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Effect of Divalent Metals on Hg(II) Uptake and Methylation by Bacteria

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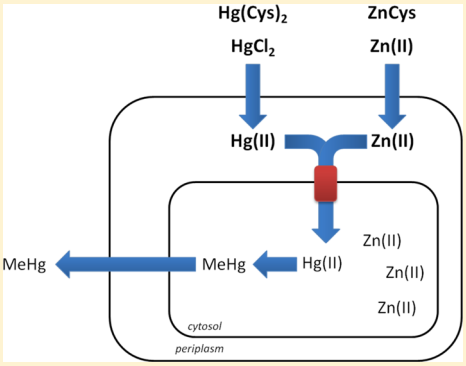
Supporting Information

ABSTRACT: The production of methylmercury by some bacteria is a key first step in the accumulation and biomagnification of this toxic substance in aquatic food webs, a major human health concern. By direct measurement of cellular Hg(II) uptake in model iron and sulfate reducing bacteria, we have observed that specific trace metals, such as Zn(II) and Cd(II), inhibit uptake and methylation in these organisms, whereas other metals, such as Ni(II), Co(II), or Fe(II), do not. The inhibition of Hg(II) methylation by Zn(II) was competitive in nature and related to the concentration of inorganically complexed Zn(II) (Zn′). The inhibition of Hg(II) methylation was alleviated by decreasing the free Zn′ concentration through complexation with nitrilotriacetic acid without altering the speciation of Hg(II). The inhibitory effect by Zn(II) was observed when either Hg-cysteine complexes or neutral $HgCl_2$ dominated the speciation of Hg(II), demonstrating that both charged and neutral species are transported into the cytosol by an active rather than passive process. We propose that Hg(II) uptake is the result of its accidental uptake by metal transporter(s), possibly one effecting the transport of Zn(II).



INTRODUCTION

Hg methylation by anaerobic bacteria represents a key first step in the production and bioaccumulation of the neurotoxin methylmercury.[1] Our understanding of the mechanisms responsible for mercury methylation by bacteria has improved significantly over the past few years. Notably, two proteins, the corrinoid HgcA and the ferredoxin HgcB, which are essential for Hg methylation in the iron reducer *Geobacter sulfurreducens* and the sulfate reducer *Desulfovibrio desulfuricans* ND132, have now been identified.[2] The HgcA protein, which is believed to directly methylate Hg(II), contains four putative transmembrane domains with the corrinoid binding group likely facing the cytosol,[2] further supporting the importance of cellular uptake in regulating the amount of methylmercury (MeHg) produced. Previous research has proposed that neutral $HgCl_2$[3,4] and $Hg(HS)_2^0$ species[5,6] passively diffuse into the cell, where they are methylated by bacteria. However, several studies have questioned that hypothesis.[7−9] In fact, recent kinetic experiments have established that in the presence of thiols, Hg(II) uptake is an active process, and that cellular uptake, methylation, and export of MeHg are tightly coupled. Despite these advances, we still know little of the possible physiological function of Hg methylation or of the mechanisms responsible for Hg(II) uptake and MeHg export in methylating bacteria.

In our previous work, we established the importance of thiol complexation for mercury uptake and methylation.[9,10] In particular, we observed high uptake and methylation rates when Hg(II) was present predominantly as an Hg−-cysteine complex; enhanced Hg(II) uptake in the presence of cysteine[8] or histidine[7] has likewise been observed in *Escherichia coli*. Hg(II) uptake in *G. sulfurreducens* was dependent on the structure of the thiol moiety, not on amino acid chirality, the presence of either amino or acidic functional group, nor did we observe any competitive inhibition by neutral or cationic amino-acids (e.g., valine, leucine, or glutamate),[10] as had been observed in mammalian cells.[11] We thus hypothesized that Hg(II) uptake is effected by a divalent metal transporter rather than a cysteine/cystine or other amino acid transporter. Here we provide a first order test of this hypothesis by studying the effect of divalent metals on Hg(II) uptake and methylation in *G. sulfurreducens* and *D. desulfuricans* ND132, and provide evidence in support of a common active transport mechanism for Hg(II) uptake of both Hg−thiol and Hg−chloride complexes. We also attempt to localize the key transport proteins by removing the external membrane from *G. sulfurreducens* and performing uptake and methylation experiments with the resulting spheroplasts.

MATERIALS AND METHODS

Cultures and Media. *G. sulfurreducens* PCA (DSM 12127) and *D. desulfuricans* ND132 (gratefully obtained from C. Gilmour[12]) were grown by fumarate reduction at 30 °C as described in Schaefer et al.,[10] except that the Cu(II) concentration in growth medium for *G. sulfurreducens* was lowered 10-fold to 40 nM total Cu. Cells were grown to midlog phase and harvested by centrifugation (7000*g* for 8 min) in an

