Detailed Assessment of the Kinetics of Hg-Cell Association, Hg Methylation, and Methylmercury Degradation in Several *Desulfovibrio* Species

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The kinetics of inorganic Hg [Hg(II)_i] association, methylation, and methylmercury (MeHg) demethylation were examined for a group of *Desulfovibrio* species with and without MeHg production capability. We employed a detailed method for assessing MeHg production in cultures, including careful control of medium chemistry, cell density, and growth phase, plus mass balance of Hg(II)_i and MeHg during the assays. We tested the hypothesis that differences in Hg(II)_i sorption and/or uptake rates drive observed differences in methylation rates among *Desulfovibrio* species. Hg(II)_i associated rapidly and with high affinity to both methylating and nonmethylating species. MeHg production by Hg-methylating strains was rapid, plateauing after ~3 h. All MeHg produced was rapidly exported. We also tested the idea that all *Desulfovibrio* species are capable of Hg(II)_i methylation but that rapid demethylation masks its production, but we found this was not the case. Therefore, the underlying reason why MeHg production capability is not universal in the *Desulfovibrio* is not differences in Hg affinity for cells nor differences in the ability of strains to degrade MeHg. However, Hg methylation rates varied substantially between Hg-methylating *Desulfovibrio* species even in these controlled experiments and after normalization to cell density. Thus, biological differences may drive cross-species differences in Hg methylation rates. As part of this study, we identified four new Hg methylators (*Desulfovibrio aes-poeensis*, *D. alkalitolerans*, *D. psychrotolerans*, and *D. sulfodismutans*) and four nonmethylating species (*Desulfovibrio alcohol-ivorans*, *D. tunisiensis*, *D. carbinophilus*, and *D. piger*) in our ongoing effort to generate a library of strains for Hg methylation genomics.

Microbial mercury methylation is the main driver of risk associated with Hg pollution. Methylmercury production is an anaerobic process that occurs in saturated soils and wetlands (26, 44, 45, 53), decaying periphyton mats (1, 14, 31), aquatic bottom sediments (16, 27, 33, 36), and anaerobic bottom waters (56). Early investigations, prior to the advent of modern methylmercury (MeHg) analyses, reported a wide variety of aerobic and anaerobic Gram-positive and Gram-negative bacteria (30, 49, 55, 58) and fungi (55) to be capable of MeHg production. However, subsequent studies with pure cultures have conclusively demonstrated a role only for sulfate-reducing bacteria (SRB) (4, 8, 11, 13, 20, 23, 38, 50) and iron-reducing bacteria (FeRB; principally *Geobacter* spp.) (21, 37), all belonging to the *Deltaproteobacteria*. Many field studies, using selective microbial stimulants (1, 10, 26, 44, 57), inhibitors (1, 16, 24, 26, 59), and biogeochemical correlates (6, 39, 40, 45), have buttressed the paradigm of SRB and FeRB as the dominant Hg methylators in natural aquatic systems (16, 24, 59), though recent studies have hypothesized that methanogens may be significant in some systems (31).

Only a subset of SRB and FeRB are capable of Hg methylation (11, 23, 37, 50), but why this is the case remains unclear. Early work by Choi and Bartha (13) suggested that Hg methylation was a “metabolic mistake” of SRB utilizing the acetyl coenzyme A (acetyl-CoA) pathway for carbon metabolism. Subsequent studies, however, indicated that Hg methylation capability is not restricted to SRB possessing the acetyl-CoA pathway (20). At present, it is not possible to conclusively identify the methyltransferase or methyl donor in SRB (or other *Deltaproteobacteria*) responsible for *in vivo* Hg methylation. Hg methylation occurs intracellularly (23), and significant effort has therefore been devoted to elucidating the mechanism(s) of Hg uptake by Hg-methylating bacteria.

Passive diffusion of neutral HgS species has been hypothesized to control Hg uptake in SRB (4, 18), while more recent work suggests a role for facilitated transport of specific Hg-amino acid complexes in sulfide-free solutions (51, 52). Further muddying this picture are recent studies demonstrating that HgS or HgS-dissolved organic matter nanoparticles or clusters are bioavailable to Hg-methylating bacteria (29, 60). The mechanism of uptake of these Hg-S species is not yet known. To summarize, significant gaps exist in our understanding of the diversity of microorganisms capable of Hg methylation, the bioavailability of Hg for uptake and the mechanism of uptake, and the mechanism(s) of enzymatic Hg methylation.

In this study, we compared methylation rates among a group of *Desulfovibrio* species. *Desulfovibrio* spp. are prevalent in the environment; *Desulfovibrio* is the best-studied genus of Hg methylators, and a wide variety of *Desulfovibrio* type strains are available in culture collections. The strains used in this study include *Desulfovibrio* species previously tested for MeHg production potential, plus several newly tested strains. Importantly, the rate measurements were done in highly controlled, chemically defined, short-

Received 1 June 2012 Accepted 31 July 2012

Published ahead of print 10 August 2012

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Supplemental material for this article may be found at <http://aem.asm.org/>.

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doi:10.1128/AEM.01792-12

term washed-cell assays. Because medium chemistry, cell density, and cell growth stage may dramatically affect MeHg production, development of a standardized assay for MeHg production was critical for valid comparison of MeHg production potentials among species or strains. In developing a robust data set for Hg methylation in *Desulfovibrio* spp., we aimed to improve coverage of the phylogenetic tree of known *Desulfovibrio* spp. in order to provide targets for comparative genomics that will aid in uncovering the basis of microbial Hg methylation.

Our second major objective was to provide high-temporal-resolution kinetic data for inorganic Hg [Hg(II)_i] association and methylation and MeHg degradation for a set of methylating and nonmethylating *Desulfovibrio* species. One goal of the kinetic studies was to assess whether Hg sorption and/or uptake rates are related to the ability to produce MeHg. Another goal was to measure demethylation rates in *Desulfovibrio* with and without the ability to produce MeHg, investigating whether very rapid demethylation might mask MeHg production in some strains. Overall, the kinetic measurements were designed to advance understanding of the Hg association, uptake, methylation, and demethylation processes in *Desulfovibrio*.

