Environmental Science & Technology

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Effect of Thiols, Zinc, and Redox Conditions on Hg Uptake in *Shewanella oneidensis*

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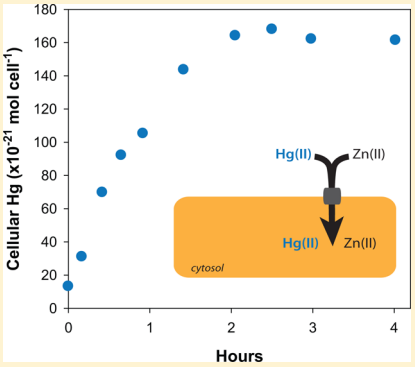
Supporting Information

ABSTRACT: Mercury uptake in bacteria represents a key first step in the production and accumulation of methylmercury in biota. Previous experiments with mercury methylating bacteria have shown that Hg uptake is enhanced by some thiols, in particular cysteine, and that it is an energy-dependent process through heavy metal transporters [Schaefer et al. *Environ. Sci. Technol.* **2014**, *48*, 3007]. In this study, we examine Hg uptake in the nonmethylating facultative aerobe, *Shewanella oneidensis*, under both anaerobic and aerobic conditions. Our results demonstrate similar characteristics of the Hg uptake system to those of the Hg methylating strains: (1) uptake is enhanced in the presence of some thiols but not others; (2) uptake is energy dependent as evidenced by inhibition by a protonophore, and (3) uptake is inhibited by high Zn(II) concentrations. Initial cellular uptake rates in *S. oneidensis* were remarkably similar under aerobic and fumarate-reducing conditions. These data support a similar Hg(II) uptake mechanism within the proteobacteria of accidental Hg(II) transport through heavy metal transporters with similar rates of uptake but differences in the ability to take up Hg bound to different thiols.



INTRODUCTION

Mercury is a global pollutant which readily bioaccumulates as methylmercury (MeHg) in aquatic food chains, posing health problems to fish consumers. The production of MeHg appears to be a specialized process restricted to strictly anaerobic microorganisms[2,3] carrying the *hgcAB* gene cluster.[4] The uptake of Hg(II) into cells represents the initial step in the production of MeHg, such that factors affecting Hg(II) bioavailability and cellular transport influence the cycling of Hg(II) and MeHg in the environment.

Although evidence that Hg(II) uptake is not simply a passive process has been available for over a decade,[5] very little research has focused on understanding the nature and variety of processes that exist. Recent studies with the Hg methylating bacteria, *Geobacter sulfurreducens* and *Desulfovibrio* sp. ND132, have demonstrated that Hg(II) uptake is enhanced by cysteine[6] and is energy dependent[7] apparently as a result of accidental import through heavy metal transporters, such as those responsible for Zn(II) uptake.[1] Once inside the cell, however, Hg(II) is rapidly methylated and exported from the cell.[6] Thus, uptake, methylation, and export are tightly coupled in these bacteria suggesting that Hg methylation/export may be part of a mercury detoxification pathway designed to maintain low intracellular Hg concentrations.[7] In some aerobic microorganisms, Hg resistance is mediated by the *mer* operon which encodes for specific Hg(II) importers coupled to Hg(II) reduction and volatilization of Hg(0), thereby decreasing the local external and internal Hg concentration.[8] However, this operon has not been observed in obligate anaerobic organisms including those capable of methylating mercury.[9]

The strict segregation between the two known gene clusters specifically devoted to dealing with mercury, the *hgcAB* cluster found only in some anaerobes and the *mer* operon found only in some aerobes, brings up the question of the possible environmental or physiological reasons for the corresponding differences in the biochemical processing of Hg. In particular, the differences in the chemical speciation of Hg(II) in anaerobic and aerobic media may be reflected in different uptake mechanisms and different rates of accumulation. For instance, gene expression patterns are different depending upon electron acceptor utilization, including expression levels of transporters and outer membrane proteins,[10,11] which could result in differences in uptake kinetics and surface sorption within the same strain depending upon growth conditions.[10,11] In addition, it is possible that Hg(II) uptake via heavy metal transporters in methylating bacteria may be due to specific adaptations to life in highly reduced habitats allowing these organisms to acquire essential metals, such as Zn(II), that may be bound to reduced sulfur groups,[1] resulting in a fundamental difference in Hg uptake mechanisms and/or the rates of Hg accumulation between methylating and nonmethylating anaerobic bacteria.

