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Cysteine Inhibits Mercury Methylation by *Geobacter sulfurreducens* PCA Mutant Δ*omcBESTZ*

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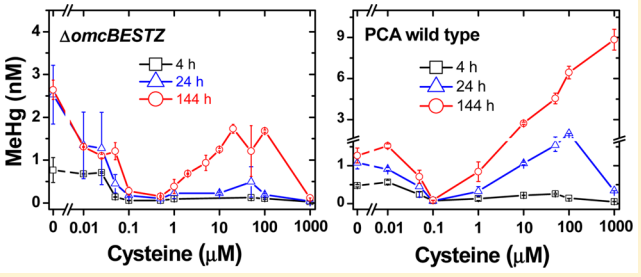
Supporting Information

ABSTRACT: Cysteine enhances Hg uptake and methylation by the *Geobacter sulfurreducens* PCA wild-type (WT) strain in short-term assays. The prevalence of this enhancement in other strains remains poorly understood. We examined the influence of cysteine concentration on time-dependent Hg(II) reduction, sorption, and methylation by PCA WT and its *c*-type cytochrome-deficient mutant (Δ*omcBESTZ*) in phosphate-buffered saline. Without cysteine, the mutant methylated twice as much Hg(II) as the PCA WT, whereas addition of cysteine inhibited Hg methylation, regardless of the reaction time. PCA WT, however, exhibited both time-dependent and cysteine concentration-dependent methylation. In a 144 h assay, nearly complete sorption of the Hg(II) by PCA WT occurred in the presence of 1 mM cysteine, resulting in our highest observed level of methylmercury production. The chemical speciation modeling and experimental data suggest that uncharged Hg(II) species are more readily taken up and that this uptake is kinetically limiting, thereby affecting Hg methylation by both the mutant and WT.



INTRODUCTION

Mercury (Hg) is a global pollutant of concern because of its bioaccumulation in the form of neurotoxic methylmercury (MeHg) in the food chain.[1−5] Certain anaerobic bacteria convert inorganic Hg to MeHg,[6−11] and this process is influenced by many environmental factors, notably, pH, sulfide, and complexing organic ligands such as naturally dissolved organic matter (DOM) and thiols in freshwater systems.[12−15] Some thiol compounds, such as cysteine (Cys), are found to enhance the uptake and methylation of mercuric Hg(II) species by *Geobacter sulfurreducens* PCA wild type (WT).[13−15] Because of the highly thiophilic nature of Hg,[16] cysteine is also known to form strong complexes with Hg(II)[16−18] and induce anaerobic oxidation of dissolved elemental Hg(0),[10,19,20] thus affecting Hg chemical speciation and interactions in biological systems.[16] The formation of strong Hg(II)−cysteine (Hg−Cys) complexes not only affects Hg(II) adsorption and desorption due to ligand-exchange reactions but also inhibits Hg(II) reduction by *G. sulfurreducens* PCA cells.[21]

Active transport of Hg−Cys complexes is the proposed mechanism that facilitates the cellular uptake and methylation of Hg(II) by *G. sulfurreducens* PCA WT cells,[14,15] and only certain Hg−Cys complexes (e.g., 1:1 or 1:2 Hg:Cys ratios) are preferentially taken up by the cells. Hg(II) and cysteine form complexes with varying stability constants (log β = 14−60) and numbers of coordination, ranging from 1:1 to 1:4 Hg:cysteine ratios, of which the 1:2 complex, $Hg(Cys)_2$, is thought to be the most important.[16−18] At high excess cysteine concentrations (i.e., high Cys:Hg ratios), the formation of the tris complex, $Hg(Cys)_3$, was found to inhibit Hg(II) uptake and methylation in 2 h assay studies,[15] although the inhibitory mechanism is not fully understood.