MATERIALS AND METHODS

Cell cultivation and maintenance. Cultures of *Desulfovibrio aesopaeensis*, *D. alcoholivorans*, *D. alkalitolerans*, *D. tunisiensis*, *D. africanus* (strain Benghazi), *D. carbinophilus*, *D. desulfuricans* (strain Essex6), *D. piger*, *D. psychrotolerans*, and *D. sulfodismutans* were purchased from the Leibniz-Institute German Collection of Microorganisms and Cell Cultures (DSMZ) as freeze-dried stocks and revived according to DSMZ instructions. *D. desulfuricans* ND132 and Chesapeake Bay isolates T2 and X2 were isolated from Chesapeake Bay bottom sediments (25).

Cultures were maintained on either freshwater or estuarine sulfate lactate growth medium (FSL or ESL). ESL is identical to the SRM medium previously described (23), except that it was amended with 0.5 mM L-cysteine. FSL contained only 17 mM NaCl. Cells were grown anaerobically in Hungate tubes or serum bottles at 31°C. Representative growth curves for each strain on these media are provided in Fig. S1 in the supplemental material.

Washed-cell assays for determining MeHg production potential. Hg methylation potentials were determined in short-term washed-cell assays in chemically defined minimal medium (51), similar to the approach used in our previous study (29). Cultures were prepared for MeHg production assays by growing cells to mid-log phase (generally to an optical density at 600 nm [OD₆₀₀] of 0.1 to 0.2) and harvesting by centrifugation (15 min at 3,000 × g). Inside an anaerobic glove bag (Coy Laboratory Products; atmosphere of 5% H₂, 95% N₂), cells were resuspended with either FSL or ESL wash buffer. The wash buffer contained 4.1 mM KH₂PO₄, 7.5 mM NH₄Cl, 9.9 mM KCl, 17.1 mM (FSL) or 171 mM (ESL) NaCl, 1 mM Na₂SO₄, 1 mM sodium lactate, 5 mM MOPS, 0.5 mM L-cysteine, and 1 mg/liter resazurin. The pH of the wash buffer was adjusted to 7.50. Cells were reharvested by centrifugation (15 min at 3,000 × g) and then resuspended in wash buffer a second time. Tubes remained clear during all manipulations, indicating minimal oxygen transfer during the cell-washing procedure. Methylation assays were initiated by spiking washed-cell suspensions with 50 nM stable-isotope-enriched ²⁰¹HgCl₂ (98.11% ²⁰¹Hg; Oak Ridge National Laboratory). All methylation assays were performed in triplicate. Aliquots were removed immediately after spiking for determination of optical density, total cell protein, pH, and sulfide; washed-cell suspensions were then incubated for 3 h at 31°C inside the anaerobic glove bag. The 3-h incubation period was selected based on detailed kinetic investigations (see below). Following the 3 h of incubation, samples were collected for measurement of total Hg (THg) and MeHg (preserved in 0.5% HCl), optical density, total cell protein, pH, and

sulfide concentration. Sulfide production was used to estimate the sulfate reduction rate (SRR). Cell-free controls were run periodically to confirm the lack of abiotic methylation by medium components.

Kinetics of inorganic Hg association, methylation, and demethylation. We measured the kinetics of Hg(II)_i-cell association (sorption and/or uptake), methylation, and MeHg demethylation for a subset (*n* = 6 for association and methylation and *n* = 3 for demethylation) of *Desulfovibrio* species. Cells were grown to mid-log phase on estuarine medium with pyruvate as an electron donor and fumarate as an electron acceptor (EPF; 171 mM NaCl) or freshwater pyruvate-fumarate medium (FPF; 17.1 mM NaCl). Cells were washed as described above with minimal FPF (17.1 mM NaCl) or EPF (171 mM NaCl) wash buffer. Pyruvate-fumarate medium and wash buffer recipes are provided in the work of Graham et al. (29); this medium supports respiratory growth of many *Desulfovibrio* species without sulfate reduction and resultant sulfide production. *Desulfovibrio* spp. do degrade cysteine provided in the EPF/FPF medium (29), however, resulting in ~10 to 15 μM inorganic sulfide produced in these experiments.

The kinetics of Hg(II)_i association and methylation were measured for six *Desulfovibrio* strains: *D. desulfuricans* ND132, Chesapeake Bay isolate X2, *D. psychrotolerans*, *D. carbinophilus*, *D. desulfuricans* Essex6, and *D. alcoholivorans*. Experiments were conducted at 31°C in an anaerobic glove bag by spiking hand-agitated cell suspensions with ~1.0 nM ²⁰¹HgCl₂. Demethylation kinetics were examined for three species, *D. desulfuricans* ND132, *D. carbinophilus*, and *D. desulfuricans* Essex6. Washed-cell suspensions were spiked with ~1.0 nM stable isotope enriched Me¹⁹⁸Hg (synthesized in-house by reaction of methylcobalamine with 92.78% enriched ¹⁹⁸HgCl₂ from Oak Ridge National Laboratory; the synthesis method was modified from the work of Bancon-Montigny et al. [2]). Samples were withdrawn over time for measurement of unfiltered and filter (0.2 μm)-passing THg and MeHg in the medium. Measurement of unfiltered THg and MeHg was critical for establishing mass balance and accounting for potential losses of THg or MeHg (e.g., precipitation and/or sorption to bottle walls). For all kinetic experiments, cell density, pH, and sulfide levels were periodically monitored over the duration of the experiments (~8 h). In these experiments, we inferred Hg-cell association (either Hg(II)_i or MeHg) based on the difference between the total measured Hg or MeHg in the cultures and the filter-passing THg or MeHg. Abiotic controls were performed to quantify the extent of Hg(II)_i precipitation and account for abiotic losses of filter-passing Hg(II)_i. Our estimation of cell-associated Hg does not permit discrimination of sorption at the cell-surface from intracellular uptake (51, 52); for that reason, we use the term “Hg-cell association” to describe the combined processes of sorption and uptake.