To begin answering these questions, we examined Hg uptake by *Shewanella oneidensis*, a facultative aerobic, iron-reducing γ-proteobacterium that does not methylate Hg(II).[12] Our goals

Received: February 6, 2015
Revised: May 3, 2015
Accepted: May 18, 2015
Published: May 18, 2015

DOI: 10.1021/acs.est.5b00676
Environ. Sci. Technol. 2015, 49, 7432−7438

are to shed light on the influence of redox conditions on Hg accumulation by comparing data obtained under anaerobic and aerobic conditions. Both *S. oneidensis* and the Hg methylator, *G. sulfurreducens*, are quite phylogentically divergent and yet have similar plasticity in electron acceptor utilization and occupy similar ecological niches. Comparing the results obtained under similar redox conditions in *S. oneidensis* to those previously obtained for the Hg methylator *G. sulfurreducens* gives insight into the potential differences in the Hg uptake mechanisms of methylating and nonmethylating strains.

MATERIALS AND METHODS

Cultures and Media. *Shewanella oneidensis* MR-1 was grown at 30 °C in complex medium adapted from Myers and Nealson[13] containing (mM): $(NH_4)SO_4$ (3.8), K_2HPO_4 (5.7), KH_2PO_4 (3.3), $MgSO_4$ (2.0), $CaCl_2$ (0.48), sodium lactate (20), yeast extract (0.5 g/L), tryptone (1 g/L), and trace metals (10 mL/L) at pH 7.1. Aerobic cultures were shaken at 220 rpm in Erlenmeyer flasks. Anaerobic cultures were grown statically in N_2-flushed serum bottles in the presence of 40 mM sodium fumarate rendered anoxic by boiling and bubbling with O_2-free N_2.

Cells were harvested at mid log phase by centrifugation at 7000*g* for 7 min at 12 °C. They were washed twice and resuspended with assay buffer[6] containing (mM), MOPS (10), NH_4Cl (0.1), lactate (1), KCl (1.3), NaCl (0.12), $MgSO_4$ (0.12), and NaH_2PO_4 (5). Cultures grown under anaerobic conditions were likewise harvested in an anaerobic glovebox (Coy Lab, Grass Lake, MI) containing a N_2:H_2 (95:5) atmosphere. All buffer solutions were prepared under anaerobic conditions by boiling and cooling under N_2 gas.

Hg Uptake Assays. Washed cell assays for Hg uptake experiments were prepared in glass vials containing assay buffer exposed to similar redox conditions in which cells were grown. Aerobic assays were shaken at 220 rpm in loosely covered Erlenmeyer flasks. Anaerobic assays contained 1 mM fumarate as an electron acceptor and were incubated statically in N_2-flushed serum bottles sealed with Teflon septa. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added from a 20 mM stock prepared in ethanol to a final concentration of 20 μM. Control assays, lacking CCCP, contained 0.1% (*v*/*v*) ethanol to mimic +CCCP additions.

Prior to the addition of cells, assays were pre-equilibrated with Hg(II) at 30 °C for 1 h. Assays were initiated by the addition of cells at a density 5−22 × 10^9 cells/L. Intracellular Hg(II) uptake was monitored over time by periodically collecting cells by filtration as described previously.[7] Briefly, at each time point, 5 mL assay volume was filtered through 0.2 μm pore-sized polycarbonate filters and sequentially washed with a oxalate-EDTA-KCl buffer (pH 7) and two glutathione-ascorbic acid (pH 7) solutions for 5 min each. Dissolved Hg species were collected by syringe filtration (0.22 μm) into amber glass vials sealed with Teflon septa. All Hg fractions, total Hg, cellular Hg, and dissolved Hg, were digested overnight in 0.02 N BrCl.

Hg Analysis. Following digestion, excess BrCl was reduced by the addition of 0.002% (w/v) hydroxylamine-HCl. Hg was analyzed by $SnCl_2$-reduction and trapped onto gold-coated beads (Tekran, Toronto, Canada) followed by thermal desorption and fluorescence detection on a Tekran 2500 or Brooksrand MERX total system (Seattle, WA).[14]

Cell Number and Protein Measurements. Cell density was measured in washed cell suspensions by acridine orange direct count (AODC)[15] using a Carl Zeiss Axioskop MC 80 Microscope. Cell counts (n = 3) obtained for each growth condition were normalized to OD_{660}. Conversion factors for aerobic and fumarate-reducing conditions were 5.5 × 10^{11} and 1.3 × 10^{12} cells L^{-1} $OD_{660}{}^{-1}$, respectively. The lower cell density of aerobically grown cells per OD_{660} is due to increased cell size relative to fumarate-reducing conditions (data not shown).

