bacterial biomass under the experimental conditions. In so doing, we conclude that the sites also play an important role in detoxification of Cd by sequestering the metal on EPS molecules and away from the cell walls where internalization could occur. Cd^{2+} ions can readily bind to dissolved thiols, and the conversion in solution of free Cd^{2+} or weak Cd complexes to Cd-thiol complexes can dramatically reduce the toxicity of Cd to bacteria (Murata et al., 1985). Similarly, the binding of Cd to the sulfhydryl sites on cell-bound EPS of *P. putida* can also reduce the toxicity of Cd. Because of the strength of the Cd-S bond, the adsorbed Cd is likely relatively inert within the EPS matrix, and remains so until the EPS molecule that hosts the sulfhydryl site is degraded. In fact, previous studies (Rubino, 2015) find that CdS is the only Cd product for the degradation of Cd-metallothionein complexes and no free Cd^{2+} is released during the whole degradation, indicating that the adsorbed Cd onto the sulfhydryl sites of EPS likely remains nontoxic after the degradation of EPS molecules at least in some cases. In contrast, the complexes between Cd^{2+} and other binding sites (e.g., carboxyl and phosphoryl sites) are significantly weaker (Yu and Fein, 2015), and hence the adsorbed Cd^{2+} that is bound to these non-sulfhydryl sites can easily desorb and thus remains potentially toxic to the bacterial cells. As a result, we observed no correlation between the extent of Cd toxicity and the concentrations of non-sulfhydryl sites on *P. putida* cell surfaces (Figs. 4C and S4).

P. putida cells possess multiple types of detoxification systems, enabling them to survive a range of extreme or contaminated environments (Murata et al., 1985; Chen et al., 1995; Shamim et al., 2014). This study elucidates one of these methods of detoxification. *P. putida* cells produce EPS molecules that are bound to the bacterial cells and which contain high concentrations of sulfhydryl binding sites. When *P.*

putida biomass is exposed to aqueous Cd, our experiments demonstrate that the Cd becomes bound to these EPS-hosted sulfhydryl sites (Fig. 2), decreasing the toxicity of the Cd to the *P. putida* cells. Biomass from different growth stages contains different amounts of EPS (Petry et al., 2000; Lbarburu et al., 2007), and hence different concentrations of sulfhydryl sites (Fig. 3). Our experiments demonstrate that the extent of Cd toxicity to the bacterial cells is inversely related to the concentration of sulfhydryl sites on bacterial cell surfaces (Fig. 4b). In addition to Cd, the sulfhydryl sites on the cell surfaces of *P. putida* likely can reduce the toxicity of a range of other chalcophile or similar metals as well, such as Hg, Zn, Au and Cu, because these metals also strongly bind to sulfhydryl sites (Guine et al., 2006; Pokrovsky et al., 2012; Nell and Fein, 2017; Yu and Fein, 2017b). It is unclear at this time how widespread this detoxification strategy is among bacterial species, but sulfhydryl sites have been detected on the EPS molecules within the biofilms that are produced by some pathogenic bacteria (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*) and biofilm formation bacteria on drinking water pipes (*Pleomorphomonas oryza NM1* and *Acidovorax ebreus* NM25) (Lin et al., 2014), with the roles of their EPS sulfhydryl sites unexplored. Therefore, further studies focusing on the potential role of cell surface sulfhydryl sites of these bacterial species in detoxification would be important for addressing multiple key environmental issues, such as water disinfection and drinking water pipe protection. Some species, such as *Shewanella oneidensis*, produce EPS molecules with much lower concentrations of sulfhydryl sites than does *P. putida*, and cell envelopes with much higher concentrations (Yu and Fein, 2016), and hence likely rely on other strategies than EPS metal sequestration for metal detoxification. However, it is noteworthy that the sulfhydryl sites on EPS molecules of *P. putida* could also contribute to protect other microbial species from toxic metals in natural environments, where mixed microbial species form aggregates such as biofilms and microbial mats via the production of EPS.

The decrease in sulfhydryl site concentration in the biomass samples from 24 h to 72 h (Fig. 3) suggests that, under the experimental conditions studied, EPS molecules can mobilize from planktonic bacterial cells upon death and degradation of the cells. However, EPS molecules within biofilms are likely to be more stable and remain immobile until the whole biofilm collapses, especially those EPS molecules located in the biofilm interior. The degree of mobilization may greatly affect the stability and lifespan of EPS-associated sulfhydryl binding sites. For example, previous studies found that although sulfhydryl sites on small molecules such as cysteine oxidize rapidly when exposed to air (Hird and Yates, 1961), but significantly less oxidation occurs when the cysteine is present in the outer layers of a biofilm, and no oxidation of cysteine occurs in the inner layers of biofilms (Lin et al., 2014). Therefore, sulfhydryl sites within the bacterial biofilms that are ubiquitous in natural environments likely play a more important and long-lasting role in metal detoxification than what we observed in our experiments for planktonic cells. In order to determine the prevalence of EPS metal sequestration as a strategy for metal detoxification in natural and engineered systems, it is crucial to expand study from isolated bacterial species to natural bacterial consortia and biofilms to measure sulfhydryl site concentrations on cells relative to that on EPS molecules, and to determine if a relationship exists, as we have observed for *P. putida*, between sulfhydryl site concentrations and metal toxicity response.

CRediT authorship contribution statement

Qiang Yu: Conceptualization, Methodology, Investigation, Visualization, Writing - original draft. **Bhoopesh Mishra:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Jeremy B. Fein:** Conceptualization, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

QY was supported through the Center for Environmental Science and Technology at University of Notre Dame. The research was funded in part by U.S. National Science Foundation grants EAR-1424950 and EAR-1904192. Two journal reviews were helpful and significantly improved the presentation of this work.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.122209.

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