Analytical methods. Optical density was measured at 660 nm using a Shimadzu UV-Vis spectrophotometer. Total cell protein was measured using the Bradford assay (9). Sulfide was measured using an ion-specific electrode on samples preserved in sulfide antioxidant buffer, calibrated against a Pb-titrated Na₂S standard (15). For most kinetic experiments, cell densities were also determined with a Coulter Counter (Beckman Coulter Multisizer 4). Samples were diluted in Isoton II (Beckman Coulter) and analyzed by counting a 50-μl volume of diluted sample using a 20-μm aperture tube with aperture current set to 600 μA and preamp gain set to 4.0. The aperture tube was calibrated with National Institute of Standards and Technology (NIST)-certified latex beads with a diameter of 2 μm (coefficient of variation < 8%). For *D. psychrotolerans*, cells lysed upon transfer from freshwater medium into Isoton II diluent; cell enumeration was therefore accomplished using epifluorescence microscopy with acridine orange staining.

Methylmercury was measured by isotope dilution (ID) gas chromatography (GC) inductively coupled plasma mass spectrometry (ICP-MS), following aqueous-phase distillation and ethylation with sodium tetraethylborate (34, 45). Derivatized distillates were analyzed with a Brooks Rand MERX automated methylmercury system interfaced to a Perkin Elmer Elan DRCII ICP-MS. Isotope-enriched Me¹⁹⁹Hg (synthesized from

TABLE 1 Results of washed-cell Hg methylation assays^a

Isolate	Source	Growth or assay medium	pH	SRR ($\mu\text{mol H}_2\text{S}_\text{T}$ mg protein^{-1} h^{-1})	MeHg production rate ^b		
					nmol $\text{liter}^{-1} \text{h}^{-1}$	pmol mg protein^{-1} h^{-1}	% Hg methylated ^{b,c}
<i>D. aespoeensis</i> DSM 10631	Deep granitic aquifer, Sweden	ESL	7.59 $\pm$ 0.01	1.5 $\pm$ 0.1	1.16 $\pm$ 0.02	41.4 $\pm$ 0.7	11.5 $\pm$ 1.3
<i>D. alcoholivorans</i> DSM 5433	Distillery waste fermenter, France	ESL	7.41 $\pm$ 0.01	9.7 $\pm$ 3.6	<0.001	<0.2	<0.02
<i>D. alkalitolerans</i> DSM 16529	Alkaline waters of heating system, Denmark	ESL	7.27 $\pm$ 0.02	<0.1	0.71 $\pm$ 0.07	83.5 $\pm$ 10.2	9.0 $\pm$ 1.1
<i>D. desulfuricans</i> ND132	Chesapeake Bay bottom sediments, Maryland, USA	ESL	7.41 $\pm$ 0.01	2.0 $\pm$ 0.1	6.03 $\pm$ 0.60	236 $\pm$ 21	53.3 $\pm$ 5.6
<i>D. tunisiensis</i> DSM 19275	Oil refinery exhaust water, Tunisia	ESL	7.27 $\pm$ 0.01	0.093 $\pm$ 0.005	<0.001	<0.02	<0.01
Chesapeake Bay isolate T2	Chesapeake Bay bottom sediments, Maryland, USA	ESL	7.31 $\pm$ 0.02	2.5 $\pm$ 0.1	3.06 $\pm$ 0.33	190 $\pm$ 9	27.1 $\pm$ 3.0
Chesapeake Bay isolate X2	Chesapeake Bay bottom sediments, Maryland, USA	ESL	7.33 $\pm$ 0.01	1.4 $\pm$ 0.1	7.34 $\pm$ 0.55	109 $\pm$ 9	62.2 $\pm$ 13.7
<i>D. africanus</i> DSM 2603	Well water, Libya	FSL	7.35 $\pm$ 0.01	0.31 $\pm$ 0.08	1.09 $\pm$ 0.22	63.3 $\pm$ 12.3	12.9 $\pm$ 5.1
<i>D. carbinoliphilus</i> DSM 17524	Gas condensate contaminated aquifer, USA	FSL	7.38 $\pm$ 0.01	4.2 $\pm$ 0.6	<0.001	<0.04	<0.01
<i>D. desulfuricans</i> strain Essex 6; DSM 642	Tar-sand mixture in clay, United Kingdom	FSL	7.43 $\pm$ 0.01	3.3 $\pm$ 0.4	<0.001	<0.04	<0.01
<i>D. piger</i> DSM 749	Human intestines	FSL	7.39 $\pm$ 0.01	3.1 $\pm$ 0.2	<0.001	<0.05	<0.01
<i>D. psychrotolerans</i> DSM 19430	Himalayan lake mud, India	FSL	7.38 $\pm$ 0.01	1.9 $\pm$ 0.1	1.34 $\pm$ 0.02	26.7 $\pm$ 0.4	15.3 $\pm$ 0.9
<i>D. sulfodismutans</i> DSM 3696	Freshwater mud, Germany	FSL	7.41 $\pm$ 0.01	4.1 $\pm$ 0.6	0.135 $\pm$ 0.001	21.5 $\pm$ 3.6	1.9 $\pm$ 0.1

^a Washed-cell assays were carried out at 31°C for 3 h in minimal medium amended with 50 nM ²⁰¹HgCl₂ and 0.5 mM L-cysteine.^b Lower detectable limit calculated based on corresponding method detection limit for MeHg (2.2 pM Me²⁰¹Hg).^c Fraction of Hg methylated calculated based on measured total Me²⁰¹Hg and ²⁰¹THg in medium at the end of 3 h of incubation.

