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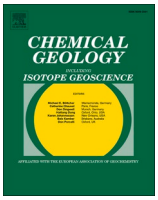

The role of cell surface sulfhydryl and amine binding sites in the removal of Cr(VI) from solution by *Bacillus subtilis* bacterial cells

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ABSTRACT

A range of bacterial species are known to be capable of reducing soluble Cr(VI) to Cr(III), causing the precipitation of Cr(III) phases from solution, but the mechanism responsible for the initial interaction between anionic aqueous Cr(VI) and the negatively charged cell surface has not been identified. Our study examines Cr(VI) removal from solution by the Gram-positive soil bacterial species *Bacillus subtilis*. We measured the kinetics of Cr (VI) removal as a function of pH in *B. subtilis* suspensions with different Cr(VI):cell ratios, and we measured the effect of pH on the extent of Cr(VI) removal at a fixed time in experiments with *B. subtilis* biomass (cells plus exudates) and in experiments involving *B. subtilis* exudates alone. The roles of sulfhydryl and amine binding sites in Cr(VI) removal were constrained using site–specific blocking molecules, and the impact of the site blocking on Cr(VI) removal was studied as a function of pH. Our results indicate that Cr(VI) removal by *B. subtilis* cells under the experimental conditions is at least partially non-reversible and is dependent on binding site concentration, pH, and metal loading. Our results are consistent with a two step removal process: Cr(VI) first adsorbs reversibly onto a cell wall binding site, followed by Cr(VI) reduction to Cr(III) likely via electron transfer from cell wall electron transport chain molecules. *B. subtilis* exudates are capable of removing a relatively small fraction of Cr (VI) from solution, and hence our results indicate that the dominant mechanism of Cr(VI) removal requires interaction with bacterial biomass. The presence of either the sulfhydryl-specific blocking molecule or the amine-specific blocking molecule or both in the experimental systems dramatically reduces the extent of Cr(VI) reduction, especially under circumneutral pH conditions, strongly suggesting that both sulfhydryl and amine binding site types participate in the initial attachment of Cr(VI) onto the cell surface. The experiments with either sulfhydryl or amine sites blocked both exhibited a similar reduction in Cr(VI) removal to the experiments with both types of sites blocked, strongly suggesting that both types of sites are involved simultaneously in binding Cr (VI) species to the cell surface. For example, these sites could both be involved in forming a bidentate bond with Cr(VI) species, or the positive charge of an amine site in close proximity to a sulfhydryl site could be necessary in order to enable the Cr(VI) species to approach the cell wall and to bind to a sulfhydryl site. The results of this study are the first to propose a viable mechanism that can explain the binding of anionic Cr(VI) onto an overall negatively charged cell surface as a first step in the reduction to Cr(III) and subsequent removal of Cr from solution.

1. Introduction

Chromium is a widespread heavy metal pollutant in soils, surface waters, and groundwater, and it poses significant safety hazards to humans and ecosystems. Chromium is commonly released into surface water as waste from electroplating, leather tanning, and textile industries (Fishbein, 1981; Avudainayagam et al., 2003; Johnson et al., 2006) and common sources of chromium contamination of soils include the disposal of commercial products and coal ash (Nriagu and Pacyna, 1988). The fate and transport of chromium in contaminated waters is controlled primarily by its oxidation state and the solution pH (Barceloux, 1999). The common oxidation states of chromium under typical environmental conditions are Cr(III) and Cr(VI) (Nieboer and Jusys, 1988; Shupack, 1991; Avudainayagam et al., 2003). Cr(VI) is much more soluble, bioavailable, and carcinogenic than Cr(III), therefore remediation typically focuses on removal of Cr(VI) or reduction of Cr (VI) to the less toxic and less mobile Cr(III) (Rai et al., 1987; Liu et al., 2012; Nur-E-Alam et al., 2020). Several methods have been developed

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for remediation of Cr(VI) contaminated water and industry effluent including ion exchange, membrane separation, and chemical precipitation (Fathima et al., 2005; Nur-E-Alam et al., 2020). These Cr(VI) remediation approaches produce highly concentrated waste that is difficult to dispose of and has high risk for secondary pollution (Lian et al., 2019). Bioremediation of Cr(VI) uses Cr(VI)-reducing bacteria to facilitate Cr(VI) reduction, and leads to efficient removal of chromium from the system as Cr(III) (Thacker et al., 2007; Liu et al., 2012; da Rocha Junior et al., 2018; Hossan et al., 2020). For example, Cr(VI) reducing microbial strains have been used to remediate contaminated soils through bacterial soil amendment, and this method is both effective and has a relatively low environmental impact (Kamaludeen et al., 2003).

There are several possible mechanisms involved in microbially-mediated Cr(VI) reduction depending on the bacterial species involved, and a common motif observed across several species is the involvement of chromate reductase enzymes (e.g., Campos-Garcia et al., 1997; Thatoi et al., 2014; Paul et al., 2020). For example, enzymatic Cr (VI) reduction in *Bacillus subtilis* is mediated by the chromate reductase **nfrA2**, which facilitates the reduction of flavin mononucleotide (FMN, a sugar commonly involved in metabolic processes), cellular toxins, such as nitro-organic compounds, azo dyes, and chromate (Campos-Garcia et al., 1997; Ackerley et al., 2004; Morokutti et al., 2005). Cr(VI) reduction can occur in different locations, including on the cell membrane, within the cytosol, and exterior to the cell facilitated by bacterial exudates (Cheung et al., 2006; Cheung and Gu, 2007). For example, Fein et al. (2002) demonstrated that microbially-mediated Cr(VI) reduction by *Bacillus subtilis* cells without the presence of an electron donor in the experiments is a surface phenomenon with kinetics driven by solution pH such that higher rates of Cr(VI) removal are associated with lower pH. Whether Cr(VI) reduction occurs on attached EPS material or on the cell membrane, Cr(VI) must bind onto the bacterial molecules in order for electron transfer to occur. Although previous studies have documented the capacity for bacterial biomass to reduce Cr(VI) to Cr(III), the mechanisms by which Cr(VI) attaches to the molecules involved in electron transfer are poorly understood. Depending on pH, Cr(VI) is present in aqueous solutions predominantly as the negatively charged $HCrO_4^-$, $Cr_2O_7^{-2}$, or CrO_4^{-2} species (Shupack, 1991). Bacterial surfaces are overall negatively charged under neutral pH conditions (Mozes et al., 1987; Wilson et al., 2001; Jiang et al., 2004), leading to significant electrostatic repulsion between Cr(VI) anions and bacterial surface molecules.

Sulfhydryl binding sites (-SH) or amine binding sites ($-NH_2$), or both could be responsible for Cr(VI) binding onto bacterial surfaces. Sulfhydryl sites are present on bacterial surfaces in significantly lower concentrations than other metal binding sites (Yu et al., 2014), but they have a much higher affinity for binding chalcophile elements through the formation of strong covalent bonds (Yu and Fein, 2015; Yu et al., 2018; Fein et al., 2019). Under low metal loading conditions, where metal cation concentrations are less than cell surface sulfhydryl site concentrations, metal-sulfhydryl binding controls metal adsorption onto bacteria for a range of chalcophile cations such as Zn (Guiné et al., 2006), Cd (Mishra et al., 2010; Yu and Fein, 2015; Yu et al., 2020), Hg (Yu and Fein, 2017b), Cu (Pokrovsky et al., 2012), and Au (Song et al., 2012). In addition, sulfhydryl binding sites are responsible for the binding of anionic chalcophile elements onto cell surfaces. For example, the reduction of aqueous Se(IV) in the form of selenite (SeO_3^{-2}) to Se(0) mediated by *Bacillus subtilis* is strongly inhibited when the sulfhydryl sites on *B. subtilis* are inactivated with a sulfhydryl-specific blocking molecule, emphasizing the importance of sulfhydryl binding of Se(IV) as a first and necessary step in the reduction process (Yu et al., 2018). Similarly, tellurite (TeO_3^{-2}) binding onto *Escherichia coli* K-12 bacterial cells occurs predominantly onto sulfhydryl sites located on cell-associated extracellular polysaccharide molecules (Goff et al., 2021).

