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Controls on the Concentration of Bacterial Cell Surface Sulfhydryl Sites: Effects of Inorganic Nutrients

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ABSTRACT

Understanding the controls on bacterial surface sulfhydryl site concentrations is crucial for modeling the effects of bacterial cells on the fate and transport of chalcophile metals in the environment. In this study, we investigate the effects of changing the concentrations of $MgSO_4$, NaCl and $NaNO_3$ in a growth medium on the concentration of cell surface sulfhydryl sites on the Gram-positive bacterial species *Bacillus subtilis*, as measured using a potentiometric titration approach. Our results show that the concentration of $MgSO_4$ in a M9 minimal medium exerts a strong influence on bacterial surface sulfhydryl site concentrations under low $MgSO_4$ concentrations but that this effect plateaus with increasing $MgSO_4$ concentrations, indicating that a minimum amount of a sulfur source is required for bacteria to produce cell surface sulfhydryl sites. Although the sulfhydryl site concentration was below the detection limit on biomass samples that were grown in a M9 minimal medium containing 0.1 mM $MgSO_4$, increasing the concentration of either NaCl or $NaNO_3$ in the medium yielded biomass samples with measurable sulfhydryl site concentrations on the cell surface. In contrast, adding $NaClO_4$ to the M9 medium did not increase cell surface sulfhydryl site concentrations. The NaCl and $NaNO_3$ effects likely arise because increasing the concentration of NaCl or $NaNO_3$ in a M9 minimal medium can promote the production of EPS by bacteria, and because the surface sulfhydryl sites of *B. subtilis* are located mostly on its EPS molecules. The results of this study suggest that bacterial nutrients and water chemistry composition are key controls on the abundance of bacterial surface sulfhydryl sites.

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Bacillus subtilis; bacterial sulfhydryl site; $MgSO_4$; NaCl; qBBr

Introduction

Metal adsorption onto bacteria can affect metal mobility and bioavailability (Beveridge and Murray 1976; Fein 2017; Mullen et al. 1989), and therefore plays an important role in controling the fate, transport, and influence of metals in aquatic ecosystems. A comprehensive understanding of metal adsorpton onto bacteria is crucial not only for understanding the global cycling of metals, but also for optimizing engineering applications such as the bioremediation of metal contaminated sites and the development of biosorbents for metal removal and recovery (Bailey et al. 1999; Mullen et al. 1989; Volesky and Holan 1995).

The adsorption of metals onto bacteria occurs through binding of metal ions onto various functional groups, such as carboxyl, phosphoryl, amino and sulfhydryl groups, on bacterial cell surfaces (Beveridge and Murray 1980; Jiang et al. 2004; Mishra et al. 2010; Ngwenya et al. 2003; Yu and Fein 2015). Under high metal loading conditions (metal:bacteria ratios >10 µmol of aqueous metal/gram of wet biomass), carboxyl and phosphoryl groups are the most important metal bindings sites on bacterial cell surfaces, largely because they are present on the surfaces at higher concentrations than other binding site types (Beveridge and Murray 1980; Jiang et al. 2004; Ngwenya et al. 2003). However, recent studies have demonstrated that sulfhydryl sites, a type of low abundance, high affinity site that is present on a wide range of bacterial cell surfaces, become the dominant binding sites for many chalcophile metals under low metal loading conditions (Guine et al. 2006; Mishra et al. 2010; Pokrovsky et al. 2012; Yu and Fein 2015). Here, chalcophile metals are defined as metals such as Hg, Cd, Zn, Pb and Au that have stronger interactions with sulfur than with oxygen, and most of these metals are of high environmental concern due to their high toxicity and occurrence as industrial and mining contaminants. The reported stability constant values of chalcophile metal-sulfhydryl complexes on bacterial cell surfaces are typically 2–3 orders of magnitude higher than corresponding stability constants for bacterial surface complexes between the metals and oxygen-bearing binding sites (Nell and Fein 2017; Yu and Fein 2015), e.g., carboxyl and phosphoryl sites. Therefore, although sulfhydryl sites typically accout for only ~10% of the total binding sites on bacterial cell surfaces (Yu and Fein 2017; Yu et al. 2014), they can control the adsorption of chalcophile metals onto bacteria under low metal loading conditions (Mishra et al. 2010; Nell and Fein 2017; Yu and Fein 2015). In addition to controlling adsorption, bacterial

