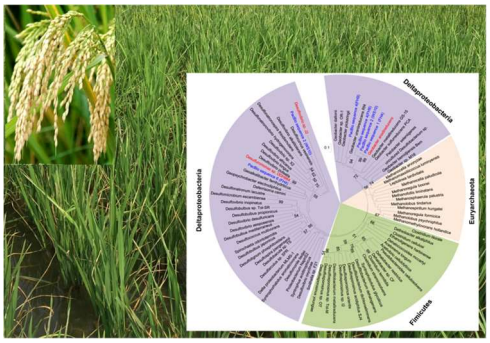
Deltaproteobacteria
Euryarchaeota
Firmicutes

Unraveling microbial communities associated with methylmercury production in paddy soils

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ABSTRACT

Rice consumption is now recognized as an important pathway of human exposure to the neurotoxin methylmercury (MeHg), particularly in countries where rice is a staple food. Although the discovery of a two-gene cluster *hgcAB* has linked Hg methylation to several phylogenetically diverse groups of anaerobic microorganisms converting inorganic mercury (Hg) to MeHg, the prevalence and diversity of Hg methylators in microbial communities of rice paddy soils remain unclear. We characterized the abundance and distribution of *hgcAB* genes using 3rd-generation PacBio long-read sequencing and Illumina short-read metagenomic sequencing, in combination with quantitative PCR analyses in several mine-impacted paddy soils from southwest China. Both Illumina and PacBio sequencing analyses revealed that Hg methylating communities were dominated by iron-reducing bacteria (i.e., *Geobacter*) and methanogens, with a relatively low abundance of $hgcA^+$ sulfate-reducing bacteria in the soil. A positive correlation was observed between the MeHg content in soil and the relative abundance of *Geobacter* carrying the *hgcA* gene. Phylogenetic analysis also uncovered some *hgcAB* sequences closely related to three novel Hg methylators, *Geobacter anodireducens*, *Desulfuromonas* sp. DDH964, and *Desulfovibrio* sp. J2, among which *G. anodireducens* was validated for its ability to methylate Hg. These findings shed new light on microbial community composition and major clades likely driving Hg methylation in rice paddy soils.

INTRODUCTION

Mercury (Hg) is a growing environmental concern because it can be converted to neurotoxic methylmercury (MeHg) that can bioaccumulate and biomagnify up the food chain.[1,2] The consumption of foods contaminated with MeHg threatens human health globally.[3, 4] Seafood, especially large predatory fish, is widely considered as the primary route for human exposure to MeHg,[5] but recent studies show that rice may constitute another important exposure route, particularly in countries where rice is a staple of the everyday diet.[6] According to the United Nations Food and Agriculture Organization, an estimated 3.5 billion people worldwide are consuming rice. We now know that rice grains bioaccumulate MeHg, originating from microbial conversion of inorganic Hg to MeHg in paddy soils,[7-9] although currently little is known about microbial community composition and diversity associated with Hg methylation. Delineating major microbial communities and their roles in methylating Hg in these soils is thus needed for improved understanding of MeHg production in paddy soils and bioaccumulation in rice grains.

MeHg in the environment is produced primarily by anaerobic microorganisms including Deltaproteobacteria, Firmicutes, and the methylotrophic Methanomicrobia.[10-12] For more than two decades, sulfate-reducing bacteria were considered to be the primary Hg methylators in aquatic environments,[13-15] although other studies have shown that iron-reducing bacteria and methanogens also play roles in MeHg production.[16-20] So far no direct correlations have been observed between microbial community composition and Hg methylation potential since Hg methylation is strain-specific.[11, 21] Two genes, *hgcA* and *hgcB*, were recently identified to be essential for microbial Hg methylation.[11] These genes can be used as biomarkers for determining the diversity and distribution of Hg methylating microorganisms in the environment.[22-27] However, phylogenetically diverse *hgcA* genes were reported to exist in a broad range of environments based on analysis of publicly available microbial metagenomes.[10, 25] Additionally, microorganisms containing *hgcAB* genes were shown to have distinctly different efficiencies in methylating inorganic Hg, ranging from about 0.6% to 75% of Hg,[10] but to date no apparent correlations were reported between *hgcAB* transcript levels and Hg methylation rates.[28-29] Several studies reported the analysis of Hg methylating communities by targeting only the known *hgcA* gene in paddy soils,[30, 31] but these analyses

were limited by a low sequencing depth and did not include the second essential gene *hgcB*, which is typically found immediately downstream of *hgcA*.[11] The high similarity of *hgcB* to other ferredoxins could potentially lead to false positives if targeted independently of *hgcA*. Therefore, more comprehensive studies and new techniques are needed to further understand microbial community composition and diversity and to determine the linkages between microbial communities, MeHg production and its subsequent bioaccumulation in paddy soils.

The main objective of this study was to determine microbial community composition and diversity in several mine-impacted paddy soils from southwest China. Using recently developed high throughput sequencing technologies and broad-range primer pairs,[24] we combined metagenomics, PacBio sequencing and real-time quantitative PCR (qPCR) to determine the distribution and abundance of *hgcAB* genes and their phylogenetic relationships. These complementary techniques provide unbiased microbial community composition and insights into *hgcAB* gene diversity (based on Illumina metagenomic and PacBio sequencing) and *hgcA* gene abundance (by qPCR). In particular, the longer read lengths of PacBio sequencing compared to second-generation sequencing techniques enabled contiguous assembly of full-length *hgcAB* gene sequences and allowed us to comprehensively characterize Hg methylators in the microbial communities.

MATERIALS AND METHODS

Site description and sample collection

Paddy soil samples were collected from two historical Hg mining sites (Figure S1), Fenghuang (FH) and Wanshan (WS), in Southwest China in August 2016. Fenghuang is located in Hunan Province, and Wanshan in East Guizhou Province, and both sites are the main rice production areas in China.[8] Historical discharge from Hg mining operations and ongoing atmospheric deposition led to high concentrations of both total Hg (THg) and MeHg in the soils around these areas (Table S1). A total of 72 paddy soil samples (0-15 cm depth) were collected from 24 sites (in triplicate). They were collected under saturated or reducing conditions during the rice growing season and appeared greyish in color due to the presence of ferrous Fe(II), as previously described.[9] The basic soil properties are listed in Table S1. We also collected three upland soils (with maize crops) under unsaturated or oxic conditions

surrounding the paddy fields as controls for comparisons of microbial community characteristics. All samples were sealed in sterile plastic bags, and immediately shipped to our laboratory on ice. Sub-samples were stored at -20 °C for microbial analysis, while the remaining samples were freeze-dried, homogenized, and passed through a 2 mm sieve for total Hg (THg) and MeHg analyses.