Cell surfaces also contain amine group binding sites (e.g., Beveridge and Murray, 1980), which are proton active and positively charged at pH values below their pK_a values. Beveridge and Murray (1980) estimated an amine site concentration of 0.3 mmol/g on the *B. subtilis* cell wall. The concentration of surface sites in *B. subtilis* with pK_a around 8.9 (suggesting amine) was estimated potentiometrically to be 0.075 mmol/g biomass, and similar methods were used to estimate a site concentration of 0.11 mmol/g biomass in *Shewanella oneidensis* with pKa 9.4 ± 0.5 (Fein et al., 2005; Mishra et al., 2010). Although the exact concentration of amine sites on bacterial surfaces remains ambiguous, their presence there and their likely positive charge at circumneutral pH suggests that amine sites could play a role in binding anionic species, such as CrO_4^{2-}, to cell surfaces. Several studies have analyzed Cr(VI) binding onto cell surfaces with FTIR and measured peak shifts associated with the binding in ranges corresponding to the amine stretching bond (N—H) (e.g., Banerjee et al., 2019; Zakaria et al., 2007; Hossan et al., 2020), although unique assignment of an FTIR signal to a specific binding mechanism can be problematic for surfaces as complex as the bacteria-water interface (Kenney and Gorzsás, 2019). To our knowledge, there have been no studies that have used more direct analytical methods, such as X-ray absorption spectroscopy, to characterize the binding of Cr(VI) with cell surface amine sites. At present, although the presence of amine binding sites has been documented on bacterial cell walls and there is likely electrostatic attraction between these sites and anionic aqueous Cr(VI), the role of these sites in bacterial bioavailability of Cr(VI) has not been determined.

Bacterial exudates are dissolved organic compounds that are released by cells actively and/or passively, and they can influence important geochemical processes such as microbially-mediated metal reduction (e.g., Ohnuki et al., 2007; Seders and Fein, 2011; Kenney et al., 2012; Sullivan et al., 2022). Exudates contain proton-active functional groups that are likely responsible for interactions between exudates and metals (Seders and Fein, 2011), and exudates can exert a significant impact on the environmental behavior of metals, including promoting the reduction of anionic metalloids such as aqueous Se(IV) species (Sullivan et al., 2022). Previous characterization of *Bacillus subtilis* exudates by Seders and Fein (2011) determined the major components to be polysaccharides and DNA. Because of the similarities between Se(IV) and Cr(VI) aqueous species (i.e., both are present in solution as anions) it is possible that bacterial exudates could influence Cr(VI) reduction, but the importance of exudate-promoted reduction relative to cell surface-promoted reduction is unknown.

In this study, we use sulfhydryl- and amine-specific blocking molecules to test whether these binding site types are involved in Cr(VI) reduction by *Bacillus subtilis*, a common Gram-positive soil bacterial species. Cr(VI) removal was measured as a function of time, Cr loading, and pH, with and without the use of sulfhydryl and amine binding site blockers to constrain Cr(VI) removal due to both types of binding sites. Sulfhydryl binding sites were blocked using monobromo(trimethylammonio)bimane bromide (qBBr), which has been demonstrated to bind sulfhydryl sites on cell surfaces selectively and irreversibly (Joe-Wong et al., 2012; Yu et al., 2014). N-hydroxysulfosuccinimide acetate (sulfo-NHS acetate) selectively binds to primary amines and renders them inactive by capping them with an acyl group (Wan et al., 1999). In addition to determining the role of sulfhydryl and amine binding sites in the reduction of Cr(VI) by bacterial cells, we also examined the role of *B. subtilis* exudates in Cr(VI) reduction, the effects of pH and metal loading on the kinetics of Cr(VI) removal, and the reversibility of Cr(VI) removal. Our results indicate that bacterial exudate molecules play a relatively minor role in Cr(VI) reduction, at least for *B. subtilis* biomass grown under our experimental conditions, and that the reduction likely involves a reversible Cr(VI) binding step followed by a less reversible reduction to Cr(III). Furthermore, our results indicate that both sulfhydryl and amine binding sites on the bacterial surface are involved in the initial binding of Cr(VI) onto negatively charged bacterial cell surfaces and that this binding likely controls the bacterial bioavailability of Cr (VI).

2. Methods

2.1. Bacterial biomass growth and preparation

Bacillus subtilis cells were grown and prepared following a previously established method (Fein et al., 1997; Yu et al., 2014; Yu and Fein, 2017a). Briefly, *B. subtilis* cells were cultured in a growth medium consisting of autoclaved trypticase soy broth (TSB, 30 g/L) with yeast extract (5 g/L). Initially, 3 mL of growth medium in a test tube was inoculated with *B. subtilis* cells, and the test tube was gently agitated for 24 h in an incubating shaker (32 °C, 100 rpm). The test tube contents were then added to 1 L of fresh growth medium (50 g/L glucose, 30 g/L TSB, 5 g/L yeast extract) which was prepared by autoclaving 750 mL of TSB and yeast extract (40 g/L, and 6.67 g/L respectively) and adding 250 mL of a 200 g/L glucose solution that was vacuum filtered using a 0.2 μm nylon membrane filter (Corning® #430515) to remove contaminant cells. Glucose was added to yield biomass with elevated cell surface sulfhydryl site concentrations (Yu and Fein, 2017a). The inoculated growth medium was gently agitated for another 24 h in an incubating shaker (32 °C, 100 rpm).

B. subtilis biomass was prepared for the experiments using the following washing method. The biomass was first separated from the growth solution by centrifugation (11,060 *g*, 5 min), and then rinsed using 0.1 M NaCl three times, removing the biomass from the NaCl rinse by centrifugation (8100 *g*, 5 min) after each wash. The biomass was then centrifuged twice (8100 *g*, 30 min), and the liquid that separated from the biomass after each centrifuge cycle was removed from the pre-weighed container, thereby yielding the 'wet' biomass weight used for biomass concentration that we report for the experiments. The ratio of wet biomass to dried *B. subtilis* biomass is approximately 4.7:1 (Yu et al., 2014; Yu and Fein, 2015).

2.2. Chromium removal experiments

We probed Cr(VI) reduction by *B. subtilis* biomass by measuring the extent of Cr(VI) removal from solution as a function of time, pH, and Cr loading onto the biomass in batch experiments without the presence of an electron donor. The reduction of Cr(VI) to Cr(III) results in removal of total dissolved Cr from solution because of the low solubility of Cr(III), which causes precipitation of Cr(III) from the system as $Cr(OH)_{3(s)}$ (Rai et al., 1987). The experimental systems used in this study are closed batch systems and Cr loss only occurs as precipitated or adsorbed Cr is removed by filtration. Therefore, the concentration of total dissolved Cr in our experiments was measured in each sample, and the extent of Cr (VI) reduction that occurred in each system was quantified by the decrease in concentration of dissolved Cr. The solubility of Cr(III) hydroxide solid is $<10^{-7}$ M under circumneutral pH but increases with decreasing pH (Rai et al., 1987), suggesting a potential for release of reduced Cr(III) into experimental solutions under low pH conditions. Additionally, precipitated Cr(III) hydroxide particles smaller than 0.45 um that are not attached to bacterial cells could remain suspended in solution after filtration and preparation of samples for ICP-OES analysis (see Kinetics Experiments), which could also cause the inclusion of any Cr(III) in solution in the measurement of the total Cr concentration remaining in solution. However, the product of bacterial Cr(VI) reduction is typically Cr(III) oxide nanoparticles which remain bound to cell surfaces (e.g. Daulton et al., 2002; Li et al., 2023a) and would be removed during filtration. Hence, our results represent minimum rates of Cr(VI) reduction under the experimental conditions. We anticipate that the extent of Cr(III) dissolution is small, especially above pH ~4 where the solubility of Cr(III) oxide is low.

For all experiments, the Cr(VI)-bearing solutions were prepared by diluting a commercial volumetric Cr(VI) standard solution (1000 ± 5 ppm) with 0.01 M NaCl. An aliquot of the diluted Cr(VI) solution was collected prior to the addition of biomass in each experiment in order to measure the initial dissolved Cr concentration. Biomass-free and exudate-free abiotic controls (Supplementary S1) showed no significant Cr(VI) removal from solution in the pH range of 3.1–9.5 after 4 h or when reacted for 4 h with qBBr and sulfo-NHS acetate (see *Site Blocking Experiments*). Duplicates for our kinetics experiments (see below) were conducted to demonstrate experiment reproducibility and to estimate the extent of natural variation in these biological systems. Our reversibility experiments were run in triplicate, and the experiments conducted as a function of *pH* with and without site blocking treatments (see below) were repeated with different bacterial growths at least twice for each treatment (Supplementary S4). The exudate experiments were conducted twice using exudates extracted at two different solution pH with high reproducibility observed between the two treatments. All treatments conducted in duplicate reflect a high level of reproducibility in our biological systems and therefore, duplicates rather than triplicates are used to probe the uncertainty in our data due to natural biological variation.

