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Mercury Methylation by Novel Microorganisms from New Environments

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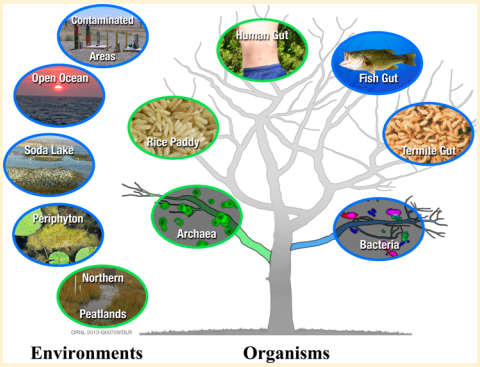
Supporting Information

ABSTRACT: Microbial mercury (Hg) methylation transforms a toxic trace metal into the highly bioaccumulated neurotoxin methylmercury (MeHg). The lack of a genetic marker for microbial MeHg production has prevented a clear understanding of Hg-methylating organism distribution in nature. Recently, a specific gene cluster (*hgcAB*) was linked to Hg methylation in two bacteria.[1] Here we test if the presence of *hgcAB* orthologues is a reliable predictor of Hg methylation capability in microorganisms, a necessary confirmation for the development of molecular probes for Hg-methylation in nature. Although *hgcAB* orthologues are rare among all available microbial genomes, organisms are much more phylogenetically and environmentally diverse than previously thought. By directly measuring MeHg production in several bacterial and archaeal strains encoding *hgcAB*, we confirmed that possessing *hgcAB* predicts Hg methylation capability. For the first time, we demonstrated Hg methylation in a number of species other than sulfate- (SRB) and iron- (FeRB) reducing bacteria, including methanogens, and syntrophic, acetogenic, and fermentative *Firmicutes*. Several of these species occupy novel environmental niches for Hg methylation, including methanogenic habitats such as rice paddies, the animal gut, and extremes of pH and salinity. Identification of these organisms as Hg methylators now links methylation to discrete gene markers in microbial communities.



INTRODUCTION

Most of the harm caused by mercury to humans and ecosystems[2] derives from MeHg bioaccumulation. Microbes are primarily responsible for MeHg production in nature, predominantly in anaerobic sediments, soils, and bottom waters, with sulfate- (SRB) and iron- (FeRB) reducing bacteria being the major contributors.[3] To date, using appropriate analytical methods, Hg methylation has only been demonstrated in ~30 SRB or FeRB species, all within the *Deltaproteobacteria* (Supporting Information, Table S1). Importantly, only a subset can produce MeHg, with no apparent commonality based on either phylogeny or environmental niche. Although a biochemical pathway was proposed almost 20 years ago,[4] until this year, no consistent relationship between specific genes and the ability of some SRB to methylate mercury had been identified.[5,6]

The lack of a confirmed genetic marker has prevented a clear understanding of Hg methylation potential of various microbial taxa and their contribution to the MeHg present in different environments. The role of microbial community structure on methylation rates remains poorly understood, especially in comparison with the important effects of Hg complexation on its bioavailability for methylation.[7] Further, recent demonstrations of net MeHg production where sulfate and iron reduction are limited, including methanogenic periphyton[8] and surface ocean waters[9,10] suggest that Hg-methylator diversity is much broader than previously understood.

Here we tested the recently identified gene pair (*hgcAB*), necessary for MeHg production in two model bacteria (*Desulfovibrio desulfuricans* ND132 and *Geobacter sulfureducens*)[1] as a reliable predictor of Hg methylation ability by other bacteria with orthologues. The gene *hgcA* encodes a corrinoid-dependent protein that presumably functions as a part of a methyltransferase similar to the corrinoid iron−sulfur protein (CFeSP) of the reductive acetyl-CoA pathway. The gene *hgcB* encodes an associated ferredoxin protein that potentially reduces the corrinoid center of HgcA.[1] Strains tested for Hg methylation ability were selected based on a phylogenetic distribution of *hgcAB* among all of the microorganisms with available genome sequences.

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Table 1. Strains Tested for Methylation, and Culture Conditions Used in Methylation Assays

kingdom	group	genus and species	source	*hgcAB* homologue	modified from medium	carbon substrate	e- acceptor	pH	growth temp, °C
Archaea	*Methanomicrobia*	*Methanomethylovorans hollandica*	DSMZ 15987	Y	DSM Medium 503	MeOH/ TMA	CO_2	6.5	35
		Methanolobus tindarius	DSMZ 2278	Y	DSM Medium 233	MeOH	CO_2	6.0	25
		Methanoculleus bourgensis	ATCC 43281	N	DSM Medium 119	formate	CO_2	7.3	37
		Methanobrevibacter smithii	ATCC 35061	N	DSM Medium 119	formate	CO_2	7.1	37
Bacteria	*Firmicutes*	*Ethanoligenens harbinense*	DSM 18485	Y	DSM Medium 1057	glucose		3.8	35
						lactate/ pyruvate/			
		Dethiobacter alkaliphilus	DSM 19026	Y	DSM Medium 1104	EtOH	thiosulfate	9.7	31
		Desulfosporosinus acidiphilus	DSM 22704	Y	DSM Medium 1250	fructose	sulfate	5.5	31
		Desulfitobacterium metallireducens	DSM 15288	Y	DSM Medium 838	lactate	FeIII citrate	7.3	31
		Desulfosporosinus youngiae	DSM 17734	Y	DSM Medium 641	lactate	sulfate/ thiosulfate	7.7	31
		Desulfitobacterium dehalogenans	DSM 9161	Y	DSM Medium 663	pyruvate	thiosulfate	7.1	31
		Acetonema longum	DSM 6540	Y	DSM medium 515	glucose/ casitone		7.2	30
		Clostridium thermocellum	DSM 1237	N	MTC	Avicel/ cellibios		7.4	55
	Deltaproteobacteria	*Geobacter bemidjiensis*	DSM 16622	Y	DSM Medium 579	acetate	FeIII citrate	7.2	31
		Desulfonatronospira thiodismutans	DSM 19093	Y	DSM Medium 1101	lactate	sulfate	10.1	31
		Desulfomicrobium baculatum	DSM 4028	Y	DSM Medium 63	lactate	sulfate	7.4	31
		Anaeromyxobacter dehalogens K	Robert Sanford	N	NBAFYE Medium 67	acetate	fumarate	7.1	37
		Geobacter daltonii	Joel Kostka	Y	NBAFYE	acetate	fumarate	7.0	27
		Syntrophus acidotrophicus	M. McInerney	Y	McInerney medium	crotonic acid		7.2	30

