Mercury Methylation by Dissimilatory Iron-Reducing Bacteria[▽]

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The Hg-methylating ability of dissimilatory iron-reducing bacteria in the genera *Geobacter*, *Desulfuromonas*, and *Shewanella* was examined. All of the *Geobacter* and *Desulfuromonas* strains tested methylated mercury while reducing Fe(III), nitrate, or fumarate. In contrast, none of the *Shewanella* strains produced methylmercury at higher levels than abiotic controls under similar culture conditions. *Geobacter* and *Desulfuromonas* are closely related to known Hg-methylating sulfate-reducing bacteria within the *Deltaproteobacteria*.

Methylmercury (MeHg) concentrations in most sediments are controlled by in situ net microbial methylation (1, 13). Environmental mercury methylation is an anaerobic microbial process generally driven by dissimilatory sulfate-reducing bacteria (DSRB) (1). However, recent research suggests that dissimilatory iron-reducing bacteria (DIRB) may play a role in environmental methylation (10, 27). Further, Fleming et al. (10) demonstrated Hg methylation by a *Geobacter* strain isolated from Clear Lake, CA. Consequently, we designed an experiment to screen a phylogenetically diverse group of DIRB cultures for Hg-methylating capability in order to develop insight into in situ biological methylation controls and to further investigate the phylogenetic distribution of methylating bacteria.

Two studies have demonstrated net MeHg production in sediments where iron was the dominant terminal electron acceptor (10, 27), but another demonstrated inhibition of methylation by iron (23). Iron could potentially influence Hg methylation rates either through changes in DIRB activity or via the impact of iron on Hg complexation and bioavailability. In a study of estuarine wetland sediment slurries from San Francisco Bay, CA, Mehrotra and Sedlak (23) observed decreases in Hg methylation rates with the addition of 30 mM Fe(III), and they suggested that this effect was caused by decreases in dissolved Hg and sulfide due to complexation with iron. However, Warner et al. (27) found measurable methylation in sediments where iron reduction was the dominant terminal electron acceptor, although rates of methylation were lower than those observed in sulfate-reducing or methanogenic sediments. Similarly, in sediments from Clear Lake, CA (10), where microbial Fe(III) reduction was apparent, chemical inhibition of sulfate reduction did not result in complete inhibition of Hg methylation. This decoupling of Hg methylation from sulfate reduction suggests that another process (i.e., iron reduction) may be responsible for some amount of in situ Hg methylation.

Mercury methylation by a *Geobacter* strain isolated from Clear Lake (10) further supports this hypothesis.

Phylogenetic relationships between members of the *Geobacteraceae* and the Hg-methylating DSRB also suggest a possible role for DIRB in environmental Hg methylation. The *Geobacteraceae* are found in the *Deltaproteobacteria*, branching phylogenetically between the orders *Desulfovibrionales* and *Desulfobacterales* (15), both of which contain DSRB with methylating capability (2, 3, 9, 16). A wide variety of bacteria and archaea are capable of dissimilatory Fe(III) reduction (19, 20, 22), including *Shewanella*, which is in the γ subclass of *Proteobacteria*. This phylogenic distribution of DIRB implicates *Geobacteraceae* as possible Hg methylators and provides strains that are phylogenetically distant from the DSRB, which may give insight into the phylogenetic distribution of Hg methylation.

To assess the role of DIRB in Hg methylation, pure cultures of *Desulfuromonas palmitatis* SDBY-1 (8), *Geobacter hydrogenophilus* (7), *Geobacter metallireducens* GS-15 (21), *Geobacter sulfurreducens* (6), *Shewanella alga* BrY (5), *Shewanella oneidensis* MR-1 (26), and *Shewanella putrefaciens* CN-32 (18) were tested for the ability to methylate inorganic Hg while growing on a variety of electron donors and acceptors, including Fe(III), nitrate, and organic substrates (Table 1). Cultures were grown in media modified from the work of Bond and Lovley (4) with electron donors and acceptors as described in Table 1, using previously described trace elements and vitamins (17).