It is unclear whether the inhibited Hg uptake at high excess cysteine levels results from the cell's inability to take up the Hg−Cys tris complex or from slow reactions between cells and aqueous Hg−Cys complexes. In the latter case, short-term assay studies cannot reveal the time-dependent cell sorption and uptake of Hg species. Recent studies have shown that, in the absence of cysteine, Hg(II) methylation by PCA WT cells is positively correlated with Hg(II) species and its sorption, but negatively correlated with Hg(0) that is formed from bacterial Hg(II) reduction.[12] This result was further demonstrated by reactions between Hg(II) and the deletion mutant of PCA (Δ*omcBESTZ*),[22] which had the five outer membrane *c*-type cytochromes (OmcB−OmcE−OmcS−OmcT−OmcZ) removed and was thus impaired in its ability to reduce metal ions such as Hg(II).[12,23] Without these cytochromes, the mutant was less efficient in Hg(II) reduction, resulting in higher levels of Hg(II) uptake and methylation.[12] These findings suggest that Hg chemical speciation and MeHg production are closely coupled, and we therefore hypothesized that the coupled interactions among cells, Hg(II), and high-affinity ligands, such as cysteine in solution, are time-dependent and are affected by cell type, such as the mutant strain with impaired ability to reduce Hg(II). To test this hypothesis, we

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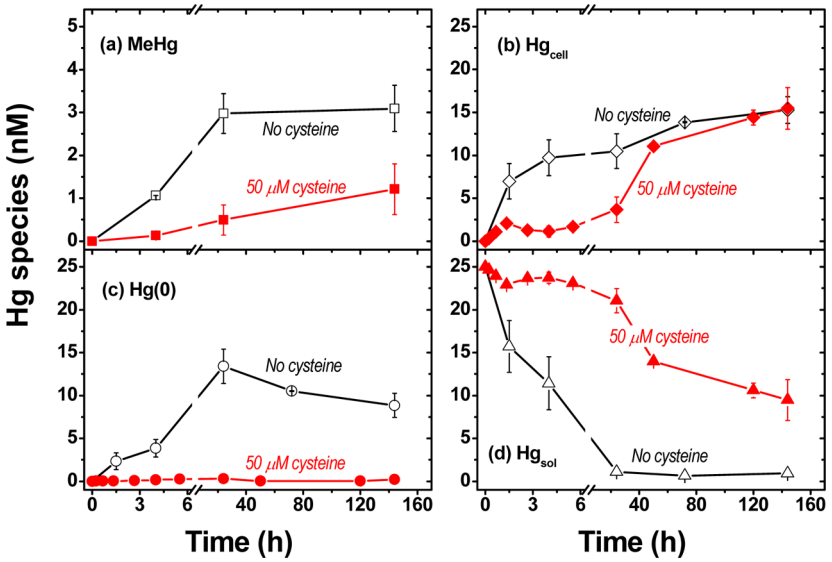

Figure 1. Production of (a) methylmercury (MeHg), (b) cell-associated or sorbed Hg_{cell}, (c) reduced or purgeable Hg(0), and (d) soluble Hg_{sol} (0.2 μm filter passing solution) as a function of reaction time between Hg(II) (as $HgCl_2$ at 25 nM) and washed cells of *G. sulfurreducens* PCA mutant Δ*omcBESTZ* (10^8 cells/L) in phosphate-buffered saline (pH 7.4). Experiments were performed in the absence or presence of 50 μM cysteine. Error bars represent one standard deviation of replicate samples (n = 2 or 3) from different batch experiments (n = 2 or 3).

conducted kinetic experiments and speciation modeling to determine the time-dependent Hg(II) reduction, sorption, and methylation by cells affected by cysteine concentrations in washed cell assays using both *G. sulfurreducens* PCA and the Δ*omcBESTZ* mutant.

MATERIALS AND METHODS

G. sulfurreducens PCA wild type (WT) and its mutant strain Δ*omcBESTZ* were cultured anaerobically in nutrient broth basal salts (NB) medium at 30 °C.[12,21] NB medium contained 20 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor at pH 6.8. Cells were harvested at the exponential growth stage with an optical density of 0.4−0.5 at 600 nm by centrifugation (1500*g* for 10 min at 25 °C) in an anoxic glove chamber (Coy) containing 98% N_2 and 2% H_2. Cells were then washed three times with a deoxygenated phosphate-buffered saline (PBS) assay medium, consisting of 0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4 at pH 7.4. All washing steps and subsequent Hg(II) assays were conducted in PBS in the glove chamber.[21,24]