METHODS

Identification of hgcAB orthologues. A set of genes related to corrinoid iron–sulfur protein (CdhD) domain-containing proteins, the HgcA family, is characterized by distinctive features: a conserved motif N(V/I)WCA(A/G)GK, predicted C-terminal transmembrane helices and, importantly, the presence of a distinct ferredoxin-encoding gene (*hgcB*) immediately downstream (or in rare cases one gene apart) from *hgcA*.[1] These criteria were used to screen for *hgcA* among the microbial genomes and the available protein sequences of CdhD proteins, using BlastP, Pfam domain search followed by sequence alignments and inspection of the presence of *hgcB*. For phylogenetic analyses, a concatenated alignment of HgcA and HgcB was constructed using Muscle[11] and further refined manually. A maximum likelihood phylogeny was calculated using RAxML[12] (v.7.3) using PROTCAT with RTREV substitution model. Node support was calculated by 100 bootstraps under the same criteria.

Mercury Methylation Assays. To assess the ability to produce MeHg, we measured the conversion of an isotopically enriched 10 nM inorganic ^{201}Hg spike (obtained from ORNL at >98% purity) to Me^{201}Hg during batch growth of cultures, along with measures of cell growth and metabolism. The enriched ^{201}Hg spike was added to the culture medium prior to inoculation. The use of a ^{201}Hg spike as a methylation substrate obviates issues with background Hg and MeHg contamination.

The cultures tested, media, and growth conditions are listed in Table 1 (details in Supporting Information). Cultures included strains with *hgcAB* orthologues and related strains without orthologues. All cultures were confirmed for purity based on 16S rRNA gene sequences. For methylation assays, media recipes were amended with 500 μM cysteine. The Hg-cys complex is highly bioavailable to Hg-methylating *Deltaproteobacteria*.[13,14] Cell density, protein concentration, and, where appropriate, sulfide, Fe(II), and methane concentrations were measured to assess growth. Some culture media contained added sulfide; in other media, cells produced sulfide from added cysteine. Analytical methods are described in the Supporting Information.

Since small amounts of MeHg can be produced chemically,[15,16] it is critical to assess MeHg production in appropriate controls. Because redox and sulfur chemistry are important in Hg bioavailability, we matched redox conditions in cultures and controls. For each organism, controls consisted of uninoculated medium (fresh medium), and filtrate of mature cultures (spent medium). Spent media were prepared by filtration of late log or stationary cultures under O_2-free N_2. Organisms without *hgcAB* were used as negative control strains. We characterized cultures as Hg methylators when they produced significantly higher amounts of MeHg than appropriate fresh and spent medium controls. All cultures and controls were carried out in triplicate.

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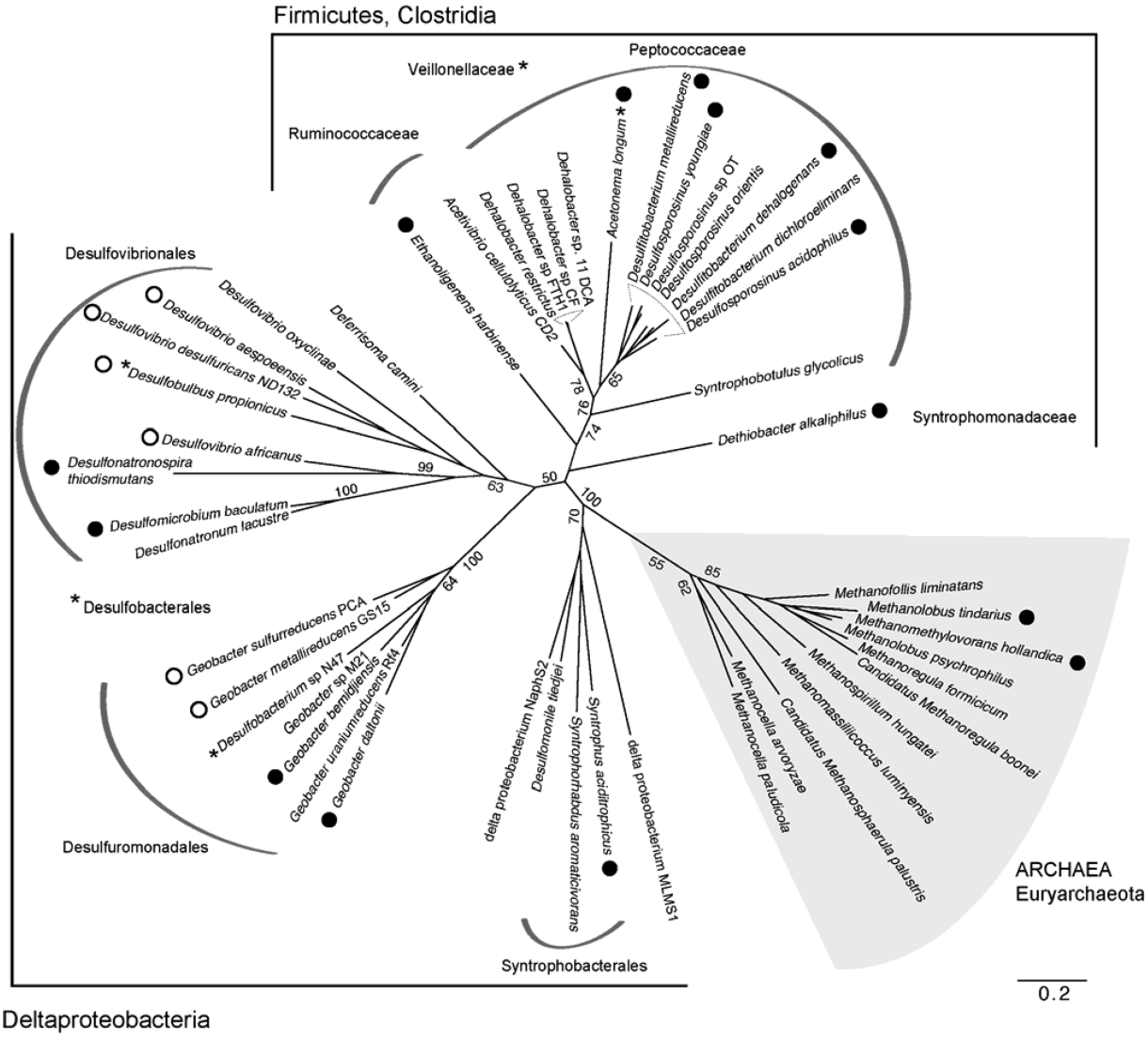