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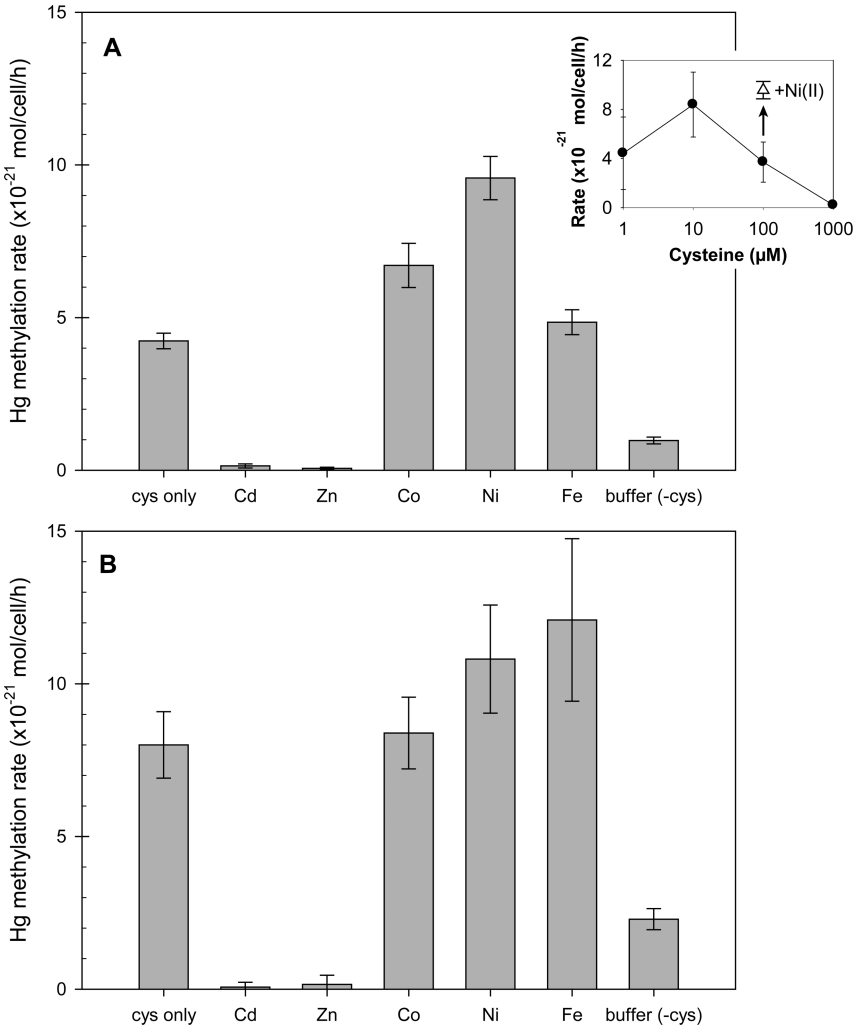

Figure 1. The response of various divalent metals (50 μM total) on Hg(II) methylation rates in 1 h washed cell incubations of (A) *G. sulfurreducens* and (B) *Desulfovibrio* sp. ND132. Except where indicated, all conditions contain 5 nM Hg(II) total, 100 μM cysteine, and 50 μM of one of the following divalent metals: Cd(II), Zn(II), Co(II), Ni(II), or Fe(II). Vials without additional trace metal (excepting Hg(II)) include the controls "cys only" and "buffer (-cys)" which contain Hg(II) as the sole trace metal in the presence and absence of 100 μM cysteine, respectively. (Inset) Rate of Hg methylation as a function of cysteine concentration. The arrow indicates the increase in the methylation rate upon addition of 50 μM Ni(II) (open triangle). Error bars indicate ±1 stdev of triplicate assays.

anaerobic glovebox with 5% H_2 in N_2 atmosphere. Cells were washed once with assay buffer containing 1 mM acetate (GsAB for *G. sulfurreducens*) or 1 mM pyruvate (Dd-AB for ND132), as the electron donor.[10] Washed cells were resuspended in the same buffer supplemented with 1 mM fumarate (filter-sterilized) as the electron acceptor. All media and buffers were rendered anoxic by boiling and bubbling with ultrahigh purity N_2 (O_2-free) gas.

Whole Cell Methylation Assays in the Presence of Trace Metals. Hg(II) methylation assays, as described in Schaefer et al.,[10] were conducted in glass serum bottles prepared under anoxic conditions with 9 mL assay buffer supplemented with 1 mM fumarate, closed with Teflon stoppers, and crimp-sealed. Thiol and trace metal stock solutions were prepared fresh daily with previously boiled and N_2-bubbled Milli-Q water. Vials were additionally flushed with N_2 gas for 5 min following thiol or trace metal additions, prior to the addition of Hg(II). Assays were then spiked with 5 or 10 nM Hg(II) and allowed to equilibrate at 30 °C for ≥1 h. Longer pre-equilibration times (1−4 h) were not found to increase methylation rates; however, long pre-equilibration periods were avoided (~24 h) to prevent loss of cysteine. The assays were started by the addition of 1 mL washed cell suspension (final assay ≈ 10^8 cell/mL) and incubated in the dark at 30 °C. After a 1 h incubation period, the assay vials were cooled quickly in an ice bath, stored frozen at −20 °C, and analyzed for MeHg.