Protein was quantified in washed cell suspensions by the Bio-Rad Protein Assay (Hercules, CA) using a bovine serum albumin standard solution (Thermo Scientific Pierce). Cells were lysed by pulsed sonication (6W; 15 s intervals) on ice for a total of 3 min. Conversion factors for aerobic and fumarate-reducing conditions were 340 and 240 mg protein L^{-1} $OD_{660}{}^{-1}$, respectively.

Chemicals and solutions. Hg(II) was added as $Hg(NO_3)_2$ from a diluted High Purity Standards 1000 μg/mL stock. Each reduced thiol stock was prepared less than 30 min before assay preparation using high purity water previously boiled and bubbled under N_2. Thiol reagents were replaced approximately every six months. Zn(II) was added as $ZnSO_4$.

Speciation Calculations. MINEQL+ 4.6 (Environmental Research Software, Hallowell, ME) and stability constants reported by Lenz and Martell[16] and Cardiano et al.[17] were used to calculate the speciation of Zn and Hg complexes in solution. Cysteine concentrations were chosen to ensure full complexation with Hg(II) over chloride.

RESULTS

Effect of Redox Conditions on Hg Uptake. Hg uptake experiments were carried out under aerobic and fumarate-reducing conditions in a nonmethylating bacterium, *Shewanella oneidensis*. Micromolar concentrations of cysteine were added to guarantee identical speciation of Hg under both conditions. As shown in Figure 1, cysteine promoted Hg(II) uptake under both redox conditions. Cysteine has been shown to facilitate uptake under anaerobic conditions in Hg methylating strains (*Geobacter sulfurreducens* and *Desulfovibrio* sp. ND132)[6,7] and under aerobic conditions in *Escherichia coli*.[18,19] Interestingly, the initial rates observed for Hg uptake under fumarate-reducing conditions were remarkably similar between *S. oneidensis* and the Hg methylating bacterium, *G. sulfurreducens* (Figure 1B). The rate of uptake was approximately constant over the first 1.5 h in *S. oneidensis*, much longer than observed for the Hg methylators, *G. sulfurreducens* and *Desulfovibrio* sp. ND132[7] which plateaued after ∼20 min. This difference in the length of time for Hg uptake results in higher overall cellular accumulation levels in *S. oneidensis* as compared to the Hg methylating strains.[7]

Individual *S. oneidensis* cells took up more Hg(II) under aerobic conditions than under fumarate-reducing conditions (Figure 2A). However, microscopic observation clearly showed that aerobically grown cells had a larger cellular size and volume than those growing under anaerobic conditions (data not shown). Thus, when Hg uptake was normalized to protein content (Figure 2B), this difference in cellular uptake between aerobic and fumarate-reducing conditions became less significant and showed the opposite trend of slightly higher uptake rates under fumarate-reducing than aerobic conditions.

Effect of Cysteine on Hg Uptake. Previous experiments with *G. sulfurreducens* have shown that Hg uptake (and methylation) increases with cysteine (CYS) concentration, reaching a maximum at [CYS] = 10 μM and decreasing at