Figure 1. Phylogenetic tree using amino acid sequences from all microorganisms with available genome sequences containing *hgcAB* orthologues. White dots signify species formerly established as Hg-methylators, black dots show species newly established as Hg-methylators here.

To compare methylation rates, MeHg production (%MeHg) was normalized to the total [^{201}Hg] in the medium, including cells. This is important because Hg spikes can be sorbed to bottle walls and Hg partitioning may be different among media and cultures.[14,17] We also compared MeHg production normalized to protein in cultures.

Hg and MeHg Analysis. Total Hg was quantified in digested samples using isotope dilution, with stannous chloride reduction coupled online to ICP−MS.[18−20] Total Hg in medium (unfiltered samples) and particulate THg (on filters) samples were digested in hot 7:4 v/v HNO_3/H_2SO_4 (1:2 v/v sample digest acid) until vapors turned colorless and were preserved with 1% v/v BrCl. Filter-passing THg samples were digested overnight with 1% v/v BrCl at room temperature (RT). THg measurements were made using a Brooks Rand MERX automated THg system (Seattle, WA) interfaced to a Perkin-Elmer Elan DRC II ICP−MS (Shelton, CT). Excess [^{201}THg] were calculated using isotope abundances after correcting for impurities in the isotope tracer.[20] The terms "^{201}Hg" or "Me^{201}Hg" imply excess enriched isotope concentration above natural abundance. Detection limits for total ^{201}Hg specific to each methylation assay are given in Supporting Information, Table S1.

MeHg was determined by isotope dilution (ID), gas chromatography (GC), and inductively coupled plasma mass spectrometry (ICP−MS) following aqueous phase distillation and ethylation.[17,20] The detection limit for methylation was ~0.04% of aqueous ^{201}Hg. Explicit detection limits are in Supporting Information, Table S1. Spent medium controls averaged 0.12 ± 0.03% methylation, and fresh, uninoculated medium controls averaged 0.05 ± 0.02%. Stable isotope enriched Me^{199}Hg was synthesized in-house with $^{199}HgCl_2$ (Oak Ridge National Laboratories, 91.95% enriched in ^{199}Hg) with methylcobalamin[21] and was used as the ID spike. MeHg concentrations of ambient and tracer (Me^{201}Hg) were calculated on the basis of isotope abundances after corrections were made for impurities in the ID spike and ^{201}Hg tracer. All MeHg measurements were made using a Brooks Rand MERX automated MeHg system (Seattle, WA) interfaced to a Perkin-Elmer Elan DRC II ICP-MS (Shelton, CT).

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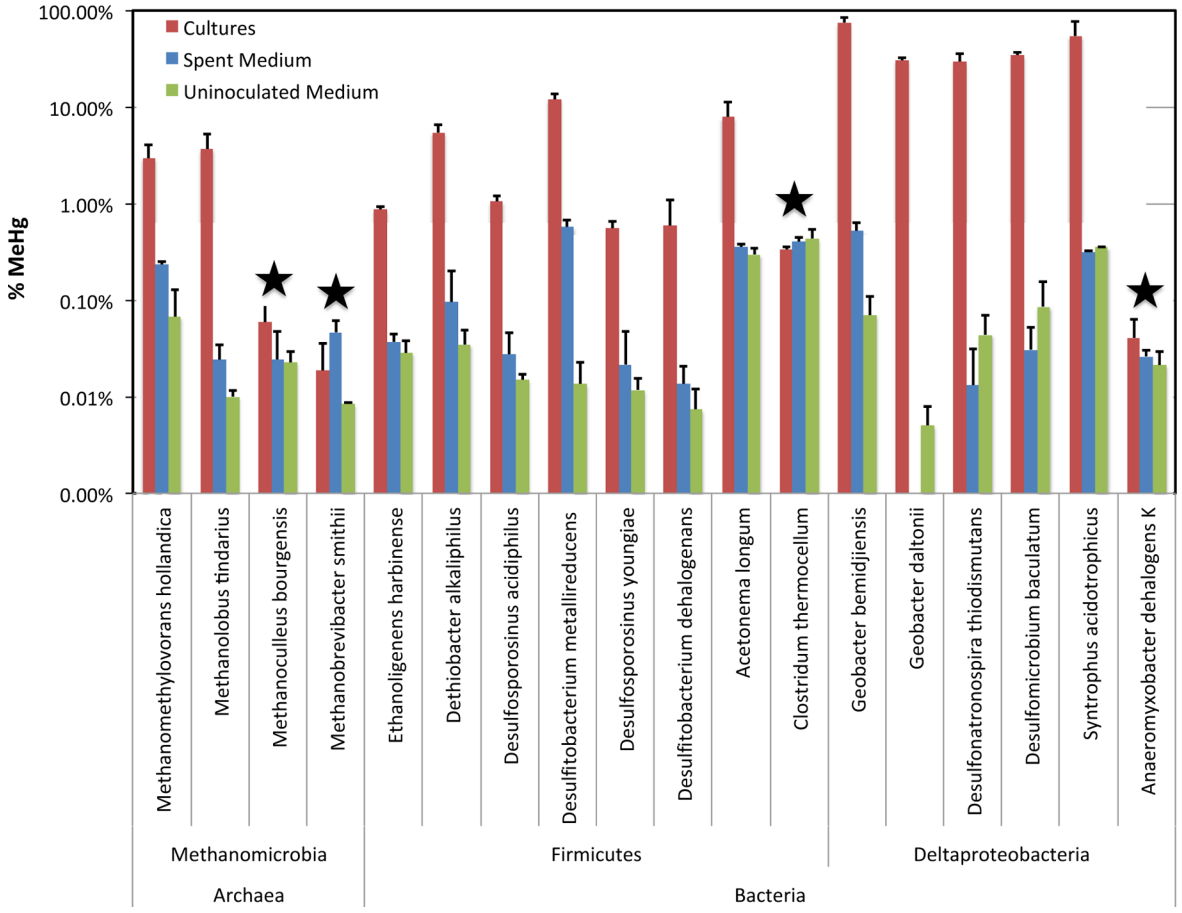