Hg Uptake and Methylation Rates in Spheroplasts and Whole Cells. Spheroplasts were generated in *G. sulfurreducens* according to the method described in Coppi et al.[13] The spheroplasts were collected by centrifuging at 20 000*g* for 10 min and resuspended in 10 mL of WMS buffer at pH 6.8 (g/L): KH_2PO_4 (0.42), K_2HPO_4 (0.22), KCl (0.38), MOPS (2.10), and sucrose (120) to a protein concentration ~0.1−0.2 mg/mL. Whole cells were simply washed in WMS buffer in place of the solutions needed to generate spheroplasts in order to directly compare spheroplasts to intact whole cells. The quality of the spheroplast preparation (fixed with 10% formalin) was verified by microscopic observation of acridine orange stained cells on dark 0.22 μm polycarbonate filters.[14] Spheroplasts represented >99% of the total bacterial population by direct counting methods. Hg uptake and methylation assays were prepared in 20 mL glass scintillation vials (Thermo Scientific) in an anaerobic chamber (5% H_2 in N_2) with an isotonic buffer of 9 mL WMS

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buffer (pH 6.8) supplemented with 1.5 mM $MgCl_2$, 1 mM each fumarate and acetate, 10 μM L-cysteine, and 50 nM $HgCl_2$, unless otherwise noted (e.g., C-starved cells lacked both fumarate and acetate). In inhibitor experiments with spheroplasts, the protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), was added from a 20 mM stock solution prepared in ethanol. Ethanol (0.1% final) was added to control assays (−CCCP) to replicate conditions to those containing CCCP; no effect of the added ethanol on Hg(II) methylation was observed. The assays were started by the addition of 1 mL cells (spheroplasts or whole cells), representing 10% the assay volume. Final cell densities in assays were (1−4) × 10^8 cells/mL or ∼10−30 μg/mL protein for spheroplast and 40−50 μg/mL protein for whole cell assays. We note that inconsistent results with Zn(II) addition were obtained at spheroplast concentrations corresponding to ≥30 μg/mL protein, likely due to Zn(II) binding to cellular material in the preparation, and thus spheroplast experiments with and without added Zn(II) contain 7−14 μg/mL protein. Subsamples were taken from each assay over time to follow Hg(II) uptake and methylation from a single assay vial. Cellular Hg contents were measured by filtering cells (1 mL) onto 0.2 μm nitrocellulose membranes followed by sequential washing with a oxalic acid-EDTA and two glutathione-ascorbic acid washes.[10] This wash strategy was necessary to maintain a low and yet highly reproducible background Hg concentration on the filters, such that uptake rates could be reliably determined. Subsamples for MeHg (1 mL) were collected in amber glass vials containing 0.02% H_2SO_4 (final pH < 2), chilled on ice, and frozen at −20 °C until analysis. In parallel assays, subsamples (1 mL) were collected for malate dehydrogenase activity measurements, harvested by centrifugation (10 min at 18 000*g*, 4 °C) and the spheroplasts lysed by vortexing cell pellets in a hypotonic solution of 0.96 mL phosphate buffer (100 mM, pH 7.5). Malate dehydrogenase (MDH) activity was determined at room temperature by monitoring the oxaloacetate-dependent oxidation of NADH at A_{340} (ε = 6.22 μmol^{-1} cm^{-1} mL^{-1}).[15] Protein concentration was determined by the BCA protein assay kit (Pierce, Thermo Scientific) in cell preparations pelleted by centrifugation, resuspended in 10 mM Tris and 0.2% SDS (pH 7), and lysed by boiling for 5 min.

Hg and MeHg Analyses. Cellular Hg (as washed filters) and total Hg samples were digested overnight in 0.5% BrCl. Excess BrCl was reduced with hydroxylamine-HCl. Digested Hg(II) was reduced to Hg(0) by $SnCl_2$ in round-bottom flasks and the Hg(0) collected onto gold-coated sand traps (Tekran) upon bubbling with N_2. The trapped Hg(0) was desorbed by heating and detected by fluorescence on a Tekran 2500 Hg analyzer.[16] Samples for MeHg analyses were first distilled on a Tekran 2750 Methyl Mercury Distillation System, derivitized with 0.001% tetraethylborate in 15 mM acetate buffer (pH 4.5), and analyzed on a Tekran 2700 Methyl Mercury Analysis System (EPA Method 1630).[17] The detection limit for MeHg was ≤20 pM and ≤50 pM in the presence of 5 and 50 nM Hg(II), respectively, primarily due to production of MeHg during distillation.

Chemical Reagents and Standards. High concentrations of $HgNO_3$ and MeHgCl standard solutions were purchased from High Purity Standards and Alfa Aesar, respectively and diluted as needed. Thiol reagents such as L-cysteine and reduced L-glutathione (GSH) were purchased in small quantities from Sigma-Aldrich and replaced after 1 year (<6 months for GSH).

Speciation Calculations. All speciation calculations were conducted using MINEQL+ 4.6 and stability constants provided with the software as well as those provided by Lenz and Martell, 1964 (Zn-cysteine species), and Cardiano et al. 2011 (Hg-cysteine species).[18,19] A full list of all relevant stability constants are provided in Supporting Information (SI) Table S1.

