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Electron Donor Effects on Bacterial Surface Sulfhydryl Site Concentrations

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ABSTRACT

In this study, we examined two effects on bacterial surface sulfhydryl site concentrations and distributions: (1) the effect of glucose concentration on the distribution of sulfhydryl sites between the bacterial cell surface and surface-associated extracellular polymeric substance (EPS) molecules, and (2) the effect of electron donor identity and concentration on sulfhydryl site concentrations on bacterial biomass. In each set of experiments, the total site concentration was measured using a potentiometric titration approach, and sulfhydryl site concentrations were determined using a site-specific blocking technique. The measurements were conducted with and without the removal of cellular EPS. For the first set of experiments, our results indicate that the two Gram-positive bacterial species, *Bacillus subtilis*, and *Bacillus licheniformis*, and one of the Gram-negative bacterial species studied, *Pseudomonas putida*, each have a greater concentration of sulfhydryl sites on their EPS molecules than is present on their cell surfaces, possibly serving to sequester toxic metals away from the cell surface. Conversely, for the Gram-negative bacterial species *Shewanella oneidensis*, the concentration of sulfhydryl sites on the cell surface is greater than that on its EPS molecules. *S. oneidensis* can gain metabolic energy through metal reduction, and hence enhancing the extent of metal binding to the cell wall through the formation of sulfhydryl binding sites may be more beneficial than the risk of metal toxicity. In the second set of experiments, increasing the concentration of two electron donors, pyruvate and glucose, in the growth medium of *B. subtilis* led to an increase in the percentage of total sites represented by sulfhydryl sites, but the concentration of the two other electron donors, glycerol and fumarate, had no effect on the percentage of sulfhydryl sites. Our results indicate that both the identity and the concentration of electron donors significantly influence the formation of sulfhydryl binding sites on bacterial cell surfaces. In addition, our results suggest that the total energy availability of a specific electron donor-bacterial species pairing affects both the ability of bacterial cells to produce sulfhydryl binding sites, and the distribution of those sites between the cell surface and its associated EPS molecules.

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Bacterial sulfhydryl sites; electron donor; extracellular polymeric substances; qBBr

Introduction

Bacteria are ubiquitous in near-surface environments, and bacterial adsorption can affect the transport and bioavailability of a range of heavy metal contaminants (Beveridge and Murray 1976; Beveridge 1989; Fein 2017). Bacterial metal adsorption occurs onto proton-active organic acid functional groups located within the cell envelope and on surface-attached extracellular polymeric substance (EPS) molecules (Fein et al. 2019), and the most important site types are carboxyl, phosphoryl, hydroxyl, amine, and sulfhydryl (Beveridge and Murray 1980; Guiné et al. 2006; Yu et al. 2014). Although carboxyl and phosphoryl groups control heavy metal binding under high metal-loading conditions (e.g., Boyanov et al. 2003; Fang et al. 2009; Ueshima et al. 2008), sulfhydryl sites dominate metal binding under more environmentally-relevant low metal loadings (Mishra et al. 2010; Yu and Fein 2015). Sulfhydryl sites are typically present on bacterial surfaces at lower concentrations than other binding site types (Yu et al. 2014), but they exhibit much higher affinities for binding chalcophile metals (Nell and Fein 2017; Yu and Fein 2015). Furthermore, the distribution of sulfhydryl sites, between being located on the cell wall and on extracellular polymeric substance (EPS) molecules that are bound to the cell, varies between bacterial species (Yu and Fein 2016) and may be controlled by a range of factors. In some species, sulfhydryl sites on the cell wall may facilitate metal acquisition by the cell; in other species, it may be advantageous for sulfhydryl sites to be located on EPS molecules in order to decrease metal bioavailability to the cell and to decrease the potentially toxic effects of the metal (Yu et al. 2020). Because sulfhydryl sites can control the bacterial adsorption and bioavailability of chalcophile elements, it is crucial to elucidate the factors that control the concentration and distribution of these sites.

In oxygenated environments, because the thermodynamically stable form of sulfur is oxidized sulfur (e.g., sulfate), sulfhydryl site concentrations on the cell surface are limited likely due to energy requirements of the cell to produce

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reduced sulfur. Increasing the concentration of glucose in the growth medium yields higher concentrations of sulfhydryl sites within the cell envelope of some bacterial species, but has no significant effect on others (Yu and Fein 2017), supporting the conclusion that energy supply limits sulfhydryl site production at the cell surface and that different electron donors may exert different effects between bacterial species. Here, we examine both of these possibilities, measuring the effect of glucose concentration in the growth medium of two Gram-positive and two Gram-negative bacterial species on the distribution of sulfhydryl sites between the cell walls and cell-associated EPS molecules. In addition, we varied the electron donor used in *Bacillus subtilis* growth media to determine if electron donor identity affects the concentration and/or the distribution of sulfhydryl sites between the cell wall and EPS molecules.