Figure 2. Maximum MeHg production in pure culture growth experiments by microbial species whose genomes contain *hgcAB* close orthologues, plus control species without orthologues (stars). $Me^{201}Hg$ production in cultures (red bars) is shown as a percentage of the measured total ^{201}Hg in the medium at the time point where %MeHg was maximal, generally the end of log phase growth. Blue and green bars show %MeHg in spent medium and fresh medium, respectively, after an incubation time equivalent to each cultures growth curve. Error bars are standard deviations for triplicate incubations. Methylation data are normalized to protein content in Supporting Information, Figure S13.

RESULTS AND DISCUSSION

Phylogenetic Distribution of hgcAB. Among the several thousand sequenced microbial genomes available at this writing, only 70 contain orthologues of both genes (Supporting Information, Table S2). A phylogenetic reconstruction of *hgcAB* distribution using amino acid sequences (HgcAB) shows that the genes and proteins are confined to five distinct clades (Figure 1). These include species within three groups of *Deltaproteobacteria*: (1) iron-reducing *Geobacter,* (2) sulfate-reducing *Desulfovibrionales* and *Desulfobacterales*, and (3) a newly identified clade of syntrophic SRB. The other two new clades are evolutionarily distant; a methanogen group and a mainly *Clostridia* group. Although all species containing *hgcAB* orthologues fall into these five clades, the gene pair is still relatively rare within most clades.[14,17,22] To directly test whether species containing *hgcAB* orthologues are capable of Hg methylation, we measured MeHg production in 13 species (Table 1), including species isolated from novel niches. As controls, related strains without *hgcAB* orthologues were also tested.

Results of Methylation Assays. We report Hg methylation capability in 15 previously untested bacteria with *hgcAB* homologues, including 2 methanogens, 5 *Deltaproteobacteria*, and 8 metabolically diverse *Firmicutes*. We observed significant MeHg production above controls in all tested strains with *hgcAB* orthologues, while none of the 3 tested strains without *hgcAB* homologues produced MeHg above controls (Figure 2).

Figure 3 shows as an example the methylation assay for *Desulfomicrobium baculatum.* $Me^{201}Hg$ production in cultures was significantly above $Me^{201}Hg$ in spent and fresh medium controls with matched chemistry. Methylation occurred during log phase growth and stopped at or before the stationary phase. Total aqueous [^{201}Hg] (Hg in culture medium and cells) was measured explicitly during growth and used to calculated % MeHg. Normalized to protein concentration, MeHg production was generally strongest in the early log phase, consistent with prior observations.[5,17,23]

Our survey of Hg methylation in a diverse group of organisms encoding *hgcAB* strongly supports the idea that all microbes whose genomes include this gene pair, including organisms outside of the *Deltaproteobacteria*, are capable of MeHg production. With this new survey, Hg methylation capability has been demonstrated in 26 of the 70 known organisms with *hgcAB* homologues.

Methylation by Deltaproteobacteria. This class of *Proteobacteria* has been the most widely tested for methylation (Supporting Information, Table S2). Here we report five new Hg-methylating species. *Desulfomicrobium baculatum* (Figure 3), is a new species in a genus from which other Hg-methylating isolates have been identified. *Desulfonatronospira thiodismutans* (Supporting Information, Figure S1) is the first

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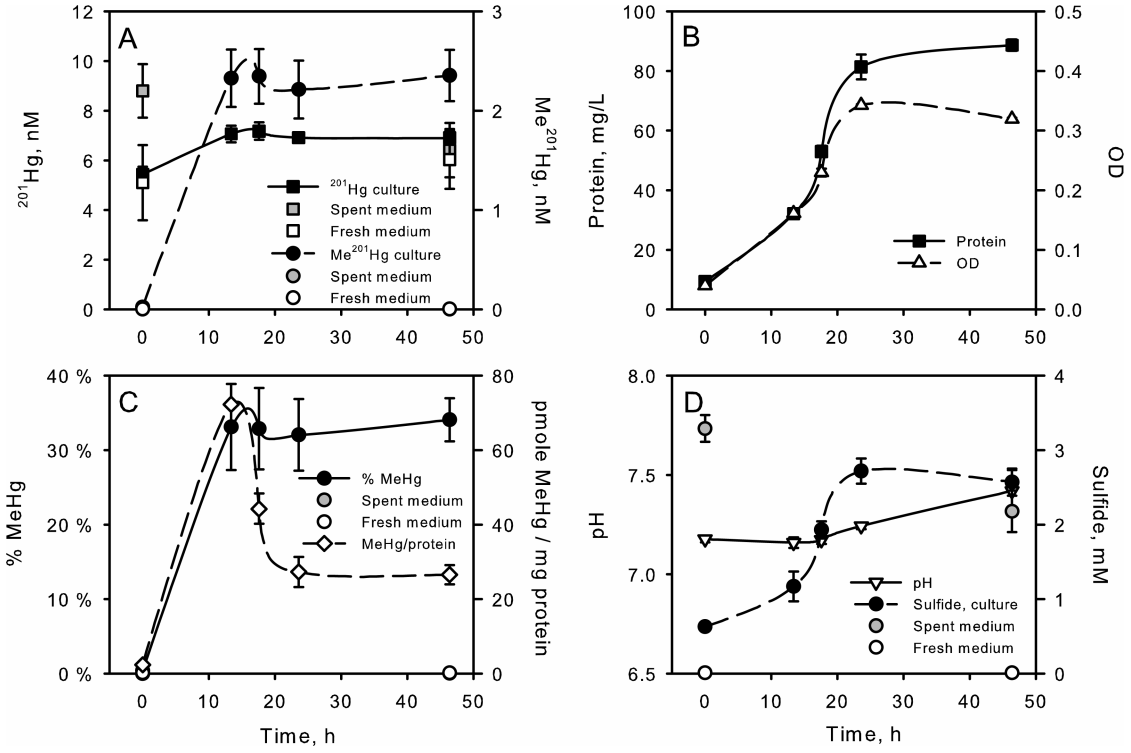