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surface sulfhydryl sites can strongly affect other metal-bacteria interactions, such as metal reduction and detoxification (Yu et al. 2018, 2020). These results suggest that sulfhydryl sites have a stronger influence on the fate and transport of chalcophile metals in aquatic ecosystems via metal-bacteria interactions than other binding site types on the bacterial cell surface under environmentally-relevant metal loading conditions.

In order to assess the role of bacterial surface sulfhydryl sites in metal adsorption and other metal-bacterial interations, the concentration of bacterial surface sulfhydryl sites under different geological settings must be determined. While different bacterial species contain similar concentrations of surface sulfhydryl sites when these bacterial species are grown in a nutrient-rich trypticase soy broth (TSB) medium (Yu et al. 2014), the concentrations of cell surface sulfhydryl sites of these bacterial species vary significantly from one another when the cells are grown in a M9 minimal medium (Yu and Fein 2017). Sulfhydryl sites contain reduced S atoms, and hence cells must expend metabolic energy in order to create sulfhydryl sites from aqueous sulfate under aerobic growth conditions. Therefore, factors that influence the energy and nutrients that are available to bacterial cells during growth are likely to affect the ability of the cell to produce sulfhydryl binding sites. The concentration and identity of the electron donor that is present in the growth medium have been identified as factors that affect the concentration of cell surface sulfhydryl sites for several bacterial species (Butzen and Fein 2021; Yu and Fein 2017; Yu et al. 2020), however the effects of inorganic nutrient availability on the creation of cell surface sulfhydryl sites have not been examined.

The objective of this study was to examine the potential effects of the sulfur source and aqueous anions on bacterial surface sulfhydryl site concentrations, using *Bacillus subtilis* as a model bacterial species. We first tested the cells that were grown in a M9 minimal medium containing different concentrations of $MgSO_4$ in order to assess the effect of the sulfur source concentration on the sulfhydryl site concentration. We then varied the concentrations of chloride, nitrate or perchlorate in the growth media containing a low concentration of $MgSO_4$ in order to test the effects of these anions on the sulfhydryl site concentration. Cell surface sulfhydryl site concentrations were measured using a potentiometric titration approach (Yu and Fein 2017; Yu et al. 2014). Our results show that the concentrations of sulfate, chloride, and nitrate in a M9 minimal medium can strongly affect the concentration of cell surface sulfhydryl sites on *B. subtilis*.

Materials and methods

Bacterial cell preparation

Gram-positive *B. subtilis* cells were grown in a M9 minimal medium (Cold Spring Harbor Laboratory 2010) with concentration adjustments of certain components of interest. The standard M9 minimal medium consists of 6.78 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.01 g of $CaCl_2$, 0.012 g of $MgSO_4$, 10 mL of a vitamin solution (Table S1) and 10 mL of a trace element solution (Table S2) per 1 L of ultrapure 18 MΩ water, with solution pH adjusted to 7.3–7.4 using a 1 M NaOH solution. The procedures for growth, washing, and weighing of the bacterial cells were similar to those described previously (Yu et al. 2014). Briefly, bacteria were first cultured aerobically in 3 mL of a TSB medium containing 30 g/L of trypticase soy broth and 5 g/L of yeast extract at 32 °C for 24 h. This 'seed' culture was then transferred to 1 L of a M9 minimal medium in a 2 L flask, and was cultured aerobically in an incubator shaker at a speed of 100 rpm at 32 °C for 48 h in order for the bacterial cell suspensions to reach early stationary phase, as determined by bacterial growth curves (data not shown). $MgSO_4$ is the primary sulfur source in the M9 medium, and a concentration of 0.1 mM $MgSO_4$ was used in most experiments unless otherwise specified. The experiments that determined the effect of sulfate concentration on the production of sulfhydryl sites were conducted by varying the concentration of $MgSO_4$ in the medium from 0.1 to 5 mM. The experiments that tested the NaCl effect were conducted by varying the NaCl concentration from 8.5 to 171 mM (0.5 to 10 g/L), and a comparative experiment for different anions was conducted by adding 77 mM of NaCl, $NaNO_3$ or $NaClO_4$ to the standard M9 medium. The experiments that tested the glucose effect were conducted in the presence of 27.8 and 278 mM (5 and 50 g/L) of glucose.