Materials and methods

Bacterial growth

For the glucose experiments, we measured the concentration of sulfhydryl sites on two Gram-positive bacterial species, *B. subtilis* and *Bacillus licheniformis*, and two Gram-negative bacterial species, *Pseudomonas putida* and *Shewanella oneidensis*, each grown with two different concentrations of glucose. The cells in each case were first cultured in a trypticase soy broth (TSB) medium with 0.5% yeast extract and ultimately grown in an M9 minimal medium with varying extents of glucose amendment . Each species was initially transferred from an agar growth plate by a loop to 3 mL of a TSB growth medium consisting of 5 g/L Bacto^{TM} yeast extract and 30 g/L BBL^{TM} trypticase soy broth. After 24 h of growth at 32 °C, the solution was transferred to a 4 L flask containing 1.5 L of an M9 minimal medium with either 25 g/L or 50 g/L glucose, and incubated at 32 °C with shaking at 100 rpm. The concentration of glucose in the growth medium has been shown to affect the concentration of sulfhydryl site within bacterial biomass (Yu and Fein 2017), but the distribution of those sulfhydryl sites between bacterial cells and associated EPS molecules has not been measured. *B. subtilis*, *B. licheniformis*, and *P. putida* cells were incubated for 48 h, and *S. oneidensis* cells were incubated for 72 h for the bacterial populations to reach early stationary growth phase, as determined by growth curve measurements (Yu and Fein 2017). The M9 medium consisted 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 $CaCl_2$, and 0.24 g of $MgSO_4$ per 1 L of ultrapure 18 MΩ water (M9 minimal medium (standard) 2010). In addition, 10 mL of a vitamin solution (Table S1) and 10 mL of a trace element solution (Table S2) per 1 L medium were added in order to promote growth (e.g., Lovley and Phillips 1988; Yu and Fein 2017). After the glucose was added to each, the pH values of the growth media were adjusted to 7.3–7.4 using aliquots of 1 M NaOH, and the media were filter sterilized before adding bacterial cells.

When early stationary phase was reached, the bacterial cells were harvested by centrifugation for 5 min at 10,970 g, after which the supernatant was discarded, and the pelleted biomass was transferred to centrifuge tubes. The biomass was washed three times by suspending the biomass in 0.1 M NaCl, centrifuging for 5 min, and then discarding the supernatant before resuspending in fresh 0.1 M NaCl. After washing, the biomass was centrifuged twice at 8100 g for 30 min to remove any excess liquid, pouring any supernatant off between runs. All bacteria masses reported in this study correspond to this wet biomass weight. The biomass was then set aside for EPS removal, except for a portion of *B. licheniformis* which was retained for potentiometric titrations of biomass with intact EPS. Site concentrations for *B. subtilis*, *S. oneidensis*, and *P. putida* with intact EPS were previously determined by Yu and Fein (2017).

For the experiments in which the electron donor identity and concentration was varied, we measured the concentration and distribution of sulfhydryl sites on *B. subtilis* biomass only, grown with three different carbon sources other than glucose. The four electron donors examined (including glucose from the previous set of experiments) yield different amounts of energy for cellular metabolism, as they enter the glycolysis-TCA cycle at different stages. For these experiments, *B. subtilis* cells were grown and harvested as above; however, the growth media contained different electron donors in place of glucose, and three different concentrations were tested for each electron donor. Either sodium pyruvate, sodium fumarate, or glycerol was added to the M9 growth medium to achieve carbon concentrations of 0.3, 0.8, or 1.7 mol-C/L. Conversion of these values to g/L is accomplished using the mol of C per gram of each electron donor, *e.g.,* 0.03 mol-C/g for glucose. These electron donor concentrations represent equivalent C concentrations to the glucose concentrations of 10 g/L (0.3 mol-C/L), 25 g/L (0.8 mol-C/L), and 50 g/L (1.7 mol-C/L) which were used by Yu and Fein (2017) in their study of the effect of glucose concentration on the total sulfhydryl site concentrations in the produced biomass. The *B. subtilis* cells for these experiments were incubated at 32 °C with 100 rpm shaking for 48 h, 72 h, or 96 h for the glycerol, sodium fumarate, and sodium pyruvate systems, respectively, in order for the bacterial cells to reach early stationary growth phase as determined by bacterial growth curve measurements (Figure S1). Half of the resulting biomass in each case was then treated with qBBr (see below) for determination of sulfhydryl site concentrations.

EPS removal

We determined the concentration of sites located on EPS molecules by measuring total proton-active site concentrations with and without EPS removal from the biomass. A cation exchange resin was used to remove the EPS for the glucose experiments. A previous study by Yu and Fein (2016) confirmed the removal of EPS material from biomass using this same procedure, with no significant cell damage after resin treatment as measured via LIVE/DEAD staining and SEM imaging. AMBERLITE® HPR1100 sodium ion exchange resin, 20–50 mesh (purchased from Sigma-Aldrich) was added to a beaker to achieve a 30 g resin to 1 g biomass ratio and was washed three times with ultrapure

water and once with 0.1 M NaCl. The biomass was then transferred to the beaker containing the resin, and the system was topped off with 0.1 M NaCl solution to yield a resin to solution ratio of 1 g to 1 g. The system was stirred for 2 h using a Teflon-coated magnetic stir bar, after which the system was allowed to sit undisturbed to allow the resin beads, with bound EPS, to settle. The biomass was harvested by pouring the liquid into centrifuge tubes, removing any resin beads which may have been poured with the biomass. After centrifuging for 5 min at 8100 g, the supernatant was decanted, and the system was washed three times with 0.1 M NaCl, centrifuging for 5 min at 8100 g and decanting the supernatant after each resuspension. The system was then centrifuged at 8100 g for 30 min twice to remove excess liquid which was decanted. After EPS removal, half of the biomass was transferred to a separate centrifuge tube for potentiometric titrations, while the other half was treated using qBBr (see below) prior to potentiometric titrations in order to determine the sulfhydryl site concentration of the sample.

qBBr treatment

In this study, we measured the concentration of sulfhydryl binding sites on biomass samples indirectly, using a technique developed by Yu et al. (2014) which involves the treatment of the biomass by monobromo(trimethylammonio)bimane bromide (qBBr), a thiol-specific labeling molecule. Control experiments have shown that qBBr effectively blocks the protonation of sulfhydryl sites on molecules, and does not interact with non-sulfhydryl sites (Yu et al. 2014). A detailed description of the interaction between qBBr and sulfhydryl sites can be found in Yu et al. (2014). In brief, when exposed to qBBr, the sulfur group in sulfhydryl sites forms a covalent bond with the qBBr molecule in place of the bromine. In this way, the covalently bonded sulfhydryl site becomes inert to protonation, as confirmed in control experiments (Yu et al. 2014). Since qBBr itself does not protonate or deprotonate, the concentration of sulfhydryl sites can be determined by calculating the difference in total measured site concentrations between qBBr-treated and untreated biomass.