DOI: 10.1021/acs.est.5b00676
Environ. Sci. Technol. 2015, 49, 7432−7438

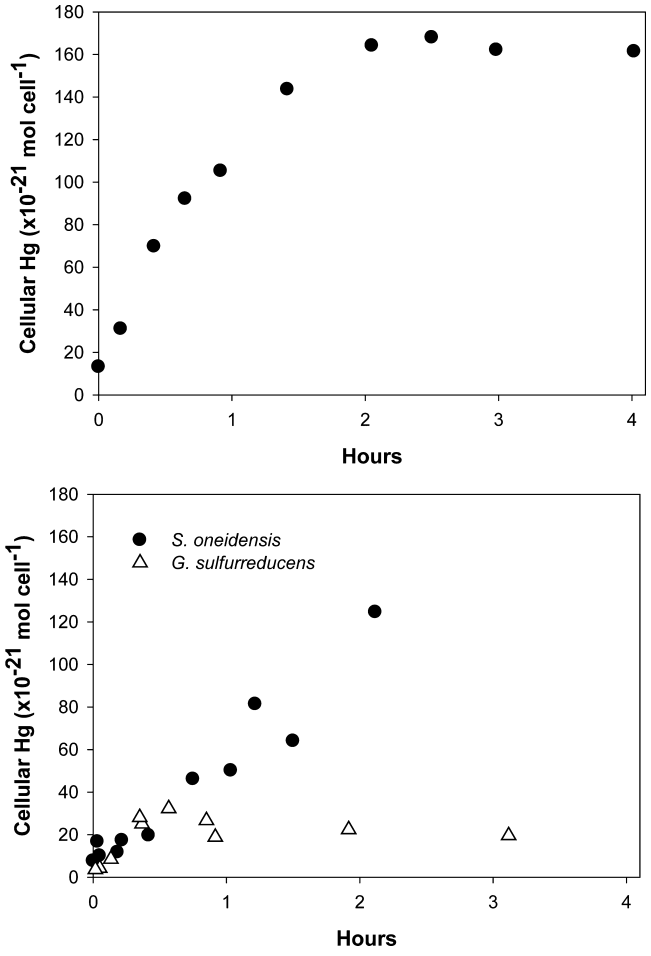


Figure 1. Cellular Hg uptake by washed cell suspensions of (●) *S. oneidensis* and (△) *G. sulfurreducens* under (A) aerobic and (B) fumarate-reducing conditions. Assays contain 10 nM Hg(II) and either (△) 10 or (●) 20 μM cysteine.

concentrations above 100 μM.[6,7] This was found to also be the case for *S. oneidensis*, albeit with a particularly sharp peak in Hg uptake rate at [CYS] = 40 μM (Supporting Information, SI, Figure S1) under aerobic conditions. This sudden peak at 40 μM CYS was not observed in fumarate-reducing conditions, as Hg uptake rates remained relatively constant between 10−40 μM CYS. This may reflect physiological differences between growth conditions. At higher CYS concentrations (60−100 μM), Hg uptake rates declined in *S. oneidensis* as observed in *G. sulfurreducens*, which has been attributed to the unavailability of the Hg-CYS_3 complex.[1,20]

Active Transport of Hg(II). In the Hg methylating bacteria *G. sulfurreducens* and *Desulfovibrio* sp. ND132, Hg(II) uptake has been shown to occur via an active transport mechanism.[1] In order to determine whether this is also the case in a nonmethylating organism, we performed Hg(II) uptake experiments with washed cell suspensions of *S. oneidensis* in the presence of 20 μM CYS under aerobic and fumarate-reducing conditions. Under both conditions, Hg(II) uptake was completely suppressed by the protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), demonstrating that transport in *S. oneidensis* is an energy requiring process (Figure 3). Thus, active transport of Hg(II) appears to be phylogenetically widespread and not restricted to either methylating bacteria or anoxic conditions.