Figure 3. Methylation assay for *Desulfomicrobium baculatum*, a Hg-methylating sulfate-reducer in the *Deltaproteobacteria*. (A) Total ^{201}Hg and Me^{201}Hg in cultures, spent, and fresh medium. Error bars represent standard deviation among triplicate cultures or controls. (B) Protein and optical density of cultures (absorbance at 660 nm). (C) Excess Me^{201}Hg shown as a fraction of measured total excess ^{201}Hg in cultures, spent and fresh medium; and Me^{201}Hg normalized to the protein content of cultures. (D) Culture pH and sulfide in cultures, spent, and fresh medium.

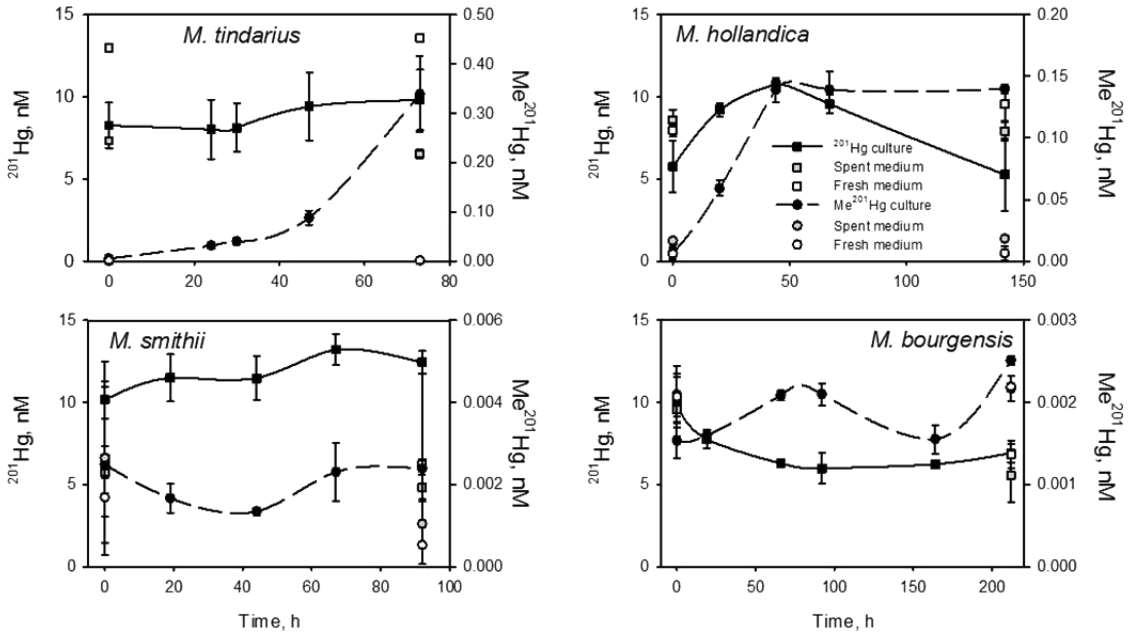

Figure 4. Comparison of batch culture Hg-methylation assays for two methanogens with *hgcAB* close orthologues (*Methanolobus tindarius* and *Methanomethylovorans hollandica*; top) and two methanogens without (*Methanobrevibacter smithii* and *Methanoculleus bourgensis*; bottom). Panels show total excess ^{201}Hg concentrations and excess Me^{201}Hg produced in cultures and in spent and fresh medium controls. Note the different scales. *M. tindarius* and *M. hollandica* produced increasing amounts of MeHg through time, at levels significantly above spent and fresh medium controls. *M. smithii* and *M. bourgensis* are not Hg-methylators. In these cultures, MeHg did not rise during growth, nor did it ever exceed concentrations in spent and fresh medium controls.

example of a Hg-methylating extremophile. This obligate alkaliphile from a hypersaline soda lake[24] is also the first reported Hg methylator in the family *Desulfohalobiaceae*. Both of the *Geobacter* tested here, *G. daltonii* FRC-32,[25] and *G. bemidjensis*[26] (Supporting Information, Figure S2), were strong methylators, the latter accomplishing almost complete con-