The washed biomass was treated with a qBBr to biomass ratio of 100 µmol to 1 g. At the glucose concentrations used here, cell sulfhydryl site concentrations range from 0–93 ± 8 µmol/g (Yu and Fein 2017), and hence the qBBr to biomass ratio used here is sufficient to saturate all sulfhydryl sites present. The qBBr was transferred to a Teflon bottle and 0.1 M NaCl solution, buffered to pH 7.0 ± 0.1 using a 1.8 mM Na_2HPO_4/18.2 mM NaH_2PO_4 buffer solution, was added to achieve a qBBr to electrolyte solution ratio of 1 mg to 1 g. The bacteria were then transferred to and suspended in the bottle. The bottle was capped and covered in tin foil, and rotated at 20 rpm for 2 h, adequate time for the qBBr binding onto sulfhydryl sites to occur (Yu et al. 2014). The solution was then centrifuged at 8100 g for 5 min, the supernatant was decanted, and the biomass was washed and dried as described above.

Potentiometric titrations

We measured the total proton-active site concentrations and acidity constants for each type of biomass grown using potentiometric titrations coupled with surface complexation modeling. The concentration of sulfhydryl sites within the biomass was calculated as the difference in the total site concentrations for the qBBr-treated and the untreated biomass samples. Potentiometric titrations were performed by suspending approximately 0.3 g of biomass in 10–11 mL of 0.1 M NaCl that had been bubbled for at least an hour with N_2 gas in order to purge atmospheric CO_2 from the solution. This resulted in a bacteria concentration of approximately 30 g/L. A T70 autotitrator from Mettler Toledo, Inc. was used for all titrations and a Teflon-coated magnetic stir bar homogenized the solution for the entire titration process. In addition, N_2 gas flowed into the headspace of the capped titration vessel in order to prevent air infiltration. The titrations were performed in two steps: after an initial acidification to pH 2.7 using aliquots of a 1.000 ± 0.005 M HCl standard, the pH was raised to pH 9.7 using aliquots of a 1.000 ± 0.005 M NaOH standard. Titrations were conducted in triplicate for each set of conditions studied, with samples prepared individually from harvested biomass. Sterile cell-free control titrations were conducted before each set of biomass titrations.

Surface complexation modeling

We modeled the potentiometric titration data using FITEQL 2.0 (Westall 1982) in order to determine the number of site types, site acidity constants, and site concentrations using a non-electrostatic discrete site surface complexation approach (Fein et al. 2005). Note that here 'site type' refers to the family of sites that exhibit similar acidity constants, and does not necessarily refer to a chemically distinct site composition (e.g., carboxyl, sulfhydryl). The deprotonation reaction for any monoprotic binding site type, A_i, can be modeled as the following equilibrium reaction:

$$R - A_iH^0 \leftrightarrow R - A_i^- + H^+ \quad (1)$$

where '*R*-'represents the molecule to which the organic acid functional group A_i is bound, whether the site exists on the cell surface or on an EPS molecule. The acidity constant for a site type, A_i, can be expressed as:

$$K_{a,i} = \frac{[R - A_i^-]a_{H^+}}{[R - A_iH^0]} \quad (2)$$

where $K_{a,i}$ represents the acidity constant for the *i*-th site type, [*R*–A_i-] is the concentration of deprotonated sites of functional group *i*, [*R*–A_iH^0] is the concentration of protonated sites of functional group *i*, and a_{H^+} is the proton activity in the bulk solution. Each titration yields hundreds of measurements while FITEQL is limited to 40 serial data points. Therefore, data points were selected for the modeling approximately every 0.2 pH units from the second titration step for the titration that raised pH from 2.7 to 9.7, beginning at pH 3.0. We tested one-, two-, three-, four- and five-site models, and selected the model which best fit the data.

The best fitting model was selected as the one with a FITEQL variance value (V(Y)) closest to 1.0, or as the one with the best visual fit to the data when V(Y) values could not differentiate between multiple models.

The concentration of sulfhydryl sites, for each biomass sample was calculated by subtracting the average total site concentration from triplicate experiments of qBBr-treated biomass from the average total site concentration from triplicate experiments of untreated biomass. We applied the Student's t-test to our modeling results in order to determine whether a statistically-significant difference exists between the two measured total site concentrations. The calculated sulfhydryl site concentration value was considered to be above the detection limit of the procedure when the p-value was <0.05. A higher p-value was taken to indicate that the difference between the two measured total site concentrations was not significant, and thus the sulfhydryl site concentration was below the detection limit of the method. The standard deviation associated with the sulfhydryl site concentration, σ_{SH}, is reported as the standard error of the difference:

$$\sigma_{SH} = \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}} \quad (3)$$

where σ_1 and σ_2 are the standard deviations for the total binding sites for qBBr-treated and untreated biomass, respectively, and n_1 and n_2 are the corresponding number of replicate titrations conducted for each condition.

Results

Potentiometric titrations

Representative titrations and associated model fits for biomass grown in glucose with and without the EPS removed, and with and without qBBr treatment, are shown in Figure S2. The figure demonstrates that blocking sulfhydryl sites with qBBr diminishes the proton buffering capacity of the biomass somewhat, but that the effect is small compared to the overall buffering capacity of the biomass in general. This result is consistent with the sulfhydryl site concentration representing a relatively low proportion of the total concentration of proton-active sites. In addition, the removal of EPS molecules also causes only a small effect on the mass-normalized buffering capacity of the biomass. Each titration model provides an excellent fit to the titration data that was used to generate it. The calculated average site acidity constants and site concentration values from all of the titrations are compiled in Tables S3–S5.