91.95% ¹⁹⁹HgCl₂ from Oak Ridge National Laboratory) was used as the ID spike. Concentrations of MeHg were calculated based on the isotope abundances of the isotope tracer (either Me²⁰¹Hg or Me¹⁹⁸Hg), the ID spike (Me¹⁹⁹Hg), and ambient Hg (using Me²⁰²Hg) after correcting for impurities in the tracer and ID spike solutions (34). Total Hg (THg) samples were digested in hot 7:4 (vol/vol) HNO₃/H₂SO₄ (1:2 [vol/vol] sample, digest acid) until vapors turned colorless; digests were preserved with 1% (vol/vol) BrCl. Filtered THg samples were digested overnight with 1% (vol/vol) BrCl at room temperature. THg analyses were performed by SnCl₂ reduction and detection of Hg(0) vapor by flow-injection ICP-MS or cold vapor atomic fluorescence spectrometry (CVAFS; Tekran model 2600 spectrometer). The CVAFS analysis was used in the detailed kinetic experiments and does not provide isotope information; however, ICP-MS analysis demonstrated that levels of ambient Hg were generally negligible (i.e., concentrations of excess tracer Hg $\approx$ total Hg). Analytical precision, reproducibility, and instrument detection limits for Hg analyses are summarized in Table S1 in the supplemental material.

Equilibrium speciation calculations. Inorganic Hg(II) speciation in washed-cell assays was modeled using MINEQL+, version 4.5 (Environmental Research Software). All thermodynamic data for input into MINEQL+ were identical to those used by Graham et al. (29) and are given in Table S2 in the supplemental material.

RESULTS AND DISCUSSION

Comparison of Hg methylation rates by *Desulfovibrio* spp. in washed-cell assays. Of the 13 *Desulfovibrio* assayed in this study, 8 were capable of significant MeHg production in short-term washed-cell assays on sulfate-reducing medium (Table 1). Of the eight previously unevaluated *Desulfovibrio* spp., we identified four Hg methylators (*D. aespoeensis*, *D. alkalitolerans*, *D. psychrotolerans*, and *D. sulfodismutans*) and four nonmethylating species (*D. alcoholivorans*, *D. tunisiensis*, *D. carbinoliphilus*, and *D. piger*).

Among the *Desulfovibrio* strains tested here, the three strains isolated from Chesapeake Bay sediments stood out as exceptionally efficient Hg methylators. Under the conditions of these assays, these strains methylated 50 to 60% of Hg in the culture medium within 3 h.

Both MeHg production rates and sulfate reduction rates during the methylation assays varied significantly among the strains. Mercury methylation rates, measured as the absolute amount of MeHg produced, varied $\sim$ 50-fold (0.14 to 7.3 nmol liter⁻¹ h⁻¹) among the Hg-methylating strains. Variability among strains was lower when MeHg production was normalized to either the amount of Hg in the medium (30-fold spread, as some Hg is lost to bottle walls, where it is presumably unavailable to bacteria) or to protein concentrations (10-fold spread). This highlights the importance of careful examination of Hg partitioning and cell density, factors not always considered in previous studies of Hg methylation by bacterial cultures.

Importantly, sulfate reduction rates (SRR) and MeHg production rates were not correlated among the strains tested (see Fig. S2 in the supplemental material). Protein-normalized SRR were within the range reported for other SRB at similar cell densities (38). The lack of a positive correlation suggests that general metabolic activity is a poor predictor of MeHg production rates among Hg-methylating species. However, the relationship between SRR and MeHg production is complicated by the opposite influence of sulfide on inorganic Hg(II) speciation and bioavailability (4, 5, 7). Inorganic Hg(II) bioavailability to Hg-methylating bacteria is believed to decline with increasing sulfide concentration, owing to formation of nonbioavailable HgS species [HgS(s) and/or negatively charged HgS species] (4, 5).

Previous studies showed that cysteine enhances Hg methylation rates in cultures of both *Desulfovibrio* (29, 52) and *Geobacter* (51, 52). We therefore added 0.5 mM L-cysteine to our methylation assays to promote maximal Hg methylation rates. Equilibrium speciation calculations suggest that 0.5 mM L-cysteine was insufficient to prevent metacinnabar [β -HgS(s)] precipitation under our assay conditions in sulfide-producing cultures (total Hg added $[\text{Hg}]_T = 50 \text{ nM}$, $[\text{H}_2\text{S}]_T = 10 \text{ to } 500 \text{ }\mu\text{M}$; pH = 7.4; see Fig. S3 in the supplemental material). The speciation calculations are sensitive to the value of K_{SO} for β -HgS(s), which has uncertainty spanning 4 orders of magnitude (17), but β -HgS(s) precipitation is predicted for $10 \text{ }\mu\text{M} \leq [\text{H}_2\text{S}]_T \leq 1 \text{ mM}$, whether a high (40.0) or low (36.0) value for $\log K_{\text{SO}}$ is selected. We observed that Hg was highly bioavailable in our assays, however, with up to 60% of Hg being methylated within 3 h by the most efficient strains (e.g., *D. desulfuricans* ND132 and isolate X2).

The addition of cysteine has a dramatic effect on Hg(II)_i bioavailability, even under conditions where complexes or precipitates of Hg with inorganic sulfide are predicted to dominate Hg speciation. At similar Hg and sulfide concentrations, Gilmour et al. (23) observed only ~5% Hg methylation over 3 days by *D. desulfuricans* ND132 in cysteine-free solutions in batch growth experiments. Previous work in our laboratory demonstrated that dissolved organic matter (DOM), a relatively weak ligand for Hg(II)_i compared to sulfide, could substantially enhance Hg bioavailability in solutions supersaturated with respect to β -HgS(s) (29). We attributed this finding to the retardation of β -HgS(s) growth and aggregation by DOM (17, 22) and hypothesized that DOM-stabilized β -HgS(s) nanoparticles may be bioavailable to Hg-methylating bacteria, a supposition recently corroborated by Zhang et al. (60). While previous work has hypothesized facilitated or active transport of Hg-cysteine complexes (51, 52), it is also likely that cysteine enhances Hg methylation by retarding growth and aggregation of β -HgS(s), as low-molecular-weight thiols are effective inhibitors of metal sulfide particle growth (28).