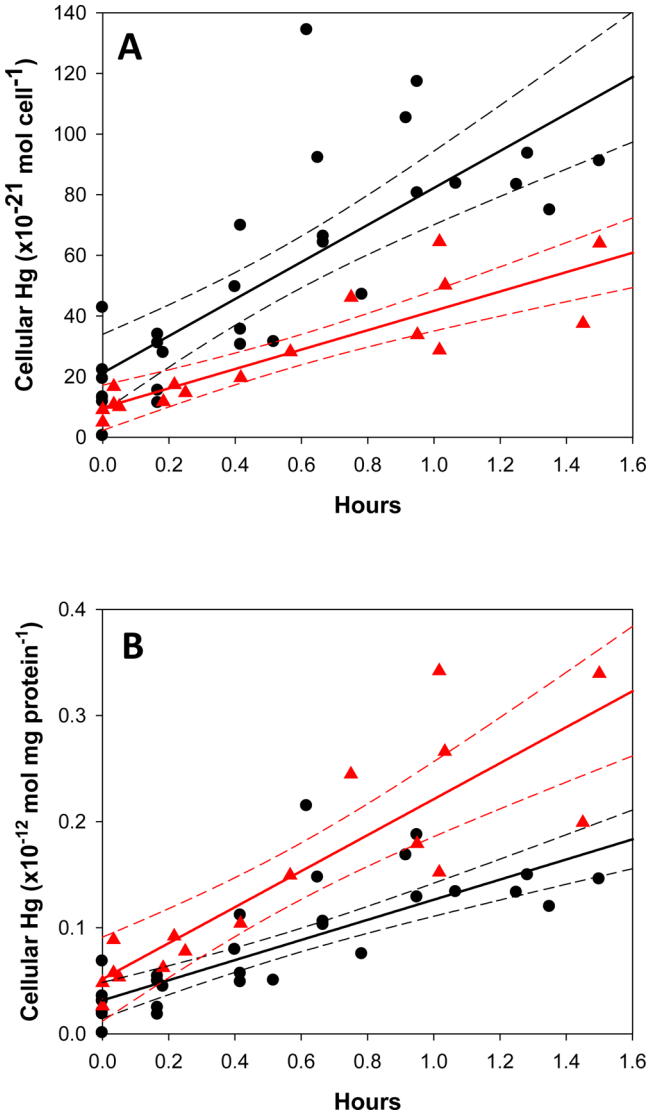


Figure 2. Hg uptake over time of replicate assays containing 20 μM cysteine and 10 nM Hg under (●) aerobic and (red triangle) fumarate-reducing conditions when normalized (A) per cell and (B) per mg protein. Solid lines and dashed lines indicate linear regression and 95% confidence intervals, respectively. The 95% confidence intervals for the uptake rates were 0.0012−0.0020 and 0.0019−0.0037 $\times 10^{-12}$ mol Hg mg $protein^{-1}$ h^{-1} for aerobic and fumarate-reducing conditions, respectively.

Effects of Zn(II) on Hg Uptake. Previous research showed that Zn(II) and Cd(II) concentrations in the μM range severely inhibit Hg uptake in Hg methylating bacteria.[1] This effect was not seen with other divalent metals such as Co(II), Ni(II), or Fe(II), indicating that a Zn transporter is likely involved in Hg uptake in these organisms. To determine whether this is also the case for *S. oneidensis*, we measured cellular Hg(II) uptake in cell suspensions containing 20 μM Zn(II) and an excess of cysteine (40 μM). Under these conditions, the concentration of Zn′ (Zn′ = sum of free and inorganically complexed Zn) was calculated to be 3.8 μM while the remainder was complexed to cysteine as a mono (93%) or bis (7%) complex. This concentration of Zn completely inhibited Hg(II) uptake in *S. oneidensis* under both anaerobic and aerobic conditions (Figure 4) suggesting the role of a Zn transporter in Hg uptake by this nonmethylating strain.

DOI: 10.1021/acs.est.5b00676
Environ. Sci. Technol. 2015, 49, 7432−7438

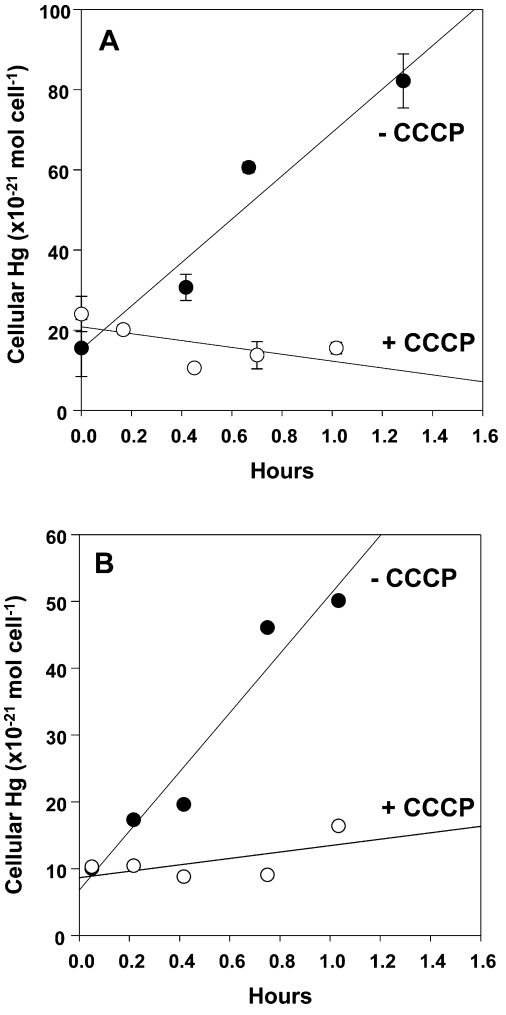


Figure 3. Cellular Hg uptake in washed cell assays under (A) aerobic and (B) fumarate-reducing conditions containing 20 μM cysteine and 10 nM Hg(II) with and without the protonophore, CCCP. Error bars in (A) represent the range in duplicate assays.