Effect of EPS removal on total site and sulfhydryl site concentrations

There is no consistent trend in total site concentrations between the Gram-positive and the Gram-negative species studied. When grown in the presence of 50 g/L glucose, the Gram-positive species exhibit higher total site concentrations than the Gram-negative species, but this trend is not clear for the cells grown in the presence of 25 g/L glucose (Figure 1).

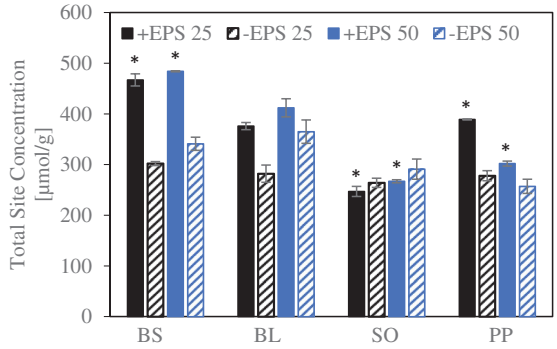

Figure 1. Mass-normalized total site concentrations for *B. subtilis* (BS), *B. licheniformis* (BL), *S. oneidensis* (SO) and *P. putida* (PP) biomass grown in 1.5 L of M9 minimal medium with the addition of either 25 g/L glucose (black bars) or 50 g/L glucose (blue bars). The solid bars represent values for the untreated biomass that includes EPS molecules (+EPS); the striped bars depict values for the resin-treated EPS-free biomass (−EPS). Totals are the averages of triplicate measurements with one standard deviation error bars. (*) The values for the untreated biomass for BS, SO, and PP are from Yu and Fein (2017).

Although the number of species studied here is limited, our results suggest that the structure of the cell wall is not a primary control on total site concentrations under the conditions of these experiments.

For *B. subtilis*, *B. licheniformis*, and *P. putida*, removal of EPS from the biomass leads to a significant decrease in the total proton-active site concentration compared to the concentration measured for the untreated cells at both glucose concentrations studied (Figure 1). The effect is largest for *B. subtilis* for which total site concentrations decreased from 467 ± 12 μmol/g to 302 ± 20 μmol/g for the biomass samples grown with 25 g/L glucose, and from 484 ± 1 μmol/g to 341 ± 7 μmol/g for the biomass samples with 50 g/L glucose added. Conversely, EPS removal from the *S. oneidensis* biomass resulted in no significant change in total site concentrations in the biomass samples grown with 25 g/L glucose and resulted in an increase in total site concentrations from 267 ± 3 μmol/g to 291 ± 6 μmol/g for the biomass samples grown with 50 g/L glucose.

The Gram-positive species studied, *B. subtilis* and *B. licheniformis*, exhibit significantly higher sulfhydryl site concentrations with EPS intact than the Gram-negative species studied, *S. oneidensis* and *P. putida*, and this holds for both the 25 and the 50 g/L glucose experiments (Figure 2). For the two Gram-positive species studied, EPS removal from biomass samples grown in the presence of either 25 g/L or 50 g/L glucose leads to a significant decrease in the measured sulfhydryl site concentration (Figure 2). For *B. subtilis* the decrease was larger at the higher glucose amendment, with sulfhydryl site concentrations in biomass grown with 25 g/L glucose decreasing from 54 ± 14 μmol/g in the EPS-bearing biomass (hereafter referred to as '+EPS') to 32 ± 16 μmol/g for the biomass samples with EPS removed (referred to as '−EPS'). Corresponding values for the *B. subtilis* biomass grown with 50 g/L glucose are 79 ± 13 μmol/g for the + EPS biomass and 13 ± 8 μmol/g for the − EPS biomass. Conversely, for *B. licheniformis* the sulfhydryl site concentration decrease was larger at the lower glucose amendment, with sulfhydryl site concentrations decreasing from 82 ± 14 (for the + EPS biomass) to 36 ± 8 μmol/g (for the − EPS biomass) and from 90 ± 13 μmol/g (+EPS) to

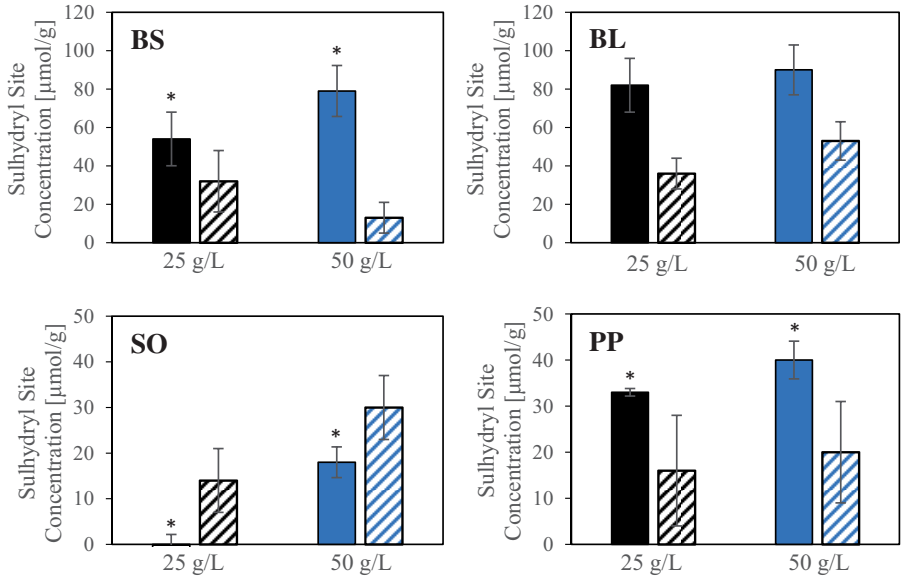

Figure 2. Mass-normalized sulfhydryl site concentrations for *B. subtilis* (BS), *B. licheniformis* (BL), *S. oneidensis* (SO) and *P. putida* (PP) biomass grown in 1.5 L of M9 minimal medium with the addition of either 25 g/L glucose (black) or 50 g/L glucose (blue). The solid bars represent values for the untreated biomass that includes EPS molecules (+EPS); the striped bars depict values for the resin-treated EPS-free biomass (−EPS). The reported values are the average of triplicate measurements, and the error bars represent the standard error of the difference. (*) The values for the untreated biomass for BS, SO, and PP are from Yu and Fein (2017). Note that the Y-axis scale for BS and BL is different than the Y-axis scale for SO and PP.

