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Contribution of Coexisting Sulfate and Iron Reducing Bacteria to Methylmercury Production in Freshwater River Sediments

Ri-Qing Yu,*[,†] J. R. Flanders,[‡] E. Erin Mack,[§] Ralph Turner,[⊥] M. Bilal Mirza,[†] and Tamar Barkay[†]

[†]Department of Biochemistry and Microbiology, Rutgers University, New Brunswick, New Jersey 08901, United States

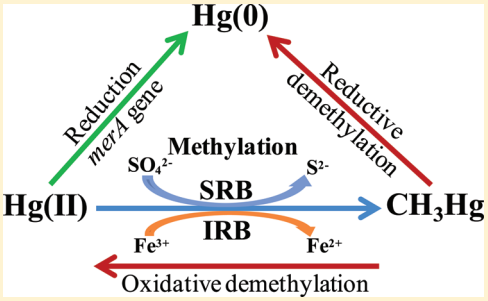
[‡]URS Corp., Fort Washington, Pennsylvania 19034, United States

[§]DuPont Company, Corporate Remediation Group, Wilmington, Delaware 19805, United States

[⊥]RT Geosciences Inc., Squamish, BC V8B 0A4, Canada

Supporting Information

ABSTRACT: We investigated microbial methylmercury (CH_3Hg) production in sediments from the South River (SR), VA, an ecosystem contaminated with industrial mercury (Hg). Potential Hg methylation rates in samples collected at nine sites were low in late spring and significantly higher in late summer. Demethylation of $^{14}CH_3Hg$ was dominated by $^{14}CH_4$ production in spring, but switched to producing mostly $^{14}CO_2$ in the summer. Fine-grained sediments originating from the erosion of river banks had the highest CH_3Hg concentrations and were potential hot spots for both methylation and demethylation activities. Sequencing of 16S rRNA genes of cDNA recovered from sediment RNA extracts indicated that at least three groups of sulfate-reducing bacteria (SRB) and one group of iron-reducing bacteria (IRB), potential Hg methylators, were active in SR sediments. SRB were confirmed as a methylating guild by amendment experiments showing significant sulfate stimulation and molybdate inhibition of methylation in SR sediments. The addition of low levels of amorphous iron(III) oxyhydroxide significantly stimulated methylation rates, suggesting a role for IRB in CH_3Hg synthesis. Overall, our studies suggest that coexisting SRB and IRB populations in river sediments contribute to Hg methylation, possibly by temporally and spatially separated processes.



INTRODUCTION

Mercury (Hg) methylation converts inorganic Hg into methylmercury (CH_3Hg) and increases Hg toxicity because of the trophic transfer of CH_3Hg, a potent neurotoxicant, in aquatic food chains. Although Hg methylation in the environment has been studied for over 40 years,[1−4] a few key issues regarding CH_3Hg production and degradation are still not resolved. For example, potential rates of CH_3Hg degradation (demethylation) as the competing process to methylation, have not always been integrated with potential methylation rates in assessing the dynamics of CH_3Hg production in freshwater systems.[5−7] Likewise, the lack of knowledge regarding the genes involved in microbial methylation hinders application of metagenomic approaches that could directly link biotic Hg methylation to methylating microbes. Currently metagenomic approaches have used 16S rRNA or dissimilatory sulfite reductase genes which specify sulfate reduction to address this question. Such studies are crucial for identifying potential methylators, providing insight into methylation processes in natural environments and supporting remediation efforts.[8−12]

From 1929 to 1950, an industrial facility in Waynesboro, VA, located on the banks of the South River (SR), used mercuric sulfate as a catalyst to produce acetate fiber. Thousands of kilograms of Hg waste were released into the surrounding landscape and SR.[13] River bank erosion has continued to transport inorganic Hg into the river and led to persistent contamination of fish for decades, with Hg levels that exceed the USEPA criterion of 0.3 $\mu g\ g^{-1}$ by a factor of 4 to 13.[14] This contamination has also resulted in the trophic transfer of Hg from aquatic food chains to birds and other biota in adjacent terrestrial ecosystems.[15] River sediments may be a major sink for Hg, and are likely sites where microbial Hg methylation and CH_3Hg demethylation activities occur. Although the kinetic balance between Hg methylation and CH_3Hg demethylation determines net accumulation of CH_3Hg in aquatic ecosystems, these processes and their dynamics have not been examined in the SR.