Reported Hg methylation rates vary widely, even for a single organism. These differences likely reflect interacting dependencies on Hg concentration, cell density and/or growth stage, temperature, and medium chemistry (4, 11, 20, 21, 38, 50). Such variability complicates efforts to make comparisons across organisms or studies. Although Hg complexation and bioavailability have been studied somewhat systematically, Hg mass balance and partitioning are rarely monitored during assays, and so rate calculations can be obfuscated. Further, there are few controlled studies on the influences of cell growth rates or growth phase (46). Both medium chemistry and growth phase may impact methylation. Illustrating that point, we observed Hg methylation rates in washed-cell assays that were 1 to 2 orders of magnitude higher than rates previously reported for the same bacteria in batch growth assays in a similar medium and at a similar Hg(II)_i concentration. For example, Hg methylation rates of ~0.02 to 0.08 $\text{nmol liter}^{-1} \text{ h}^{-1}$ were observed for three Chesapeake Bay isolates (T2, X2, and *D. desulfuricans* ND132) in batch growth assays (23) compared to ~3 to 7 $\text{nmol liter}^{-1} \text{ h}^{-1}$ for the same bacteria in short-term washed-cell assays with 0.5 mM cysteine addition. The higher Hg methylation rates we report here probably reflect the shorter duration of the assays as well as the greater bioavailability of Hg(II)_i in the presence of cysteine.

Given the cost and effort associated with MeHg analyses, MeHg production rates are routinely calculated based on a single

measurement of Hg methylation after some arbitrarily selected incubation time, and the full time course of MeHg production over the assay duration is rarely monitored (see our detailed time courses below). The single measurement approach may underestimate Hg methylation rates if Hg methylation activity levels off prior to when the measurement is taken. Further, medium chemistry, especially at the end of methylation assays, is rarely measured in sufficient detail to calculate the complexation of Hg. As SRB produce sulfide from sulfur sources other than sulfate—including cysteine—measurement of sulfide is critical to evaluating Hg bioavailability in methylation assays.

A major objective of the present work was to develop a standardized experimental approach that would permit quantitative comparison of methylation rates between different bacterial species. Our goal was to make comparisons at the same growth phase and cell density and with the best possible control of Hg(II)_i speciation, and bioavailability. Under those controlled conditions, we still observed significant differences in Hg methylation rates among *Desulfovibrio* strains with the ability to produce MeHg, suggesting that there are intrinsic biological differences in their abilities to methylate Hg. Moving forward with studies of bacterial Hg methylation, we advocate the adoption of a more standardized and rigorous approach, which will allow researchers to make more meaningful comparisons of Hg methylation among bacteria and across environmental conditions. At a minimum, we stress that future studies of bacterial Hg methylation should report Hg mass balance, key variables (especially sulfide concentration) that permit estimation of Hg(II)_i speciation, and measurements of cell density and activity.

An updated phylogeny of Hg methylation by *Desulfovibrio*. To date, 23 of 59 *Desulfovibrio* species available in pure culture (39%) have been phenotyped for Hg methylation capability, representing by far the most comprehensive effort to understand Hg methylation at the subgenus level. Eleven of the 23 species evaluated (48%) are capable of Hg methylation, and Hg-methylating *Desulfovibrio* species are distributed throughout the phylogenetic tree, with small subclades of methylators being apparent (see Fig. S4 in the supplemental material). Consistent with earlier phylogenetic analyses of Hg methylation (23, 50), the ability to methylate Hg is species specific. This finding implies that linking microbial community structure to *in situ* rates of MeHg production in field soils or sediments (54) will be extremely difficult without an Hg methylation-specific genetic probe, as it is impossible to generalize about Hg-methylating capability even at the genus level.

The observed taxonomy of Hg methylation to date shows that this trait is focused in the *Deltaproteobacteria* (4, 8, 11, 13, 20, 21, 23, 37, 38, 50). Most of the identified methylators are in two genera, *Desulfovibrio* and *Geobacter*. Additional Hg-methylating species have been identified in three other less thoroughly studied families within the *Deltaproteobacteria*, specifically the *Desulfobacteraceae*, *Desulfobulbaceae*, and *Desulfomicrobiaceae* (as summarized by Ranchou-Peyruse et al. [50]). However, among most of these families, the ability to methylate Hg is scattered, and there are often more strains without the ability to produce MeHg. No Hg methylators have been identified to date in pure culture outside the *Deltaproteobacteria*. In the case of the *Desulfovibrio*, the ability to produce MeHg appears to be somewhat more common among estuarine and marine than freshwater bacteria. The pattern that we observed, i.e., loosely related or unrelated small clades of Hg methylators, suggests horizontal gene transfer among a

FIG 1 Comparison of Hg sorption/uptake and methylation rates for methylating *Desulfovibrio* species following addition of ~ 1 nM HgCl_2 to cell suspensions. Right panels show enlargements of first 30 min of each time course. Filter-passing total Hg (THg) or MeHg passed a $0.2\text{-}\mu\text{m}$ filter. Particulate THg is the difference between mean total THg in the medium and measured filter-passing THg.

group of anaerobes utilizing the same types of habitat. The taxonomy of Hg-methylating microbes provides a starting point for bioinformatic queries on the genetic basis of Hg methylation.

Kinetics of Hg-cell association. The rate at which Hg partitioned to cells was examined for six *Desulfovibrio* strains, including three with and three without the ability to produce MeHg. These kinetic experiments were done in washed-cell assays, using cultures grown on pyruvate-fumarate medium, thus limiting sulfide production to the low-micromolar levels ($13.7 \pm 3.6 \mu\text{M}$ across experiments) produced from cysteine in the medium. All cells were in early log phase, but cell densities in the assays ranged from $\sim 1 \times 10^7$ to 7×10^8 cells/mL.