Mercury and methylmercury analyses

For THg analysis, the soil was digested in aqua regia at a HCl to HNO_3 ratio of 1:3 (v/v) in a water bath at 95 °C for 2 h. An aliquot was used to determine THg using cold-vapor atomic fluorescence spectrometry (CVAFS). The detection limit was 0.2 $ng \cdot mL^{-1}$. Standard Hg solutions and a soil standard reference material GSS-9 (THg: 32±3 $ng \cdot g^{-1}$) were included in the analysis for quality assurance and control. Typical recoveries of THg from GSS-9 were between 90.7% and 95.2%. For MeHg analysis, a previously established method of $CuSO_4$-methanol/solvent extraction was used.[32, 33] Typically, 0.30 to 0.40 g soil was used for MeHg extraction. An aliquot of the extract (20-50 μL) was then transferred to a 40 mL amber glass vial, mixed with a citrate buffer and sodium tetraethylborate (1% $NaBEt_4$, w/w), and the concentration of MeHg was determined by an automated MeHg analyzer (TEKRAN 2700 GC-CVAFS). Analytical blanks and a certified sediment reference material (ERM-CC580, 75.5±3.7 $ng \cdot g^{-1}$) were also included in analyses for quality control. The recovery of MeHg from the ERM-CC580 reference was 86.2–107.4%.

Soil DNA extraction and real-time quantitative PCR (qPCR)

The total microbial DNA was extracted from 0.30 g of soil using the MO BIO PowerSoil DNA isolation kit (QIAGEN Inc., USA) following the manufacturer recommended protocols. The concentration and quality of isolated DNA were validated using a NanoDrop® ND-2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). The abundance of the *hgcA* gene was quantified using the clade-specific degenerate primer pairs ORNL-Delta-HgcA and ORNL-Archaea-HgcA for the Deltaproteobacterial and Archaeal methylators,[24] respectively, by amplification of the *hgcA* sequences from extracted DNA with an iCycler iQ5 thermocycler (Bio-Rad, USA). The primers for Deltaproteobacteria were ORNL-Delta-HgcA-F (5'-GCCAACTACAAGMTGASCTWC-3') and ORNL-Delta-HgcA-R

(5'-CCSGCNGCRCACCAGACRTT-3'). Primers of ORNLArchaea-HgcA-F (5'-AAYTAYWCNCTSAGYTTYGAYGC-3') and ORNL-Archaea-HgcA-R (5'-TCDGTCCCRAABGTSCCYTT-3') were used for Archaea. The 25-µl reaction mixture contained 12.5 µl SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan), 0.5 µl (10 mM) of each primer, and 2 µl of 10-fold diluted DNA template (1-10 ng). Thermal-cycling conditions for the Deltaproteobacterial primers were as follows: 3 min initial denaturation at 95°C, 40 cycles of 15 s at 95°C, 15 s at 50°C, and 15 s at 55°C, 4 min at 72 °C, followed by a plate read at 83°C. For the Archaeal primers, the thermal-cycling conditions were as follows: 3 min initial denaturation at 95°C, 40 cycles of 15 s at 95°C, 30 s at 50°C, and 25 s at 55°C, 4 min at 72 °C, followed by a plate read at 83°C. Five replicates of 25-µl reaction mixtures without template were inserted as negative controls. To verify amplicon sequences and to generate a standard curve for quantification, PCR amplicons of *hgcA* genes from Deltaproteobacteria and Archaea were ligated to a pGEM-T Easy vector (Promega, USA) and transformed into *Escherichia coli* JM109 cells. Positive clones containing the target gene insert were sequenced, and the most abundant one was used for plasmid DNA extraction. After measuring the concentration of the purified plasmid DNA with a Nanodrop spectrophotometer, a 10-fold dilution series was analyzed with qPCR in triplicate to generate an external standard curve. The *hgcA* gene abundance from Firmicutes was not quantified since no amplification was obtained from clade-specific PCR products using the ORNL-SRB-Firm-HgcA primers, as previously reported.[24, 30]

Shotgun metagenomic analysis by Illumina sequencing

Seven paddy soils and three upland soils were selected for comparative metagenomic analysis. Five replicates were conducted for each sample to obtain >1 µg DNA for shotgun metagenomic sequencing. Sequencing was performed using an Illumina MiSeq (Illumina Inc., USA) at Majorbio in Shanghai, China. Raw reads (PE150, 150 bp paired end reads) were trimmed to remove low quality reads as follows. First, the SeqPrep software (https://github.com/jstjohn/SeqPrep) was used to remove the adapter sequences. Second, the library sickle (https://github.com/najoshi/sickle) was used to trim the reads from the 5' end to 3' end using a sliding window (size 50 bp, 1 bp step). If the mean quality of bases inside a

window drops below 20, the remainder of the read below the quality threshold was trimmed. Quality-trimmed reads that were shorter than 50 bp or containing N (ambiguous bases) were discarded. As a result, a total of 901,610,484 clean reads were generated with an average of 90,161,048 reads per sample.

Profile hidden Markov models (HMMs) were used to search metagenomic data for *hgcA* homologs directly using the HMMER package.[34] Search methods based on profile HMMs are preferable in identifying distantly related homologs compared to other methods such as BLAST. A profile HMM of the entire HgcA sequence (HgcA trim), including the predicted cobalamin binding domain and distinct transmembrane domain, was generated from 65 representative HgcA sequences (Table S2). To remove high-scoring false positives in HMM search results, we used the FrameBot toolset.[35, 36] The reads were validated for correct open reading frames (ORF) using a set of known HgcA protein sequences as a reference. Only those reads with an ORF spanning the entire *hgcA* sequence were retained. HgcA sequences obtained in the HMM search results were then confirmed by validating the presence of conserved sequence domains (cap helix and transmembrane region).[11] The resulting *hgcA* homolog hits were further validated by BLASTX searches[37] with a set of known HgcA sequences from predicted Hg methylators (http://www.esd.ornl.gov/programs/rsfa/data.shtml). Similarly, a profile HMM of HgcB was generated from an alignment of 65 known HgcB sequences shown in Table S2. In addition, the short reads from all soil samples were assembled using SOAPdenovo2 with default parameters. Searching assembled contigs (> 500 bp) using HMMs produced only several *hgcA* homologs due to the loss of reads during assembly. The assembled contigs were processed for taxonomic assignment using BLASTP (BLAST Version 2.2.28+)[37] with an E-value cutoff of 1×10^{-5} against the NR nucleotide database maintained by the National Center for Biotechnology Information (NCBI). Based on the results of the taxonomic assignment, taxon abundances were assigned to different taxonomic ranks.