2.3. Kinetics experiments

The kinetics of Cr(VI) removal from solution were measured by removing aliquots of solution from batch bacterial biomass suspensions as a function of time. Washed and weighed *B. subtilis* biomass was suspended in Cr(VI)-bearing solutions of three different initial concentrations and three different pH values (0.1 ppm Cr at pH 6, 1 ppm Cr at pH 6, and 2 ppm Cr at pH 4, 6, and 8) to reach a biomass concentration of 25 g wet biomass/L. The solution pH was adjusted to approximately 4, 6, or 8 using additions of small volumes of 0.1 and/or 0.5 M HCl, and/or 0.1, 0.2, and/or 1 M NaOH. 8 mL aliquots of the solution were removed at 0.5, 1.5, 2.5, and 4 h, and then every 4–8 h after that until 48 h after initiation of the experiment. Aliquots were removed while agitating the solution to minimize changes in biomass concentration throughout the kinetics trials, and each was immediately placed into a polypropylene 50 mL centrifuge tube and centrifuged (10 min, 8100 *g*). The supernatant was filtered using a 0.45 μm nylon syringe filter (VWR #514–1267) and saved for total dissolved Cr analysis (see below. The pH of the experimental system solution was measured every 15 min for the first 2 h, every 30 min for the next 2 h and after the removal of each sample, and the pH was re-adjusted if necessary to the starting pH using 0.1 and/or 0.5 M HCl, and/or 0.1, 0.2, and/or 1 M NaOH. The systems at pH 4 and 6 increased in pH over time, requiring addition of NaOH, while the system with solution pH 8 decreased in pH over time. The most significant drift in pH in these systems was observed at early times (< 4 h), and changes in pH were minimal throughout the rest of the experiment duration. The reaction container was attached to and spun on a test tube rotator (25 rpm) between collection of samples. The results from these kinetics trials indicate that Cr(VI) is continuously removed from solution throughout the course of these experiments. Continuous growth of bacteria throughout the duration of these kinetics experiments is unlikely since the biomass is suspended in solution without an electron donor source. All subsequent experiments were run for 4 h to standardize reaction time and to observe the other factors that influence the Cr(VI) removal rates.

2.4. pH effects

The pH dependence of Cr(VI) removal by *B. subtilis* was measured in batch experiments in which bacterial cells were suspended in 0.1 ppm or 1 ppm Cr(VI)-bearing solutions at different pH values, allowed to react for 4 h, and then sampled and analyzed for total dissolved Cr. Washed and weighed *B. subtilis* biomass was suspended in the Cr(VI)-bearing solution to achieve a bacterial concentration of 25 g wet biomass/L. The suspension was vortexed to ensure homogeneity, and then separated into 10 mL aliquots in individual polypropylene containers. The pH of each suspension was adjusted within a range of 3–9 using small additions of 0.1 and/or 0.5 M HCl, and/or 0.1, 0.2, and/or 1 M NaOH and then all of the experimental systems were rotated at 25 rpm. 30 min and

1 h after the initial pH adjustment, the pH was checked and re-adjusted to the desired pH, and the test tubes were allowed to react for a total of 4 h. The final pH of each suspension was measured, and each test tube was centrifuged (10 min, 8100 *g*) and the supernatant of each was filtered and saved for measurement of total dissolved Cr, as previously described.

2.5. Reversibility experiments

The reversibility of Cr(VI) removal by *B. subtilis* was measured using pH adjustment trials, in which the biomass was first reacted in a Cr(VI)-bearing solution at a pH of approximately 5 for 4 h before the pH was increased to approximately 8, and the system was allowed to react for another 4 h prior to sampling. First, washed and weighed biomass was suspended in 1 ppm Cr(VI) solution to obtain a biomass concentration of 25 g wet biomass/L. 20 mL aliquots of the suspension were removed quickly while agitating the bulk suspension to keep homogeneity in the solution and added to 50 mL polypropylene containers. The solution in each system was adjusted to a pH of 5.0 ± 0.5 using 10 μL additions of 0.1 and/or 0.5 M HCl, and/or 0.1, 0.2, and/or 1 M NaOH. Every container was placed on a test tube rotator (25 rpm) for 4 h. After 30 min and 1 h of reaction time, the pH of each container was measured, and adjusted back to the initial pH if necessary. After 4 h, the pH of the solution was measured and a 10 mL aliquot was removed and added to a 50 mL polypropylene centrifuge tube, centrifuged, and then filtered as described above, and kept for total dissolved Cr analysis. The remaining experimental suspension was adjusted to a pH of 8.0 ± 0.5 using small volumes of 0.2 and/or 1 M NaOH before being rotated again and allowed to react for an additional 4 h with pH adjustments at 30 min and 1 h. After the additional 4 h of reaction, the pH was measured before a final 10 mL aliquot was collected and analyzed for total dissolved Cr analysis as described above. The reversibility experiments were repeated, except with an initial pH adjustment of 8.0 ± 0.5, and no pH adjustment at 4 h to serve as a control with no pH change and a total reaction time of 8 h.

2.6. Exudate experiments

Similar Cr(VI) reduction experiments were conducted using exudates isolated from *B. subtilis* suspensions (Seders and Fein, 2011; Sullivan et al., 2022; Sullivan et al., 2024) in order to compare the reduction rates of the exudates alone to those of the biomass in general. The exudate solutions were collected from either a pH 5 or a pH 9 suspension (20 g wet biomass/L) of washed and weighed *B. subtilis* biomass in 0.01 M $NaNO_3$, after 72 h of 100 rpm agitation on a platform mixer. Then, the suspension was centrifuged (10 min, 8100 *g*) and the supernatant containing the dissolved exudate molecules was filtered through a 0.45 μm nylon syringe membrane, weighed, and then spiked to achieve a final Cr (VI) concentration of 0.1 ppm. A 10 mL sample was immediately removed, filtered, and saved for total dissolved Cr analysis. The remaining Cr-bearing exudate solution was separated into 10 mL volumes. The pH of each of these systems was adjusted to different values in the range of 3 to 10, the systems were allowed to react for 4 h, and then the pH of each solution was measured before they were filtered and analyzed for total dissolved Cr as described previously.

2.7. Site blocking experiments

We tested the role of sulfhydryl and amine binding sites in the reduction of Cr(VI) by conducting similar Cr(VI) removal experiments to those described above, but using *B. subtilis* biomass that had been treated with either a sulfhydryl-specific blocking molecule, monobromo(trimethylammonio)bimane bromide (qBBr), an amine-specific blocking molecule, N-hydroxysulfosuccinimide acetate (sulfo-NHS acetate), or both. After growing and washing *B. subtilis* using identical procedures to those described above, the biomass was suspended in 0.1 M NaCl (15 g wet biomass/L), and one or both of the site blockers was added to the suspension so that the total blocker:site ratio was approximately 2:1. For the qBBr treatment, we used a blocker:site ratio of 180 μmol qBBr/g wet biomass, following the results of Yu et al. (2014). The concentration of amine sites on the cell wall has not been directly measured for *B. subtilis*, but we estimated an approximate maximum amine site concentration of $1.1 \times 10^{-4} \pm 0.6$ mol/g wet biomass based on the concentration of sites with a pKa value of 9.4 ± 5 reported by Mishra et al. (2010), yielding a treatment ratio of 0.0570 g sulfo-NHS acetate/g wet biomass. The experiments completed with amine blockers were repeated with a blocker:site ratio of 3:1 to confirm saturation of amine sites, and no significant differences were observed with the increased sulfo-NHS acetate treatment concentration (Supplementary S1). In each experiment involving only one type of site blocker, the bacterial suspension with the blocker was shaken vigorously by hand before the pH was adjusted to 7.0 ± 0.1 using additions of small volumes of NaOH. The mixture was secured to a test tube rotator and allowed to equilibrate for 2 h, which has been shown to be a sufficient length of time for the blocking reaction to go to completion (Yu et al., 2014). In experiments using both types of site blockers, both were added and equilibrated simultaneously as preliminary experiments demonstrated that the order of addition had no significant effect on the results (Supplementary S1). In order to reduce the breakdown of qBBr or NHS molecules under light exposure, the addition of the site blockers was conducted in the dark, and the reaction container was wrapped with Al foil and placed in the dark during the reaction period. After the blocking treatment, the treated biomass was washed three times with 0.1 M NaCl to remove excess unreacted sulfo-NHS acetate and/or qBBr, as described above (see *Bacterial biomass growth and preparation)*, weighed to obtain the wet biomass, and 4 h Cr (VI) removal experiments were conducted as a function of pH, as described above (see *pH effects*).