Sulfate-reducing bacteria (SRB) have long been considered as the principal Hg methylators in estuarine and freshwater ecosystems[2,3] and more recently pure cultures of iron reducing bacteria (IRB) were shown to methylate Hg.[16,17] However, the relative importance of SRB and IRB for in situ CH_3Hg production and the ecological significance of IRB to methylation in environmental habitats such as in highly Hg-

Received: September 25, 2011
Revised: December 5, 2011
Accepted: December 13, 2011
Published: December 13, 2011

contaminated riverine ecosystems have been rarely reported. Distinguishing dominant microbial pathways that lead to CH_3Hg production by amendments with metabolic stimulators and inhibitors, and the molecular characterization of Hg methylating communities are two key approaches to understand the process of Hg methylation. The objectives of this study were first to estimate the relative rates of Hg methylation and CH_3Hg degradation in SR sediments; second, to identify possible habitats within the ecosystem where CH_3Hg is likely to be produced; third, to relate methylation rate potentials to microbial community structure; last, to test hypotheses regarding pathways of methylation in the SR ecosystems.

MATERIALS AND METHODS

Sampling and Analytical Methods. The SR, located in northern Virginia, is a sinuous and steep gravel-bed river. The cohesive near-bank sediments are mostly silt and clay with some sand and gravel. The ten study sites were located along a 21-km reach of the SR downstream from the Hg contamination source, a previous textile manufacturing facility in Waynesboro, VA, at the origin (0 km; Supporting Information Figure S1). These sites, labeled as RRD2.6 to 20.6 (Relative River Distance, in km, downstream from the origin), included five distinct habitat types (Supporting Information Text S1.1 and Table S1). Fine-grained sediment (FGS) deposits (RRD10.0 and 20.6) refer to in-channel deposits of mud and sand caused by reduced flow velocity due to bank obstructions. Surface sediment samples (0 to 7 cm depth) were collected in May (sediment temperature of 15.1−16.5 °C) and August 2008 (19.7−20.6 °C) by using a 5-cm polycarbonate core (3.3 mm wall thickness) or a plastic hand-operated bilge pump method when the coring method could not be used for sampling.[18] Samples collected for CH_3Hg analysis were immediately placed on dry ice. Samples for measuring potential methylation (M) and demethylation (D) rates were kept in sterile mason jars without headspace, shipped on ice, and stored at 4 °C for less than one week before assaying, and samples for RNA extractions were shipped on dry ice and stored at −80 °C in the lab prior to analyses. An additional set of samples were collected in May 2010 from RRD10.0, RRD14.0, and RRD20.6 for potential M measurements in the presence of metabolic stimulators and inhibitors.

Total Hg (THg) concentrations in sediment samples were analyzed by cold-vapor atomic fluorescence spectroscopy (CVAFS) detection following extraction, oxidation and volatilization.[19,18] CH_3Hg in wet sediment was separated by a solvent (methylene chloride) extraction procedure, and measured following aqueous ethylation with sodium tetraethylborate, purging and trapping, adsorption and desorption, separation by gas chromatography at 100 °C, reduction by a pyrolytic column, and detection by CVAFS.[20] Quality control analyses with reference sediment material (IAEA-SL-1) showed an average of 103% recovery rates for THg and of 90−95% for CH_3Hg. Measurements of pH, sulfate, and nitrate in porewater, as well as Fe(II), microbially reducible Fe(III) and acid volatile sulfide (AVS) in whole sediment, and other biogeochemical parameters are described in Supporting Information Text S1.2.

Mercury Methylation and Amendment Experiments. Sediments were manually homogenized with a Teflon spatula in an anaerobic chamber (Coy Laboratories, MI) and processed under strictly anoxic conditions. Potential methylation rates in sediment samples were measured by spiking $^{203}HgCl_2$ (Eckert & Ziegler Isotope Products, CA) at trace levels (Supporting Information Text S1.3) as previously described.[6,10] To investigate which microbial guilds were involved in Hg methylation, molybdate (Na_2MoO_4) as an inhibitor and sulfate (Na_2SO_4) as a stimulator of sulfate reduction were added to slurries (total 6 mL) of RRD10.0, 14.0, and 20.6 at either 400 or 1,000 μM each, representing 2- or 5-fold increase over the average porewater sulfate concentration (200 μM) in the May 2010 samples (Supporting Information Text S2.1, 2.2). Additions of Fe(III) to sediments were by 1/2, 1, and 2 fold above measured ambient microbially reducible Fe(III) concentrations (Supporting Information Text S2.2). Fe(III), as $Fe(OH)_3$, was added to slurry incubations at amended concentrations of 0.80, 1.60, and 3.21 mg g^{-1} dry weight (dwt) sediment for Site RRD10.0, 2.54, 5.11, and 10.11 mg g^{-1} dwt for RRD14.0, and 1.44, 2.87, and 5.74 mg g^{-1} dwt for RRD20.6, respectively. Fresh amorphous iron(III) oxyhydroxide was synthesized according to the methods described by Cornell and Schwertmann.[21] All slurries for methylation were incubated at ambient temperature (~22 °C) in the dark for two days.