Community analysis of *hgcAB* genes by PacBio sequencing

3rd-generation PacBio sequencing was subsequently used to characterize *hgcAB* gene distribution and community composition of selected paddy soil samples. The PacBio single

molecule,[38] real-time (SMRT) sequencing technology has the ability to cover nearly the entire length of *hgcAB* (818-1020 bp) overcoming major limitations of 2nd-generation sequencing technologies (e.g., Illumina MiSeq) using short reads, which can introduce potential biases, particularly for the identification of homologs with high sequence variability such as *hgcAB* genes. The *hgcAB* genes were amplified using the broad-range degenerate PCR primer set ORNL-HgcAB-uni-F and ORNL-HgcAB-uni-R,[24] following a touchdown protocol, consisting of 30 s at 95°C, annealing for 30 s at temperatures from 70 to 55°C over 15 steps (1°C decremental steps), and ending with an extension step at 72°C for 60 s. This protocol was repeated over a total of 45 cycles. The PCR amplification reactions were performed in triplicate, and each 25 µl mixture consisted of 10 ng DNA, 1 µl of each primer set (10 µM), and 20 µl of sterilized double-distilled deionized water. PCR amplicons were extracted from 2% agarose gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Biosciences LLC., USA). Purified amplicons were pooled in equimolar concentrations within each plot and then sequenced on a PacBio RS II system (Pacific Biosciences of California, USA) using C4 chemistry and standard protocols. Data were processed and analyzed following a previously-published procedure,[39] where raw FASTQ data files were quality filtered with QIIME (version 1.7.0) using the corresponding default parameters. Subsequent read processing was performed by USEARCH and clustered at 60% identity cutoff using CD-HIT-EST.[40] The rarefaction curves approached a plateau demonstrating sufficient sequencing depth of our reads (Figure S2). The metagenomic sequences have been deposited with the NCBI under accession number PRJNA450451.

To generate taxonomic assignments, the resulting *hgcAB* sequences (818-1020 bp) were subjected to local BLAST searches with predicted Hg methylators (http://www.esd.ornl.gov/programs/rsfa/data.shtml), as well as BLAST searches on GenBank. We found that some *hgcAB* sequences shared a high similarity with sequences of three new species (i.e., *Geobacter anodireducens*, *Desulfuromonas* sp. DDH964 and *Desulfovibrio* sp. J2), which have not yet been identified as Hg methylators. These potential methylators were subsequently confirmed to contain both HgcA and HgcB sequences using the methods described above. To explore evolutionary relationships of the translated HgcA sequences in our samples and those from published and the newly identified Hg methylators, we conducted

a phylogenetic analysis based on a multiple sequence alignment of the representative HgcA sequences from our samples with a set of openly available HgcA sequences. All HgcA amino acid sequences from the alignment were truncated to 120 residues, starting with the highly conserved 'G(I/V)NVWCA(A/G)' motif and ending on a highly conserved arginine. This set of truncated amino acid sequences was aligned with ClustalW within the MEGA6 suite,[41] and gaps and ambiguously aligned positions were deleted. After alignment, a phylogenetic tree was constructed using the Jones-Taylor-Thornton (JTT) model, the maximum likelihood method, and bootstrapping using 100 replicates for each sequence.

RESULTS AND DISCUSSION

qPCR and metagenomic data analyses of *hgcA* gene abundance

Results of quantitative real-time PCR (qPCR) analyses showed the presence of *hgcA* genes in all paddy soils from Fenghuang (FH) and Wanshan (WS) areas, with gene copy numbers ranging from 1.06×10^7 to 3.28×10^8 per gram dried soil (Figure 1a and Figure S3). This result is in contrast to the unsaturated oxic upland soils (controls), where no *hgcA* genes from either Deltaproteobacteria or Archaea could be detected (Figure 1a), consistent with the requirement for anaerobic conditions common to all known methylators.[10,11] Overall *hgcA* abundance varied depending on the site and appeared slightly higher in the WS than the FH paddy soils. We quantified *hgcA* abundances using two clade-specific primer pairs [24] and found that *hgcA* gene copy numbers for the Archaea were slightly higher than for the Deltaproteobacteria. However, neither the total Hg nor MeHg concentrations were found to be significantly correlated to *hgcA* abundances in these paddy soils ($p > 0.05$) (Figure S4). This result is not surprising because DNA-based community analysis alone cannot explicate MeHg concentrations and enzymatic activities of the Hg methylators in the field, as previously suggested.[28, 42] Additionally, net MeHg production could be affected not only by site-specific geochemical conditions but also by MeHg degradation in the environment.[6]

The qPCR results agree well with metagenomic sequencing analyses evaluating the occurrence and distribution of *hgcA* genes in seven paddy soils and three upland soils (Figure

1b). Homologs of *hgcA* were identified using HMMs, constructed from a set of known gene variants (Table S2), and confirmed by protein sequence alignments and identification of conserved HgcA sequence motifs. On average, 12 *hgcA* hits per Gbp metagenome size were detected in the paddy soils, which is much higher than the average 0.2 hits per Gbp in the metagenomes of unsaturated oxic upland soil controls. The result again indicates the importance of predominantly anoxic conditions for Hg methylating microorganisms in paddy soils. So far, no aerobic microbes have been identified as capable of methylating Hg although Hg methylation has been reported in settling particles of oxic water columns.[43] As expected, the relative abundance of *hgcA* genes obtained from metagenomes showed similar trends across all sites when compared to the abundances of *hgcA* genes among Deltaproteobacteria and Archaea derived from qPCR data (Figure 1a), confirming the effectiveness of the primer pairs used for *hgcA* genes. The relative abundance of identified *hgcA* genes in paddy soils is also comparable to data obtained from metagenome analyses of thawing permafrost soils from Bonanza Creek, Alaska, and tropical forest soils from Puerto Rico, United States.[25]

Diversity of *hgcA* homologs in soil metagenomes

The *hgcA* homologs identified by metagenomes in the paddy soils were taxonomically diverse (Figure 1c). Most of the *hgcA* sequences were closely related to sequences from previously reported Hg methylating genera within the Deltaproteobacteria, Firmicutes, and the methylotrophic Methanomicrobia.[10,11,25] This finding is expected since the profile HMM used to identify *hgcA* homologs was constructed from *hgcA* sequences of known methylators. The result showed that *hgcA* homologs associated with *Geobacter* spp. and *Methanoregula* spp. were dominant in the metagenomes in these paddy soils (Figure 1c). The metagenome data also revealed significant variability in total community composition based on the taxonomic assignments of assembled contigs across the sites (Figure S5). Variability in the relative abundance of phyla and classes (including both $hgcA^+$ and $hgcA^-$ taxa) potentially related to Hg methylation was observed, in which the relative abundance of the class Deltaproteobacteria was much higher than the phyla Euryarchaeota and Firmicutes.

While statistically insignificant, soil MeHg contents appeared to increase with increasing relative abundance of all clades containing methylators (Figure 2a; $r = 0.70$, $p < 0.08$). When

normalized to the relative abundances of *hgcA*$^{+}$ *Geobacter* spp, we observed a significant correlation between the relative abundance of *hgcA*$^{+}$ *Geobacter* and soil MeHg contents (Figure 2b; r = 0.82, $p < 0.05$) in the paddy soils, but no clear correlation was found between the abundance of *hgcA*$^{+}$ *Methanoregula* spp. and MeHg (Figure 2c). This result suggests potentially important roles of *Geobacter* spp. in methylating Hg in the paddy soil, although additional transcript studies are required to validate their activities in the field.