After incubation, the bacterial cells were harvested by centrifugation at 10,970 g for 5 min. The biomass pellets were rinsed with a 0.1 M NaCl solution, followed by centrifugation at 8,100 g for 5 min, and the same process was repeated three times. The biomass pellets were then transferred into pre-weighed test tubes and centrifuged for two 30-min intervals at 8,100 g. After decanting the supernatant, the wet weight of the cells was used to calculate the bacterial concentrations in the subsequent experiments, and the bacterial concentrations that are reported in this study are expressed in terms of these wet weights. The ratio of wet mass to dry mass is 4.7 for *B. subtilis* biomass (Yu et al. 2014).

Determination of concentrations of sulfhydryl sites

The approach used to determine the concentrations of bacterial surface sulfhydryl sites was the same procedure that we developed and described in previous studies (Yu and Fein 2017; Yu et al. 2014). In this approach, we used monobromo(trimethylammonio)bimane bromide (qBBr) to selectively block the surface sulfhydryl sites on bacterial cells, and we used potentiometric titrations and surface complexation modeling to determine the concentration of total surface binding sites on bacterial biomass samples before and after the qBBr treatment. qBBr effectively blocks the protonation ability of sulfhydryl sites, but does not react with other binding sites such as carboxyl or phosphoryl groups, and qBBr itself is not proton-active (Joe-Wong et al. 2012; Yu et al. 2014). Therefore, the concentration of bacterial surface sulfhydryl sites can be determined by measuring the

decrease in the concentration of total surface binding sites after the qBBr treatment. We used the Student's T-test to determine if the concentration of total binding sites decreased significantly after qBBr treatment. A p value $<$ 0.05 indicates a significant difference between the signals from the biomass samples with and without qBBr treatment, and this difference represents the concentration of sulfhydryl sites on the biomass sample. Otherwise, a p value $>$0.05 suggests that the concentration of sulfhydryl sites is too low to be determined by this method.

qBBr was purchased from Toronto Research Chemical, Inc. In order to block sulfhydryl sites, the bacterial pellets were suspended 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, and the mixture was allowed to react for 2 h at room temperature under continuous shaking on a rotating plate at 60 rpm. Potentiometric titrations of cells with and without qBBr treatment were conducted using an autotitrator assembly with ~10 mL of a 0.1 M NaCl cell suspension containing 30 g (wet mass) of cell per liter. Prior to each titration, the 0.1 M NaCl solution was purged with N_2 for at least 1 h in order to remove dissolved CO_2. All the titrations were conducted in a closed vessel under a N_2 headspace, and each suspension was stirred continuously with a magnetic stir bar. For each titration, two steps were conducted: (1) acidifying the bacterial suspension from the original pH down to pH 3.0 by adding 1 M HCl standard; and (2) a forward titration from pH 3.0 to pH 9.7 by adding 1 M NaOH standard. The 1 M HCl and 1 M NaOH standards were purchased from Honeywell Research Chemicals. Only these forward titration data were used for the surface complexation modeling to calculate the total sulfhydryl site concentrations using a four-site non-electrostatic surface complexation model and FITEQL 2.0 (Westall 1982). All titrations were conducted in triplicate. The site concentrations that we report are the average values of the three titrations, and the uncertainties that we report represent one standard deviation from the average.