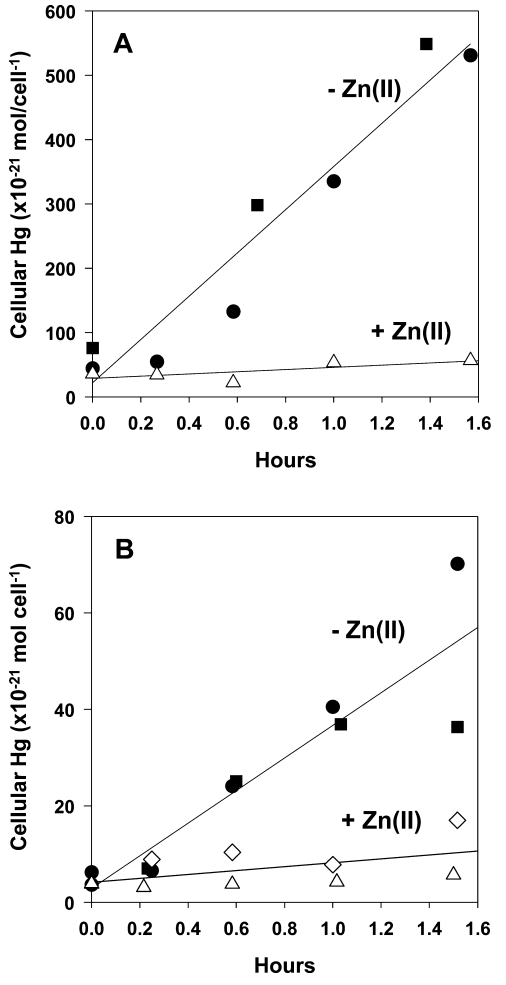


Figure 4. Effect of Zn(II) addition (20 μM) on cellular Hg uptake in washed cell assays under (A) aerobic and (B) fumarate-reducing conditions containing 40 μM cysteine and 10 nM Hg(II). Unique symbols represent replicate experiments.

Effect of Thiol Ligand Structure on Hg Uptake and Availability. Hg(II) availability to bacteria has been shown to depend upon ligand structure in *G. sulfurreducens*[7] but less so in the sulfate-reducing bacterium, *Desulfovibrio* sp. ND132. The availability of different Hg(II)-thiol complexes were similarly investigated in *S. oneidensis* by comparing Hg(II) uptake under aerobic and anaerobic conditions in washed cell assays containing a variety of thiol ligands at 20 μM concentration. Three of the chosen thiols, penicillamine (PEN), 2-mercaptopropionic acid (2MPA), and cysteine (CYS), vary by the degree of methyl substitution on the thiol carbon, a key factor determining the degree of Hg(II) availability to *G. sulfurreducens*. As shown in Figure 5, the structure of the Hg(II) binding ligand also has a significant impact on the bioavailability to *S. oneidensis* (see also SI Figure S2). Similar to another iron-reducer, *G. sulfurreducens*, Hg-GSH and Hg-PEN complexes were largely unavailable to *S. oneidensis*, although in one replicate some uptake (9%) may have occurred in the presence of GSH under fumarate-reducing conditions. Determination of total, dissolved, and cellular Hg pools showed that Hg(II) remained largely in the soluble phase and was neither significantly adsorbed on the cellular surface, nor appreciably taken up by the cells (SI Figure S3 and Table S1).

DISCUSSION

Our data on Hg uptake by the nonmethylating, iron-reducing bacterium *S. oneidensis* are qualitatively remarkably similar to those obtained with the methylating iron reducer *G. sulfurreducens*: Hg uptake is an active process in both organisms, and it is enhanced when Hg(II) is complexed to cysteine but inhibited in the presence of other complexing thiols such as PEN and GSH. These similarities imply that a similar Hg uptake mechanism may be at play. Further, in both species, relatively modest Zn(II) concentrations inhibit Hg uptake, indicating the possible involvement of a Zn transporter in the process.