2.8. Total dissolved Cr analyses

The samples obtained from our experimental bacterial suspensions formed a white cloudy precipitate when acidified likely due to the presence of proton-active bacterial exudate organic molecules, and so the samples were not acidified prior to analysis. All total dissolved Cr analyses were conducted within 2 days of each of the Cr(VI) removal experiments, except for kinetics experiments which were analyzed within 6 days. Samples were refrigerated for the entire duration between filtration and before analysis on the ICP-OES. Repeat analysis of Cr-containing aqueous samples (Supplementary S1) showed no significant loss of Cr from solution for at least the 6 day period tested. The concentration of total dissolved Cr in each sample was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES), analyzing at a wavelength of 267.716 nm. Matrix-matched standards were prepared over a range of 0 to 0.2 ppm for the experiments with a total dissolved Cr concentration of 0.1 ppm; over a range of 0 to 2 ppm for the experiments with a total dissolved Cr concentration of 1 ppm; and over a range of 0 to 3 ppm for the 2 ppm Cr experiments. A yttrium standard (analyzed at a wavelength of 371.029 nm) was added to each sample and standard in order to check for instrument drift throughout the run, and the magnitude of drift throughout the run was used to correct the intensity at the wavelength associated with Cr (267.716 nm). Most of the analyses had less than ±10% drift in the Y standard throughout the run (Supplementary S1). All analyses were run in triplicate, and the standard deviation of triplicate measurements for most samples was <3% (Supplementary S3).

3. Results and discussion

3.1. Chromium removal by Bacillus subtilis cells and exudates

In contrast to cation adsorption onto bacterial cells which typically reaches equilibrium within 1–2 h (e.g., Fein et al., 1997; Yu and Fein,

2017b), Cr(VI) sorption by *Bacillus subtilis* cells is relatively slow and takes at least 24 h to reach steady-state (Fig. 1). The biomass removes higher concentrations of Cr(VI) from solutions with higher Cr(VI) concentrations within a given amount of time (Fig. 1A), however the Cr(VI): biomass ratio does not impact the proportional rate of Cr(VI) removal or the proportion of Cr(VI) removed within 48 h (Fig. 1B). For example, in solutions with 0.1, 1, and 2 ppm Cr, 80–90% of the initial Cr(VI) concentration is removed after 24 h (Fig. 1B). Therefore, the system is undersaturated in Cr(VI) relative to binding site availability on the biomass at a pH of 6. *B. subtilis* biomass removes Cr(VI) more quickly and to a greater extent in solutions with lower pH (Fig. 1C). For example, after 10 h of reacting *B. subtilis* biomass in a 2 ppm Cr solution at pH 4, 80% of the initial Cr(VI) is removed, while only 70% is removed in the same time in a solution at pH 6, and 50% in a solution at pH 8. Additionally, the maximum proportion of Cr(VI) removed by *B. subtilis* biomass within the experiment duration decreases with increasing pH, with a maximum of approximately 50% Cr(VI) removed from the pH 8 solution, compared to a maximum removal of 80–90% by pH 4 and 6 solutions (Fig. 1C).

Both the pH 4 and pH 6 systems exhibited an initial relatively rapid rate of Cr(VI) removal over the first few hours of the experiment, with continuously slowing rates of removal over the next ~24 h until a steady-state was reached (Fig. 1C). In contrast, the pH 8 experiment exhibited a nearly constant rate of Cr(VI) removal over the first 15–24 h, followed by a decrease in removal rate after 24 h, and even a reversal of the rate such that the concentration of Cr that was present in solution after 24 h increased with time until the end of the experiment (Fig. 1C). We hypothesize that the release of Cr after 24 h in the pH 8 system occurs due to the release of organic exudate molecules by *B. subtilis* cells. The total organic carbon (TOC) concentration increases throughout the experiment duration (Supplementary S1), indicating the release of organics over time in our experiments. Dissolved organic molecules contain binding sites that compete with cell surface sites to bind Cr(III), increasing the soluble fraction of Cr(III) through the formation of Cr(III) aqueous complexes (Gustafsson et al., 2014). Therefore, as the concentration of these molecules increases in the system over 48 h, higher proportions of Cr(III) will be bound in the soluble exudate fraction, and consequently the dissolved concentration of Cr also increases. The effect of these exudates is largest at pH 8, likely because the functional groups on the exudate binding sites are more highly deprotonated at this pH relative to pH 4 and 6 conditions (Seders and Fein, 2011).

We tested the ability of several kinetics models to fit the first 24 h of Cr(VI) removal data (see Supplementary S2 for modeling details and results), testing first and second order kinetics models (Harcourt and Esson, 1865), and nonlinear pseudo first- and second-order (PFO and PSO, respectively) models (Weber and Morris, 1963; Ho and McKay, 1999; Revellame et al., 2020). The PSO model yields the best fit to the datasets as a whole. The calculated non-linear PSO rate constants ranged from 6.58×10^{-5} to 1.68×10^{-4} g/mg min. These values are

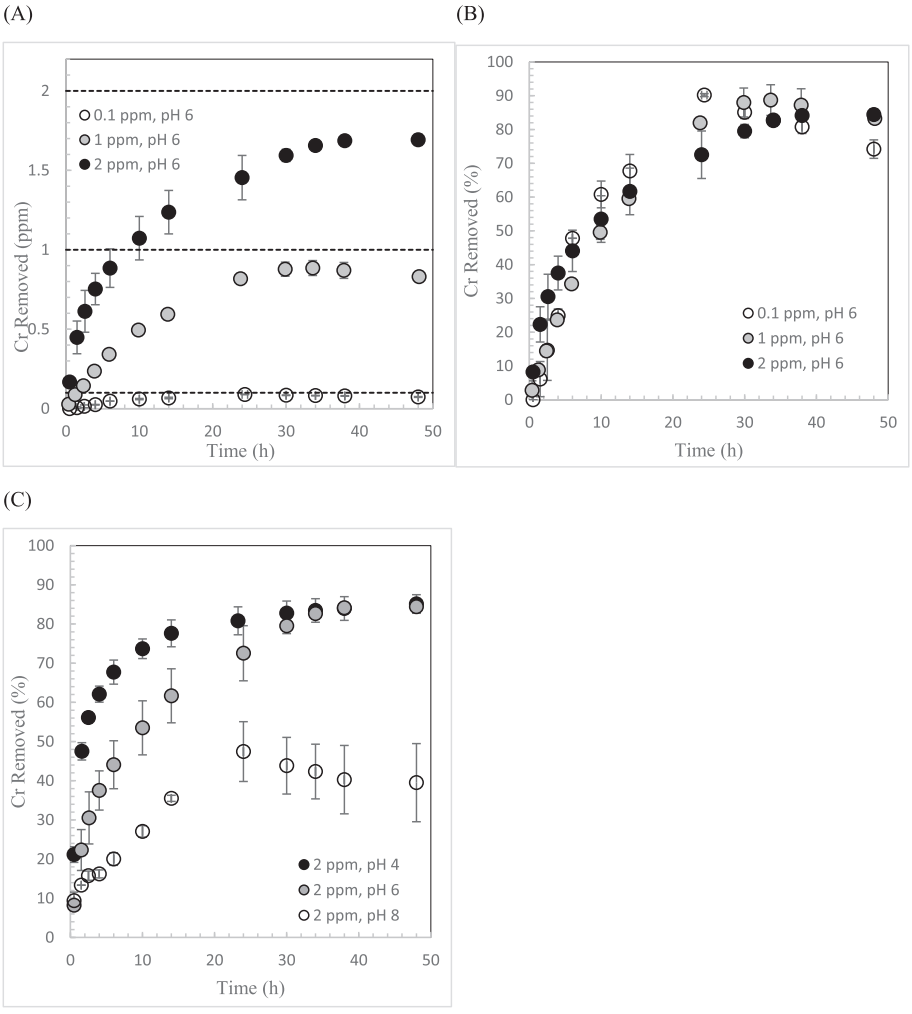

Fig. 1. A) Chromium removed over time by 25 g/L *B. subtilis* in systems with 0.1 ppm (empty squares), 1 ppm (2 replicate experiments in grey triangles and grey circles, labeled 'A' and 'B', respectively), and 2 ppm (black squares) Cr(VI) at pH 6 ± 0.5; B) proportion of Cr removed in the same system (pH 6 ± 0.5) over time; and C) proportion of Cr removed over time by 25 g/L *B. subtilis* in systems with 2 ppm Cr(VI) at pH 4 (black circles), pH 6 (grey circles), and pH 8 (2 replicate experiments in white circles and white triangles, labeled 'A' and 'B', respectively). Error bars represent the range of duplicates.

significantly lower than the PSO rate constants that describe the kinetics of metal cation adsorption onto bacterial surfaces. For example, the rate constant value for a non-linear PSO model of Cd^{+2} adsorption onto a gram-negative bacterial species is 0.0465 g/mg min (Li et al., 2023b). The significantly slower rate of removal that we observed in the Cr(VI) experiments compared the rate of metal cation removal due to bacterial adsorption alone strongly suggests that Cr(VI) removal involves more than a simple bacterial surface binding mechanism.