Determination of Potential Demethylation Rates. Sediment slurry microcosms were prepared and incubated for 24 h as above (see methylation experiments) before spiking $^{14}CH_3HgCl$ (Amersham Corp., England). Spiking levels were comparable to, or lower than, the in situ sediment CH_3Hg concentrations. Potential rates of demethylation were determined by methods modified from previous studies.[6,22] Details of the experiments and trapping for $^{14}CO_2$ and $^{14}CH_4$ are in Supporting Information Text S1.4.

RNA Extraction, cDNA Synthesis, PCR Amplification, and Sequencing of 16S rRNA Genes. Molecular characterizations of microbial communities were performed with the 2008 sediment samples from site RRD10.0, RRD14.0, and RRD20.6. RNA was extracted from sediment samples using a modification of the Hurt method.[23] RNA in extracts was separated by a RNA/DNA Mini Kit (Qiagen, CA) according to the manufacturer's protocol. Total RNA was then purified using a RNeasy kit (Qiagen, CA). DNA in extracted RNA preparation was removed with RQ1 RNase-free DNase (Promega, WI). RNA was reverse-transcribed to cDNA by the Superscript III First-Strand Synthesis System (Invitrogen, CA).

The 16S rRNA genes representing active bacteria in sediments were amplified from cDNA by using the bacterial universal primer set 27f and 910r[24] in a GeneAmp PCR System 9700 (Applied Biosystems, CA). Reactions (25 or 50 μL each) included 0.4 μM PCR primers, $MgCl_2$ at 1.5 mM (final concentration), 1× PCR buffer provided by the polymerase manufacturer, 0.2 nM of each deoxynucleoside triphosphate, 0.25 mg of bovine serum albumin ml^{-1}, 50 to 250 ng of cDNA, and 0.025 U of *Taq* polymerase (Denville, NJ). PCR conditions were an initial 5-min hot start at 95 °C, 35 cycles of 94 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min and 30 s, concluding with a final extension at 72 °C for 12 min. Methods for construction of clone libraries were described by Yu et al.[10] 16S rRNA gene inserts were sequenced using primer 27r by Genewiz. Inc. (Piscataway, NJ).

Phylogenetic and Statistical Analyses. DNA sequences of clone libraries were edited using MEGA (http://www.megasoftware.net/), and the sequence similarity of 16S rRNA genes of clones to those in all databases was compared using BlastN (http://www.ncbi.nih.gov/). Edited sequences were aligned by ClustalW.[25] Phylogenetic trees were constructed by PAUP* (version 4.0 beta 10; Sinaur Associates, MA) and

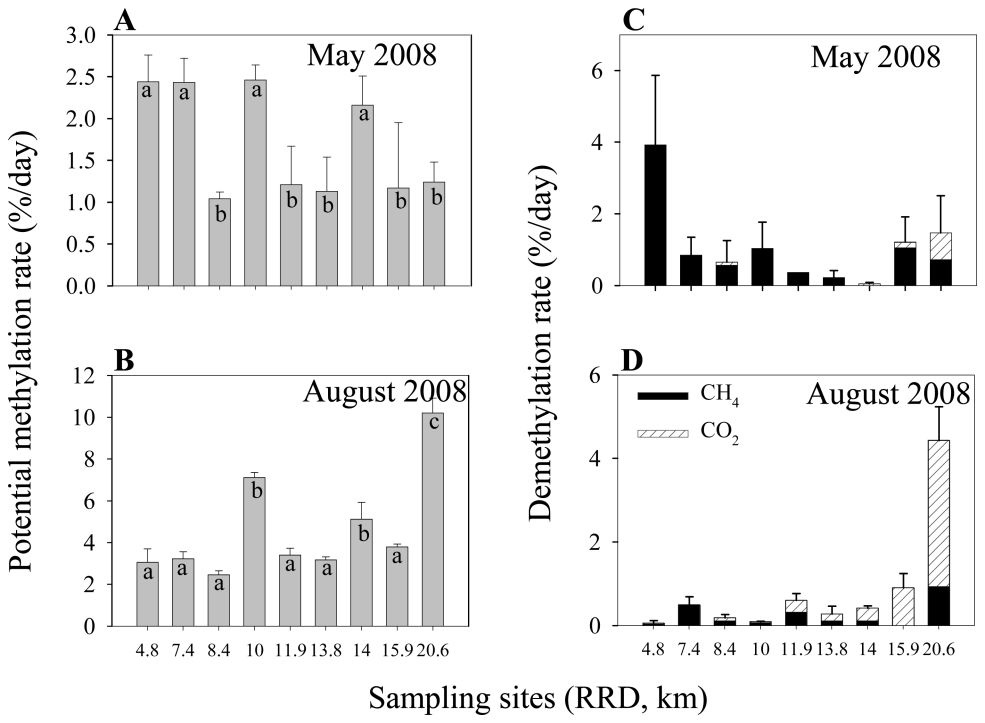


Figure 1. Potential methylation (A and B) and demethylation rates (C and D) by slurry incubations of sediments collected at nine sites downstream from the source of Hg contamination in the South River, VA. RRD = relative river distance (km) from the source. Bars with different letters in A and B indicate significant differences (ANOVA, $p < 0.05$).