Cell−Hg interaction assays were performed in a series of 4 mL amber glass vials.[10,21,24] For the time-dependent experiments, washed cells (both PCA WT and Δ*omcBESTZ* mutant) were first added to the PBS containing 50 μM cysteine. A stock Hg(II) solution (as $HgCl_2$) was added to give a final concentration of 25 nM. The final volume was 1 mL, and the cell concentration was 10^8 cells/mL. Unless otherwise specified, acetate and fumarate (1 mM each) were added once at time zero as the respective electron donor and acceptor. Analyses during incubation with PCA WT cells showed that acetate was not consumed, whereas fumarate was mostly consumed by day 6, indicating that cells were stressed but metabolically active (Figure S1 of the Supporting Information). All vials were immediately sealed with PTFE-lined silicone screw caps and equilibrated on a rotary shaker in the dark in the glove chamber. At selected time intervals, a set of sample vials (two or three) was taken out of the chamber and analyzed for Hg chemical speciation (described below). For MeHg, separate sample vials (two or three) were taken and preserved in trace metal grade H_2SO_4 [0.2% (v/v)] and kept at −20 °C until the contents were analyzed. Similar experiments were performed for cysteine concentration-dependent studies, in which cysteine concentrations varied from 0.01 to 1000 μM, and samples were taken and analyzed at 4, 24, and 144 h. The chemical speciation of Hg(II) at different cysteine concentrations (0−1 mM) was calculated using MINTEQ computer code, and the formation constants (log β) between Hg(II) and cysteine are listed in Table S1 of the Supporting Information.[16] All biotic and abiotic controls were performed in a similar manner, and data presented in Figures 1−3 represent an average of four to six sample measurements from at least two independent batch experiments.[12,24] Error bars represent one standard deviation.

All samples were analyzed for (1) purgeable elemental Hg(0), (2) total nonpurgeable Hg(II) (Hg_{NP}) remaining in cell suspension after purging, (3) soluble Hg (Hg_{sol}) after purging and filtration with 0.2 μm filters, (4) total Hg (Hg_{tot}) without purging and filtration, and (5) total MeHg without purging and filtration. The cell-associated Hg(II) (Hg_{cell}), including both Hg(II) adsorbed on the cell surface and intracellular Hg(II), was calculated by subtracting Hg_{sol} from Hg_{NP} (i.e., Hg_{cell} = Hg_{NP} − Hg_{sol}). Analytical procedures have been described in detail elsewhere.[12,24] In brief, Hg(0) was first quantified via a Hg(0) gaseous analyzer (Lumex 915+, Ohio Lumex) by purging the sample with ultrapure N_2 for 2 min (detection limit of 0.05 ng). After purging, Hg_{Total}, Hg_{NP}, and Hg_{sol} were analyzed, first by oxidizing the samples in BrCl [5% (v/v)] overnight, then by reducing Hg(II) via $SnCl_2$, and trapping and measuring Hg(0) using a cold-vapor atomic fluorescence spectrometer (CVAFS) (Tekran 2600, Tekran Instruments) (detection limit of 0.02 ng).[12,21] A modified version of EPA Method 1630 was used for MeHg analysis with enriched $Me^{200}Hg$ used as an internal standard.[4,10,12] MeHg was first extracted from the sample matrix via distillation, ethylation, and trapping in a Tenax column via N_2 purging. Thermal desorption and separation by gas chromatography were performed prior to the detection of Hg by an inductively

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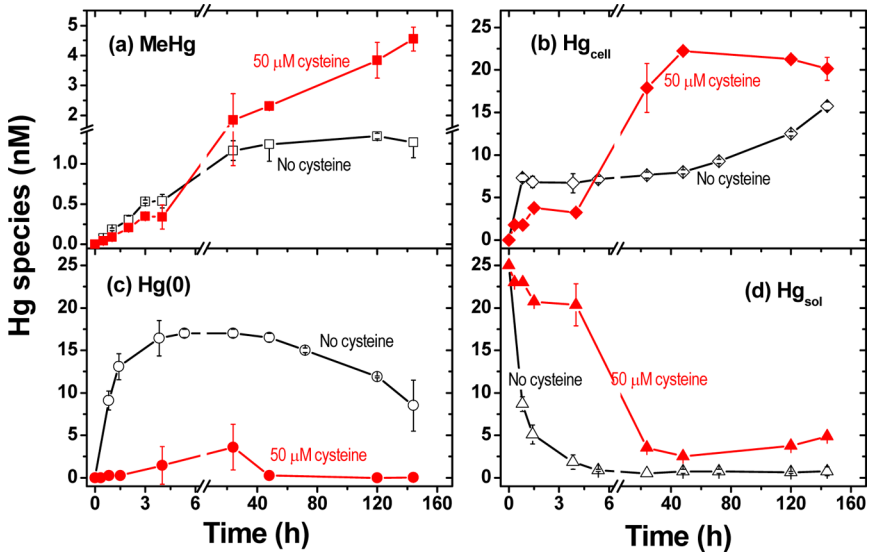