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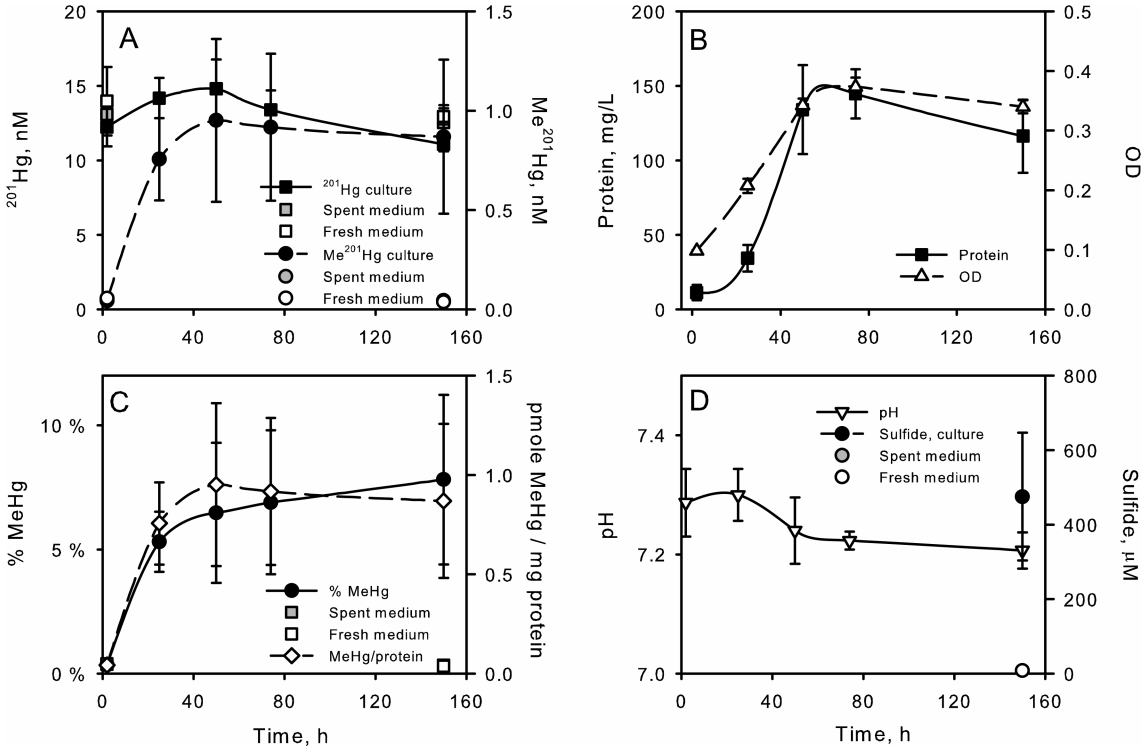

Figure 5. Methylation assay for *Acetonema longum,* a Hg-methylating *Firmicute*. (A) Total ^{201}Hg and Me^{201}Hg in cultures, spent and fresh medium. Error bars represent standard deviation among triplicate cultures or controls. (B) Protein and optical density of cultures (absorbance at 660 nm). (C) Excess Me^{201}Hg shown as a fraction of measured total excess ^{201}Hg in cultures, spent and fresh medium; and Me^{201}Hg normalized to the protein content of cultures. (D) Culture pH and sulfide in cultures, spent, and fresh medium.

version to MeHg. *Anaeromyxobacter dehalogenans* (Figure S3) was used as the *Deltaproteobacteria* negative control. This metabolically diverse metal-reducer is deeply nested in the order *Myxococcales*, but its "mosaic" genome shares genotypic traits with many other *Deltaproteobacteria*.[27]

Predicted methylators in a third clade of Hg-methylating *Deltaproteobacteria (Syntrophobacterales)* include a reductive dechlorinator (*Desulfomonile tiedjei*), and two syntrophs (*Syntrophus aciditrophicus* and *Syntrophorhabdus aromaticivorans*), both isolated from syntrophic cultures with SRB.[28,29] We tested the Hg methylation ability of *Syntrophus aciditrophicus*, and found it to be a strong methylator (Figure 2). This clade also includes the deep-sea vent thermophile *Desulfacinum hydrothermale*. Malcolm et al.[30] found it to be a weak methylator; however, the genome sequence is not available.

Methylation by Methanogens. Within the *Archaea*, we demonstrated Hg methylation by two *hgcAB*+ methylotrophic *Methanosarcinaceae, Methanomethylovorans hollandica* (Supporting Information, Figure S4) and *Methanolobus tindarius* (Figure S5). During log phase, methylation was 1−4% of the ^{201}Hg spike (panel A). Both cultures contained measurable sulfide, *M. tindarius* produced MeHg with ∼100 μM sulfide. Methylation occurred significantly above spent and fresh medium controls. In contrast *Methanoculleus bourgensis* and *Methanobrevibacter smithii*, which do not encode *hgcAB*, did not produce MeHg above controls (Figures S6 and S7). Figure 4 compares MeHg production across all four methanogens tested, highlighting the need for careful controls in order to confirm microbial methylation. Mercury methylation by *hgcAB*+ *Methanospirillum hungatei* JF-1, in the class *Methanomicrobia* was also recently demonstrated.[31]

The occurrence of *hgcAB* among *Archaea* appears confined to the *Methanomicrobia*, including methylotrophic and hydrogenotrophic species. Of the 25 sequenced *Methanomicrobia* genomes, 10 include *hgcAB* orthologues, suggesting that Hg methylation may be relatively common (although phylogenetically scattered), as with the sulfate-reducing *Deltaproteobacteria*. Mercury methylation appears more common in the E1/E2 family level clade of order *Methanomicrobiales*, in which all of the genome-sequenced members have *hgcAB* orthologues. These predicted Hg-methylators include strains from freshwater wetlands and wastewater treatment sludge (Supporting Information, Table S3); known locations of MeHg production where iron and sulfate reduction may not be dominant.[32−35]

Both *M. hollandica* and *M. tindarius* were isolated from freshwater sediments,[36,37] and metabolize methylamines, MeOH, and dimethylsulfide. A potential relationship between methylotrophy in methanogens and *hgcAB* evolution is intriguing. MeHg production was low relative to the *Deltaproteobacteria*. These low methylation rates fits the common observation of low %MeHg in methanogenic environments like rice paddy soils,[38] and Fe and sulfate-limited freshwater sediments.[32] However, these are preliminary and limited data and more work is needed to evaluate MeHg production in methanogens and methanogenic environments.