ClustalX.[26] The robustness of tree topology was tested by bootstrap resampling with 1000 iterations.

The M/D ratio was calculated by dividing the potential M by the potential D rate obtained from replicate samples of each site. Results of M and D rates were analyzed by one-way and two-way ANOVA. Specific comparisons among different treatments and sampling sites were performed by Tukey's honest significant difference (HSD) (one-way) or Tukey-Kramer (two-way) tests using SAS software (SAS Institute, Cary, NC).

Nucleotide Sequence Accession Numbers. GenBank accession numbers of SR 16S rRNA gene sequences are JN641355 to JN641731.

RESULTS AND DISCUSSION

Total Hg and CH_3Hg Concentrations and Potential for Microbial CH_3Hg Production. The highest sediment THg concentration in the ten river sites was found in site RRD8.4 (56.6 $\mu g\ g^{-1}$) from a mill race channel (Supporting Information Table S1 and S2). The fine-grained sediment deposits from sites RRD10.0 and 20.6 in May 2008 had the highest CH_3Hg concentrations (123 and 124 ng g^{-1}, respectively; Supporting Information Table S2). Sediment CH_3Hg levels in May 2008 for most sites were significantly higher than those from the August 2008 samples (Supporting Information Table S1). A three year study of Hg and CH_3Hg in soil, surface water, and sediment identified that the major nonpoint source of Hg to the SR was the particle-bound Hg form derived from bank erosion along the first 14 km reach downstream from the historic point source in Waynesboro.[18]

For samples collected in May 2008, potential methylation rates ranged from 1.0 to 2.5% per day (Figure 1A and B). August samples had significantly higher potential methylation rates (2.5−10.2% day^{-1}) than the May samples. Two-way ANOVA tests demonstrated that seasonal changes and site variations of Hg methylation potentials in the 2008 samples were highly significant ($p < 0.0001$). When the habitat types within the SR were compared, the highest methylation potentials were observed in FGS deposits (RRD10.0 and 20.6) collected along the river pool edges. Potential methylation rates in the SR sediments (1.1−10.2% day^{-1}) were significantly higher than those reported for Adirondack (NY, U.S.A.) wetland soils and sediments (0.1−1.2% day^{-1})[10] and Idrija River (Slovenia) sediments (0.5−1.5% day^{-1}),[27] but similar to those in Valdeazogues River (Almadén, Spain; 0.38−13.0% day^{-1}).[28]

Reductive degradation of $^{14}CH_3Hg$, that is, $^{14}CH_4$ production, dominated in May 2008 samples, with the highest rate (3.92% day^{-1}) occurring in site RRD4.8 ($p < 0.01$). In contrast, oxidative demethylation, production of $^{14}CO_2$, dominated in August 2008 samples (Figure 1C and D). The production of $^{14}CO_2$ in August samples was significantly higher than in May samples (two-way ANOVA; $p < 0.0001$), and showed a roughly increasing trend with distance from the Hg source, reaching the highest rate of CO_2 production (3.50% day^{-1}) in site RRD20.6. The potential demethylation rates in this study were generally lower than those reported in the Idrija River (6−8% day^{-1})[27] and Valdeazogues River sediments (0.04−17% day^{-1}).[28] Reductive demethylation may lead to the conversion of CH_3Hg to elemental Hg(0) and its transport into the gaseous phase, while oxidative demethylation with the likely production of Hg(II) may result in an infinite cycle of methylation and demethylation.[4]

Our results suggest that potential rates of Hg methylation in the SR were at the higher range of those reported from other riverine systems while potential rates of demethylation were at the lower range reported by others. Consequently, the potential for net CH_3Hg production, as indicated by the M/D ratios

(Supporting Information Figure S2), was high particularly so for the samples collected in August even though these samples had lower CH_3Hg concentrations than those collected in May. Korthals and Winfrey[29] also observed that the methylation potentials in surficial lake sediments increased from spring to late summer and decreased in the fall. FGS deposits (RRD10.0 and 20.6) had the highest rates of potential methylation and demethylation (Figure 1). These observations suggest that FGS deposits originating from eroded river banks are potential hot spots for both methylation and demethylation activities within the SR ecosystem.