Figure 2. Production of (a) methylmercury (MeHg), (b) cell-associated or sorbed Hg_{cell}, (c) reduced or purgeable Hg(0), and (d) soluble Hg_{sol} (0.2 μm filter passing solution) as a function of reaction time between Hg(II) (as $HgCl_2$ at 25 nM) and washed cells of *G. sulfurreducens* PCA WT (10^8 cells/L) in phosphate-buffered saline (pH 7.4). Experiments were performed in the absence or presence of 50 μM cysteine. Error bars represent one standard deviation of replicate samples (n = 2 or 3) from different batch experiments (n = 2–4).

coupled plasma mass spectrometer (ICP-MS) (Elan-DRCe, PerkinElmer). The recovery of spiked MeHg standards was 100 ± 10%, and the detection limit was ~6 pg of MeHg.[12,25]

RESULTS AND DISCUSSION

We previously reported that, without cysteine, the mutant strain Δ*omcBESTZ* can produce about twice as much MeHg as PCA WT because multiple deletion of the outer membrane cytochromes resulted in a decreased level of Hg(II) reduction.[12] However, addition of cysteine (50 μM) greatly inhibited Hg(II) methylation by the mutant, regardless of the reaction time (up to 144 h) (Figure 1a). The observed initial MeHg production rate decreased from $(1.24 \pm 0.19) \times 10^{-12}$ to $(0.2 \pm 0.15) \times 10^{-12}$ nM cell^{-1} h^{-1} in 24 h, and the MeHg production decreased from to 3 to 0.5 nM under our experimental conditions. No Hg(II) reduction (Figure 1c) occurred in the presence of cysteine because of strong Hg(II)–cysteine complexation, as described previously.[16,21] Coincident with a decreased rate of methylation, the cell-associated Hg concentration, Hg_{cell} (i.e., sorption + uptake), also decreased after addition of cysteine, especially within the first 6 h (Figure 1b). A large percentage of the added Hg(II) (~23 nM or 90%) remained in solution (Figure 1d), which was attributed to cysteine aqueous complexation, consequently driving competition for Hg(II) with cells. Even after 24 h, only ~3 nM Hg(II) was cell-associated, and 22 nM Hg(II) remained in solution. Hg_{cell} eventually increased to ~11 nM after 48 h and to 15 nM after 144 h. In the absence of cysteine, however, Hg_{cell} was 2–5 times higher than in the presence of cysteine in the first 24 h (Figure 1b), despite significant amounts (up to 50%) of Hg(II) being reduced to Hg(0) (Figure 1c). Little or no soluble Hg_{sol} was detected in cysteine-free assays, as compared to ~9 nM of the Hg(II) remaining in solution with cysteine present (Figure 1d).

In comparison, the PCA WT cells produced nearly 4-fold more MeHg (4.6 nM) in the presence of cysteine than in the absence of cysteine (1.25 nM) in the 144 h assays (Figure 2a). This supports the notion that cysteine promotes Hg(II) uptake and methylation through the formation of Hg–Cys complexes.[14,15] However, the time-dependent Hg(II) methylation data revealed that, in the first 4 h, MeHg concentrations were consistently 20–40% lower in the presence of cysteine than in its absence (Figure 2a), disputing previous views that cysteine generally enhances Hg(II) methylation. A decreased level of Hg(II) methylation with cysteine also coincided with decreased Hg_{cell}, which was approximately half of that in the absence of cysteine (Figure 2b), leaving a large percentage of the added Hg(II) (80%) in solution (Figure 2d). Less than 10% of the Hg(II) was reduced to Hg(0) in a 4 h period with cysteine present, compared to 66% of Hg(II) (or 16.5 nM) reduced in cysteine-free assays (Figure 2c), as observed previously.[21,24] Thus, in the short-term assay, the aqueous complexation of Hg(II) by cysteine dominates, inhibiting Hg reduction, sorption, and uptake. Following this initial delay (4 h), Hg_{cell} increased substantially, and nearly 90% (22.3 nM) of the added Hg(II) was associated with the cell after 24 h, leaving <10% of the Hg(II) in solution (consisting mostly of MeHg) (Figure 2b,d and Figure S2 of the Supporting Information). These results thus show that cysteine inhibits the initial Hg(II) sorption and methylation by PCA WT cells but enhances both sorption and methylation after 24 h.