Subsequent experiments were conducted with a 4 h reaction time period in order to measure the effects of pH and Cr(VI) loading on the removal behavior of the system and to match the Cr(VI) exposure period of previous work by Fein et al. (2002). Because these systems continue to remove Cr(VI) beyond 4 h, these results are snapshots of the system at a set time point, rather than values that represent a steady state in the system. The slow Cr(VI) removal kinetics relative to typical bacterial adsorption kinetics and the dependence of removal on pH suggest that Cr(VI) removal from solution is a multi-step process that is dependent on the concentration of bacterial sites responsible for Cr(VI) binding. Our results suggest that the rate-determining step is not the attachment of Cr (VI) to the bacterial surface, but rather a slower process, likely the reduction of Cr(VI) to Cr(III), which occurs after attachment. This two-step mechanism is consistent with previously proposed multi-step mechanisms of microbially mediated Cr(VI) reduction; for example, several bacterial species can reduce Cr(VI) to Cr(III) using membrane bound enzymes (e.g. **nfrA2** in *B. subtilis*) that facilitate the transfer of electrons to Cr(VI) (e.g., Morokutti et al., 2005; Cheung and Gu, 2007).

Both pH and Cr(VI) loading significantly influence the extent of Cr (VI) removal by *B. subtilis* biomass over 4 h of reaction time (Fig. 2). For both the 0.1 ppm and the 1.0 ppm Cr(VI) solutions, increasing pH decreases the amount of chromium removed, but there is still significant chromium removal even under the highest pH conditions tested. For example, in the 0.1 ppm Cr experiments, 89% and 49% of the Cr(VI) is removed at pH 4.6 and 9.3, respectively, and in the 1 ppm Cr experiments 84% and 18% of the Cr(VI) is removed at pH 4.6 and 8.4. Cell surface charge becomes increasingly negative as pH increases due to deprotonation of surface sites. Therefore, because Cr(VI) is present in solution as $HCrO_4^-$, $Cr_2O_7^{-2}$, and CrO_4^{-2}, electrostatic repulsion increases with increasing pH. However, if the dominant control on Cr(VI) binding to the cell surface is electrostatic, we would expect to see a much stronger pH effect. For example, adsorption studies of the negatively charged ligand EDTA onto *B. subtilis* show a dramatic decrease in EDTA adsorption with increasing pH such that EDTA adsorption is insignificant above a pH of 4 (Fein and Delea, 1999). Electrostatic repulsion between aqueous Cr(VI) and the bacterial surface likely explains the general decrease in Cr(VI) removal with increasing pH. However, because significant Cr(VI) removal is observed even under the highest pH conditions studied, our results suggest that electrostatics are not the only influence on Cr(VI) removal and that covalent bonding with one or more binding sites on the bacterial cell surface may occur. In addition, it should be emphasized that the systems represented by the data in Fig. 2 are not at equilibrium, and pH effects on the Cr(VI) reduction kinetics could explain the pH trend as well.

Adsorption of aqueous cations (e.g. Cd^{2+}, Ca^{2+}) onto bacteria is both rapid and fully reversible (Fowle and Fein, 2000), indicating that the process can be modeled successfully as a simple one-step binding mechanism (Fein et al., 1997). In order to test if Cr(VI) removal is controlled by a similar binding reaction, we measured the reversibility of Cr(VI) removal by first equilibrating a system under low pH conditions where Cr(VI) removal is most extensive, and then adjusting the pH upwards, allowing the system to re-equilibrate, and testing whether the concentration of Cr in solution increases due to desorption reactions (Fig. 3). After the initial 4 h sorption step, the extent of Cr removal is consistent with our first set of 4 h removal experiments; adjusting the pH upward and allowing the systems to react for another 4 h causes some Cr to desorb from the bacterial cells, but the extent of Cr desorption is not enough to have the systems reach the lower extents of Cr removal that we observed in the first set of Cr removal experiments. In the 1 ppm Cr system (Fig. 3), increasing pH from a range of 4.3–4.6 to a range of 7.5–8.2 decreases Cr removal by 21% (from 78% to about 57% removed). In the system reacted under the same conditions, but kept at pH 7.6–7.9 for the entire experiment, only 20% of the Cr(VI) was removed after 4 h, and 27% was removed after the full 8 h. If the Cr removal was solely a bacterial surface adsorption phenomenon, then 30% more Cr should have been released back to solution after the pH was adjusted from pH ~4.5 to pH ~8.0. In contrast, the results of these reversibility tests strongly suggest that Cr(VI) removal in the experimental systems involves more than a simple binding reaction between Cr(VI) and the bacterial surface. The reversal experiments suggest that approximately half of the Cr(VI) that is removed during the initial 4 h reaction period under low pH conditions in these experiments remains as desorbable Cr(VI), and that the remaining Cr(VI) is not available for

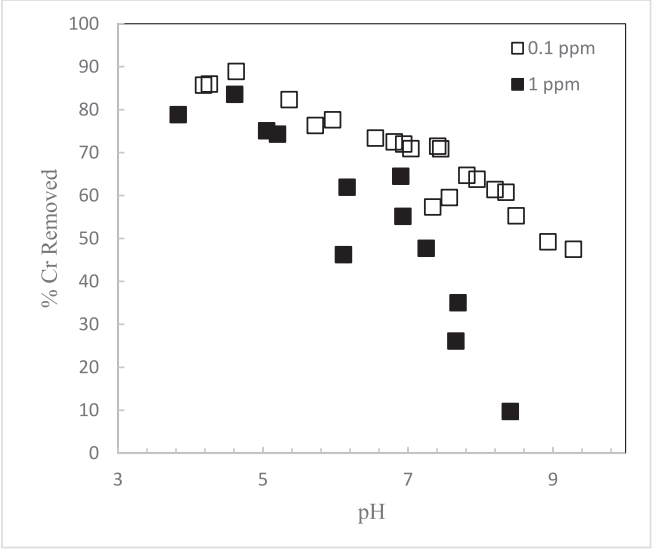

Fig. 2. The extent of Cr(VI) removed by 25 g/L *B. subtilis* biomass after 4 h of reaction. Open squares represent experiments with an initial total Cr(VI) concentration of 0.1 ppm; closed squares represent experiments with an initial total Cr(VI) concentration of 1 ppm.

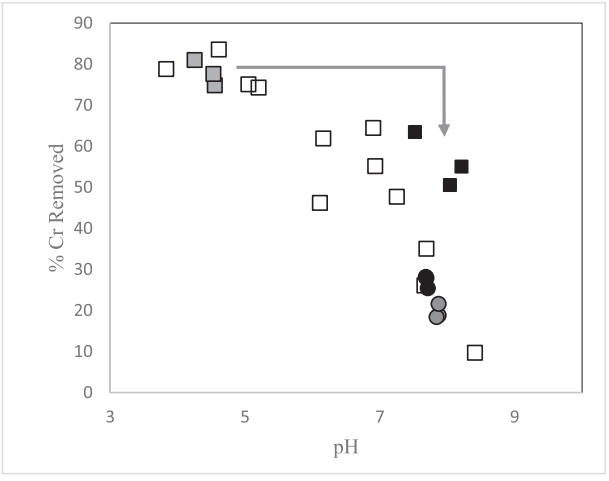

Fig. 3. Chromium removed by 25 g/L *B. subtilis* in a 1 ppm Cr-bearing system reacted for 8 h with pH increased after 4 h (see arrow, squares), and in a system reacted for 8 h with pH kept constant (circles), grey squares/circles are measured at 4 h, and black squares/circles are measured at 8 h. Also shown: chromium removed after 4 h as a function of pH (open squares, same data as shown in Fig. 2).

desorption back into solution. The results of the reversibility experiments are consistent with results from Fein et al. (2002) which showed that interaction between Cr(VI) and non-metabolizing *B. subtilis* cells leads to attachment of Cr(VI) to the cell surface followed by Cr(VI) reduction to Cr(III) after attachment. Although we do not identify the valence state of irreversibly bound Cr in our experiments, our results suggest that Cr(VI) is reduced to Cr(III), which when pH is adjusted higher in these reversibility tests, would either remain as an adsorbed Cr^{+3} cation on the highly negatively charged bacterial surface, or as a Cr (III) solid phase precipitate.