For most of the strains, Hg(II)_i was rapidly lost from solution and partitioned to cells (Fig. 1 and 2). The magnitude of the loss was directly proportional to cell density (Fig. 3). Although there was an initial Hg loss from solution in uninoculated controls, it was of lesser magnitude than in cultures (Table 2). Both cultures (Fig. 1 and 2) and controls (Fig. 4; also, see Fig. S5 in the supplemental material) showed an immediate (<2 -min) loss of ~ 0.2 to 0.4 nM (or up to 40%) Hg(II)_i from solution to the particulate phase. This loss occurred even if medium had been filtered immediately before the Hg addition (Fig. 4), suggesting loss via Hg(II)_i precipitation, presumably with sulfide.

FIG 2 Comparison of Hg sorption/uptake by nonmethylating *Desulfovibrio* species following addition of ~ 1 nM HgCl_2 to cell suspensions. Right panels show enlargements of first 30 min of time course. Filter-passing total Hg (THg) passed a $0.2\text{-}\mu\text{m}$ filter. Particulate THg is the difference between mean total THg in the medium and measured filter-passing THg. MeHg concentrations were below the limit of detection (1.4 pM).

In all active cell suspensions, except for that of *D. alcohovorans*, we observed rapid and near-complete loss of filter-passing THg beyond initial losses in uninoculated controls. After the losses that occurred in the first minute, Hg in cultures continued

FIG 3 Relationship between cell density and the initial rate of loss of filter-passing THg for methylating and nonmethylating *Desulfovibrio* species in washed-cell assays spiked with 1 nM HgCl_2 . The triangle shows the rate of loss of filter-passing THg in an abiotic control experiment. The initial rate of loss of filter-passing THg was calculated based on the loss during the interval before the first sampling (20 s to 2 min).

TABLE 2 Hg(II)_i-cell association rates and initial Hg methylation rates in *Desulfovibrio* strains^a

Strain or control	Cell density (cells/ml)	Initial loss of filter-passing THg (nM/min) ^b	Estimated Hg-cell association rate (pM/min) ^c	Initial Hg methylation rate (pM/min) ^d
Methylators				
<i>D. desulfuricans</i> ND132	2.72×10^8	0.75	92 ± 45	21.8 ± 0.3
<i>Desulfovibrio</i> sp. strain X2	6.85×10^8	2.59	70 ± 80	1.1 ± 0.2
<i>D. psychrotolerans</i>	1.17×10^7	0.32	12 ± 4	0.60 ± 0.05
Nonmethylators				
<i>D. carbinophilus</i>	1.45×10^8	0.67	33 ± 4	$<0.003^e$
<i>D. desulfuricans</i> Essex6	2.96×10^8	1.44	70 ± 30	$<0.003^e$
<i>D. alcohovorans</i>	4.23×10^7	0.16	0.9 ± 0.4	$<0.003^e$
Abiotic controls				
Unfiltered EPF assay buffer	NA ^f	0.19	0.6 ± 0.3^g	$<0.003^e$
0.2- μ m- <i>pre</i> filtered EPF assay buffer	NA	0.12	0.8 ± 0.2^g	$<0.003^e$

^a Rates were measured in Hg-methylating and nonmethylating *Desulfovibrio* strains following the addition of ~ 1 nM $^{201}\text{HgCl}_2$ to washed-cell suspensions containing 0.5 mM L-cysteine.

^b Loss of filter-passing THg between ~ 1 nM HgCl_2 and first sampling point (20 s to 2 min).

^c Based on linear regression of filter-passing THg-versus-time data for the initial portion of the time course following initial loss of filter-passing THg. Error estimates reflect 95% confidence intervals.

^d Initial Hg methylation rate determined by linear regression of the early linear portion of total MeHg-versus-time data. Error estimates reflect 95% confidence intervals.

^e MeHg production after 8 h was below the limit of detection (1.4 pM). The values are the corresponding detection limits for cell-normalized MeHg production rates.

^f NA, not applicable.

^g The rate of slow filter-passing-THg loss following the initial drop in filter-passing THg, provided as a comparison for rates of filter-passing-THg loss in cell suspensions.

to partition from the aqueous phase to the particulate phase; in contrast, filter-passing THg declined very slowly in abiotic controls (Fig. 4; also, see Fig. S5 in the supplemental material). Together, these data suggest that Hg-cell interaction (sorption and/or uptake) was the dominant removal mechanism for Hg(II)_i for the majority of the *Desulfovibrio* strains we evaluated. We also note that abiotic losses in active cell suspensions may have been less than that observed in cell-free controls due to competition for Hg between cells and inorganic sulfide. The strong dependence of the initial Hg loss on cell density (Fig. 3) and the extent of MeHg production support the idea that initial losses of Hg from solution are not solely abiotic. Because MeHg production occurs intracellularly (23), the extent of MeHg provides a direct measure of the minimum amount of Hg(II)_i uptake (29). In the case of the strong

FIG 4 Loss of filter-passing THg in an abiotic control experiment following addition of ~ 0.9 nM HgCl_2 to filtered (0.2 μm) EPF wash buffer containing 0.5 mM L-cysteine and ~ 1 μM inorganic sulfide. Loss of THg from medium represents loss to bottle walls (as Hg^0 production was not observed in *Desulfovibrio* cultures or medium [23]). Data are means and standard deviations from triplicate experiments.

methylator *D. desulfuricans* ND132 (Fig. 1a), MeHg production [and hence Hg(II)_i uptake] exceeds 0.9 nM, indicating that $>65\%$ of the loss of 0.6 nM filter-passing THg in the first minute of the experiment is due to association with cell surfaces and/or intracellular uptake. For *Desulfovibrio* sp. strain X2 (Fig. 1b), total Hg(II)_i uptake was at least 0.3 nM based on measured MeHg production, meaning that at least 20% of the near instantaneous loss of filter-passing THg is accounted for by Hg(II)_i uptake.

Minimum Hg(II)_i-cell association rates can be estimated based on linear regression of the filter-passing THg-versus-time data for the period after the initial loss in filter-passing THg. For Hg-methylating *Desulfovibrio*, we restricted regression analysis to the portion of the time course before a minimum of filter-passing THg was reached (see enlarged portions of Fig. 1), i.e., the time before cells begin to export significant filter-passing Hg as MeHg. Rates of Hg(II)_i-cell association estimated in this way ranged from 0.9 to 92 pmol liter⁻¹ min⁻¹ (Table 2). Normalized to cell density, Hg(II)_i-cell association rates ranged from ~ 0.1 to 1.0 zmol cell⁻¹ min⁻¹ (excluding *D. alcohovorans*) and were not appreciably different for Hg-methylating and nonmethylating strains.