MeHg production was assayed by measuring the amount of MeHg produced from an inorganic Hg spike during batch culture growth through stationary phase. All Hg methylation assays were conducted in 20-ml Hungate tubes with butyl-rubber stoppers under strictly anaerobic conditions at 30°C and pH 7.0. Assays using *G. metallireducens*, *G. sulfurreducens*, *S. putrefaciens*, and *S. oneidensis* were conducted using an enriched stable Hg isotope, added as $^{201}HgCl_2$, at a final concentration of 10 ng/ml. Assays using *D. palmitatis*, *G. hydrogenophilus*, and *S. alga* were conducted with natural isotopic abundance $HgCl_2$ at the same concentration. For each strain and growth condition, triplicate assays and abiotic controls were prepared.

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TABLE 1. Electron donors and acceptors used in Hg
methylation tests

Culture	Electron donor	Electron acceptor(s)
D. palmitatis	20 mM acetate	55 mM Fe(III)
G. hydrogenophilus	20 mM acetate	55 mM Fe(III)
G. sulfurreducens	20 mM acetate	55 mM Fe(III), 40 mM fumarate
G. metallireducens	20 mM acetate	55 mM Fe(III), 30 mM nitrate
S. alga	30 mM lactate	55 mM Fe(III), 30 mM nitrate
S. oneidensis	30 mM lactate	55 mM Fe(III), 30 mM nitrate
S. putrefaciens	30 mM lactate	55 mM Fe(III), 30 mM nitrate

Abiotic controls were composed of autoclaved medium spiked with inorganic $HgCl_2$.

Analysis of total MeHg was performed via distillation/ethylation (11)/cold vapor atomic fluorescence (CVAF), using a Tekran 2500 atomic fluorescence detector. For CVAF analysis, the method detection limit was determined by the method blank, which was generally <20 pg/sample. For analysis of a 20-ml culture sample at 10 ng Hg/liter, this yields a blank equivalent to roughly 0.01% methylation. Analysis of $Me^{201}Hg$ was performed by distillation/ethylation/ICP-MS (inductively coupled plasma mass spectometry), with isotope dilution (14), using a Perkin Elmer ELAN 6100 DRCII. $Me^{200}Hg$ (96.4% purity) was used as the isotope dilution standard. The concentration of $Me^{200}Hg$ was determined by reverse isotope dilution analysis against certified standards. $Me^{200}Hg$ was synthesized from $^{200}HgCl_2$ using an aqueous methylcobalamine method

(14). All enriched isotopes were purchased from Oak Ridge National Labs as HgO. Method detection limits using isotope dilution–ICP-MS were generally <1 pg $Me^{201}Hg$/sample, or <0.001% methylation.

Phylogenetics of DIRB Hg-methylating capability. Methylation of inorganic mercury significantly above that in uninoculated controls (*t* tests, $P < 0.05$, two-tailed, unequal variances) was observed on Fe-reducing medium in *G. metallireducens*, *G. sulfurreducens*, *G. hydrogenophilus*, and *D. palmitatis* but not *S. alga* or *S. putrefaciens* (Fig. 1). While growing with electron acceptors other than Fe(III), both *G. metallireducens* and *G. sulfurreducens* produced MeHg above abiotic control levels, while *S. oneidensis* and *S. putrefaciens* did not. The small percentages of methylation observed in abiotic controls are attributed to abiotic formation of MeHg in the experiment or during analysis (12).