RESULTS AND DISCUSSION

Effect of Trace Metals on Hg Methylation in Whole Cells. We carried out short-term, washed cell Hg(II) methylation experiments with *G. sulfurreducens* and *D. desulfuricans* ND132 in the presence of 5 nM Hg(II) and a large excess of cysteine (100 μM).[9,10] This condition has been shown to enhance uptake and methylation, although rates were less than optimal, as discussed below. This high concentration of cysteine was necessary; however, to ensure full complexation of Hg and cysteine, even in the presence of high concentrations of trace metals. Addition of 50 μM Zn(II) or Cd(II) resulted in quasi complete inhibition of methylation in both organisms (Figure 1). The effect of Zn(II) and Cd(II) on Hg(II) methylation is rather specific as no inhibition whatsoever, but rather a slight enhancement of methylation, was seen upon addition of 50 μM Co(II), Ni(II), or Fe(II). The inhibition of Hg(II) methylation by *G. sulfurreducens* was essentially complete at Zn(II) concentrations ≥20 μM (Figure 2, Table 1), corresponding to only 2.8 μM Zn′ (where Zn′ = sum of all inorganic Zn complexes and hydrated free ion). In the presence of 100 μM cysteine, the bulk of Zn(II) species was calculated to be present as ZnCys (77.5%) and $Zn(Cys)_2$ (8.5%) complexes (Table 1), while Hg(II) remained complexed to cysteine. This inhibitory response observed in Figure 2 is specific to methylation of Hg(II) and not a generalized toxic effect to the cells, as growth was unaffected by Zn(II) concentration as high as 100 μM (corresponding to 25 μM Zn′) (SI Figure S1). The inhibitory effect by Zn(II) was alleviated by the addition of equimolar concentrations of NTA and Zn(II) (10 μM each). At this concentration, NTA decreased markedly Zn′ from 1.3 μM (without NTA) to 0.096 μM (Table 1, Figure 2) while having no effect on Hg speciation.

To interpret the results of Figures 1 and 2, it is necessary to take into account how the complexation of Hg(II) by cysteine may be affected by the addition of the other metals. In particular, the effect of Zn(II) (and Cd(II)) on Hg methylation could conceivably be caused by competition between Zn(II) and Hg(II) for cysteine and a resulting decrease in the concentration of Hg−cysteine complexes. This explanation is highly unlikely for the high cysteine concentration (100 μM) was chosen to make sure that Hg(II) remained bound to cysteine even when other metals were added at 50 μM concentrations, although the exact speciation of the Hg−cysteine complexes is difficult to calculate because of the large range in reported formation constants and species (Table 1, SI Table S1).[18−21] While the specific choice of Hg binding constants affected the relative ratio of the different mono [$Hg(Cys)^0$ and $Hg(Cys)H^+$] and bis [$Hg(Cys)_2^{2-}$ and $Hg(Cys)_2H_2^0$] species (SI Table S1), it did not affect the sum total of all Hg-cysteine complexes (ΣHgCys, Table 1), nor the complexation of Zn(II) with cysteine.

Cysteine has a greater affinity for Ni(II), which does not inhibit methylation, than for Zn(II), which does.[19] In fact, inhibition of methylation by Zn(II) was also observed in experiments carried out in the absence of cysteine, in which Hg(II) is present chiefly as the $HgCl_2$ complex (Figure 3 and Table 1). This result demonstrates that the inhibition of methylation by Zn(II) is not mediated by cysteine or a reduction in Hg−cysteine complexation. We note that the results of Figure

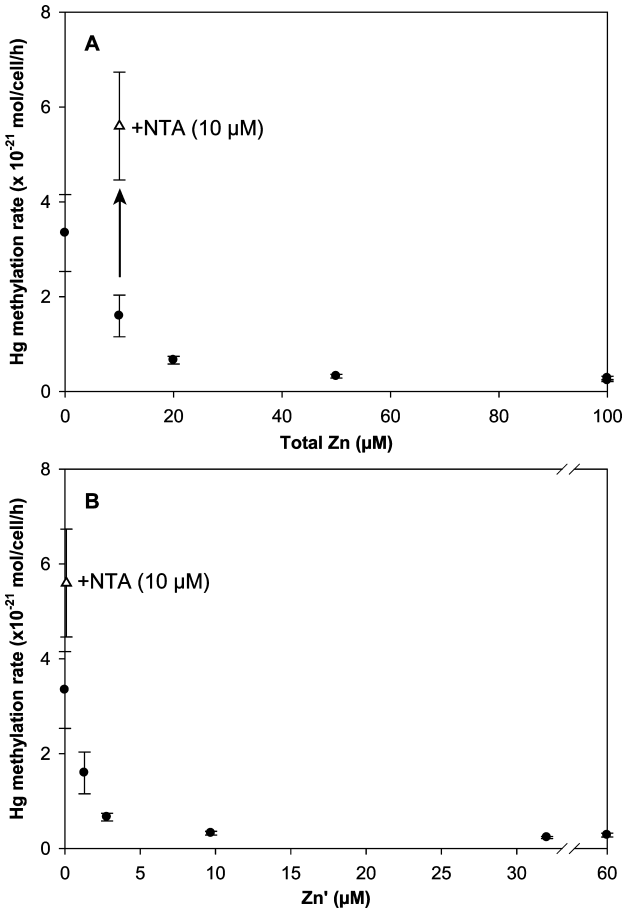

Figure 2. Hg(II) methylation rates in the presence of varied concentrations of (A) total Zn(II) and (B) Zn′ (inorganically complexed Zn) in 1 h washed cell assays of *G. sulfurreducens*. Assays denoted by closed circles contain 5 nM Hg(II), 100 μM L-cysteine and 1.6 mM Cl^- at pH 6.8. Assays denoted by the open triangle denote similar conditions supplemented with 10 μM NTA to decrease [Zn′]. The arrow indicates the increase in Hg methylation rate by the addition of NTA. Error bars indicate ±1 stdev of triplicate assays.