53 ± 10 µmol/g (−EPS) for the biomass samples grown with 25 g/L and 50 g/L glucose, respectively. For the Gram-negative species *P. putida*, sulfhydryl site concentrations also decreased with EPS removal to a similar extent for both glucose amendments. However, for the other Gram-negative species *S. oneidensis*, sulfhydryl site concentrations increased with EPS removal for both glucose concentrations, increasing from below detection limit (+EPS) to 14 ± 7 µmol/g (−EPS) for the biomass grown with 25 g/L glucose, and increasing from 18 ± 3 (+EPS) to 30 ± 7 µmol/g (−EPS) for the biomass grown with 50 g/L glucose.

Effect of electron donor on total site and sulfhydryl site concentrations

Pyruvate, fumarate, and glucose yield the greatest concentration of proton-active binding sites at carbon concentrations of 0.3 mol-C/L, 0.8 mol-C/L, and 1.7 mol-C/L, respectively (Figure 3). Thus, none of the electron donors yield a greater total site concentration across all carbon concentrations. Both glycerol and pyruvate exhibit a consistent decrease in total site concentration as the electron donor concentration increases. Biomass grown in fumarate exhibits a maximum total site concentration at 0.8 mol-C/L, with the lowest concentration produced at 1.7 umol-C/L. As was the case for the total site concentrations, there is no consistent order in resulting sulfhydryl site concentrations on the biomass between the four electron donors (Figure 4). At the lowest electron donor concentration, biomass grown in glycerol exhibits the highest concentration of sulfhydryl sites; at the highest electron donor concentration, biomass grown in pyruvate contains the highest sulfhydryl site concentration. Increasing the concentration of glycerol in the growth medium results in fewer sulfhydryl sites with site

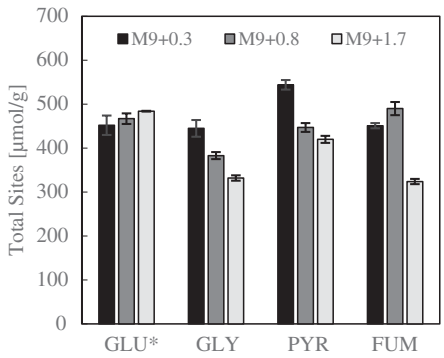

Figure 3. Mass-normalized total site concentrations for *B. subtilis* cells grown in the M9 minimal medium with the addition of glucose (GLU), glycerol (GLY), pyruvate (PYR), or fumarate (FUM) at carbon concentrations of 0.3 mol-C/L (black), 0.8 mol-C/L (dark grey), and 1.7 mol-C/L (light grey). Totals are the averages of three replicates with error bars of one standard deviation. (*) Glucose data are those reported by Yu and Fein (2017).

concentrations decreasing from 92 ± 11 µmol/g at 0.3 mol-C/L to 76 ± 8 µmol/g and 63 ± 6 µmol/g at 0.8 and 1.7 µmol-C/L, respectively. Conversely, both glucose and pyruvate exhibit the opposite trend, with sulfhydryl site concentrations increasing with increasing electron donor concentration in the growth medium. The biomass grown with fumarate exhibits a different behavior from the other electron donors, with similar sulfhydryl site concentrations produced at 0.3 and 0.8 mol-C/L, and a lower sulfhydryl site concentration produced with 1.7 mol-C/L of fumarate in the growth medium.

The percentage of the total site concentration on each biomass sample represented by sulfhydryl sites follows two distinct behaviors for the four electron donors tested (Figure 5), with increasing electron donor concentration yielding either no significant change in the percent sulfhydryl sites (for biomass grown in glycerol or fumarate) or an increase in percent sulfhydryl sites (for glucose and pyruvate). For glycerol and

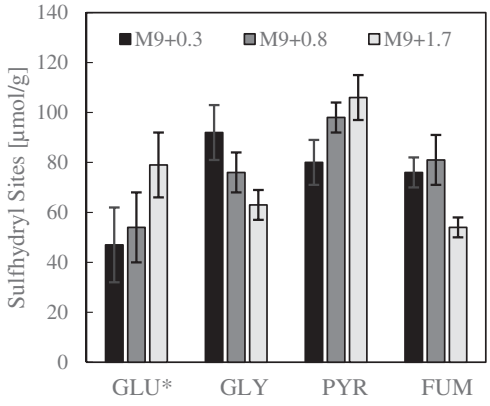

Figure 4. Mass-normalized sulfhydryl site concentrations for *B. subtilis* cells grown in the M9 minimal medium with the addition of glucose (GLU), glycerol (GLY), pyruvate (PYR), or fumarate (FUM) at carbon concentrations of 0.3 mol-C/L (black), 0.8 mol-C/L (dark grey), and 1.7 mol-C/L (light grey). The reported values are the difference between the averages of triplicate measurements of total site concentrations of biomass with and without qBBr treatment, and the error bars represent the standard error of the difference. (*) Glucose data are from Yu and Fein (2017).