Bacteria release a range of organic acids either actively as a result of metabolic processes or passively due to cell lysis, and hence these exudates are present to some extent in our experimental systems. TOC concentrations in our experimental systems increase over time (Supplementary S1), demonstrating that there is a release of dissolved organic exudates over time in our systems. Additionally, cell lysis occurring over time in our systems could allow soluble enzymes to be released, which may interact and reduce Cr(VI) anions. In order to constrain the extent to which bacterial exudate molecules affect the Cr (VI) reduction behavior that we observed in the above experiments, we conducted parallel experiments using exudate molecules isolated from 20 g/L bacterial suspensions maintained at either pH 5 or pH 9. Exudate solutions were collected after soaking *B. subtilis* biomass for 72 h, and therefore contain exuded components from both active production and from lysed cells, as is possible in our 4 h and 48 h experimental systems. The results from the exudate experiments indicate that while the exudates isolated from *B. subtilis* biomass are capable of removing Cr(VI) from solution, particularly when the solution pH is below 7 (Fig. 4), the extent of Cr(VI) removal that can be ascribed to exudate interactions is small relative to that promoted by the cells themselves. The extent of Cr removal from solution by *B. subtilis* exudates decreases from a maximum of approximately 20% removal at pH 4.5–5.0, to a negligible amount of Cr removal above pH 7. In addition, we do not observe a significant difference in the ability of the exudate solutions to reduce Cr(VI) between the two different pH conditions used to produce the exudates, suggesting that any differences in exudate production by cells under these different pH conditions does not alter the Cr(VI) reduction capability of *B. subtilis* exudates. The decrease in the observed extent of Cr (VI) removal with increasing pH that we observed in the exudate experiments mimics the decrease in Cr removal that we observed for the bacterial biomass experiments (Fig. 2), and likely reflects the increasing electronegativity associated with the organic acid molecules that comprise bacterial exudates (Seders and Fein, 2011). Our results suggest that Cr(VI) removal from solution by both bacteria and bacterial exudates is first mediated by Cr(VI) attachment to either the bacterial surface or the bacterial exudate molecule, and hence pH and electrostatics represent important factors in controlling the rate and extent of Cr removal. The results of the exudate experiments demonstrate that while exudates released by *B. subtilis* can influence Cr(VI) reduction to some extent, the interactions between Cr(VI) and the bacterial surface significantly outpace those of Cr(VI) and the exudate molecules, and that bacterial surface binding and reduction represent the dominant mechanism for Cr(VI) reduction.

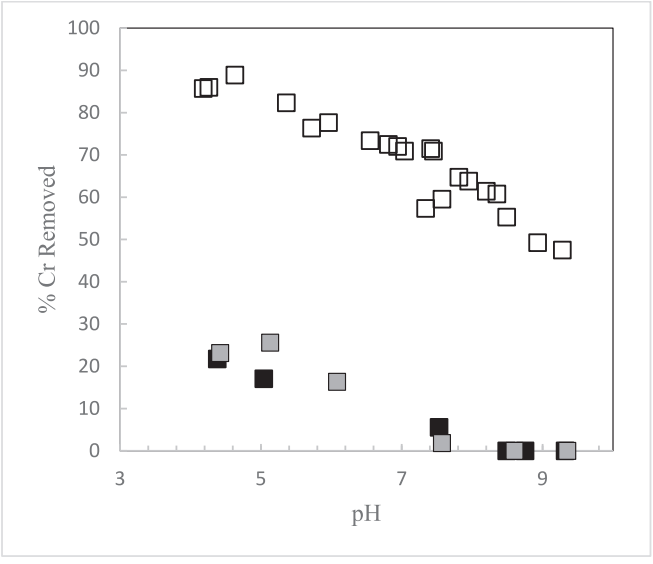

Fig. 4. Chromium removal by exudates collected from 20 g/L *B. subtilis* prepared in solutions with pH 5 (grey squares) and 9 (black squares), compared to the extent of removal that we measured in a system containing biomass after 4 h as a function of pH (open squares, same data as shown in Fig. 2) in 0.1 ppm Cr.

3.2. Sulfhydryl and amine sites involvement in Cr(VI) reduction

The slow kinetics and non-reversibility of Cr(VI) removal in our experiments suggest that Cr(VI) removal by *B. subtilis* biomass involves a more complex process than simple binding of Cr(VI) onto bacterial surface sites. Our blocking experiments probe the possible role of cell surface sulfhydryl and/or amine binding sites as the first step in Cr(VI) removal from solution (Fig. 5). Cr(VI)-sulfhydryl binding would involve covalent bonding that could offset the electrostatic repulsion between aqueous Cr(VI) anions and the predominantly negatively-charged bacterial surface. Similarly, the positively-charged amine sites, although located amidst other binding sites on the cell surface that are negatively charged, could serve as a location for anionic Cr(VI) binding as well. In experiments with either 0.1 or 1 ppm Cr(VI), qBBr treatment of the *B. subtilis* biomass dramatically decreases the extent of Cr(VI) removal from solution compared to what is observed from unblocked biomass (Fig. 2), with the effect increasing with increasing pH (Fig. 5). For example, at pH 7 in the 1 ppm Cr(VI) experiments without qBBr treatment, the *B. subtilis* biomass removes approximately 60% of the initial dissolved Cr(VI) after 4 h of exposure time, and the biomass with sulfhydryl sites blocked removes <10% of the Cr(VI) from solution (Fig. 5b). Under lower pH conditions, the effect of the qBBr blocking is diminished. For example at pH 3.8 in the 1 ppm Cr(VI) experiments, the blocking treatment reduces the extent of Cr(VI) removal from solution from 80 to 64% only. In the 1 ppm Cr(VI) experiments, the amine blocker exerts a nearly identical effect on Cr(VI) removal to what we observe for the sulfhydryl site blocker, and the 1.0 ppm Cr(VI) experiments with cells treated by both blockers also displays nearly identical Cr(VI) removal behavior as a function of pH. The 0.1 ppm Cr(VI) experiments exhibit similar behavior to that of the 1.0 ppm experiments, with nearly complete blockage of Cr(VI) removal under near-neutral pH conditions, and less of an effect with decreasing pH (Fig. 5A).