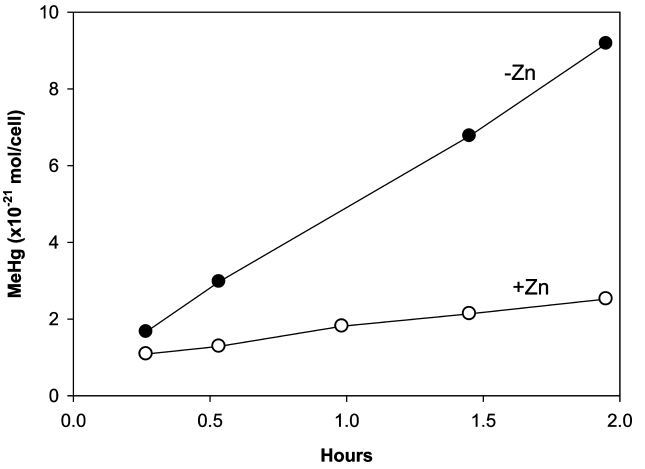

Figure 3. The effect of 50 μM Zn(II) on Hg(II) methylation in washed cell suspensions of *G. sulfurreducens* in the absence of cysteine, where $HgCl_2$ is expected to dominate the speciation of Hg(II). Assays contain 50 nM Hg(II) and 5.4 mM Cl^- at pH 6.8.

3 demonstrate directly that the bulk of the cellular Hg(II) uptake in our experiments does not occur by passive diffusion[5] across the inner membrane which would not be affected by the addition of Zn(II). This result also implies that whether complexed to thiols or chloride, Hg(II) is taken up by the same mechanism/transporter, albeit at possibly different rates and affinities. We surmise that this may also be true for Hg-sulfide complexes.

While a change in the extent of complexation of Hg(II) by cysteine does not explain the inhibition of methylation by Zn(II) or Cd(II), it likely explains the increase in methylation upon addition of other metals. A cysteine concentration of 100 μM is larger than optimal for Hg methylation, and the corresponding methylation rate decreased 60% that observed in the presence of 10 μM cysteine (Figure 1A, inset). This previously observed decrease in methylation rate at high cysteine concentration is likely due to the increase in the number of ligands bound to Hg^{2+} at increasing ligand concentrations ($Hg(Cys)_3$ was previously hypothesized[9]) rendering ligand exchange with cellular proteins

Table 1. Zn Speciation Calculations under Varying Assay Conditions[a]

cells	$Zn(II)_T$ (μM)	Cys (μM)	NTA (μM)	Cl^- (mM)	Zn′[b] (μM)	Zn-Cys[c] (μM)	$Zn(Cys)_2$[c] (μM)	ΣHg-Cys[d] (nM)	Hg meth rate (×10^{-21} mol/cell/h)[e]
Geob	0	100		1.6				5.0	3.34 (0.81)
Geob	10	100		1.6	1.3	7.8	0.9	5.0	1.59 (0.44)
Geob	20	100		1.6	2.8	15.5	1.7	5.0	0.66 (0.08)
Geob	50	100		1.6	9.7	37.5	2.9	5.0	0.32 (0.04)
Geob	100	100		1.6	32.0	65.2	2.6	5.0	0.23 (0.02)
Geob	100	50		1.6	60.0	39.4	0.51	5.0	0.28 (0.04)
Geob	10	100	10[f]	1.6	0.096	0.66	0.088	5.0	5.60 (1.14)
Geob	0	0		1.6					1.03 (0.33)
Geob	50	0		1.6	50.0				0.33 (0.11)
ND132	0	100		220				5.0	7.55 (1.56)
ND132	50	100		220	1.3	32.0	12.5	5.0	0.16 (0.30)

[a] $[Hg(II)]_T$ = 5 nM for all assays. The *G. sulfurreducens* (Geob) and *D. desulfuricans* ND132 assays were buffered to pH 6.8 and 7.1, respectively. Stability constants are provided in SI Table S1. [b] Zn′ = sum of all inorganically complexed Zn(II) and hydrated free ion. [c] Zn-cysteine species were calculated using stability constants reported in Lenz and Martell, 1964.[19] [d] ΣHg-Cys = the sum of all Hg-cysteine complexes calculated to be dominated by $Hg(HCys)_2^0$ species using stability constants reported by Cardiano et al.[18] Calculations using constants reported by Basinger et al;[28] however, suggest greater importance of the monocomplex, $Hg(Cys)^0$. Formation of the $Hg(Cys)_3$ has been suggested by Cheesman et al[21] and Jalilehvand et al 2006;[29] however, binding constants are not available. [e] Values presented as the mean (±1 stdev) for triplicate assays following 1 h incubation with Hg(II). [f] The bulk of Zn(II) is complexed to NTA as $ZnNTA^-$ (9.2 μM) while [HgNTA] is negligible (calculated <10^{-31} M).