We hypothesized that the rate of Hg sorption and/or uptake by cells might be a determinant of a strain's ability to produce MeHg. However, two of the three nonmethylating *Desulfovibrio* strains we evaluated rapidly took up Hg(II)_i from solution, and showed Hg-cell association rates similar to those observed for methylating *Desulfovibrio* strains (Fig. 2; Table 2). To our knowledge, this is the first report on the kinetics of Hg(II)_i association with nonmethylating SRB. The rate of Hg association with cells does not appear to be a determinant of a species' ability to produce MeHg.

Because MeHg production is intracellular (23), we know that some fraction of the Hg we measure as being associated with cells is taken up into the cytoplasm. However, the fraction of the cell-associated Hg(II)_i that is taken up intracellularly by the nonmethylating *Desulfovibrio* strains is unknown, and these experiments offer limited insight into Hg(II)_i uptake versus sorption in

nonmethylating *Desulfovibrio* strains. *Desulfovibrio* spp. do not appear to contain the *mer* operon (3), a genetic system that includes an active Hg transporter (3), and the mechanism of Hg uptake by methylating and nonmethylating *Desulfovibrio* strains alike remains unknown.

Kinetics of Hg methylation and export. For the Hg-methylating *Desulfovibrio* strains, MeHg was immediately and rapidly produced. Hg(II)_i-cell association rates generally exceeded initial Hg methylation rates by an order of magnitude (Table 2), and so Hg(II)_i association with the cell surface probably did not limit the overall rate of Hg methylation. However, because these experiments did not distinguish Hg(II)_i uptake from sorption, we cannot rule out the possibility that intracellular uptake was rate limiting. Interestingly, we observed no time lag in MeHg production, as previously reported by Schaefer et al. (52). Because methylation occurs intracellularly (23) the lack of a time lag in Hg methylation suggests fairly rapid intracellular Hg uptake.

MeHg was rapidly exported from cells after production. Throughout the assays, 75 to 100% of total MeHg was always in the filter-passing phase (Fig. 1). Further, we saw no evidence for the buildup of a cell-associated MeHg pool. Excreted MeHg had little affinity for the cell surface, in contrast with the extremely high affinity of Hg(II)_i for these cell surfaces. Similar findings have been observed previously in longer-term experiments (23, 29) but have yet to be adequately explained. Possible explanations for the low affinity of MeHg for the cell surface include selectivity of cell surface receptors and transporters for specific Hg(II)_i-ligand complexes compared to MeHg-ligand complexes and/or inertness of MeHg complexes with regard to ligand exchange reactions at the cell surface. MeHg generally has lower particle affinity in the environment than Hg(II)_i (32, 35).

Mass balance clearly showed the persistence of particulate Hg(II)_i that was not converted to MeHg (Fig. 1). One explanation for incomplete conversion of Hg(II)_i to MeHg is limitation in the methyl donor. We tested this idea by conducting washed-cell methylation assays with starved cells. Assays were conducted with *D. desulfuricans* ND132 in a minimal salts medium without a carbon source, electron donor, electron acceptor, or essential nutrients (only 0.171 M NaCl, 10 mM MOPS, and 0.5 mM L-cysteine). The kinetics of Hg-cell association and methylation in these starved assays were comparable to assays in more complete medium (Hg methylation rates of 0.027 ± 0.001 zmol cell⁻¹ min⁻¹ for starved cells versus 0.014 ± 0.001 zmol cell⁻¹ min⁻¹ for cells in minimal medium [see Fig. S6 in the supplemental material]). The fact that starved cells methylate Hg as fast as cells provided with energy-generating substrates suggests that energy requirements for methylation of low-nM levels of Hg(II)_i are small. Hg methylation may be supported by either endogenous metabolism or low concentrations of residual energy-generating substrates remaining after cell washing. Further, Hg methylation rates by *D. desulfuricans* ND132 in washed-cell assays were proportional to added HgCl₂ concentration over the entire concentration range examined, ~0.25 to 40 nM Hg(II)_i (see Fig. S6). Taken together, these findings argue against the interpretation that incomplete Hg methylation is due to depletion of the methyl donor(s) to Hg(II)_i.

Alternatively, uptake capacity for Hg(II)_i may be limited by precipitation of Hg(II)_i into a form unavailable for uptake and/or sorption of Hg(II)_i to cell surfaces in a location or form unavailable for intracellular uptake. Precipitation of Hg(II)_i or strong sorption of Hg(II)_i to cell surface sorption sites may serve as

FIG 5 No appreciable Hg demethylation was observed in ~8-h incubations of either an Hg-methylating strain (*D. desulfuricans* ND132) or nonmethylating strains (*D. carbinophilus* and *D. desulfuricans* Essex6). The data for this demethylation experiment were collected under conditions of growth stage, cell density, and total Hg addition (~1 nM) similar to those in the association/methylation experiments whose results are shown in Fig. 1 and 2. Filter-passing total Hg (THg) or MeHg is the THg or MeHg that passed a 0.2-μm filter.

“sinks” for Hg(II)_i, lowering the total pool of bioavailable Hg(II)_i. X-ray-based spectroscopic investigations might provide definitive information regarding the nature of Hg(II)_i interactions with SRB cell surfaces (42, 43), but significant improvements in method detection limits would be required to probe Hg(II)_i speciation at the cell surface at environmentally relevant concentrations (sub-nanomolar).