The levels of *hgcB* homologs identified by profile HMMs in metagenomes (Figure S6) were found to be much higher than levels of *hgcA* in these soils using the same technique. A recent study also reported higher levels of *hgcB* relative to *hgcA* homologs in the metagenomes of samples from Antarctic sea ice.[35] These results are attributed to the fact that the conserved [4Fe-4S] binding motif in HgcB is shared with many other ferredoxins. Therefore, it is difficult to unambiguously distinguish HgcB from other ferredoxins using profile HMMs,[25] whereas long-read sequencing techniques such as PacBio can increase the accuracy of identifying *hgcAB* in Hg methylating communities.

Characterizing community composition by PacBio sequencing of *hgcAB* genes

We used PacBio sequencing to further characterize *hgcAB* gene distribution and community composition overcoming limitations of short read lengths derived from shotgun metagenomic sequencing.[38] The full-length *hgcAB* sequences were taxonomically assigned by performing local BLAST searches using the genomes of more than 120 predicted Hg methylators.[25, 35] Results show that most of the *hgcAB* sequences were affiliated with the genera *Geobacter*, *Methanofollis*, *Methanoregula*, *Methanocella* and an unclassified *Deltaproteobacterium*, although their relative abundances differed across various sites and soil samples (Figure 3a). When these Hg methylators were grouped at phylum and class levels, average relative abundances of *hgcAB* were dominated by homologs related to the Deltaproteobacteria (42%) and Methanomicrobia (57%), with negligible amounts of Firmicutes (Figure 3b). A relatively high abundance of *hgcAB* homologs associated with the Methanomicrobia, dominated by *Methanofollis* and *Methanoregula*, is in an agreement with the metagenomic data and qPCR results (Figure 1), indicating an overall high abundance of archaeal *hgcA* genes in these soils (Figure S5). Importantly, when these methylators were

grouped into the three typical functional clades, we found a higher abundance of iron-reducing bacteria (~34%, dominated by *Geobacter* spp.) and methanogens (57%, dominated by *Methanomicrobia* spp.), while sulfate-reducing bacteria were significantly less abundant (< 3%) (Figure 3c). These observations are consistent with the results for *hgcA* homologs from the metagenomic analysis (Figure 1c, Figure S7). However, we note significant differences between relative abundances of *Desulfomonile* derived from the two methods. This variability could be attributed in part to primer preference and its limitations in covering all species equally among different environmental samples, which are known issues common to sequencing technologies. Additionally, assigning *hgcA* sequences from shotgun metagenomics to genera is based on aligning short reads with a set of known gene sequences. This approach may give rise to inaccuracies, especially if the data is evaluated down to the genus level.

Of particular interest is the observed low proportion of the *hgcA*$^+$ sulfate-reducing bacteria among the identified Hg methylators, although the total SRB population (based on the marker genes *dsrA* and *dsrB*) is relatively high (Table S3). Similarly, a recent study indicated that *Geobacteraceae*, rather than sulfate-reducing bacteria, are important members of Hg-methylating microbial communities of sediments impacted by wastewater releases.[23] However, another study suggested that sulfate-reducing bacteria are the dominant methylators in paddy soils,[31] where microbial community composition and distribution of *hgcA* were determined using conventional techniques such as clone libraries.[29,30] Here we used a combination of deep shotgun metagenomic sequencing and long-read PacBio sequencing targeting *hgcAB* genes to mutually corroborate the results showing relatively low abundances of *hgcA*$^+$ sulfate-reducing bacteria. These results suggest that sulfate-reducing bacteria likely were not the dominant players in methylating Hg in the studied paddy soils, although we acknowledge that different geochemical variables such as sulfate concentrations (Tables S1) and redox conditions may partially account for the differences observed between the current and other studies. Additionally, the DNA-based community analysis usually does not respond to the activity of clades involved in Hg methylation *in situ*,[42] and the SO_4^{2-} concentration does not always correlate with the abundance of *hgcA*$^+$ sulfate-reducing organisms since only a

small subset of sulfate-reducing organisms can methylate Hg. Some of these sulfate-reducing organisms, such as *Desulfovibrio desulfuricans* ND132, can methylate Hg in the absence of SO_4^{2-}.[44] Future transcriptomics studies are therefore recommended to further evaluate *hgcA* transcript levels in methylating taxa and to reveal possible correlations between *hgcA* gene expression and MeHg production.

Results from PacBio sequencing and analysis of phylogenetic relationships also showed that most of the identified *hgcAB* sequences are closely related to previously reported Hg methylators from diverse environments (Figure 4; Table S3),[10,25] but about 3.7% of the sequences do not match sequences from those reference taxa. Interestingly, several *hgcAB* sequences were identified in three unconfirmed methylators with recently sequenced genomes (Tables S4 and S5). These novel unconfirmed methylators include *Geobacter anodireducens*, *Desulfuromonas* sp. DDH964, and *Desulfovibrio* sp. J2, all of which contain *hgcAB* homologs based on the presence of strictly conserved sequence motifs (Figure S8 and S9). Phylogenetic analysis of translated HgcA protein sequences indicates a high level of similarity with HgcA sequences in paddy soils as determined by PacBio sequencing (Figure 4) and analysis of assembled contigs from metagenomic data (Table S6). While it is beyond the scope of this work to isolate these bacterial strains in the paddy soil, these bacteria are known to exist in anoxic environmental settings. For example, *G. anodireducens* was originally isolated from a microbial fuel cell reactor, and shown to possess significantly different physiological traits and functions from the *G. sulfurreducens* PCA strain.[45] *Desulfovibrio* sp. J2 was isolated from a deep-sea brine,[46] while *Desulfuromonas* DDH964 was recovered from an electrode enrichment culture.[47]

To confirm the ability of the newly identified Hg-methylator *Geobacter* spp. to methylate Hg (Figures 1c and 3a), we determined the Hg-methylation potential of *G. anodireducens* SD-1 in pure culture studies. A methylation assay was performed by incubating SD-1 cells with Hg (25 nM) in the presence of acetate as an electron donor and ferric citrate as an electron acceptor (see Supporting Information for additional details). Results indicate that up to 15% of the added Hg was methylated by *G. anodireducens* SD-1 within 72 h. Cells were able to produce about 6×10^{-21} mol MeHg $cell^{-1}$ within 24 h (Figure 5), with an estimated

methylation rate of 0.064 h^{-1}. This rate is comparable to that obtained for *Geobacter sulfurreducens* PCA, a widely studied iron-reducing bacterium capable of Hg methylation in the environment.[48] However, we note that, unlike *G. sulfurreducens* PCA, *G. anodireducens* SD-1 cannot grow on fumarate as an electron acceptor,[44] thus ferric citrate was used in methylation assays in the present study. The assay results nevertheless confirm *G. anodireducens* SD-1 as a novel Hg methylator and shed additional light on *Geobacter* spp. in MeHg formation in the environment. However, whether *G. anodireducens* SD-1 plays a significant role in Hg methylation in these paddy soils remains to be investigated.