The enhancement of Hg uptake by modest concentrations of cysteine originally reported in *G. sulfurreducens* by Schaefer and Morel[6] has been shown to occur in *Desulfovibrio* sp. ND132,[7] in *Escherichia coli*,[18,19] and now in *S. oneidensis*. This apparently general effect must result in part from the fact that Hg is kept in solution by complexation with cysteine, rather than adsorbing on the vessel wall or on cellular surfaces. In addition, Hg(II) bound in the Hg-CYS complex must be somehow available for uptake by all these bacterial species, most likely as a result of facile exchange of the metal between cysteine and the uptake molecules at the membrane surface, as previously suggested.[7,19] Evidence of the contrary is lacking as chirality and presence/

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Environ. Sci. Technol. 2015, 49, 7432−7438

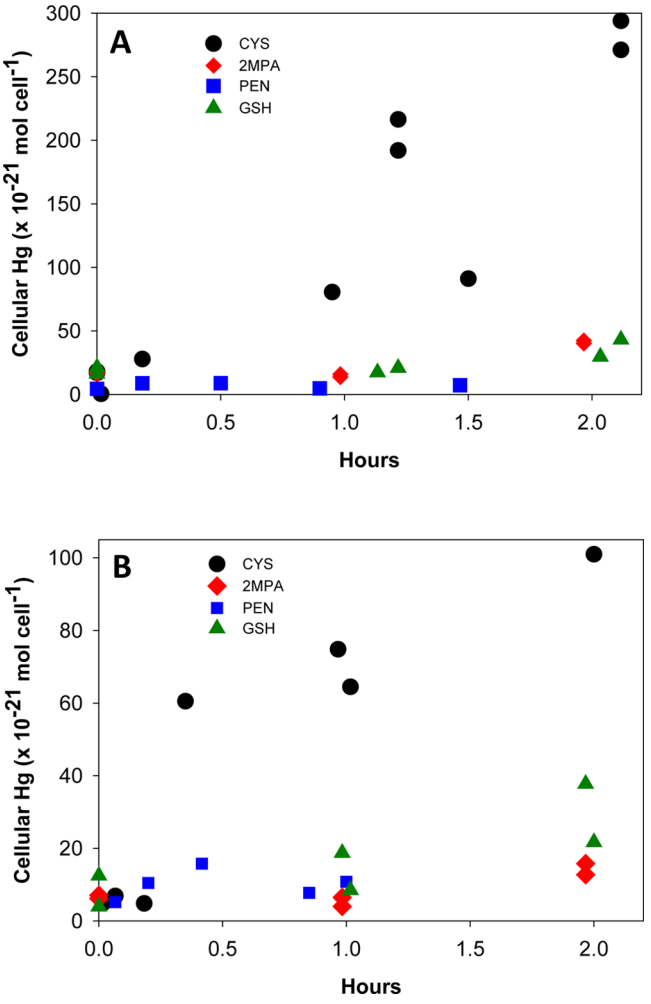

Figure 5. Effect of different thiols (20 μM) on cellular Hg(II) accumulation under (A) aerobic and (B) fumarate-reducing conditions with 10 nM Hg(II). CYS = cysteine, GSH = glutathione, 2MPA = 2-mercaptopropionic acid, and PEN = penicillamine. Calculated linear uptake rates are summarized in the SI Figure S2B.

absence of specific amino acid functional groups within the Hg ligand complex had no effect on the uptake of Hg(II).[7]

The complexation of Hg(II) to penicillamine or glutathione makes it effectively unavailable for uptake by the two facultative anaerobes and one obligate anaerobic iron reducer that have been tested so far: *E. coli*, *S. oneidensis*, and *G. sulfurreducens*.[7,18,19] This is in contrast to what has been observed in *Desulfovibrio* sp. ND132 for which PEN and GSH are as effective as CYS in promoting Hg uptake and methylation.[7] This species is not only a strict anaerobe, but a sulfate reducer isolated from a highly sulfidic, estuarine sediment.[21] The difference in the bioavailabilities of the various Hg-thiol complexes to these different species may reflect the relative abilities of the transport molecules to extract Zn(II) (and possibly other metals such as Cu(II)) from a variety of thiol complexes. We speculate that the ability of *Desulfovibrio* sp. ND132 to take up Hg(II) complexed to PEN and GSH results from divalent metal uptake systems that are adapted to a highly sulfidic environment where such metals (Zn and Cu in particular) are all complexed to a variety of strong thiol ligands. Unfortunately, similar inhibitory experiments of Hg(II) uptake by Cu(II) are complicated by the rapid catalytic oxidation and depletion of thiols by Cu.[22]

Interestingly, *S. oneidensis* is even more sensitive to the presence of a single methyl substitution on the thiol carbon than observed for *G. sulfurreducens*, as 2MPA failed to support Hg uptake in *S. oneidensis*. This observation is likely due to steric hindrance of the methyl groups near the Hg(II) binding moiety in both PEN and 2MPA. We note that neither chirality nor the presence or absence of amino acid functional groups has an effect on Hg availability in *G. sulfurreducens*.[7] The moieties most proximal to the site of Hg binding appear to be critically important for determining availability relative to more distal regions of the molecule. Since *S. oneidensis*, a facultative aerobe, lives in zones of changing redox status, the inability to take up Hg(II) from diverse Hg-thiol complexes may represent lack of selective pressure for adaptation to a sulfidic environment as hypothesized for the sulfate-reducer ND132.