more difficult, as has been shown to occur for Zn(II) uptake for phytoplankton.[22]

Upon addition of 50 μM Ni(II), nearly half the cysteine becomes complexed to Ni(II), decreasing the free cysteine concentration and enhancing the proportion of Hg complexes bound to fewer cysteine ligands. In the case of Co(II), which has a lower affinity for cysteine, the same effect is less pronounced, as expected. There are no thermodynamic data available for the formation of Fe(II)−Cys complexes but the effect of Fe(II) on methylation is not statistically significant anyway. We note that since Zn(II) should have an effect on Hg(II) complexation intermediate between those of Ni(II) and Co(II), its inhibition of Hg(II) methylation, which contrasts with the enhancement by the other metals, is all the more striking.

Effect of Zn(II) on Hg(II) Uptake in Whole Cells. Since the addition of Zn(II) caused no inhibition of growth in *G. sulfurreducens* (SI Figure S1), its effect on Hg(II) methylation is likely caused by an inhibition of Hg(II) uptake. This was confirmed in Hg(II) uptake experiments where cellular accumulation and MeHg production were monitored in individual assays over time. As seen in Figure 4, the addition of 50 μM Zn(II) in the uptake assays caused a 65% and 72% decrease in the Hg(II) uptake and methylation rate, respectively, by *G. sulfurreducens.* Similar results were observed in replicate assays following a 40 min exposure to only 5 nM Hg(II), where a 78% decrease in cellular Hg contents was observed with exposure to 50 μM Zn(II) (15.1 ± 4.6 vs $3.3 \pm 1.3 \times 10^{-21}$ mol Hg/cell).

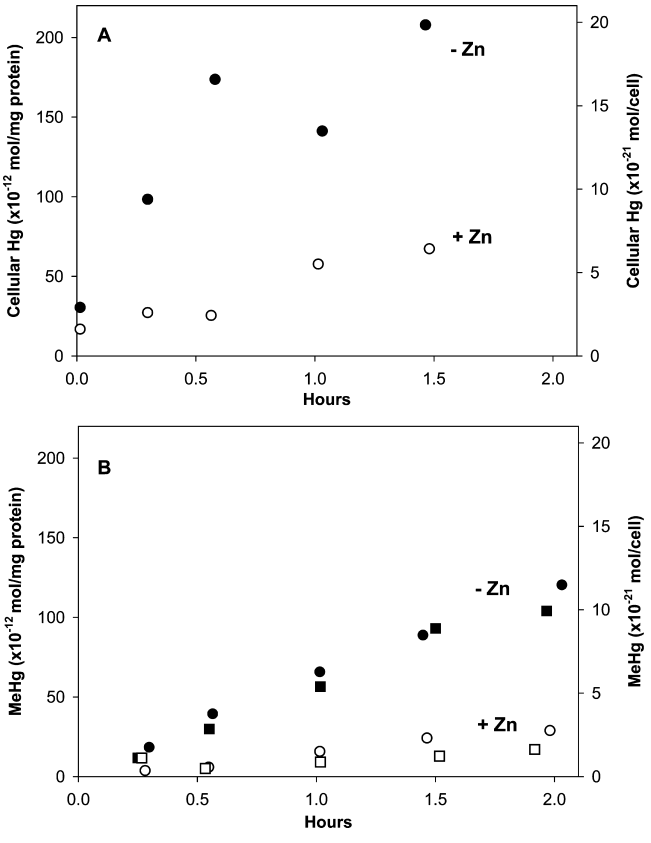

Figure 4. The effect of Zn(II) addition on the (A) uptake and (B) methylation of Hg(II) in washed cell preparations of *G. sulfurreducens* containing 100 μM L-cysteine, 50 nM Hg(II), and 5.4 mM Cl^- (pH 6.8). Filled symbols denote control assays lacking added Zn(II), while open symbols denote assays containing 50 μM total Zn (9.7 μM Zn'). Individual assay vials were followed over time and are indicated with the same symbol in (A) and (B). Data normalized to both protein (left axis) and cell number (right axis) for direct comparison with previous studies[2,3] and Figures 5 and 6.