Taken together, paddy soils represent an ideal anoxic environment for Hg methylation, leading to the bioaccumulation of the neurotoxin MeHg in rice grains, which could severely impact the health of billions of people around the globe. Compared to short-read shotgun sequencing, 3rd-generation PacBio long-read sequencing enabled targeted sequencing of nearly full-length *hgcAB* gene clusters present in Hg methylators in these soils. This study provides the first comprehensive characterization of the diversity and distribution of microbial communities associated with Hg methylation in these soils and directly compares data from different sequencing approaches. Iron-reducing bacteria and methanogens were found to be among the most abundant communities, and a good correlation was observed between the relative abundance of *Geobacter* spp. and the MeHg concentration in the studied paddy soils. Furthermore, our data revealed sequences with significant homology to three novel Hg methylators, including *G. anodireducens, Desulfuromonas* sp. DDH964 and *Desulfovibrio* sp. J2. The ability of *G. anodireducens* SD-1 to methylate Hg was validated experimentally and is comparable to the known methylator *G. sulfurreducens* PCA. Because iron oxides are among the major mineral components in temperate and subtropical soils,[49] the importance of *Geobacter* spp. for Hg methylation may have been underestimated and should be further examined, particularly in iron-rich paddy soils.

The fact that few *hgcA* genes could be detected in the unsaturated oxic upland soils corroborates the importance of anaerobic environments for all known methylators.[11] Such anoxic environments are commonly observed in wetlands where contributions of *Geobacter* spp. and *Methanoregula* spp. to Hg methylation and the complexity of methylating

communities as described herein may have increased importance. Our findings also have potential implications for further understanding syntrophic interactions of Hg methylating communities in natural ecosystems,[50] and the evolution and distribution of these organisms not only in paddy habitats but also other ecosystems such as wetlands.

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ASSOCIATED CONTENT

Supporting Information and Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supporting Materials. This material is available free of charge via the Internet at http://pubs.acs.org.

Notes

The authors declare no competing financial interest. The U.S. Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (http://energy.gov/downloads/doepublic-access-plan).

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561 **FIGURE LEGENDS:**
562
563 **Figure 1.** Abundances and distribution of *hgcA* genes from metagenomes for the selected
564 paddy and upland soils obtained from Fenghuang (FH) and Wanshan (WS) mining areas in
565 Southwest China; (a) Log_{10}-transformed qPCR data for *hgcA* gene copy number for
566 Deltaproteobacteria and Archaea in the selected paddy soils, while qPCR for *Firmicutes*
567 yielded no *hgcA*. (b) Relative abundance of *hgc*A genes (from shotgun metagenomic
568 sequencing) normalized to metagenome size (log Gbp); (c) Assignments of *hgcA* genes from
569 shotgun metagenomic sequencing at the genus level. Circle size represents the relative
570 abundance of *hgcA* sequences assigned to a specific genus in each sample. Samples FHA-U,
571 FHB-U and WSA-U were from upland fields.
572
573 **Figure 2.** (a) Correlations between relative abundances of total Hg methylators (contains
574 *hgcA* genes from each Gbp metagenome data) and MeHg concentrations in seven paddy soils,
575 and between the relative abundances of dominant Hg-methylating genera (b) *Geobacter* spp.
576 and (c) *Methanoregula* spp. and soil MeHg concentrations.
577
578 **Figure 3.** (a) Closest genera assignments for *hgcAB* genes from PacBio sequencing. Circle
579 size represents the relative abundance of taxonomically assigned *hgcAB* sequence types in
580 each sample; (b) Average relative abundance of *hgcAB* gene sequences within the three
581 functional clades, and (c) average relative abundance of *hgcAB* sequences within the three
582 major groups linked to Hg methylation.
583
584 **Figure 4.** Maximum likelihood tree of HgcA sequences including translated representative
585 PacBio sequences of *hgcAB* in this study (blue text) and predicted novel HgcA sequences (red
586 text). Sequences are compared to HgcA homologs from previously identified taxa (black text).
587 The three major phyla (class) of known Hg methylators are shown: Deltaproteobacteria
588 (purple), Euryarchaeota (yellow) and Firmicutes (green). Bootstrap support values > 50 are
589 shown, with consensus based on 100 replicates.
590
591 **Figure 5.** Methylmercury (MeHg) production by *Geobacter anodireducens* SD-1 over time.
592 The culture medium was supplemented with 10 mM sodium acetate and 20 mM ferric-citrate,
593 and 500 μM cysteine (see Supporting Information for additional details). The initially added
594 Hg(II) concentration was 25 nM, and SD-1 cell concentration was 5×10^8 cells/mL.
595