Modeling of trace metal speciation

The speciation of Zn and Cu in a M9 minimal medium with varied concentrations of NaCl, $NaNO_3$ and $NaClO_4$ was calculated using the program Visual MINTEQ 3.1 (Gustafsson 2013) and the results are presented in Table S3.

Results

Changing the $MgSO_4$ concentration in the M9 minimal medium affects the early stage of growth for *B. subtilis* cells under lower $MgSO_4$ concentration conditions, with the growth curves exhibiting longer lag phases (data not shown). However, varying the concentration of $MgSO_4$ in the growth medium from 0.1 to 5 mM does not change the 48 h biomass yield in terms of weight or the concentration of total proton-active binding sites on the *B. subtilis* cell surface ($p > 0.05$ for the Student's t-test of any two total site concentrations). Although the concentration of $MgSO_4$ does not affect total site concentrations on the biomass, it dramatically affects the concentration of the sulfhydryl sites on the cell surface (Figure 1 and Table S4). These results suggest that although the concentration of surface sulfhydryl sites on the *B. subtilis* surface is affected by the concentration of sulfate in the growth medium, the growth of the cells is not inhibited at any of the three sulfate concentrations studied. When *B. subtilis* cells were grown in the presence of 0.1 mM $MgSO_4$, the measured total site concentrations before and after qBBr treatment for blocking sulfhydryl sites was 364 ± 31 and 363 ± 29 µmol/g, respectively, indicating that the bacterial cells that were grown under this condition have a cell surface sulfhydryl site concentration that is below the detection limit of the potentiometric titration approach. In contrast, the *B. subtilis* cells that were grown in the presence of 0.8 mM $MgSO_4$ have 26 ± 13 µmol/g of sulfhydryl sites on their cell surface. A further increase of the $MgSO_4$ content from 0.8 to 5.0 mM slightly increases the cell surface sulfhydryl site concentration to 35 ± 16 µmol/g, but the Student's t-test shows that the increase from 0.8 to 5.0 mM $MgSO_4$ does not yield a change in the cell surface sulfhydryl site concentration that is statistically significant ($p > 0.05$).

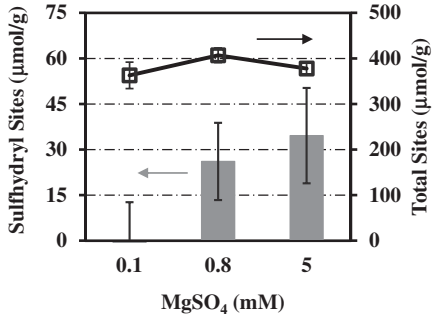

Figure 1. The effect of $MgSO_4$ concentration in a M9 minimal medium on the concentration of sulfhydryl sites (bars) and total sites (open squares) on the *B. subtilis* surface. The concentrations of NaCl and glucose in the medium were 8.5 mM and 27.8 mM, respectively.

The concentration of NaCl in a M9 minimal medium, like the $MgSO_4$ concentration, strongly influences the surface sulfhydryl site concentration of *B. subtilis*. Although the concentration of sulfhydryl sites was below the detection limit for *B. subtilis* cells that were grown in a M9 minimal medium containing 0.1 mM $MgSO_4$ and 8.5 mM NaCl, increasing the NaCl concentration to 34 mM yielded biomass samples containing 48 ± 19 µmol/g of cell surface sulfhydryl sites (Figure 2 and Table S4). However, increasing the NaCl concentration further, to 86 mM or 171 mM in the growth medium, did not cause a further significant increase in sulfhydryl site concentration, yielding biomass samples containing 55 ± 8 and 40 ± 12 µmol/g of cell surface sulfhydryl sites, respectively (Figure 2). The Student's t-test indicates that the increase in NaCl concentration from 8.5 to 34 mM yielded a change in cell surface sulfhydryl site concentration that is statistically significant ($p < 0.05$), but the increase from 34 to 171 mM NaCl did not ($p > 0.05$).