The most parsimonious explanation of our data on the effect of divalent metals on Hg(II) methylation is that Zn(II) and Cd(II) (but not Ni(II), Co(II), or Fe(II)) are competitive inhibitors for the uptake of Hg(II), a metal with which they share the same outer electronic configuration. The inhibition of Hg(II) uptake could occur through competitive reactions with proteins at the outer membrane, in the periplasm, or at the inner membrane of our model gram negative bacterium. The HgcA protein, which is thought to be the methylating enzyme, possesses four transmembrane domains and methylation could potentially occur in the periplasm rather than in the cytosol, although bioinformatics suggest that the active center of the enzyme should be oriented inward.[2]

Hg(II) Uptake and Methylation in Spheroplasts. To constrain the localization of the Hg/Zn interaction and, hence, that of the methylation reaction, we performed methylation and uptake experiments with spheroplasts of *G. sulfurreducens.* The spheroplasts took up and methylated Hg(II) in the presence of cysteine in a pattern similar to whole cells, albeit at a higher uptake rate (Figure 5). Increased uptake rates have been observed in cells with a destabilized outer membrane through insufficient Mg(II) or Ca(II) cations;[23] thus, the increased uptake rates in these cells are not surprising. Regardless, the cells methylated Hg(II) at a rate remarkably similar to whole cells (SI Table S3) demonstrating their ability to maintain metabolic functions in the presence of an external energy source, as shown previously.[13] Since the spheroplasts lack all soluble periplasmic components, these data demonstrate that Hg−cysteine complexes can serve as a direct substrate to the inner membrane transporter and do not require metal exchange with a periplasmic component prior to uptake. It is possible that Hg−cysteine may pass through the outer membrane as an intact complex, and exchange directly with the inner membrane transporter. As in intact cells, uptake and methylation were inhibited in the absence of electron donor and acceptor, as well as in the presence of the proton uncoupler, CCCP (Figure 5). Hg(II) is thus taken up by spheroplasts by active transport, as it is in whole cells. Furthermore, the MeHg produced by spheroplasts did not remain associated with the spheroplast as 100% of the total MeHg produced was measured in the filtrate (Figure 5B), as has been observed for whole cells.[9] Similar to our findings with whole cells (Figure 4), the addition of 50 μM Zn(II) inhibited Hg(II) uptake and methylation in spheroplasts (Figure 6). As shown by malate dehydrogenase activity assays, the metabolic integrity of the spheroplasts were not affected by the Zn(II) addition (SI Figure S2) with and without cysteine. This result further supports a specific inhibitory effect of Zn(II) on Hg(II) uptake rather than a generalized toxic response for both whole cells and spheroplasts. Our experimental results with spheroplasts of *G. sulfurreducens* provide strong support to the notion that Hg(II) methylation by bacteria occurs intracellularly: (i) the observation of methylation by spheroplasts rules out a mechanism occurring on the surface of the outer membrane or in the periplasm; and (ii) the demonstration that Zn inhibits both uptake and methylation and that Hg(II) is taken up actively by spheroplasts makes it highly unlikely that methylation occurs on the outside surface of the inner membrane. These results coincide nicely with the bioinformatic study localizing the Hg(II) methylating enzyme, HgcA to the inner membrane with the corrinoid binding site facing the cytosolic compartment.[2] The role of HgcA

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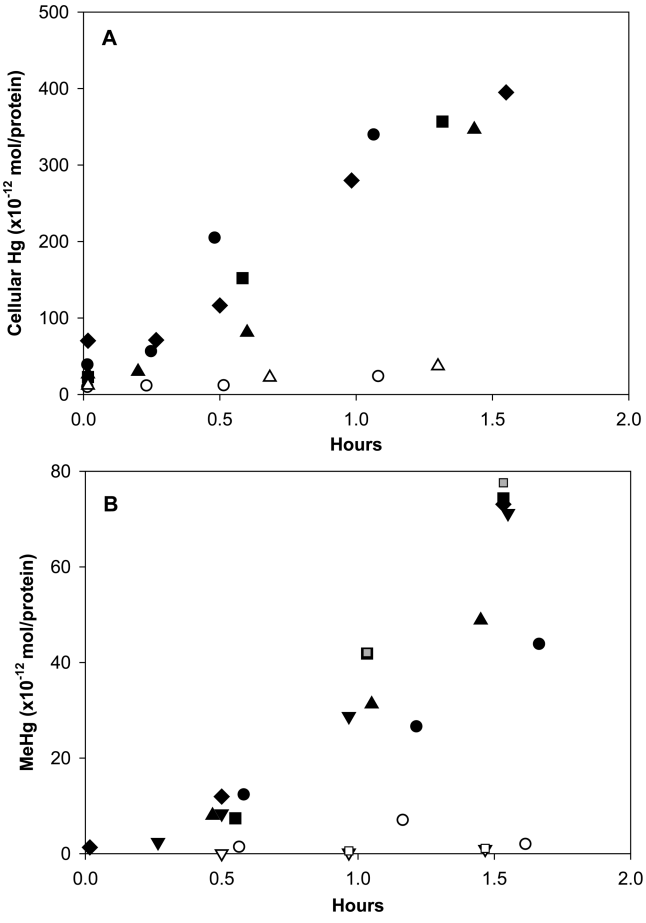

Figure 5. Hg uptake (A) and (B) methylation of Hg(II) in spheroplast preparations of *G. sulfurreducens* containing 10 μM L-cysteine and 50 nM Hg(II). Open symbols indicate either spheroplasts lacking e^- donor/acceptor (−C, circles) or with 20 μM CCCP (triangles); control assays with e^- donor/acceptor as well as 0.1% ethanol (closed triangles only) conducted on the same day as experimentals are shown as solid symbols with identical shape. Small gray squares in (B) indicate dissolved MeHg concentrations in control assays (compare to solid black squares for total MeHg, one of which is overlapping). Individual assay vials were followed over time and are indicated with the same symbol in (A) and (B). Data are normalized only to protein content, as spheroplast cell density was not determined.