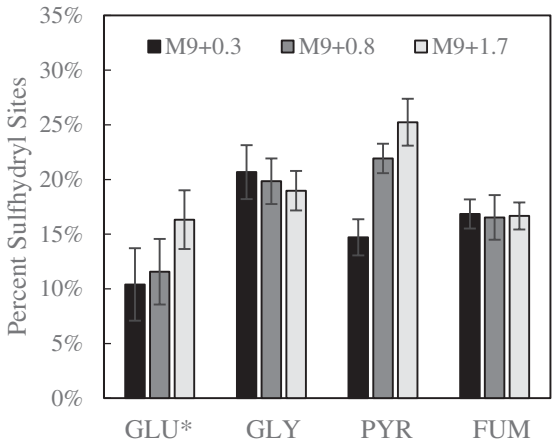

Figure 5. The percentage of total proton-active binding sites represented by sulfhydryl sites for *B. subtilis* cells grown in the M9 minimal medium with the addition of glucose (GLU), glycerol (GLY), pyruvate (PYR), or fumarate (FUM) at concentrations of 0.3 mol-C/L (black), 0.8 mol-C/L (dark grey), and 1.7 mol-C/L (light grey). The reported values are the difference between the averages of triplicate measurements of total site concentrations of biomass with and without qBBr treatment, and the error bars represent the standard error of the difference. (*) Glucose data are from Yu and Fein (2017).

fumarate, sulfhydryl sites comprise 20 ± 2% and 17% ± 2% of the total site concentrations, respectively, across all concentrations of electron donor added. For pyruvate, the sulfhydryl site percentage increases from 15 ± 2% at 0.3 mol-C/L to 22 ± 1% at 0.8 mol-C/L, to 25 ± 2% at 1.7 mol-C/L, an even bigger effect than is exhibited by glucose, with corresponding percentages of 10 ± 3%, 12 ± 3%, and 16 ± 3% for the 0.3, 0.8, and 1.7 mol-C/L samples, respectively.

Discussion

Relative concentrations of total and sulfhydryl sites between the cell surface and EPS

A change in the measured mass-normalized site concentration after removal of EPS from the biomass indicates different concentrations of sites within the EPS compared to the cell surface. A decrease in site concentration with EPS removal suggests that there is a greater mass-normalized concentration of sites within the EPS compared to the cell surface, while an increase in site concentration with EPS removal suggests that a greater mass-normalized concentration of sites exists on the cell surface. Previous studies of proton binding onto biomass with and without EPS present have yielded conflicting results, with some studies indicating a higher concentration of proton-active binding sites on the EPS molecules than on cell surfaces, some studies indicating the opposite, and some studies indicating no significant difference in proton-active binding site concentrations between the EPS molecules and the cell surface (e.g., Baker et al. 2010; Butzen and Fein 2019; Tourney et al. 2008; Wei et al. 2011).

Bacillus subtilis exhibits similar total site concentration behavior at both glucose concentrations, while all of the other bacterial species show a significant change in one or more characteristics (Figure 1). The total site concentrations for *B. subtilis* biomass grown with 25 g/L or 50 g/L glucose are similar, with EPS removal, suggesting that the total site concentration is relatively insensitive to glucose concentration over this glucose concentration range. These results are consistent with those of Yu and Fein (2016), who also observed no significant change in total site concentration within *B. subtilis* biomass, without EPS removal, as a function of glucose concentration in the growth medium over this concentration range, although they did observe a significantly lower total site concentration on biomass grown with only 5 g/L glucose present. The total site concentration for *B. subtilis* decreases significantly with EPS removal for the biomass samples grown in both 25 and 50 g/L of glucose, suggesting a greater concentration of proton-active binding sites on the EPS molecules attached to the *B. subtilis* cells compared to those on the cell surface. For *B. licheniformis*, the other Gram-positive species considered, EPS removal also leads to a decrease in total site concentration, though the effect is smaller at the higher glucose concentration studied. Removal of EPS molecules from the *S. oneidensis* biomass grown with 25 g/L of glucose does not lead to a significant change in total site concentration, suggesting an equal concentration of sites on the EPS and on the cell surface. The Gram-negative *S. oneidensis* biomass that was grown with 50 g/L glucose has a similar concentration of total sites to the biomass grown with 25 g/L glucose, but the removal of the EPS molecules leads to an increase in mass-normalized total site concentration, suggesting that the total concentration of binding sites on the cell surface exceeds that on the EPS molecules under these conditions. The other Gram-negative bacterial species, *P. putida*, shows a decrease in the total site concentration with EPS removal at both 25 and 50 g/L glucose, suggesting a greater total site concentration on the EPS of *P. putida* relative to the total site concentration on the cell surface, similar to the behavior observed for the Gram-positive species studied. Changing from 25 to 50 g/L glucose in the growth medium leads to a decrease in the total site concentration on the biomass, and hence the decrease in total sites that we observed between the 25 and

50 g/L glucose treatments could be caused by a decrease in sites on the EPS of *P. putida*.

B. subtilis, *B. licheniformis*, and *P. putida* exhibit similar trends in sulfhydryl site distribution between cell surfaces and EPS molecules, while *S. oneidensis* exhibits the opposite trend (Figure 2). The measured concentrations of sulfhydryl sites on *B. subtilis*, *B. licheniformis*, and *P. putida* biomass samples grown in either 25 or 50 g/L glucose all decrease after EPS removal, suggesting that sulfhydryl sites are distributed between the EPS molecules and the cell surfaces for these species, with a greater concentration within the EPS molecules. In contrast, the measured concentration of sulfhydryl sites on *S. oneidensis* biomass grown in either 25 or 50 g/L glucose increase after EPS removal, suggesting a higher concentration of sulfhydryl sites on the surface of the cells relative to on the EPS molecules. These data are consistent with the results reported by Yu and Fein (2016) who measured a greater concentration of sulfhydryl sites on the EPS of *P. putida* compared to its cell surface, and a greater concentration of sulfhydryl sites on the cell surface of *S. oneidensis*, compared to its EPS, for biomass grown in a different medium than studied here.