It is possible that the NaCl effect arises from aqueous chloride complexation, and a subsequent decrease in

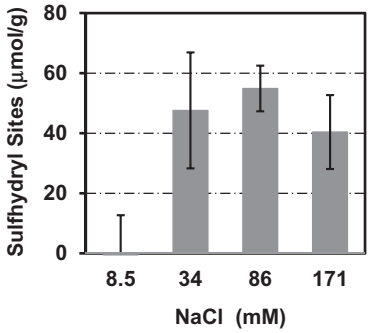

Figure 2. The effect of NaCl concentration in a M9 minimal medium on the concentration of sulfhydryl sites on *B. subtilis* biomass samples. The concentrations of glucose and $MgSO_4$ in the medium were 27.8 mM and 0.1 mM, respectively.

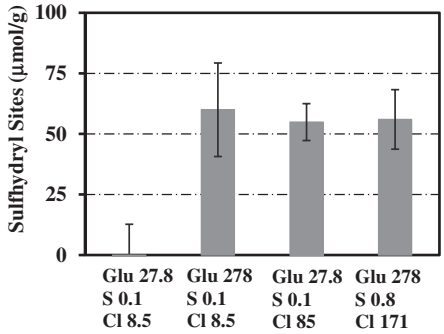

Figure 4. The combined effect of glucose (Glu), $MgSO_4$ (S) and NaCl (Cl) in a M9 minimal medium on the concentration of sulfhydryl sites on *B. subtilis* biomass samples. The concentrations of glucose, $MgSO_4$ and NaCl shown on the horizontal axis is mM.

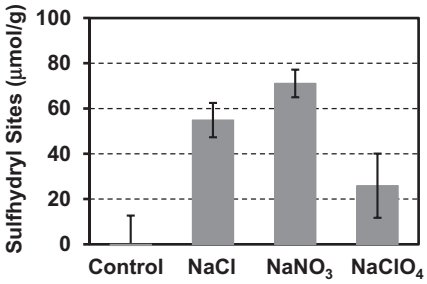

Figure 3. The effect of adding aqueous anions (77 mM) to a M9 minimal medium on the concentration of sulfhydryl sites on *B. subtilis* biomass samples. The M9 medium contains 8.5 mM of NaCl, 0.1 mM of $MgSO_4$ and 27.8 mM of glucose. No additional aqueous anion was added to the M9 medium in the control experiment.

bioavailability (Babich and Stotzky 1978) of essential trace elements in the growth medium, and that the cells respond to this decrease in bioavailability by producing a higher concentration of sulfhydryl sites to compete for binding of the trace elements. In order to test this hypothesis, we measured cell surface sulfhydryl site concentrations after replacing NaCl in the growth medium with either $NaNO_3$ or $NaClO_4$. NO_3^- is similar to Cl^- in its ability to form aqueous complexes with trace metals in the growth medium, such as Zn^{2+} and Cu^{2+}, while ClO_4^- does not form these complexes to a significant extent (Powell et al. 2013; Smith and Martell 1976). Consistent with our hypothesis, the influences of $NaNO_3$ and $NaClO_4$ on bacterial surface sulfhydryl site concentrations are significantly different from each other (Figure 3 and Table S5). The addition of $NaNO_3$ to the growth medium had a similarly strong effect to what we observed for NaCl on the surface sulfhydryl sites of *B. subtilis*, yielding biomass samples containing 71 ± 6 µmol/g of surface sulfhydryl sites. In contrast, the *B. subtilis* biomass samples that were grown in the presence of $NaClO_4$ contain only 26 ± 14 µmol/g of sulfhydryl sites on their cell surface, and this concentration is not statistically different from the sulfhydryl site concentration of the cells in the control experiment ($p > 0.05$). However, calculations of the speciation of the trace metals in the M9 medium suggest that >99% of Zn and Cu in our experiments were present in solution as aqueous complexes with NTA, and that adding 77 mM of NaCl, $NaNO_3$ or $NaClO_4$ to the M9 medium did not significantly change the aqueous speciation of Zn or Cu (Table S3). Therefore, the observed effects of NaCl and $NaNO_3$ on bacterial surface sulfhydryl sites cannot be explained by forming complexes between trace metals and Cl^-/NO_3^-. Moreover, because we added the same molar concentration of Na salt to the medium in each of these experiments, the ionic strengths of the solutions in these NaCl, $NaNO_3$, and $NaClO_4$ experiments did not vary, and were higher than that of the control experiment. Hence, ionic strength effects cannot explain the enhanced concentration of sulfhydryl sites that we measured in the NaCl and $NaNO_3$ experiments relative to the control and the $NaClO_4$ experiments. Rather, the difference must arise from differences in the properties and behaviors of the individual aqueous anions or their effects on the cell properties.