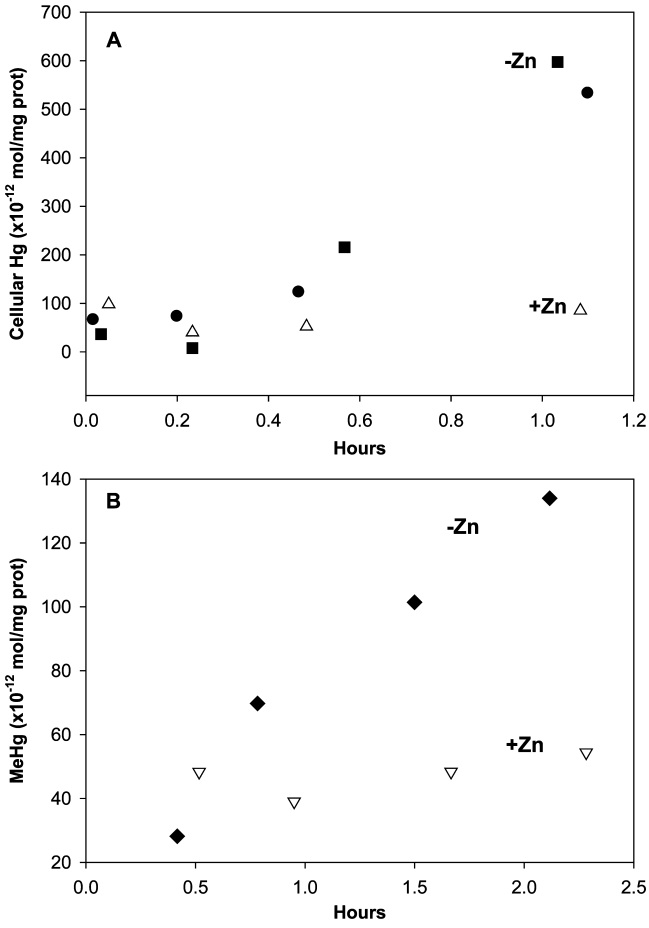

Figure 6. The effect of Zn(II) on Hg(II) uptake (A) and (B) methylation in spheroplast preparations of *G. sulfurreducens* containing 100 μM L-cysteine and 50 nM Hg(II). Assays with and without 50 μM Zn(II) are denoted as open and solid symbols, respectively. Individual assay vials were followed over time and are indicated with the same symbol.

in the transport of Hg(II) or export of MeHg remains to be examined.

Updated Model for Hg(II) Uptake in Bacteria. Our data indicate the likely involvement of heavy metal transporter(s) in the uptake of Hg(II) into cells. This is supported by the specific inhibition of Zn(II) and Cd(II) on the uptake and methylation of Hg(II) by two methylating strains of bacteria. This, coupled with the lack of inhibition by neutral amino acids or diaminopalmitate (a cystine analog)[10] further militate against the involvement of amino acid transporters in the uptake of Hg(II). Amino acid functional groups in Hg-binding ligands were also not found to be required for full rates of Hg(II) methylation in these organisms, (e.g., ligands such as 3-nitrobenzoicmercaptan, chloride, and those containing either amino or acid functional groups but not both).[9,10] Thus, the mechanism of Hg(II) transport in bacteria is clearly different from that observed in mammalian cells.[11,24] Instead, we hypothesize that the transport of Hg(II) into bacterial cells is the result of accidental uptake during the acquisition of an essential trace metal such as Zn(II).

Further, the inhibition by Zn(II) of Hg(II) uptake and methylation of both neutral $HgCl_2$ and charged Hg−cysteine complexes strongly suggest that a shared uptake mechanism exists for both of these species, and possibly Hg−sulfide species as well. Clearly, the passive diffusion model of Hg(II) uptake is not consistent with our data.

Since Hg(II) uptake is facilitated by cysteine, these results suggest that thiols may also be involved in the uptake of Zn(II) itself, and possibly other trace metals in anoxic environments. Little is known about how obligate anaerobes acquire their metals in sulfidic habitats where dissolved metal concentrations may be low.[25] Divalent uptake systems are not completely specific,[26] such that the accidental uptake of Hg(II) via another metal transporter may be widespread. In view of the very high toxicity of Hg(II), it is possible that its methylation and export (as MeHg) serves to detoxify inadvertently accumulated Hg(II) in some anaerobic bacteria, as previously argued.[10] This possibility may merit further study despite the fact that experiments have thus far not shown increased Hg(II) tolerance in methylating organisms.[27]

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■ ASSOCIATED CONTENT

Supporting Information

(Table S1) Thermodynamic stability constants used in this study; (Table S2, S3) MeHg production rates in assays; (Figure S1) growth measurements in the presence of zinc; (Figure S2) malate dehydrogenase activity in spheroplasts following zinc exposure. 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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