The differences that we observed in sulfhydryl site distribution between species may reflect different roles that sulfhydryl sites play for different bacterial species. All bacteria require trace amounts of some chalcophile and similar metals such as Co, Cu, and Zn for growth and metabolic functions, and some bacterial species, such as *S. oneidensis*, promote the reduction of chalcophile and similar metals such as Fe, V, Cr, and Co as electron acceptors (Carpentier et al. 2003; Liu et al. 2002; Lovley 1991; Middleton et al. 2003). Sulfhydryl sites located primarily on the cell surface may serve as adsorption sites that attract these nutrients to the cell for use, making them bioavailable to the cell (Fein 2017; Fein et al. 2019). However, many of the chalcophile and similar elements can be toxic to bacterial cells, and sulfhydryl sites located on EPS molecules bind these elements and sequester them away from the cell surface, decreasing bioavailability and providing greater resilience in toxic environments (Yu et al. 2020). Our results suggest that species such as *S. oneidensis*, which require metals for respiration, may benefit from higher concentrations of sulfhydryl sites on the cell surface, but species that do not respire metals (e.g., *B. subtilis*, *B. licheniformis*, and *P. putida*) do not have a need for higher concentrations of chalcophile elements at the cell surface and these species benefit from having a higher concentration of sulfhydryl sites on their EPS molecules.

Effects of electron donor type on sulfhydryl and total site concentrations

We did not observe any simple trends in either total (Figure 3) or sulfhydryl site concentrations (Figures 4 and 5) as a function of electron donor properties at any of the electron donor concentrations studied. Because sulfhydryl sites contain reduced sulfur, the cell must expend energy under aerobic conditions to produce these moieties, and this energy requirement likely is the reason that sulfhydryl sites are present on the cell surface at much lower concentrations than other less energy-intensive sites, even though sulfhydryl sites have a higher affinity to bind metals than those site types (Mishra et al. 2010; Yu and Fein 2015; 2016). Hence, we would expect that sulfhydryl site concentrations increase with increasing electron donor concentration due to the added energy availability. This conclusion is supported by the results of Yu and Fein (2017), who observed an increase in bacterial sulfhydryl site concentrations with increasing glucose concentration in the growth medium for *B. subtilis*, *S. oneidensis*, and *P. putida*. Our results with a wider range of electron donors indicate that sulfhydryl site concentrations are not a simple function of the amount of thermodynamic energy available from an electron donor. Although increasing the concentration of both pyruvate and glucose in the growth medium of *B. subtilis* leads to increased sulfhydryl site concentrations (Figure 4), the results for glycerol, and fumarate are not so straightforward. Glycerol yields the highest concentration of sulfhydryl sites at the lowest electron donor concentration studied, and fumarate shows a similar trend in which and the concentration of sulfhydryl sites is lowest at the highest concentration of fumarate.

Some of this variation in sulfhydryl site concentrations can be explained by the difference in total site concentrations (Figure 3). For example, on *B. subtilis* biomass grown with glycerol amendments, both the total and the sulfhydryl site concentrations decrease with increasing glycerol provided, yielding no significant change in the percentage of total sites represented by sulfhydryl sites (Figure 5). Figure 5 illustrates two effects of electron donor concentration: increasing the concentration of glucose or pyruvate in the growth medium yields an increase in the percentage of total sites represented by sulfhydryl sites; but in contrast the concentration of fumarate or glycerol exerts no effect on the sulfhydryl site percentage. The first behavior is consistent with sulfhydryl site formation being energy limited, while the second behavior suggests that the structure of the electron donor and the ability of the cell to metabolize the electron donor are other factors which affect sulfhydryl site formation.

The metabolic utilization pathway of each of the electron donors studied influences its thermodynamic energy potential and hence the potential for a bacterial species to convert the energy into cell surface or EPS sulfhydryl sites. Glucose is broken down through the complete glycolysis cycle followed by the TCA cycle. In contrast, glycerol is catalyzed to form glyceraldehyde 3-phosphate (G3P) by glycerol kinase, and the G3P enters glycolysis at an intermediate step, followed by the TCA cycle. Pyruvate is one product of glycolysis which is then decarboxylated to Acetyl-CoA before entering the TCA cycle. Finally, fumarate is an intermediate in the TCA cycle which is hydrated by fumarase to form L-malate, and therefore is the electron donor with the least thermodynamic potential energy of the electron donors studied. In general, the electron donors studied here are arranged in the following order of potential energy for cellular metabolism: glucose > glycerol > pyruvate > fumarate.

If energy alone was a limiting factor in producing sulfhydryl sites within the biomass, then we would expect sulfhydryl site concentrations to increase uniformly with increasing electron donor concentration in the growth medium. Further, we would expect biomass grown in glucose, glycerol, pyruvate, and fumarate at equal molalities of C per L to contain decreasing concentrations of sulfhydryl sites. However, these relationships between sulfhydryl site concentrations and electron donor characteristics are not what we observed (Figure 4). At the lowest carbon concentration of 0.3 mol-C/L, glycerol yields the greatest concentration of sulfhydryl sites, pyruvate and fumarate produce similar, intermediate concentrations of sulfhydryl sites, while glucose yields the lowest concentration of sulfhydryl sites. At the intermediate carbon concentration added, 0.8 mol-C/L, pyruvate results in the greatest concentration of sulfhydryl sites, glycerol and fumarate produce similar, intermediate concentrations of sulfhydryl sites, and glucose produces the fewest sites. At the highest carbon concentration of 1.7 mol-C/L, pyruvate also produces the highest concentrations of sulfhydryl sites, followed by glucose, glycerol, and fumarate with the fewest sulfhydryl sites. These inconsistent trends suggest that, in addition to energy provided, the structure of the carbon source and the ability of a bacterial species to metabolize a specific electron donor also affect the ability of bacteria to produce cell surface sulfhydryl sites.