Our blocking experiments suggest that most, but not all, of the binding sites on the cell surface that bind Cr(VI) anions are blocked using qBBr and sulfo-NHS acetate. Therefore, both amine and sulfhydryl binding sites appear to play a role in Cr(VI) removal. Blocking either sulfhydryl or amine sites, or blocking both sites simultaneously, yield similar effects in terms of decreasing the overall Cr(VI) removal by the system in a given period of time. One possible mechanism controlling Cr (VI) reduction is that Cr(VI) binding onto either sulfhydryl or amine sites can lead to Cr(VI) reduction and that the sites operate independent from each other. If this were the case, then our results would suggest approximately similar concentrations of amine and sulfhydryl sites on the cell surface because blocking of one type of site yields a decrease in the extent of Cr(VI) removal that is approximately equal to the decrease in Cr(VI) removal that is caused by blocking the other site type. However, the experiment that involved both blocking molecules suggests that the sites are not operating independently. If Cr(VI) was binding onto both sulfhydryl and amine sites independently, then the use of both blocking molecules in the same experiment should lead to an additive effect. That is, the experiment with both blocking molecules should

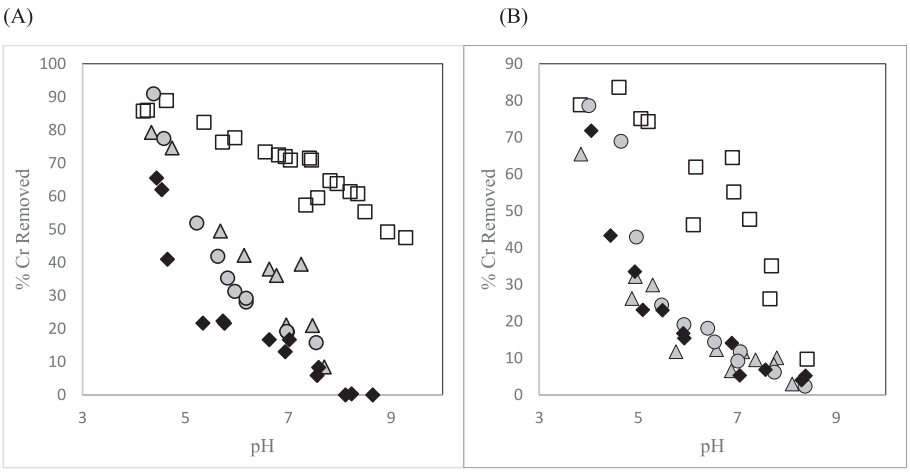

Fig. 5. Chromium removal by 25 g/L *B. subtilis* over 4 h in systems with initial total aqueous Cr(VI) concentrations of: A) 0.1 ppm or B) 1 ppm. Experiments with no blocker treatment (open squares, same data as shown in Fig. 2), qBBr blocking of sulfhydryl sites (grey triangles), sulfo-NHS acetate blocking of amine sites (grey circles), and simultaneous qBBr and sulfo-NHS acetate treatments (black diamonds).

exhibit significantly less removal than was observed in either of the single blocker experiments. The observation that the presence of either type of blocking molecule, or the presence of both blocking molecules, all led to similar effects and not an additive effect suggests that *both* types of sites are involved and required simultaneously in the binding and removal of Cr(VI). Thus, blockage of either binding site type, or both types of sites, hinders the capability of *B. subtilis* to reduce Cr(VI) to the same extent.

Simultaneous contributions by different types of binding sites have been demonstrated previously to control metal binding to cell surfaces. For example, Mishra et al. (2020) describe the attachment of Zn^{2+} to plant cell surfaces and the subsequent accumulation of the metal in plant tissue as a dynamic process involving both amine and carboxyl binding sites. Under enzymatically driven Cr(VI) reduction, both amine sites and sulfhydryl sites could be involved simultaneously in Cr(VI) attachment to the reducing enzyme or protein. Simultaneous binding of metals by sulfhydryl and amine sites is common in protein structures. For example, sulfhydryl sites on cysteine and an amine site on histidine act simultaneously to bind trace metals such as Zn^{+2} within proteins (Klug et al., 1979; Pace and Weerapana, 2014). Additionally, cysteine and histidine residues in cysteine-rich proteins are important for binding Zn(II), Cd (II), and Ni(II) in eukaryotic intracellular processes (such as detoxification), demonstrating the potential for sulfhydryl and amine interactions when binding metal ions (Kulon et al., 2007; Lukács et al., 2021). Little is known about the complexation of Cr(VI) species with these amino acids, or with sulfhydryl and amine binding sites in general. It could be that both site types are involved simultaneously in forming bidentate Cr(VI) complexes, or that binding occurs only on the sulfhydryl site with the amine site facilitating the approach of anionic Cr(VI) to the sulfhydryl due to the positive electric field surrounding the amine site. For example, the reductase found in *Bacillus subtilis* (**nfrA2**) contains multiple amines in the ligand binding site from both histidine and arginine residues, and a sulfhydryl group from a cysteine residue outside of the ligand binding site (Morokutti et al., 2005). It is therefore possible that both amine and sulfhydryl binding sites in **nfrA2** must interact with chromate at some point during enzymatic Cr(VI) reduction. The exact mechanism of interaction between Cr(VI) and both amine and sulfhydryl binding sites on *B. subtilis* during Cr(VI) reduction remains unconstrained by our work. However, the observation that the presence of both blocker types yields the same result as the presence of either of the blocker types alone strongly suggests that both sites play a role in Cr(VI) binding simultaneously.

Blocking both sulfhydryl and amine sites does not fully stop Cr(VI) removal by *B. subtilis*, particularly at low pH (Fig. 5). The reactions between qBBr and sulfhydryl binding sites and sulfo-NHS acetate and amine binding sites are most favorable under circumneutral pH conditions (e.g. Kosower and Kosower, 1987). For example, Salvucci (1993) demonstrated that sulfo-NHS acetate causes inactivation of the enzyme **ATPase** within minutes at pH 7 by binding to an amine site in the active site of the enzyme, and blocking further binding of substrates to the active site. However, the low functionality of site blockers observed in our experiments under low pH conditions is not likely caused by these trends because biomass was treated with qBBr and/or sulfo-NHS acetate prior to division of the biomass suspension into separate experiments, and therefore the biomass in each pH experiment had the same extent of sites blocked prior to pH adjustment. It is possible that there is another type of binding site responsible for binding Cr(VI), but we propose that there are sulfhydryl and amine binding sites in the complex three-dimensional network of the cell surface that are not accessible to binding by the relatively large qBBr and sulfo-NHS acetate blocking molecules, and therefore remain unreacted in our blocking experiments. These unblocked sites appear to remain accessible to the smaller Cr(VI) anion and provide a means for Cr(VI) to bind to the surface even during our site-blocked experiments. Yu et al. (2018) observed similar Se(IV) removal behavior in response to qBBr blocking of sulfhydryl sites on the *B. subtilis* surface. In that study, qBBr treatment of the cells decreased Se (IV) removal under all pH conditions tested, but the effect was diminished under lower pH conditions. In both cases, the blockers used in this study appear to block most, but not all, cell surface sulfhydryl and amine sites, especially under near-neutral pH conditions. The ability of the unblocked sites to bind Cr(VI) or Se(IV) is minimal under near-neutral pH conditions but increases with decreasing pH.

The decrease in the effectiveness of the blocking molecules with decreasing pH likely results from a combined effect of the speciation of Cr(VI) in solution, the charge of the bacterial cell surface, and Cr(VI) reduction kinetics. Above pH 5.5–6.0, Cr(VI) exists in solution predominantly as CrO_4^{2-}, and at lower pH it exists as $HCrO_4^-$ (Ramsey et al., 2001). The cell surface has an overall negative charge that becomes more negative with increasing pH due to the deprotonation of binding sites such as phosphoryl and carboxyl sites on the cell surface. Because of these pH effects, electrostatic repulsion between the Cr(VI) anion and the negatively charged bacterial surface increases with increasing pH, making it more difficult for Cr(VI) to bind to sulfhydryl and/or amine binding sites on the cell surface and hence decreasing Cr(VI) removal with increasing pH (Fig. 2). Overall, these combined effects act to reduce the rate of Cr removal with increasing pH. At circumneutral pH, the relative inaccessibility of cell surface binding sites to anionic Cr(VI) species effectively makes the process a site-limited one, and blocking a

large majority of sulfhydryl and/or amine sites causes a significant decrease in the ability of *B. subtilis* to bind and reduce Cr(VI), even if a fraction of binding sites remain unblocked and accessible to Cr(VI) anions. Similarly, decreasing pH leads to a decrease in electrostatic repulsion between aqueous anionic Cr(VI) species and the cell surface as the overall negatively charged electric field surrounding the cell surface becomes weaker. The effect of changes in electrostatic favorability is to increase the rate and extent of Cr binding to the cell surface as it becomes more favorable for Cr(VI) to reach the cell surface. In addition to these electrostatic effects, the rate of electron transfer from the cell surface to bound Cr(VI) may occur faster under lower pH conditions (Yan and Chen, 2019), and therefore could contribute to the lack of effectiveness of the blocking molecules under low pH conditions. Since Cr(VI) binding and reduction occur much faster under low pH conditions, binding site availability is no longer a strong limiting factor at low pH, and even the small fraction of sites remaining unblocked after the blocking treatment allow for significant Cr(VI) removal to occur in our systems. These effects thereby reduce the overall effectiveness of the blocking molecules under the low pH conditions studied here.