We subsequently compared Hg(II) sorption and methylation by both mutant and PCA WT cells at different cysteine concentrations, ranging from 0.01 to 1000 μM (or Cys:Hg molar ratios of 0.4–40000) (Figure 3). We found that the rate of MeHg production by the mutant was consistently lower than when cysteine was absent, regardless of its concentration (Figure 3a). Although the rate of MeHg production increased over time (up to 1.7 nM at cysteine concentrations of 20–100 μM at 144 h), very low levels of MeHg were detected in the presence of 0.1–1 and 1000 μM cysteine.

For the PCA WT cells, addition of cysteine at a level of 0.05–1 μM (equivalent to a Cys:Hg ratio of 2–40) also decreased MeHg production (~5–80% lower than that without cysteine) (Figure 3b). MeHg production was inhibited, irrespective of the incubation time (4–144 h). In the 4 h assay, Hg(II) methylation was inhibited regardless of the

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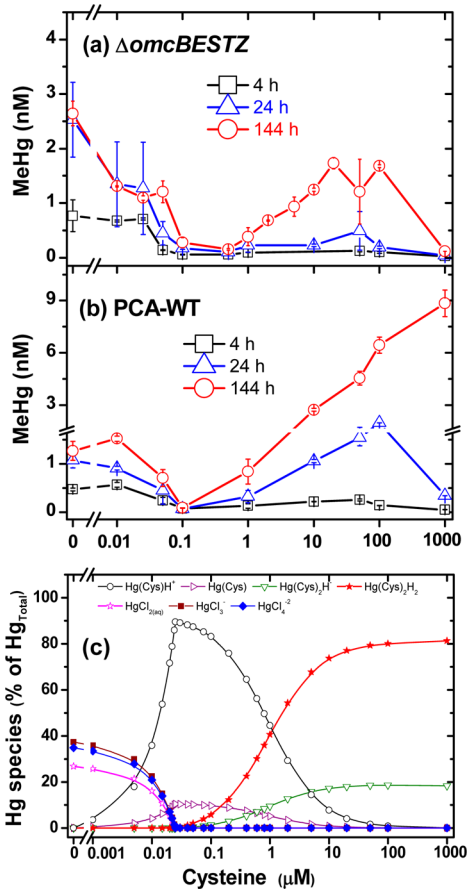

Figure 3. Production of methylmercury (MeHg) by washed cells (10^8 cells/L) of (a) *G. sulfurreducens* PCA mutant Δ*omcBESTZ* and (b) PCA WT as a function of cysteine concentration in phosphate-buffered saline (pH 7.4). The Hg(II) was added as $HgCl_2$ at a concentration of 25 nM, and samples were taken and analyzed at 4, 24, and 144 h time points. Error bars represent one standard deviation of replicate samples (n = 2 or 3) from different batch experiments (n = 2 or 3). (c) Hg(II) speciation calculations using Minteq at cysteine (Cys) concentrations of 0−1000 μM in PBS (Tables S1 and S2 of the Supporting Information).

amount of cysteine added (0.01−1000 μM), consistent with the time-dependent Hg(II) methylation assay shown in Figure 2a. Only with increasing incubation time (24 and 144 h) and at a cysteine concentration of >1 μM was the rate of MeHg production substantially increased. At 1000 μM cysteine, the 144 h assay showed a very high level of MeHg (~9 nM, 35% of added Hg), whereas in the 24 h assay, the rate of MeHg production was very low (0.35 nM); correspondingly, little Hg(II) sorption was observed (Figure S3 of the Supporting Information). The 24 h assay results are similar to previous observations, in which the rate of Hg(II) methylation by the PCA WT increased with cysteine to a plateau value, followed by decrease at a cysteine concentration of 1000 μM.[15]