A previous study (Yu and Fein 2017), conducted with a relatively high concentration of $MgSO_4$ in the growth medium (~2 mM), documented that the concentration of glucose in the growth medium exerts a strong effect on bacterial surface sulfhydryl sites. Here, we find that the concentration of glucose also has a strong effect on bacterial surface sulfhydryl site concentrations when the cells are grown in a medium with a much lower sulfate concentration (0.1 mM, Figure 4 and Table S4). Increasing the glucose concentration from 27.8 to 278 mM did not affect the growth curve of the bacteria (data not shown), but led to a significant increase in the concentration of sulfhydryl sites on the *B. subtilis* biomass samples, from an undetectable concentration on the cells from the growth medium with 27.8 mM glucose to 60 ± 10 µmol/g on the cells from the medium with 278 mM glucose. The biomass samples that were grown in a standard M9 minimal medium with either an elevated concentration of glucose (the second bar in Figure 4) or an elevated concentration of NaCl (the third bar in Figure 4) contained similar concentrations of bacterial surface sulfhydryl sites, indicating that glucose and NaCl have similarly strong effects on the concentration of bacterial surface sulfhydryl sites at the concentrations studied here. However, the glucose and NaCl effects are not additive or synergistic. As shown in Figure 4, the biomass samples that were grown in the presence of elevated concentrations of glucose, NaCl, and $MgSO_4$ together (278 mM glucose, 171 mM NaCl and 0.8 mM $MgSO_4$) contained a similar sulfhydryl site concentration to the biomass samples that were

grown in a M9 medium with only the concentration of glucose or NaCl increased.

Discussion

Our results suggest that *B. subtilis* bacterial cells require a minimum concentration of between 0.1 and 0.8 mM $MgSO_4$ in their growth medium in order to produce cell surface sulfhydryl sites under aerobic conditions, but $MgSO_4$ concentrations above this minimum concentration do not lead to higher surface sulfhydryl site concentrations (Figure 1). In a standard M9 minimal medium, $MgSO_4$ is the major sulfur source for bacterial growth and its concentration is set as 2 mM. A previous study found that ~0.1 mM of sulfate was required for preventing the exhaustion of available sulfur during the growth of *P. putida* in a minimal medium (Aderhold et al. 1996). In this study, we did not observe a lower yield of biomass in our 0.1 mM $MgSO_4$ experiments compared to the biomass that grew in the other two experiments in the presence of much higher concentrations of $MgSO_4$. Hence, our results suggest that the minimum sulfate concentration that is required for cell growth is lower than the minimum sulfate concentration that is required for the production of cell surface sulfhydryl sites. The 0.1 mM $MgSO_4$ in a M9 medium represents a condition under which the available sulfur is sufficient for the growth of *B. subtilis*, but is below the minimum sulfate concentration required for production of cell surface sulfhydryl sites, thus bacterial cells prioritize the use of sulfate for synthesizing intracellular sulfhydryl-containing proteins that are more essential for bacterial metabolism and growth than cell surface sulfhydryl sites. In contrast, the 0.8 and 5 mM $MgSO_4$ concentrations that we studied represent conditions under which the available sulfur in the growth medium is sufficient to support the synthesis of both intracellular and cell surface sulfhydryl sites. As a result, the surface sulfhydryl sites of the *B. subtilis* biomass samples increase significantly from the 0.1 mM $MgSO_4$ experiment to the 0.8 mM $MgSO_4$ experiment, but no further increase was observed when the $MgSO_4$ concentration was increased from 0.8 to 5 mM.