The near absence of an effect of redox growth conditions on Hg accumulation in *S. oneidensis* is interesting and further supports the importance of Hg speciation rather than just redox status in affecting Hg(II) transport. While Hg(II) availability at picomolar concentrations in biosensor experiments appeared higher under aerobic than anaerobic conditions in *E. coli*,[5,23] differences observed became far less significant at low nanomolar Hg concentrations used in this study. The discrepancy between these studies may be due to differences in surface sorption between aerobic and anaerobically grown cells, leading to differences in Hg(II) availability between these two conditions at trace picomolar mercury concentrations in biosensor experiments.[5] Our findings do slightly differ from that reported by Wang et al.[24] showing less Hg associated with cells under aerobic as compared to fumarate-reducing conditions, as the differences in the rates of uptake were not significantly different. However, those experiments were done at micromolar Hg concentrations in the absence of a thiol such as cysteine to minimize Hg adsorption to the cellular surface.[7] It is possible that anaerobically grown cells may have higher surface thiol contents and adsorb more Hg(II) in the absence of cysteine than aerobically grown cells.

Remarkably the rates of intracellular Hg uptake we observed in *S. oneidensis* are very similar to those observed by Schaefer and Morel[6] in *G. sulfurreducens* (Figure 1B); however, uptake is prolonged in *S. oneidensis*, taking place over 1.5 h relative to only 20 min in *G. sulfurreducens*. This extended time spent accumulating Hg(II) in *S. oneidensis* results in a doubling of cellular Hg contents in this organism relative to *G. sulfurreducens* under similar redox (fumarate-reducing) and Hg(II) speciation (Hg-CYS) conditions (Figure 1B). Lower cellular accumulation in *G. sulfurreducens* is partly limited by the production and excretion of MeHg; however, Hg methylation mutants (Δ*hgcAB*) actually accumulate slightly less Hg[25] suggesting something else may be limiting uptake in *G. sulfurreducens* besides export of MeHg.

Results presented in this paper and earlier studies[1,7] point to a common active uptake mechanism mediating the accidental intracellular Hg(II) transport through heavy metal importers in phylogenetically distant proteobacteria. While growth conditions and taxa had minimal effects on rates of Hg uptake, significant differences were observed in Hg(II) availability due to changes in Hg(II) speciation. Considering that Hg methylating organisms have recently been described within the *Firmicutes* and *Methanomicrobia*,[2,3] both of which have remarkably different membrane structures, differences in Hg(II) availability may become even more apparent in far more divergent phyla. With the discovery of the genes involved

DOI: 10.1021/acs.est.5b00676
Environ. Sci. Technol. 2015, 49, 7432−7438

in Hg methylation, *hgcAB*,[4] and development of molecular approaches to detect Hg methylating populations in environmental samples,[26−28] it is important to understand how differences in cell physiology and membrane structure across taxa is related to Hg uptake, as these parameters may be as important as the chemical speciation of Hg(II) in solution for determining the fate of Hg. For example, different bacteria have metal transport systems with varied affinities for Hg, and thus, different organisms may compete more or less effectively for the same Hg−ligand complex. Further, availability of essential trace metals and cellular nutrient requirements influence the expression of metal transporters which in turn affects the accumulation and potential methylation of Hg. Thus, it is paramount to understand how different populations respond to changing Hg speciation such that we can better predict methylmercury accumulation in the environment.

■ ASSOCIATED CONTENT

Supporting Information

(Figure S1) Effect of cysteine concentration on the uptake of Hg(II); (Figure S2) effect of thiol structure on Hg uptake rate; (Table S1 and Figure S3) distribution of Hg across dissolved and cellular pools. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b00676.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank S. Janssen for technical assistance. This research was supported by the U.S. Dept of Energy Office of Science, Biological and Environmental Research, Subsurface Biogeochemical Research Program, Project No. DE-SC0006849.

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