These results, in combination with the observation by Fleming et al. (10) of methylation by a *Geobacter* isolate, suggest that the ability to methylate Hg may be common among the *Geobacteraceae*. However, the observed lack of methylating capability among the *Shewanella* strains tested (all *Gammaproteobacteria*) shows that the ability to methylate Hg is not ubiquitous among Fe(III)-reducing bacteria. To date, essentially all strains for which Hg methylation has been demonstrated fall in the *Deltaproteobacteria* (2, 9, 16, 24). These include DSRB from the orders *Desulfovibrionales* and *Desulfobacterales*. However, it is important to note that the ability to produce MeHg is not ubiquitous among DSRB in these families. Further stud-
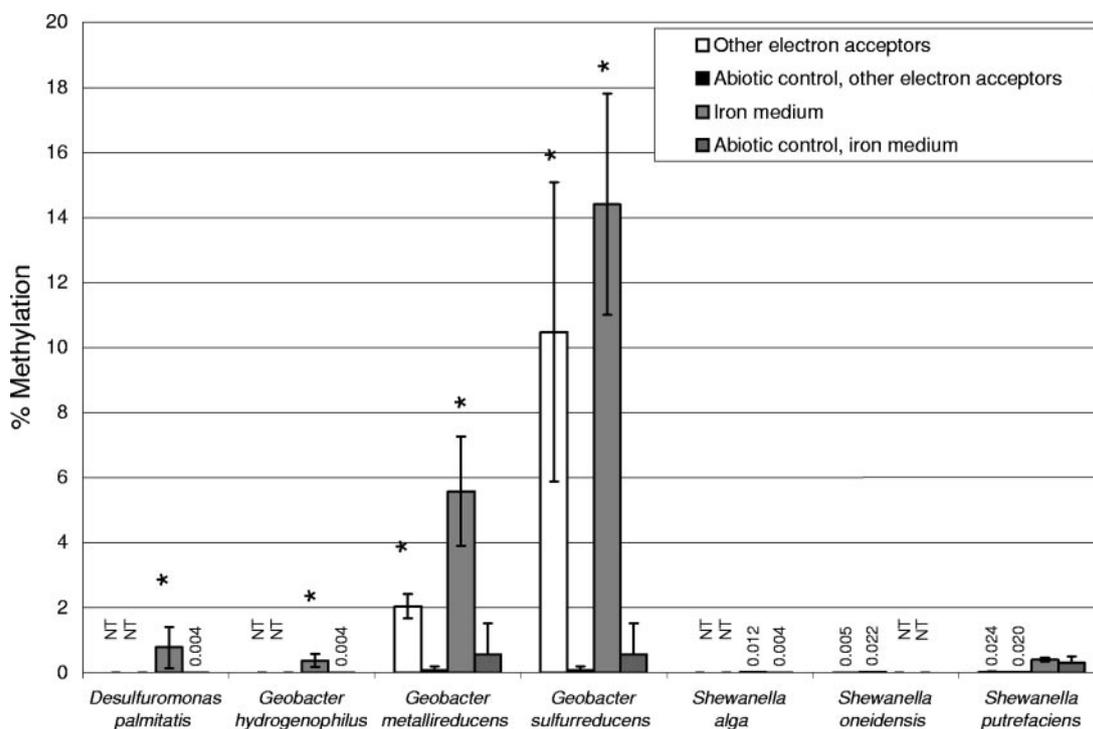
FIG. 1. Observed MeHg production by pure cultures of DIRB, expressed as the percentage of added inorganic Hg methylated. "Other electron acceptors" refers to cultures that were grown with either nitrate or fumarate as an electron acceptor; "iron medium" refers to cultures grown with Fe(III) citrate as an electron acceptor (see Table 1). Significant differences ($P < 0.05$) in MeHg production in cultures relative to matched abiotic controls are indicated by stars. Error bars represent the standard deviations for three separately prepared tubes for each sample. Culture and growth conditions that were not assayed for MeHg production are labeled "NT" (not tested).

ies are needed to ascertain whether the Hg-methylating capability is randomly distributed among *Proteobacteria* or related to phylogeny. Improved understanding of the phylogenetic distribution of Hg methylation capability may provide insight into the biochemical process of MeHg production within cells.

It is important to note that the *Geobacter* strains tested produced MeHg during growth with Fe(III) and with other electron acceptors (nitrate and fumarate). This indicates that active Fe(III)-reducing electron-transport chains are not necessary for Hg methylation in these strains. However, this experiment was not designed to quantify the effect of electron acceptors and donors on methylation rates. Further studies would be needed to quantify these effects.

Environmental significance of MeHg production by DIRB. The observation of Hg methylation by DIRB has implications for the prediction of in situ MeHg production. Due to the importance of DSRB as methylators, current models for methylation are based on relationships between methylation and sulfate reduction (1). However, the finding that DIRB can produce MeHg suggests that Hg methylation may be important in sediments and soils where these organisms are dominant, e.g., iron-rich sediments with low concentrations of sulfate (25). Iron can affect methylation by altering the chemistry of Hg (and hence its bioavailability) or by changing the activity of DIRB versus other groups of organisms, particularly DSRB (10, 23, 25, 27). The influence of iron on both Hg complexation and microbial activity will need to be considered in order to resolve how Hg methylation by DIRB will change the paradigm for in situ MeHg production.

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