Fig. 1

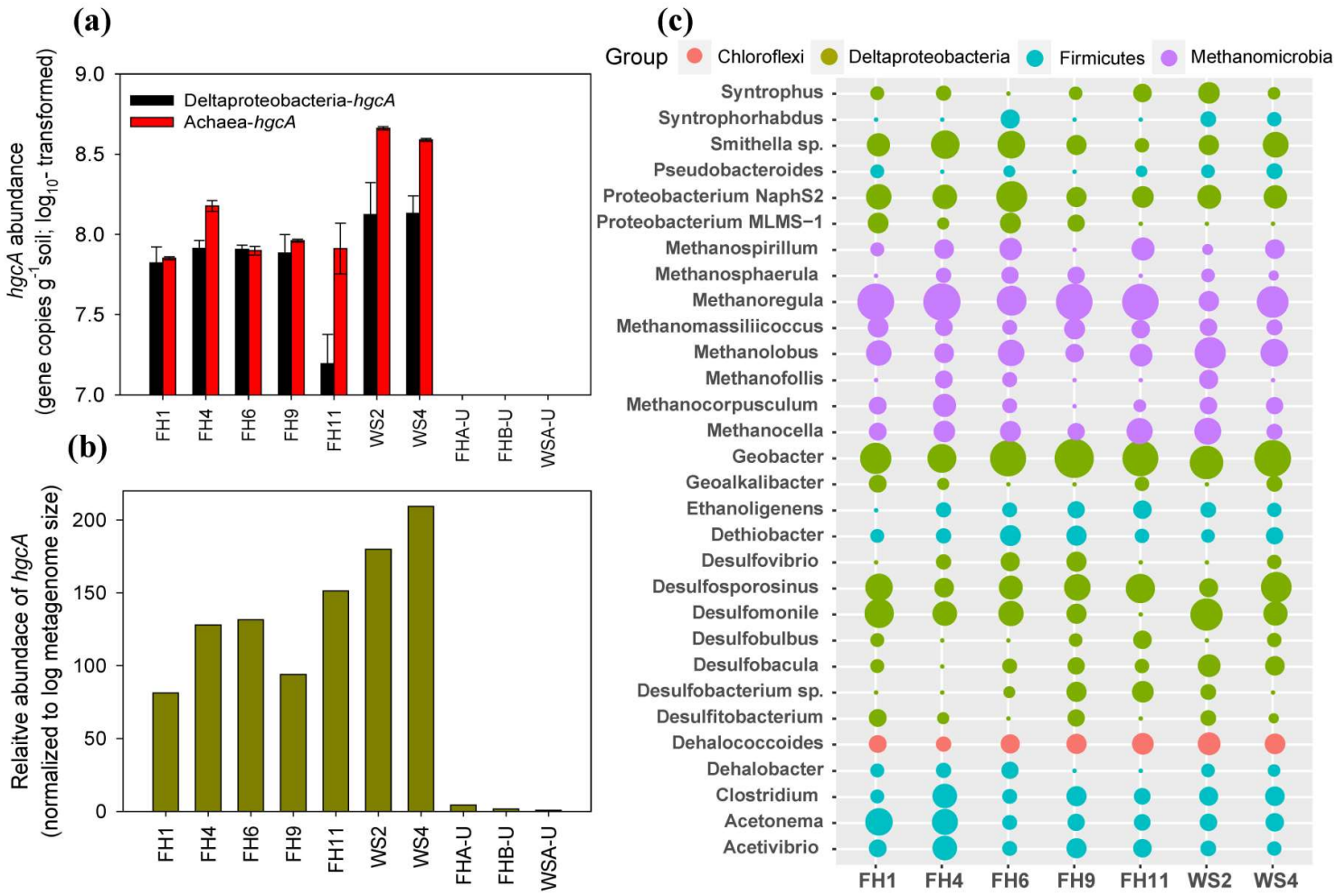

(a)
hgcA abundance
(gene copies g-1 soil; log10- transformed)
9.0
8.5
8.0
7.5
7.0
Deltaproteobacteria-hgcA
Achaea-hgcA
FH1
FH4
FH6
FH9
FH11
WS2
WS4
FHA-U
FHB-U
WSA-U
(b)
Relaitve abundace of hgcA
(normalized to log metagenome size)
200
150
100
50
0
(c)
Group
Chloroflexi
Deltaproteobacteria
Firmicutes
Methanomicrobia
Syntrophus
Syntrophorhabdus
Smithella sp.
Pseudobacteroides
Proteobacterium NaphS2
Proteobacterium MLMS−1
Methanospirillum
Methanosphaerula
Methanoregula
Methanomassiliicoccus
Methanolobus
Methanofollis
Methanocorpusculum
Methanocella
Geobacter
Geoalkalibacter
Ethanoligenens
Dethiobacter
Desulfovibrio
Desulfosporosinus
Desulfomonile
Desulfobulbus
Desulfobacula
Desulfobacterium sp.
Desulfitobacterium
Dehalococcoides
Dehalobacter
Clostridium
Acetonema
Acetivibrio
FH1
FH4
FH6
FH9
FH11
WS2
WS4

Fig. 2

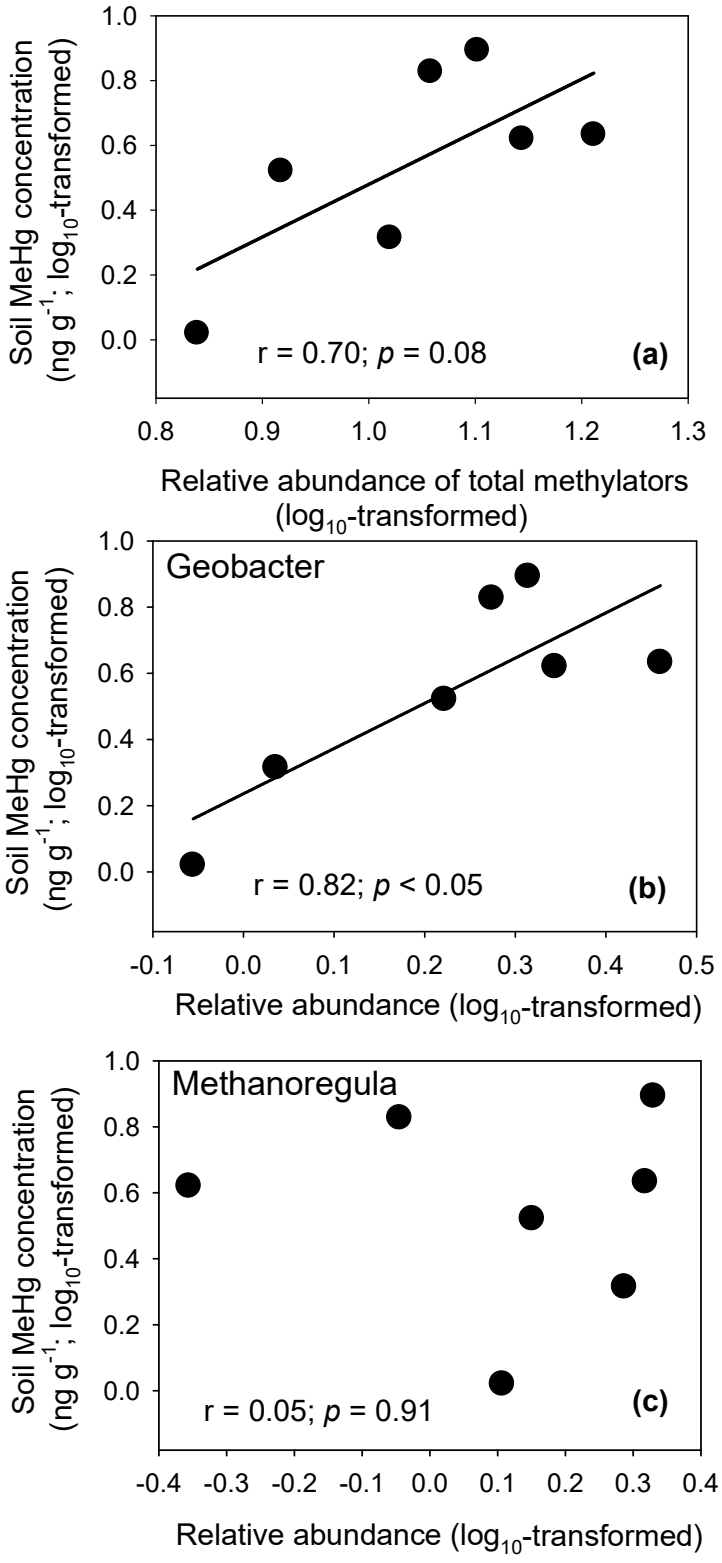

Soil MeHg concentration (ng g-1; log10-transformed)
r = 0.70; p = 0.08
(a)
Relative abundance of total methylators (log10-transformed)
Geobacter
r = 0.82; p < 0.05
(b)
Relative abundance (log10-transformed)
Methanoregula
r = 0.05; p = 0.91
(c)
Relative abundance (log10-transformed)