Firmicutes. The extension of Hg methylator phylogeny into the *Firmicutes* also broadly expands the niches and metabolisms associated with MeHg production. The predicted *Firmicute* Hg methylators include fermentative, acetogenic, and cellulolytic species, although most species with gene orthologues are sulfate- and Fe- reducing *Clostridia*. We confirmed Hg methylation in 7 *Firmicutes*. Two of the most unexpected were *Ethanoligenens harbinense* (Supporting Information, Figure

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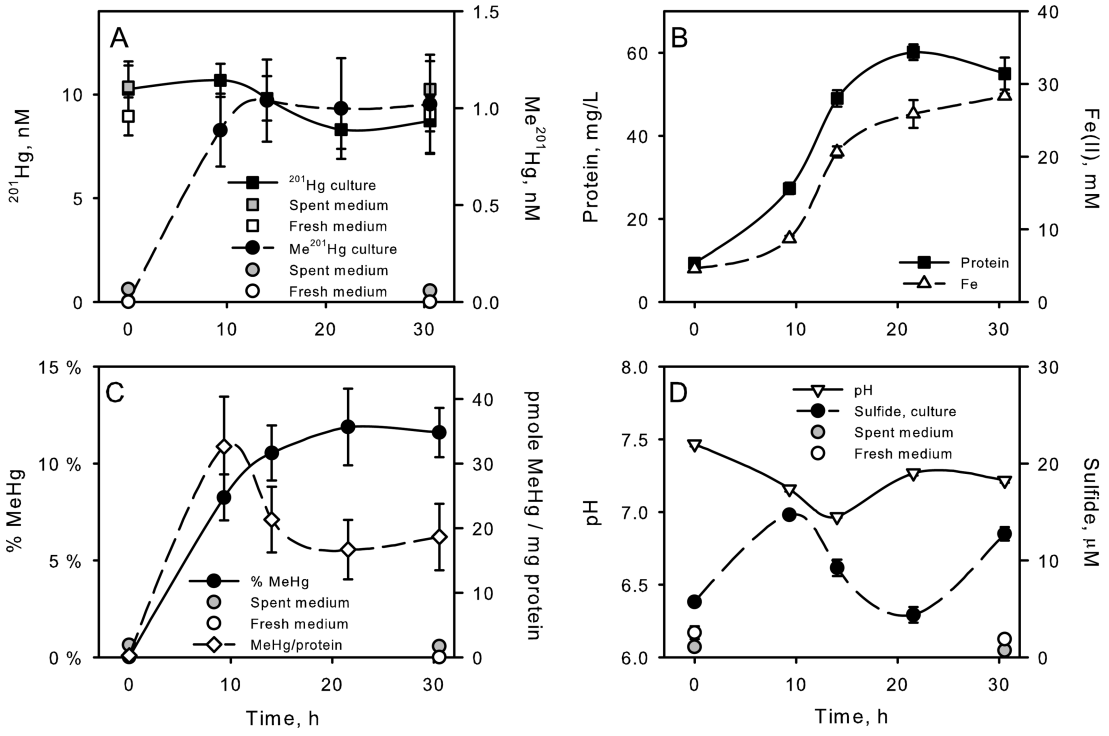

Figure 6. Methylation assay for *Desulfitobacterium metallireducens*, a Hg-methylating, Fe-reducing *Firmicute*. (A) Total ^{201}Hg and Me^{201}Hg in cultures, spent and fresh medium. Error bars represent standard deviation among triplicate cultures or controls. (B) Protein and Fe(II) in cultures. (C) Excess Me^{201}Hg shown as a fraction of measured total excess ^{201}Hg in cultures, spent and fresh medium; and Me^{201}Hg normalized to the protein content of cultures. (D) Culture pH and sulfide in cultures, spent and fresh medium.

S8), a strictly fermentative organism isolated from molasses wastewater,[39] and *Acetonema longum* (Figure 5), an acetogenic member of the *Negativicutes*, isolated from a termite gut.[40] Other strains included obligately acidiphilic SRB *Desulfosporosinus acidiphilus* (Figure S9), and *Dethiobacter alkaliphilus* (Figure S10) another obligate haloalkaliphile. Three strains utilize partially reduced sulfur species as electron acceptors, including the reductive dechlorinator *Desulfitobacterium dehalogenans* (Figure S11), and *Desulfosporosinus youngiae* (Figure S12). *Firmicute* Hg methylation was low to moderate, with the highest MeHg production (~12%) in *Desulfitobacterium metallireducens* (Figure 6), the only *Firmicute* Fe(III)-reducer tested.

Comparison of Methylation Rates among Species and Clades. MeHg production varied substantially within the clades, even when normalized to protein in cultures (Figure 2; Supporting Information, Figure S13). The *Deltaproteobacteria* were the strongest methylators, consistent with prior observations,[13,17,41−43] and with Hg methylation in nature linked to sulfate or Fe reduction.[3] Methylation was variable among the *Firmicutes*, although few species matched the rates observed in *Deltaproteobacteria* under similar conditions. The two methanogens tested had the lowest methylation rates.

Reported methylation rates vary widely in the literature, even for individual strains.[17] Methylation rate comparisons can be difficult, since they are highly dependent on growth phase, cell density, Hg concentration, and medium chemistry. Here, methylation assay conditions were matched to the extent possible across the species tested, including sampling and choice of culture chemistries. Cysteine or sulfide were likely the dominant ligand for Hg in most culture media. Nevertheless, medium chemistry differences among organisms, including organic material released by cells could affect Hg complexation and bioavailability.

Additional studies are needed to explain why observed Hg methylation rates differed among the five clades, and to confirm that the differences are biological. Methylation rates for a single organism can vary over two orders of magnitude based on culture chemistry alone. Biological differences in methylation rates may be driven by variations in the *hgcAB* genes themselves, or by other processes, including Hg association and/or uptake mechanisms. Research on *Desulfovibrio* sp. ND132 shows that *hgcAB* is necessary but not sufficient for MeHg production.[1]

Diversity of Hg-Methylating Organisms. This work provides a new understanding of a much broader diversity of Hg-methylating microorganisms in nature. Methylation in 2 methanogens, and in a wide variety of *Firmicutes* extends our knowledge of Hg-methylating microorganisms outside of the *Deltaproteobacteria* for the first time. Nevertheless, the *hgcAB* gene pair appears to be rare among bacteria, and confined within disparate groups of anaerobes. With a few interesting exceptions, the confirmed and predicted Hg methylators are dominantly heterotrophs using sulfate, iron, and now CO_2 as terminal electron acceptors. Anaerobic respiration of nitrate remains notably missing, with the exception of *G. metallireducens*.