Correlation Analyses of Sediment CH_3Hg, Potential Methylation and Demethylation Rates. Sediment CH_3Hg concentrations were significantly ($p < 0.003$) positively correlated with both porewater sulfate ($r^2 = 0.72$; Figure 2)

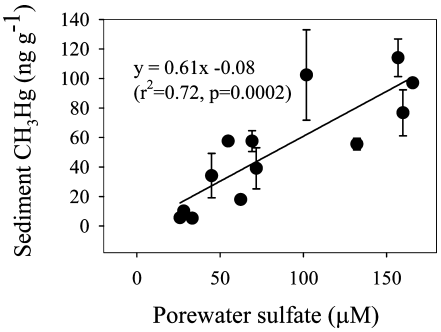


Figure 2. Regression analysis of porewater sulfate versus sediment CH_3Hg concentrations ($n = 13$) in the South River samples.

and sediment THg concentrations ($r^2 = 0.64$; Supporting Information Figure S3a). However, neither AVS nor Fe(II)/Fe(III) ratios were correlated with sediment CH_3Hg. The strongly positive correlation of sediment CH_3Hg with porewater sulfate suggested that activities of SRB contributed to Hg methylation in the SR sediments. The relationships of potential methylation rates with porewater sulfate, percent THg as CH_3Hg, AVS, and Fe(II)/Fe(III) ratios in sediments were insignificant ($p > 0.05$). The insignificant relationship between methylation rates and percent THg as CH_3Hg in sediments as compared with the positive correlation from previous studies[7,30] was likely because the SR sediment habitats were relatively open systems with higher CH_3Hg flux rates from sediment matrices into bottom water. Potential demethylation rates showed a significantly positive correlation with sediment CH_3Hg ($r^2 = 0.75$; Figure S3b). No strong relationships of potential demethylation rates with sediment THg, sediment Fe(II)/Fe(III) ratios, or porewater sulfate concentrations were observed.

Effects of Metabolic Stimulators and Inhibitors on CH_3Hg Production. In samples (May 2010) from the three most active methylation sites RRD10.0, RRD14, and RRD20.6, amendment of 0.4 or 1.0 mM sulfate significantly increased potential methylation rates by factors ranging from 1.6 to 2.6 ($p < 0.002$; Figure 3A). Only in RRD14 sediment, increasing sulfate concentrations from 0.4 to 1.0 mM significantly increased the potential methylation rate. Thus, in sediments of RRD10.0 and RRD20.6, addition of 0.4 mM sulfate resulted in conditions where Hg methylation was not limited by sulfate. Molybdate added at 0.4 and 1.0 mM significantly ($p < 0.003$) inhibited methylation rates by 27.8% and 27.2% for RRD10.0, and by 26.4 and 24.6% for RRD14, respectively (Figure 3B).

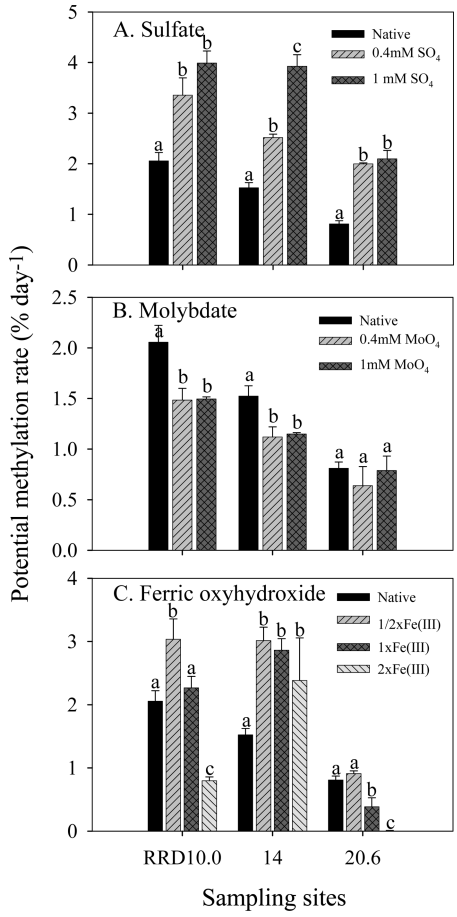


Figure 3. Effects of sulfate, molybdate, and amorphous ferric oxyhydroxide amendments on potential methylation rates. Fe(III) as $Fe(OH)_3$ was added at 1/2, 1, and 2 fold of measured microbially reducible Fe(III) concentrations in sediments. The final respective amendments of $Fe(OH)_3$ to slurries were 0.80, 1.60, and 3.21 mg g^{-1}dwt sediment for RRD10.0, 2.54, 5.06, and 10.11 mg g^{-1}dwt for RRD14.0, and 1.44, 2.87, and 5.74 mg g^{-1}dwt for RRD20.6. Different letters above bars indicate significant differences within each site (ANOVA, $p < 0.05$).

For site RRD20.6, however, molybdate additions had no significant effects on potential methylation rates. There was also no significant difference for molybdate addition between 0.4 mM and 1.0 mM in all sediment samples tested. The results indicated that at most, molybdate addition inhibited only 28% of the potential methylation rates and that further addition of molybdate did not result in a more efficient inhibition of methylation.