Our results strongly suggest that Cr(VI) removal by *B. subtilis* is a multi-step process (Fig. 6). The first step in Cr(VI) removal is the binding of aqueous Cr(VI) anions to cell surface sulfhydryl and/or amine sites, likely followed by electron transfer from cell wall electron donors to the bound Cr(VI), reducing it to Cr(III). FTIR studies of the interaction of Cr (VI) with Cr(VI)-reducing bacterial cells have suggested that Cr(VI) binds with carboxyl and phosphoryl sites on the cell surface, but these studies have not constrained the valence state of Cr (e.g., Mangaiyarkarasi et al., 2011; Banerjee et al., 2019; Prabhakaran et al., 2019). Our results indicate that the initial binding of Cr(VI) onto the cell wall involves sulfhydryl and/or amine binding sites. Therefore, the FTIR results from previous studies suggest that there is a re-arrangement of Cr atoms on the cell surface after Cr(VI) reduction occurs such that the Cr^{3+} cation that is created from Cr(VI) reduction migrates to nearby anionic carboxyl and phosphoryl sites. These sites are plentiful on the cell surface, and may exhibit a higher affinity to bind Cr^{+3} than sulfhydryl or amine sites, especially if the positively charged amine site is in close proximity to the sulfhydryl site. Cr(III) has been found to bind to carboxyl and phosphoryl binding sites on exopolymeric substances (unfiltered exudates) from *Pseudomonas aeruginosa* (Kantar et al., 2011), and similar binding likely occurs on the bacterial surface. After the re-arrangement, the Cr either remains as adsorbed Cr^{3+} or precipitates as a Cr(III) (hydr)oxide on the cell surface (Rai et al., 1987). The migration of Cr^{3+} to anionic sites would free sulfhydryl and amine sites to bind additional Cr(VI) anions, and therefore the extent of Cr(VI) reduction does not necessarily directly reflect the concentration of Cr(VI) binding sites. Our results suggest that the overall role of sulfhydryl and amine sites in Cr(VI) reduction is to bind Cr(VI) such that it is in close enough proximity to cell wall electron donors for electron transfer to occur, and hence Cr(VI) binding to these sites represents the first step in the reduction process.

4. Conclusion

Bacterially mediated Cr(VI) reduction has been documented both in laboratory and in field studies (e.g. Kamaludeen et al., 2003; Cheung et al., 2006; Thacker et al., 2007; Liu et al., 2012), but the molecular-scale mechanisms involved in the reduction have been poorly constrained. First, the chromate anion CrO_4^{2-} must cross the diffuse layer to reach the cell surface. This step becomes increasingly unfavorable with increasing pH due to increased electrostatic repulsion between the negatively charged CrO_4^{2-} anion and the negatively charged electric field surrounding the cell surface, and these electrostatic effects lead to the decreased rate of Cr(VI) removal that we observed with increasing pH (Figs. 1, 2). Next, the chromate anion must be brought into close proximity to cell wall electron donors, which requires binding of Cr(VI) to binding sites located on and within the cell wall. Cr(VI) removal by *B. subtilis* is primarily dependent on the attachment of CrO_4^{2-} to the cell wall, evidenced by the low removal capability of isolated *B. subtilis*

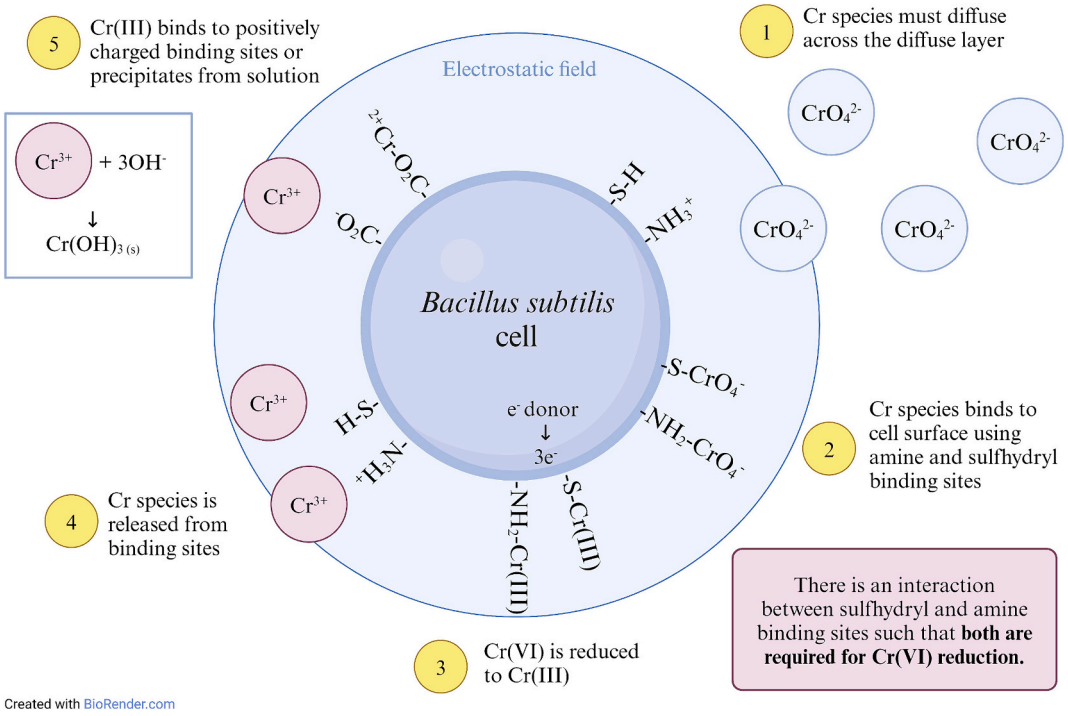

Fig. 6. The proposed mechanism of Cr(VI) removal from solution by *B. subtilis*: (1) anionic Cr(VI) species must first diffuse across an electrostatic barrier and towards the negatively charged cell surface; (2) the Cr(VI) species binds to cell surface sulfhydryl and/or amine sites; (3) Cr(VI) is reduced to Cr(III); (4) Cr(III) species are released from the site of reduction; and (5) Cr(III) is removed from the aqueous fraction by precipitation of Cr(III) (hydr)oxide or by attachment to negatively charged cell surface binding sites.

exudates, especially under circumneutral pH (Fig. 4). Our results demonstrate that both sulfhydryl and amine sites play an important role in the removal of Cr(VI) from solution, such that blocking *either* type of site significantly inhibits the capacity of Cr(VI) reduction to the same degree (Fig. 5). Our observation that blocking both sulfhydryl and amine sites at the same time yields a similar, and not an additive, effect to blocking only one type of site strongly suggests that sulfhydryl and amine sites work simultaneously to bind Cr(VI). If the sites bind Cr(VI) together, then blocking either type of site separately, or blocking both types of sites jointly, would yield similar effects. The mechanism of Cr (VI) removal, therefore, requires the attachment of Cr(VI) to both types of binding sites, likely as the initial step in the removal process. After Cr (VI) binds to cell wall sulfhydryl and amine binding sites, electrons are transferred from electron donors and Cr(VI) is reduced to Cr(III), which either either re-adsorbs to the cell surface by binding to the abundant negatively charged carboxyl and phosphoryl sites, or precipitates as a solid phase Cr(III) (hydr)oxide. The lack of full reversibility with an increase in pH (Fig. 3) suggests the presence of Cr(III) on the cell wall because increasing pH would not promote desorption of Cr^{3+} or the dissolution of solid phase Cr(III) (hydr)oxide. The relatively slow kinetics of Cr(VI) removal from solution compared to most solute adsorption reactions by bacteria suggests that the removal mechanism involves more than simple binding to the bacterial surface sites, and is consistent with a model of Cr(VI) removal from solution by a process that involves both Cr(VI) binding and reduction to Cr(III). Our results provide the first evidence that bacterial cell surface sulfhydryl and amine sites are involved in the initial binding of Cr(VI) and that these sites are involved simultaneously in binding Cr(VI) during bacterially-mediated Cr(VI) reduction. Furthermore, our findings suggest that the use of bacterial cells grown under conditions that promote enhanced concentrations of these site types on the cell wall (e.g., Yu and Fein, 2017a, 2017b) may yield faster and more complete Cr(VI) reduction in bioremediation approaches.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemgeo.2024.122299.

CRediT authorship contribution statement

Jessica C. Brown: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Quinn Mackay:** Investigation. **Qiang Yu:** Supervision, Methodology, Funding acquisition. **Jeremy B. Fein:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

Authors have no competing interests to declare.

Data availability

Data will be made available on request.

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