An alternative to interpreting our titration results in terms of metabolic pathways is to relate sulfhydryl site concentrations to the number of electrons available from each substrate. The half-cell reactions that describe glucose, glycerol, sodium pyruvate, and sodium fumarate degradation to carbon dioxide yield 4.0, 4.7, 3.3, and 3.0 electrons per mol of C in each substrate, respectively. Hence, using the known concentration of each electron donor added enables calculation of the number of electrons available for metabolic reactions per L of growth medium for each substrate. The availability of electrons could explain why glycerol results in a greater percentage of sulfhydryl sites compared to glucose, as the concentration of glycerol used in the experiments yields more electrons per mol of C for the bacterial cells to use for energy production.

In addition to being related to the energy available to the cells, the concentration of sulfhydryl sites produced by the cells could also be related to the oxygen levels in the growth medium because reduced sulfur is more stable in low oxygen systems. Although the flasks containing the growth media were agitated gently to distribute nutrients during cell growth, oxygen levels in these flasks can become depleted in the latter stages of culture growth. Yu et al. (2020) found that the concentration of sulfhydryl sites increases with increasing growth time until early stationary phase. In this study, we harvested bacteria at early stationary phase for all electron donors, which occurs at 48 h for *B. subtilis* growth in glucose and glycerol, at 72 h for growth in sodium fumarate, and at 96 h for growth in sodium pyruvate (Figure S1). Under electron donor concentrations of 0.8 and 1.7 mol-C/L, pyruvate produced the greatest concentration and greatest percentage of sulfhydryl sites, which is hard to explain with our above considerations of the metabolic energy and electron count available from each substrate. However, the systems with sodium pyruvate as the electron donor required the longest growth time to reach early stationary phase, and oxygen could have become depleted in the growth medium to a greater extent than occurred for the experiments with the other electron donors, thereby reducing the energy needed to produce reduced sulfur for sulfhydryl site production. Our results strongly suggest that there are multiple influences on sulfhydryl site production by bacteria, including the number of electrons available per mole of substrate C and oxygen levels in the growth medium.

Our data indicate that increasing the concentration of an electron donor in a bacterial growth medium does not simply lead to an increase in total sites. In fact, total site concentration does not change significantly for glucose, while total site concentration decreases with increasing carbon provided for glycerol, pyruvate, and fumarate (Figure 3). Given appropriate amounts of an energy and carbon source, coupled with appropriate nutrient levels, catabolic and anabolic reactions tend to be tightly knit, and efficient growth produces the greatest amount of biomass (Dauner et al. 2001; Russell and Cook 1995). However, if given excess carbon beyond nutrient availability, e.g., in nitrogen or phosphorous deficient environments, bacteria resort to less energy efficient metabolic strategies which produce less biomass despite the higher carbon and energy levels provided (Dauner et al. 2001; Russell and Cook 1995). This phenomenon associated with excess carbon and energy relative to nutrient availability could explain the observed changes in total site concentrations as a function of electron donor concentration. For glucose, approximately the same concentration of total sites was produced at each glucose concentration, suggesting that all three concentrations represent excess carbon conditions, and that maximum growth efficiency may occur at a lower glucose concentration. For both glycerol and pyruvate, there was a consistent decrease in total sites, suggesting that there was more of a balance between nutrients and carbon source concentrations at the lowest electron donor concentration studied and that each addition of excess carbon resulted in greater metabolic inefficiencies and hence fewer total sites. For fumarate, which contains the least potential thermodynamic energy, it appears that optimal conditions for growth, and balance between nutrients and energy inputs, occurs at approximately 0.8 mol-C/L. At the lower carbon concentration, the carbon source is the limiting input, and at the high carbon concentration, nutrients are limiting and excess carbon results in inefficiencies and reduced biomass. This result is also supported by the growth curves which resulted in less biomass at 1.7 mol-C/L compared to 0.8 mol-C/L. for *B. subtilis* grown in each of the electron donors (Figure S1).

Conclusion

In this study, we demonstrate that energy limitations affect the formation of high affinity sulfhydryl sites on bacteria, but that this relationship holds only for energy sources that a specific bacterial species can readily metabolize. For all of

the bacterial species studied, sulfhydryl site concentrations increase with increasing glucose concentration. For *B. subtilis*, *B. licheniformis*, and *P. putida*, the majority of sulfhydryl sites are located on the EPS molecules, likely in order to sequester toxic metals away from the cell surface, thereby reducing metal bioavailability. Conversely, most of the sulfhydryl sites on *S. oneidensis* biomass are located on the cell surface, possibly resulting from the need for *S. oneidensis* cells, which are able to reduce some chalcophile and similar metals, to attract oxidized metals to the cell surface. As is the case for glucose, biomass samples grown with increasing concentrations of pyruvate also contain a higher concentration of sulfhydryl sites, but both glycerol and fumarate exhibit more complex behavior as a function of electron donor concentration. Without EPS removal, the Gram-positive species in this study contain higher concentrations of sulfhydryl sites than the Gram-negative species. It is not clear from our data whether or not this is a general trend applicable to a wider range of bacterial species, but it could indicate that Gram-positive species adsorb higher concentrations of chalcophile elements from solution, especially under the low metal-loading conditions where metal-sulfhydryl binding controls metal adsorption onto bacteria. The results from our study suggest that the overall energy that is available to bacterial cells controls, to some extent, the concentration and distribution of bacterial surface sulfhydryl sites. However, complex relationships including factors such as electron availability, oxygen concentrations, and electron donor: nutrient ratios, all influence the ability of bacterial species to utilize different electron donors for sulfhydryl site production.

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