Amorphous ferric oxyhydroxide was added, calculated as Fe(III), at levels which were 1/2, 1, and 2 fold of ambient microbially reducible Fe(III). For site RRD10.0, addition of ferric oxyhydroxide at 1/2 and 1 fold obviously increased potential methylation rates by 47.6% ($p = 0.002$) and 10.3%, respectively, while the 2 fold amendment significantly decreased potential rates by 61.2% (Figure 3C). For site RRD14.0, amendment of Fe(III) at 1/2, 1, and 2 fold of background levels very significantly ($p < 0.01$) increased methylation rates by 97.9%, 87.9%, and 56.5%, respectively. In site RRD20.6, amendment at 1/2 fold as Fe(III) did not significantly increase methylation rates. In contrast, at spiked levels of 1 and 2 fold Fe(III), potential methylation rates in this site were significantly reduced by 52.4% and 100%.

dx.doi.org/10.1021/es2033718 | Environ. Sci. Technol. 2012, 46, 2684−2691

Molecular Characterization of Sediment Communities. A total of 386 clones of 16S rRNA gene transcripts, 96 to 98 clones representing sediment samples from RRD10.0 (May and August 2008), RRD14.0 (May 2008), and RRD20.6 (August 2008), respectively, were generated. These samples exhibited high methylation potentials (Figure 1A and B) or high M/D ratio (Supporting Information Figure S2). By creating clone libraries from RNA, rather than DNA, the identified microbial taxa represent the active members of the communities, rather than those that may have been present but not metabolically active at the time of sampling. Sequences similar to all classes of the *Proteobacteria* (alpha-, beta-, gamma-, and delta-) accounted for 52−60% of the active communities in the four sediments (Figure S4).

Clones representing the *Deltaproteobacteria,* the class to which most Hg-methylating sulfate and iron reducing bacteria belong,[4,17] were present in all four libraries representing 3.1%, 9.4%, 10.4%, and 11.2% of the clones from RRD10.0 (May), RRD10.0 (August), RRD14.0 (May) and RRD20.6 (August), respectively. Such clones were most abundant in site RRD20.6 (Supporting Information Figure S4), where the highest potential methylation rate was observed in August 2008 (Figure 1B). A higher representation of *Deltaproteobacteria* was obtained from site RRD10.0 in the August as compared to the May 2008 libraries, corresponding to the 2.9 fold higher potential methylation rates in the August sample (Figure 1A and B). Thus, the abundance of active bacteria affiliated with taxa known to methylate Hg was related to the potential methylation rates of sediment incubations, suggesting that active methylators were a dominant component of the microbial communities in the SR sediments. This relationship was further explored by correlating potential methylation rates with the abundance of SRB-, IRB-, and *Deltaproteobacteria*-like clones in each of the clone libraries (Figure 4). A significant

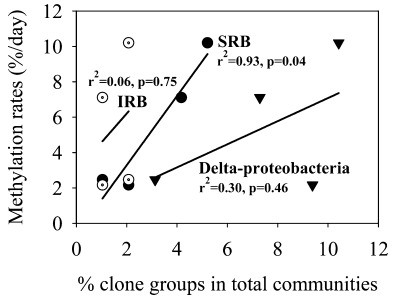


Figure 4. Relationship between potential methylation rates and the % of clones that were most similar to the 16S rRNA genes of SRB (●), IRB (⊙), and *Deltaproteobacteria* (▼) in clone libraries representing the active sediment communities from RRD10.0, RRD14.0, and RRD20.6.

correlation (r^2 = 0.93, p = 0.04) was only observed between SRB-like sequences and potential methylation rates, supporting a role of SRB in Hg methylation.

The phylogenetic analysis of cloned 16S rRNA genes with affiliation to *Deltaproteobacteria* showed that at least three groups of SRB and one group of *Geobacter*-like microbes, which were tightly clustered with known Hg methylators, were active in SR sediments (Figure 5). First, at the top of the phylogenetic tree, two clones from RRD14.0 (May) were grouped with *Desulfococcus multivorans*, a strong Hg methylator.[31] Four other clones were weakly clustered with the group of *D. multivorans* and the group of the known methylators *Desulfobacter* sp. BG8 and *Desulfobacterium* sp. BG33.[32] The second SRB group including RRD10.0Aug-25 and RRD20.6Aug-82 showed 91−96% similarity to uncultured *Desulfobulbaceae* bactereium (Supporting Information Table S3), and clustered at 100% bootstrap values with *Desulfobulbus propionicus* 1pr3, another strong Hg methylator.[31] The third SRB group of clones at the bottom of the tree was from RRD10.0 and 20.6, loosely affiliated with the methylating strains *Desulfovibrio africanus* DSM 2603[31] and *Desulfovibrio desulfuricans* ND132.[33] In the group of *Geobacter*-like bacteria, two clones (RRD10.0May-56 and RRD20.6Aug-24) were closely clustered with the methylator *Geobacter* sp. CLFeRB, a freshwater lake sediment isolate.[16] Two additional clones (RRD10.0May-28 and RRD14.0May-76) were also tightly grouped with the *Geobacter* cluster which contained two other strong methylators, *Geobacter metallireducens* and *Geobacter sulfurreducens* PCA.[17] All four clones in this cluster were 93−96% similar to uncultured *Geobacter* spp. or *Geobacter psychrophilus* strain P35 (Supporting Information Table S3).