To understand why methylation was inhibited at low cysteine concentrations (0.05−1 μM) in our experimental system, we calculated Hg(II) speciation using Minteq computer code (Figure 3c and Table S2 of the Supporting Information).[16] At pH 7.4, the equilibrium calculation indicates that $HgCl_2$ is one of the major species in the presence of <0.01 μM cysteine in PBS. The 1:1 Hg−Cys complex [as protonated $Hg(Cys)H^+$] is the dominant Hg species (up to ~90%) at cysteine concentrations between 0.01 and 1 μM, whereas the bidentate 1:2 uncharged complex [i.e., $Hg(Cys)_2H_2$] is the dominant Hg species at a cysteine concentration of >1 μM. Thus, at 0.01−1 μM cysteine, the positively charged $Hg(Cys)H^+$ complex that exists in solution may limit both the mutant and the WT of *G. sulfurreducens* PCA in Hg(II) methylation. Note that bacterial reduction of Hg(II) by the PCA WT has not been included in the chemical equilibrium calculation, but the effect of Hg(0) production on MeHg formation can be seen in the experimental data (Figure 3b and Figure S3c of the Supporting Information). At cysteine concentrations of >10 μM, $Hg(Cys)_2H_2$ species seemed to be favored for methylation by PCA WT cells, although competition between cells and aqueous cysteine for Hg(II) initially inhibited Hg(II) sorption and methylation, leaving a large percentage of the Hg(II) in solution at 4 h (Figure S3d of the Supporting Information). Previous studies also reported a sharp decrease in the rate of Hg(II) methylation from 100 to 1000 μM cysteine in a short-term, wash-cell experiment, and the effect was attributed to the formation of the tris complexes as unfavored Hg(II) species for cell uptake or methylation.[15] The tris complexes were not included in the speciation calculation, because of the lack of thermodynamic stability constants. However, our experimental results indicate that, after 144 h, cells were able to interact with the tris Hg−Cys complexes (if formed), resulting in the highest observed rate of MeHg production (~9 nM) and nearly complete sorption of the Hg(II) in the presence of 1000 μM cysteine (Figure S3 of the Supporting Information).

While the mechanism of this time-dependent Hg(II) sorption and methylation is not completely understood at present, cell uptake of Hg(II) or the Hg(II)−$(Cys)_n$ complexes or the exchange of the complexed Hg(II) between cysteine and the cell appeared to be kinetically limited. Recent studies using a nonmethylating Gram-negative bacterium, *Escherichia coli*,[26] showed that ligand-exchange reactions between cysteine and cell surface functional groups (e.g., thiols) may be responsible for Hg(II) sorption and uptake. In our study, the Hg(II) methylation curves (Figure 3b) appeared to follow the combined uncharged Hg(II) species [i.e., $Hg(Cys)_2H_2$ and $HgCl_2$] (Figure 3c), with both MeHg and the Hg(II) uncharged species at the lowest levels corresponding to 0.1 μM cysteine. These results suggest that PCA WT cells may take up $Hg(Cys)_2H_2$ over time as the carbon or energy source, especially under stress or nongrowth conditions, resulting in an increased rate of methylation. The deletion of the *omcBESTZ* genes likely altered cell physiological and surface properties (e.g., protein expression), which is similar to the observation for the methylation-deficient mutant Δ*hgcAB*,[24] and thus decreased rates of Hg(II) sorption and methylation in the presence of cysteine.

Our results demonstrate the time-dependent effect of cysteine on Hg methylation by *G. sulfurreducens* PCA cells: addition of cysteine alters Hg(II) speciation, decreasing the rates of Hg(II) sorption and reduction by cells and increasing the Hg(II) aqueous concentration. Cells in turn interact with and take up the Hg−Cys complex over time, favoring the uncharged $Hg(Cys)_2H_2$ species. These interactions are kinetically limiting and may thus control the rate of Hg(II) methylation by both the mutant and the WT. Our demonstration of the significant effect of cysteine on Hg(II) methylation by anaerobic bacteria underscores the need to further examine the effects of complexing ligands such as thiols or thiol groups in natural organic matter on methylmercury production in the environment.

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■ ASSOCIATED CONTENT

Supporting Information

Acetate and fumarate consumption, distributions of Hg species during reactions with washed cells of *G. sulfurreducens* PCA, formation constants (log β) between Hg and cysteine, and Hg speciation calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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