MeHg degradation kinetics. We examined demethylation kinetics for three *Desulfovibrio* strains (one methylator and two nonmethylators) and found no evidence for significant demethylation by any of the bacteria (Fig. 5). Previous studies (including a study with *D. desulfuricans* ND132 in our laboratory) reported demethylation rates of ~3 to 20%/day for various SRB in batch growth assays (11, 23). Given these demethylation rates, it is possible that our assays were simply too short to measure low demethylation rates (a demethylation rate of 9%/day would be

within the error of our MeHg analysis). We did not detect any production of Hg(II)_i in the aqueous phase by high-pressure liquid chromatography (HPLC)–ICP-MS (12), which provides a direct and independent verification of slow demethylation in these experiments. In the washed-cell demethylation experiments reported here, we supplied 0.5 mM L-cysteine as the dominant MeHg ligand to match the association and methylation assays. In the previously reported batch culture demethylation assays with ND132 (23), the primary ligand for MeHg (in experiments showing demethylation rates above those of abiotic controls) was inorganic sulfide.

Limited field data suggest that MeHg speciation is an important determinant of MeHg degradation rates in sediments (19), but the impact of MeHg speciation on its bioavailability has not been evaluated in controlled experiments with pure cultures. Efforts have been devoted to the study of MeHg uptake in cells with the *mer* operon (which can include genes for a MeHg transporter); however, demethylation in anaerobic settings is probably not driven by these organisms (3). The mechanism of “oxidative demethylation” (47), which appears to be the dominant MeHg degradation pathway in anaerobic sediments (41), is poorly understood. The mechanisms of microbial MeHg uptake and degradation in anaerobic sediments and soils, which are often rapid in field studies (19, 41, 47, 48), deserve attention.

Our short-term demethylation experiments confirm the low affinity of MeHg for cell surfaces noted in the methylation assays. In these assays, where MeHg was provided to cells exogenously, we observed low and comparable MeHg-cell association rates for methylating (Fig. 5a) and nonmethylating (Fig. 5b and c) *Desulfovibrio* strains alike. Taken together with the rapid export of MeHg from cells after its production, this implies a similar surface reactivity for MeHg produced *in vivo* and supplied exogenously.

The demethylation experiments were designed to evaluate the idea that we might be mislabeling some *Desulfovibrio* strains as “nonmethylators” because methylation in these strains is masked by very rapid demethylation. This does not appear to be the case, however, as rapid demethylation was not observed in any *Desulfovibrio* strain tested. This conclusion requires a word of caution. While exogenous MeHg appeared to be relatively inert with respect to both sorption/uptake and demethylation, it may be less readily taken up and demethylated than MeHg produced *de novo* by cells. However, the rapid export of MeHg from methylating *Desulfovibrio* strains (Fig. 1) argues against this.

Future directions for bacterial Hg methylation research.

Here we have presented a rich data set comparing methylating and nonmethylating SRB, belonging to the genus *Desulfovibrio*. Our results suggest that the Hg methylator phenotype is fairly common within the genus *Desulfovibrio* (~50% of *Desulfovibrio* spp. are capable of Hg methylation) and distributed throughout the phylogenetic tree. Hg methylation rates vary substantially between Hg-methylating *Desulfovibrio* spp. even in washed-cell experiments, where every effort is made to ensure similar Hg(II)_i speciation and bioavailability and rates are normalized to cell density. This finding indicates that biological differences (e.g., cell surface chemistry, gene expression and regulation) may drive cross-species differences in Hg methylation rates. This finding is consistent with recent reports concluding that growth state and gene expression can influence MeHg production by a single species (46). The dominance of biological versus geochemical con-

trols on Hg methylation in natural environments remains an open question.

These detailed kinetic experiments provide a first look at the short-term kinetics of Hg-cell association, MeHg production and degradation in *Desulfovibrio*. We conclude that Hg(II)_i associates rapidly with nearly all *Desulfovibrio* strains, even under conditions where a relatively high concentration of a strong Hg-binding ligand is present. For Hg-methylating *Desulfovibrio* spp., Hg methylation rates are rapid (initial rates of 0.002 to 0.08 $\text{zmol cell}^{-1} \text{min}^{-1}$) and do not appear to be limited by Hg(II)_i -cell association rates (0.1 to 1.0 $\text{zmol cell}^{-1} \text{min}^{-1}$), implying that Hg(II)_i binding at the cell surface is not rate limiting. Nonmethylating *Desulfovibrio* spp. do not appear to be deficient in acquisition of Hg(II)_i from solution or superior in Hg demethylation.

We conclude that a low affinity of Hg(II)_i for cell surfaces and/or rapid degradation of MeHg is an unsatisfactory explanation for why only a subset of *Desulfovibrio* species are capable of Hg methylation. The primal cause(s) of Hg methylation capability remains enigmatic, but whole-genome comparisons of known Hg methylators and nonmethylators may prove fruitful in identifying candidate genes involved in Hg methylation. Our finding that nanomolar levels of Hg(II)_i associate rapidly (and near completely in many cases) with cells of methylating and nonmethylating bacteria also raises important questions. We speculate that competition between cell surface sorption and intracellular uptake may limit the extent of Hg methylation by *Desulfovibrio*, but this hypothesis requires further evaluation. Building on this hypothesis further, if nonmethylating bacteria are competitive with methylating bacteria for Hg(II)_i acquisition (sorption plus uptake), do nonmethylating bacteria act as a sink for Hg(II)_i in soils and sediments with consortia of methylating and nonmethylating bacteria? The distribution of organisms capable of Hg methylation versus those sequestering Hg in natural environments awaits the determination of the mechanism(s) and genetic control of Hg uptake and methylation.

ACKNOWLEDGMENTS

This work was supported by the U.S. Department of Energy under the Subsurface Biogeochemical Research Program (SBR), Office of Biological and Environmental Research, Office of Science, through the Mercury Science Focus Area Program at Oak Ridge National Laboratory (ORNL), and by National Science Foundation grant DEB0351050 (C.C.G.). ORNL is managed by University of Tennessee UT—Battelle, LLC, for the Department of Energy under contract no. DEAC05-00OR22725. A.M.G. acknowledges support from the Smithsonian Institution Fellowship Program.

We thank Georgia Riedel and Tyler Bell for analytical assistance.

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