Under 0.1 mM $MgSO_4$ conditions, growing cells in a medium containing either an elevated concentration of glucose (278 mM, Figure 4), an elevated concentration of NaCl (34–171 mM, Figure 2) or 77 mM $NaNO_3$ (Figure 3) led to similarly enhanced concentrations of cell surface sulfhydryl sites. However, the enhancement mechanisms for these cases are likely quite different from each other. The concentration of glucose in the growth medium exerts a strong influence on the concentration of cell surface sulfhydryl sites that are produced by several bacterial species, including *B. subtilis* (Yu and Fein 2017). In this study, adding additional glucose to a M9 minimal medium led to an increase in cell surface sulfhydryl sites likely due to the extra energy that was available to the cells for the conversion of oxidized sulfur in sulfate to reduced sulfur in the sulfhydryl sites, thereby allowing *B. subtilis* cells to utilize a bigger proportion of the available sulfate under sulfur-limiting conditions. It should be noted that the glucose additions that were tested in this study did not yield a faster growth rate or a larger biomass yield, suggesting that all of the glucose concentrations tested were sufficient to yield maximal cell growth and that maximal production of sulfhydryl sites occurs at a higher glucose concentration than that necessary for maximal cell biomass yield. Under the aerobic conditions of these experiments, the cells must balance the energy for sulfate reduction that is essential for synthesis of sulfhydryl sites with the benefit that added sulfhydryl sites provide the cell. If energy sources are not limited, as in the case when glucose concentrations were elevated in our experiments, the cells have the energy needed to produce higher concentrations of surface sulfhydryl sites.

Unlike glucose, NaCl and $NaNO_3$ do not provide energy to the cells for reducing sulfate in order to synthesize sulfhydryl sites. Because adding 77 mM of NaCl or $NaNO_3$ to the M9 medium does not significantly affect the aqueous speciation of Zn or Cu under the experimental conditions (Table S3), the enhanced concentration of sulfhydryl sites that we observed for cells grown in the presence of 77 mM of NaCl or $NaNO_3$ cannot be ascribed to the formation of aqueous complexes between Zn/Cu and Cl^-/$NO3^-$ in our experiments. Sulfhydryl sites may serve to enhance the bioavailability of trace metals to bacteria, and the concentration of sulfhydryl sites on cell surfaces may respond to changes in trace metal aqueous speciation. However, our experiments did not probe conditions of different trace metal speciation in solution, and hence our results cannot be used to test these hypotheses. Therefore, the NaCl and $NaNO_3$ effects that we observed on the sulfhydryl site concentrations must arise from a different driving mechanism.

Most sulfhydryl sites on the cell surface of *B. subtilis* are located on extracellular polymeric substance (EPS) molecules secreted by and subsequently attached to the cells (Butzen and Fein 2021; Johnson and Fein 2019). Furthermore, increasing the NaCl concentration in the growth medium can enhance the production of bacterial EPS (Ozturk and Aslim 2010; Zou et al. 2006). Therefore, the elevated sulfhydryl site concentration that we observed on cells grown in a M9 medium with 34 mM NaCl relative to that on cells grown with only 8.5 mM NaCl likely stems from the enhanced production of EPS by *B. subtilis* in the presence of a higher concentration of NaCl in the growth medium. Adding $NaNO_3$ to the growth medium yields a similar increase in the cell surface sulfhydryl site concentration to the NaCl effect. In a standard M9 medium, NH_4Cl is the major nitrogen source for bacterial growth. Because $NaNO_3$ is a preferred nitrogen source over NH_4Cl for promoting the production of EPS (Han et al. 2017; Lupi et al. 1994), the addition of $NaNO_3$ to the M9 medium likely also promoted the production of EPS by *B. subtilis* in our experiments, and thereby led to an increase in the concentration of sulfhydryl sites on the cell surface.