Fig. 3

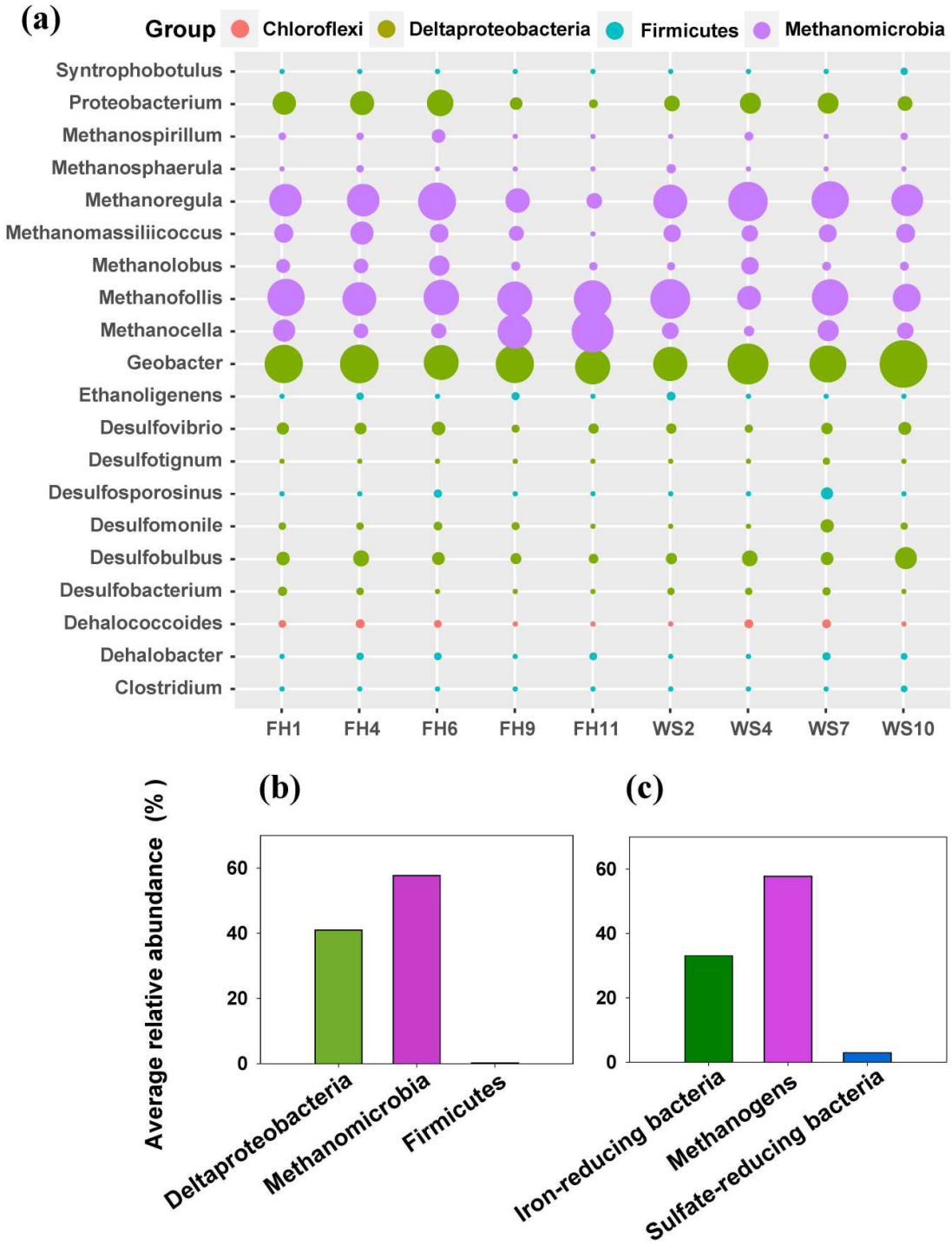

(a)
Group
Chloroflexi
Deltaproteobacteria
Firmicutes
Methanomicrobia
Syntrophobotulus
Proteobacterium
Methanospirillum
Methanosphaerula
Methanoregula
Methanomassiliicoccus
Methanolobus
Methanofollis
Methanocella
Geobacter
Ethanoligenens
Desulfovibrio
Desulfotignum
Desulfosporosinus
Desulfomonile
Desulfobulbus
Desulfobacterium
Dehalococcoides
Dehalobacter
Clostridium
FH1
FH4
FH6
FH9
FH11
WS2
WS4
WS7
WS10
(b)
(c)
Average relative abundance (%)
60
40
20
0
Deltaproteobacteria
Methanomicrobia
Firmicutes
Iron-reducing bacteria
Methanogens
Sulfate-reducing bacteria