Linking Hg Methylation Potentials to Putative Methylators. Low concentrations of AVS and high Fe(II)/Fe(III) ratios (Supporting Information Table S2) suggested that iron reduction could be the dominant terminal oxidation process in most SR sediment samples. This suggestion implies that iron reducers were probably the principle Hg methylators in the sediments. However, results of 16S rRNA cloning and sequencing partially contradicted this suggestion by showing that both iron and sulfate reducers were active in the sediments.

Sequencing results and phylogenic analysis showed that clones with high similarity to 16S rRNA genes of methylating SRB were distributed across three sediment sites (RRD10.0, 14.0 and 20.6; Supporting Information Table S3 and Figure 5) where high potential methylation rates were observed (Figure 1), suggesting that SRB were active methylators of Hg in these locations. This conclusion is consistent with the significantly positive correlations between (i) sediment CH_3Hg and porewater sulfate concentrations (Figure 2), and (ii) potential methylation rates and percentage of SRB-like clones in all 16S rRNA clones representing the sediment communities (Figure 4). However, sequences most similar to *Geobacter*-like strains, known to methylate Hg,[16,17] were also present in all clone libraries (Supporting Information Table S3 and Figure 5). Taken together, these results suggest that both iron and sulfate reducers were potential methylators in the SR sediments. However, determination of the dominant Hg methylators based only on phylogenetic analysis should be exercised with caution since the microbial ability to methylate Hg is known to be strain rather than taxon specific.[34]

Previous research showed that molybdate up to 20 mM did not inhibit reduction of iron,[35] and methylation by IRB is probably "molybdate-independent".[16] Thus, metabolism of SRB and IRB may be differentiated by molybdate and sulfate amendments. Because specific inhibitors of iron reduction are not available,[36,37] it is difficult to directly determine the contribution of IRB to Hg methylation in sediments. However, previous studies showed that additions of $Fe(OH)_3$ to freshwater river sediments could inhibit sulfate reduction by 86−100%[38] and reduce methane production by 50−90%.[39,38] Cummings et al.[40] recently indicated that amendment of ferrihydrite without molybdate to tidal sediments greatly shifted the community structure to a dominance of IRB (*Shewanella* spp.), while SRB and sulfide-oxidizing groups were simulta-

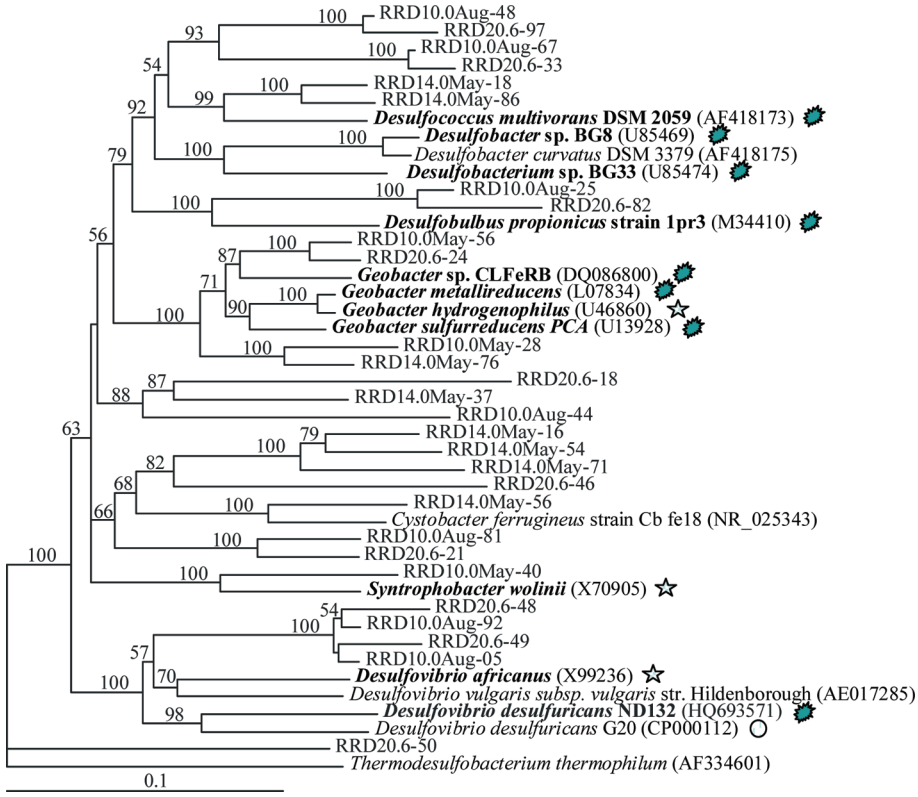


Figure 5. Phylogenetic analysis of 16S rRNA genes retrieved from South River sediments which were closely related to those of *Deltaproteobacteria* and of reference species representing strains known as strong Hg methylators (teal leaf shape), weak methylators (star), or nonmethylators (open circle). Clone designations identify the sampling site (the initial three letters followed by three numbers), sampling time (May or Aug.), and clone number (last two digits).