Although increasing the concentration of NaCl in the growth medium from 8.5 mM to 34 mM leads to a significant increase in the concentration of cell surface sulfhydryl sites on *B. subtilis*, increasing the NaCl concentration from 34 to 171 mM does not lead to any further increase in the

concentration of sulfhydryl sites. In addition, when we increased the concentration of glucose or $NaNO_3$ in the medium, the resulting biomass samples had a concentration of sulfhydryl sites similar to that of the biomass samples that were grown in the presence of 34–171 mM of NaCl, ranging from 50 to 70 µmol/g. Because we increased the concentration of only one component at a time in the medium in these experiments, we further tested whether these factors exhibit a synergistic effect on each other. We grew *B. subtilis* cells in a medium with elevated concentrations of glucose, NaCl and $MgSO_4$ together, but this approach also did not further increase the concentration of cell surface sulfhydryl sites (Figure 4). These results suggest that a maximum possible concentration of sulfhydryl sites exists that the *B. subtilis* biomass samples can host, and that this concentration limit likely was achieved in the experiments with elevated NaCl, $NaNO_3$, and/or glucose. Most sulfhydryl sites on the cell surface of *B. subtilis* are located on extracellular polymeric substance (EPS) molecules secreted by the cells (Butzen and Fein 2021; Johnson and Fein 2019). Note that our wash procedure removes all loosely-held EPS molecules from the bacterial cells, and only tightly-held EPS molecules remain attached to cells in our biomass samples. Hence, the maximum concentration of surface sulfhydryl sites of *B. subtilis* is likely limited by the amount of EPS molecules that can be tightly attached to the bacterial cells. Once the attached EPS molecules on the cells reach the maximum load that the cells can hold, increasing the concentrations of different medium components does not affect the surface sulfhydryl sites of the *B. subtilis* biomass samples. The effects of $NaClO_4$ concentration on EPS production by bacterial cells have not been studied, but our results suggest that the presence of $NaClO_4$ does not lead to as much production of EPS as do equivalent concentration of NaCl or $NaNO_3$.

Because bacterial surface sulfhydryl sites can dominate bacterial adsorption of chalcophile metals under environmentally-relevant metal-loading conditions (Mishra et al. 2010; Yu and Fein 2015), it is crucial to determine the effects of environmental parameters on the concentration of these binding sites. This study is the first to show that the concentrations of the sulfur source and of inorganic aqueous anions in the growth medium can affect the concentration of bacterial surface sulfhydryl sites. Along with the strong effect of glucose on bacterial surface sulfhydryl sites that was observed in a previous study (Yu and Fein 2017) and in this study, these results clearly indicate that bacterial nutrients and water chemistry composition are key controls on the abundance of bacterial surface sulfhydryl sites. This study also raises an important question about the limitations of using planktonic cell samples to study the controls on bacterial surface sulfhydryl sites when most of the sulfhydryl sites for some bacterial species are located on EPS molecules. Because natural bacterial consortia and biofilms typically contain more EPS than the planktonic cells studied here, the controls that were determined in this study may exert even stronger effects on bacterial surface sulfhydryl sites in natural settings. Therefore, it is crucial to expand the study of isolated bacterial species to bacterial consortia and biofilms in order to understand the controls on bacterial surface sulfhydryl sites in natural and engineered bacteria-bearing systems. A better understanding of these controls will yield more accurate predictions of the effect of bacterial cells on the fate and transport of chalcophile metals in the environment and in engineered bioremediation systems.

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