Fig. 4

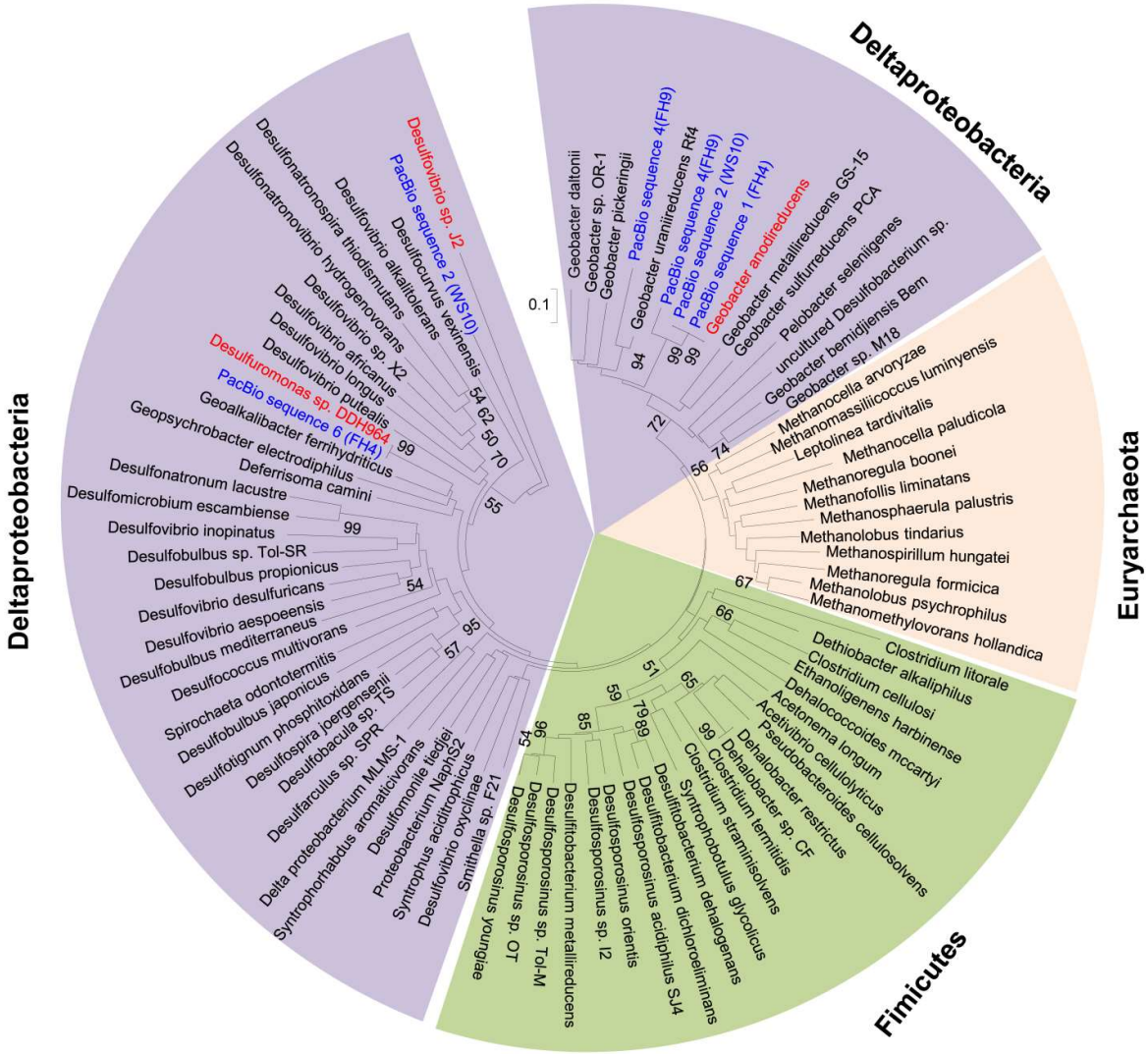
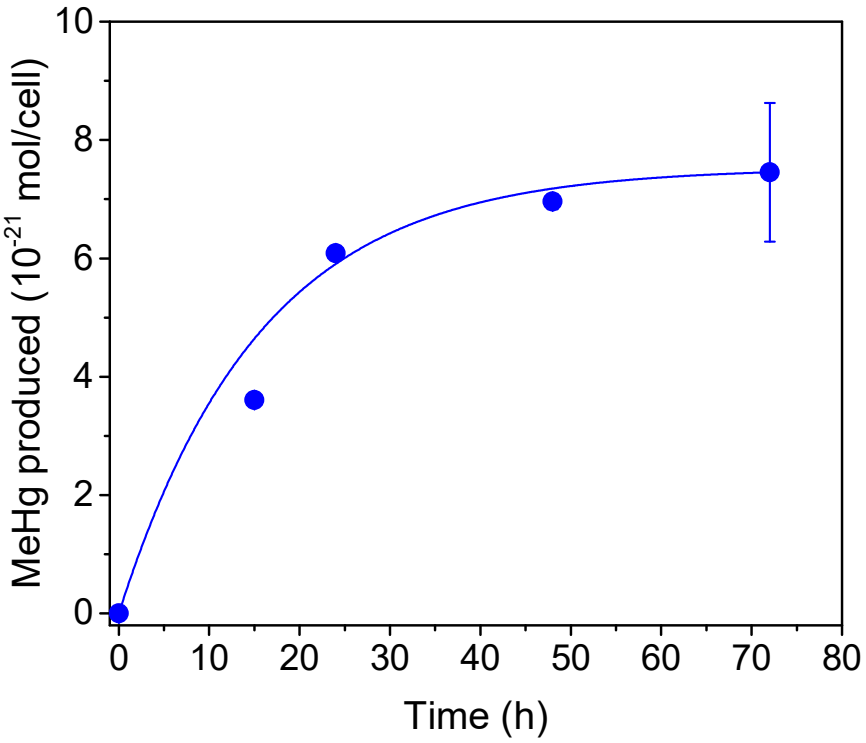

Deltaproteobacteria
Euryarchaeota
Fimicutes
Deltaproteobacteria
0.1
Geobacter daltonii
Geobacter sp. OR-1
Geobacter pickeringii
PacBio sequence 4(FH9)
Geobacter uraniireducens Rf4
PacBio sequence 4(FH9)
PacBio sequence 2 (WS10)
PacBio sequence 1 (FH4)
Geobacter anodireducens
Geobacter metallireducens GS-15
Geobacter sulfurreducens PCA
Pelobacter seleniigenes
uncultured Desulfobacterium sp.
Geobacter bemidjiensis Bem
Geobacter sp. M18
Methanocella arvoryzae
Methanomassiliicoccus luminyensis
Leptolinea tardivitalis
Methanocella paludicola
Methanoregula boonei
Methanofollis liminatans
Methanosphaerula palustris
Methanolobus tindarius
Methanospirillum hungatei
Methanoregula formicica
Methanolobus psychrophilus
Methanomethylovorans hollandica
Clostridium litorale
Dethiobacter alkaliphilus
Clostridium cellulosi
Ethanoligenens harbinense
Dehalococcoides mccartyi
Acetonema longum
Acetivibrio cellulolyticus
Pseudobacteroides cellulosolvens
Dehalobacter sp. CF
Dehalobacter restrictus
Clostridium termitidis
Clostridium straminisolvens
Syntrophobotulus glycolicus
Desulfitobacterium dichloroeliminans
Desulfosporosinus acidiphilus SJ4
Desulfosporosinus orientis
Desulfosporosinus sp. I2
Desulfitobacterium metallireducens
Desulfosporosinus sp. Tol-M
Desulfosporosinus sp. OT
Desulfosporosinus youngiae
Smithella sp. F21
Desulfovibrio oxyclinae
Syntrophus aciditrophicus
Desulfomonile tiedjei
Proteobacterium NaphS2
Desulfarculus sp. SPR
Delta proteobacterium MLMS-1
Syntrophorhabdus aromaticivorans
Desulfobacula sp. TS
Desulfospira joergensenii
Desulfotignum phosphitoxidans
Desulfobulbus japonicus
Spirochaeta odontotermitis
Desulfococcus multivorans
Desulfovibrio aespoeensis
Desulfobulbus mediterraneus
Desulfovibrio desulfuricans
Desulfobulbus propionicus
Desulfobulbus sp. Tol-SR
Desulfovibrio inopinatus
Desulfomicrobium escambiense
Desulfonatronum lacustre
Deferrisoma camini
Geopsychrobacter electrodiphilus
Geoalkalibacter ferrihydriticus
PacBio sequence 6 (FH4)
Desulfuromonas sp. DDH964
Desulfovibrio putealis
Desulfovibrio longus
Desulfovibrio africanus
Desulfovibrio sp. X2
Desulfovibrio hydrogenovorans
Desulfonatronospira thiodismutans
Desulfonatronovibrio hydrogenovorans
Desulfovibrio alkalitolerans
Desulfocurvus vexinensis
PacBio sequence 2 (WS10)
Desulfovibrio sp. J2

Fig. 5


MeHg produced (10^{-21} mol/cell)
Time (h)
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80