neously replaced by taxa known to cycle elemental sulfur. Sediments amended with both ferrihydrite and molybdate were also populated by IRB clones. Therefore, addition of $Fe(OH)_3$ should stimulate iron reduction and may also inhibit sulfate reduction and methanogenesis by directing available reducing equivalents toward iron reduction. Sulfate addition significantly increased potential methylation rates by 1.6 to 2.6 fold, and molybdate addition significantly inhibited methylation by 24.6−27.8%, clearly implicating SRB as active Hg methylators. However, more than 72% of the potential methylation rates could not be further inhibited by molybdate, indicating that SRB only partially contributed to CH_3Hg production and that other methylating microbial guilds, for example, IRB or possibly methanogens,[41] might have contributed to this process.

Potential methylation rates were significantly stimulated by the addition of $Fe(OH)_3$ at 1/2 fold of in situ microbially reducible Fe(III) levels in site RRD10.0 and at all three amendment levels in site RRD14.0, suggesting an active role of IRB in methylation. Addition at 2 fold of Fe(III) to RRD10.0 and 1 and 2 fold to RRD20.6 inhibited methylation possibly due to the inhibition of sulfate reduction by added Fe(III) as a more favored electron acceptor and/or by a reduced bioavailability of Hg(II) due to scavenging by added $Fe(OH)_3$.[42,43] To date the few studies that examined the effects by stimulating iron reduction on Hg methylation in environmental incubations reported contradicting results.[30] Warner et al.[44] reported that methylation was inhibited in riverine sediments that were preoxidized to produce Fe(III), while Jackson[42] reported that the addition of iron oxide promoted Hg methylation in lake sediments. Gilmour et al.[45] indicated that spiking Everglades sediments with soluble iron citrate did not result in any significant effects on Hg methylation. Overall, our results indicated that low levels of amorphous $Fe(OH)_3$ addition significantly stimulated microbial Hg methylation in two of the three SR sediment sites tested. However, the insignificant effects in RRD20.6 at 1/2 fold and inhibition by amendments at higher levels, suggest that the $Fe(OH)_3$ influence on Hg methylation rates was site-specific, likely due to the variations of ambient reducible Fe(III) levels and composition of sediment microbial communities.

Methylation by SRB is likely restricted to the upper layers of sediment due to their dependence on soluble electron acceptors and Hg(II). The capacity of IRB to utilize solid phase Fe(III) and adsorbed Hg(II) has been hypothesized to extend the depth of the Hg methylation zone in sediments.[16] This difference in niches where methylation takes place would enable methylation by both SRB and IRB in the same sediment samples. Additionally, in an environment where electron donors are not limiting it is possible that these processes could coexist spatially. Our results support the prior hypothesis by showing stimulation of methylation when sediment slurries were amended to enhance either sulfate or iron reduction and by the co-occurrence of active representatives of both guilds in the community. We therefore suggest that both IRB and SRB contribute to CH_3Hg production in the SR sediments probably by both temporally and spatially dependent processes. This does not rule out the possibility that other types of bacteria may also be responsible for Hg methylation in this environment. Our results indicate that Hg methylation and CH_3Hg demethylation occur throughout the SR ecosystem. However,

dx.doi.org/10.1021/es2033718 | Environ. Sci. Technol. 2012, 46, 2684−2691

the specific microbiology of methylation and demethylation appears to vary with site within this riverine environment.

■ ASSOCIATED CONTENT

Supporting Information

Methods, tables of sediment characteristics and clone list, and figures of sampling map, M/D ratio, bacterial community composition, and others. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Mailing address: Department of Biochemistry and Microbiology, Rutgers University, 76 Lipman Dr., New Brunswick, NJ 08901. Phone: (732) 932 9763 ×334. Fax: (732) 932 8965. E-mail: rqyu@rci.rutgers.edu.

■ ACKNOWLEDGMENTS

We thank Mark E. Hines for suggestions in establishing the demethylation setup, and Chu-Ching Lin for advice in measuring iron by the ferrozine assay. This project was supported by the DuPont Company and the South River Science Team and by the Office of Science (BER), U.S. Department of Energy Grant DE-FG02-08ER64544.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper published ASAP February 9, 2012 with an error in the author affiliations, and abstract. The correct version published February 13, 2012.