The phylogenetic distribution of *hgcAB* confirms and explains the observed importance of sulfate and iron-reducers in Hg methylation in nature. The relative importance of these organisms may be enhanced, given the observed high rates of methylation of many of these organisms. Interestingly, every *Geobacter* species for which a genome is available (except *G. lovleyi*) contains an *hgcAB* orthologue, and every organism

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tested in this family is capable of high MeHg production rates.[13,43,44]

Why a small subset of organisms evolved the capability for Hg methylation, and whether this capability benefits them remains unknown. Available evidence suggests that the ability to methylate Hg does not confer Hg resistance,[14] but evidence is sparse. We now know that species with the gene pair encompass a wide variety of metabolisms and habitats, including methanogens and obligate syntrophs. All are obligate anaerobes, and Hg can be substantially more bioavailable to microbes under anaerobic conditions.[18,45,46] Although MeHg is much more toxic than inorganic Hg,[2,47] Hg-methylating microbes rapidly export any MeHg produced, and do so in a form that does not reassociate with cells.[14,17,48] Whether this mechanism evolved for MeHg export, or cells use a mechanism evolved for another purpose is unknown. Further, Hg-methylating SRB and FeRB appear to sorb lower amounts of Hg than do nonmethylating strains.[17]

The phylogeny of organisms with *hgcAB* may offer hints to its evolution. Many of the Hg-methylating *Deltaproteobacteria* and the *Clostridia* also have the ability to dechlorinate organic contaminants. Mercury methylation within the *Archaea* appears to be confined to the methylotrophic methanogens. Some of the Hg-methylating syntrophs were isolated from coculture with another Hg-methylating organism. The links between these metabolisms deserve further attention. The evolutionary pathway of the *hgcAB* gene pair is a critical next step in understanding the distribution of Hg methylation in nature.

New Habitats for Hg Methylation. Confirmation of Hg methylation by methanogens, and by fermentative, acetogenic, and cellulolytic microorganisms significantly expands our knowledge of MeHg producing habitats. Identification of these new Hg-methylators provides an explanation for the observed MeHg production in niches where iron and sulfate reduction are not dominant processes, and implies that MeHg production may be occurring in a variety of previously unrecognized habitats. The observation of Hg methylation by *Desulfosporosinus acidiphilus, Dethiobacter alkaliphilus, and Desulfonatronospira thiodismutans* indicates that MeHg production may occur in alkaline and acidic habitats in addition to neutral habitats. Such geochemically distinct environments deserve attention for their potential role in the evolution of Hg methylation, and for the potential relationships with unique Hg complexation and bioavailability in these habitats.

The confirmation of Hg methylation in methanogens has potential implications for human health. For example, Hg methylation by methanogens provides a potential explanation for the observation of MeHg in rice paddies; flooded anaerobic soils that are typically methanogenic.[49] In parts of China, rice is the main route of human MeHg exposure[50] as a result of Hg methylation in rice paddy soils impacted by high levels of atmospheric Hg deposition, followed by MeHg uptake by rice plants.[38] Both of the *Methanocella* strains with *hgcAB* orthologues were isolated from rice paddies[51,52] (Supporting Information, Table S2). The presence of an *hgcAB* orthologue in *Methanomassiliicoccus luminyensis*, isolated from human feces,[53] suggests another potential human health risk, MeHg production in the gut. MeHg synthesis has been observed in the intestinal microbiota of fish and rats, but Hg exposure studies in primates have not evaluated MeHg production.[54]

Elevated Hg deposition in the Arctic has created human health and ecological risks.[55,56] MeHg production in arctic wetlands and peatlands[57,58] and marine waters[59] leads to MeHg bioaccumulation in terrestrial and marine arctic food webs. Human risk is exacerbated by consumption of traditional local foods, including fish and marine mammals. The microbes responsible for marine MeHg production remain unknown; however, methanogenesis is the dominant terminal microbial process in wet arctic tundra.[60] Three of the predicted Hg-methylating methanogens (*Methanoregula formicicum, Methanosphaerula palustris,* and *Methanolobus psychrophilus*) were isolated from northern peat lands,[61,62] including an obligate psychrophile.[63] There is thus the possibility of increased MeHg production in Arctic ecosystems as methanogenic activity increases in warming, inundated tundra soils.[64] Zhang et al.[63] observed that methanol addition supported higher levels of methanogenesis in cold-adapted wetland soils than did hydrogenotrophic methanogensis; all of the Hg-methylating methanogens are methylotrophs.

Finally, a number of the newly identified Hg-methylating strains were isolated from engineered environments like wastewater treatment systems and bioremediation sites. The ability to identify and track the abundance of Hg-methylating organisms within these managed environments could be used to devise strategies to minimize MeHg production during treatment. For example, the choice of substrate amendment, commonly used in bioremediation to stimulate the degradation of organic contaminants or the reduction and immobilization of metals,[65,66] could be manipulated to minimize MeHg production.

On the basis of the substantially expanded phylogenetic and environmental diversity of newly observed methylators, a number of important habitats deserve further attention concerning MeHg production, especially the human gut, rice paddies, anaerobic wastewater treatment systems, biological dechlorination and metal treatment systems, and northern peat lands. Confirmation that most of the isolated organisms that possess *hgcAB* are capable of methylation now definitively links Hg methylation to the presence of discrete gene markers in microbial communities. This is a necessary precursor to the development of gene probes for Hg methylation, which will allow us to evaluate the ecological distribution of Hg-methylating microbes for the first time.

■ ASSOCIATED CONTENT

Supporting Information

(Table S1) Complete list of reported Hg-methylating and non-Hg methylating organisms, provided as a separate Excel file; Detailed methods: (Table S2) MeHg production, growth measurements, and medium chemistry at the final time point in batch culture methylation assays; (Figures S1−S12) methylation assays for all of the tested cultures not shown in the manuscript; (Figure S13) MeHg production normalized to protein concentration for microbial methylation assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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