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Characterization of the *Deltaproteobacteria* in contaminated and uncontaminated stream sediments and identification of potential mercury methylators

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ABSTRACT: Microbial communities were examined in surface stream sediments at 5 contaminated sites and 1 control site near Oak Ridge, TN, USA, to identify bacteria that could be contributing to mercury (Hg) methylation. The phylogenetic composition of the sediment bacterial community was examined over 3 quarterly sampling periods (36 samples) using 16S rRNA gene pyrosequencing. Only 3064 sequences (0.85% of the total community) were identified as *Deltaproteobacteria*, the only group known to methylate Hg, using the Ribosomal Database Project classifier at the 99% confidence threshold. Constrained ordination techniques indicated statistically significant positive linear correlations between *Desulfobulbus* spp., *Desulfonema* spp. and *Desulfobacca* spp. and methyl-Hg concentrations at the Hg-contaminated sites. In contrast, the distribution of organisms related to *Byssovorax* spp. was significantly correlated to inorganic carbon, nitrate and uranium concentrations but not to Hg or methyl-Hg. Overall, the abundance and richness of *Deltaproteobacteria* sequences were higher in uncontaminated sediments, while the majority of the members present at the contaminated sites were either known potential metal-reducers/methylators or metal tolerant species. Given the abundance relative to other known Hg methylators and the association with methyl-Hg, *Desulfobulbus* spp. is considered a prime candidate for involvement in Hg methylation in these streams.

KEY WORDS: *Deltaproteobacteria* · Mercury · Methylmercury · Stream sediments

INTRODUCTION

Mercury (Hg) contamination is a concern at several U.S. Department of Energy sites, including a stream that flows through the city of Oak Ridge, TN, where high levels of both inorganic and methyl Hg have been found (Barnett et al. 1995, Han et al. 2006, Oregaard & Sorensen 2007). Hg methylation is an important biogeochemical process that generates the potent human neurotoxin monomethylmercury (MeHg) (Clarkson 1998). Net MeHg production in aquatic ecosystems is linked to environmental and geochemical parameters along with electron donor and acceptor availability (Marvin-DiPasquale et al. 2009). Previously, wetlands and floodplain lakes have been associated with MeHg production in aquatic ecosystems, but recent work in stream sediments has found significant *in situ* MeHg production that warrants further examination of streambed Hg methylation and the associated microbial communi-

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ties (Tsui et al. 2010). Most of the previous work in streams has been performed in anoxic, subsurface sediments and porewater, but MeHg can be produced in surface sediments within the anaerobic microniches in sedimentary biofilms (Bloom et al. 1999). A study of stream water and surface sediments in an Hg mining impacted watershed showed significant downstream transport of Hg from the point source and subsequent *in situ* methylation (Gray et al. 2004).

Hg methylation has been linked primarily to *Deltaproteobacteria*, including sulfate-reducing bacteria (Gilmour et al. 1992, Devereux et al. 1996, King et al. 2000, Ranchou-Peyruse et al. 2009) and more recently iron-reducing bacteria (Fleming et al. 2006, Kerin et al. 2006). Whether or not microorganisms outside the *Deltaproteobacteria* are able to methylate Hg remains an open question. A study in streambed sediments and surrounding watershed soils found *Desulfovibrio* spp. and *Desulfobacter* spp. associated with Hg methylation (Holloway et al. 2009). While these bacteria are primarily anaerobic, there are sulfate-reducing members of the class *Deltaproteobacteria* that will survive and grow under oxic conditions (Krekeler et al. 1997, Sigalevich & Cohen 2000, Sigalevich et al. 2000a). Although the aerobic metabolism of these organisms involves oxidation of sulfide and sulfite, when conditions become void of oxygen, anaerobic respiration commences, including sulfate-reduction (Sigalevich et al. 2000b), which may facilitate Hg methylation in surface sediments (Achá et al. 2005).

Close examination of microbial communities in environmental samples is difficult due to a lack of resolution (e.g. fingerprinting techniques) or low detection limits (FISH) (Ravenschlag et al. 2000, Wang & He 2011). The present study utilized high-throughput sequencing targeted to the 16S rRNA gene because it is a powerful tool for microbial ecology. Recent advances, such as the bacterial tag encoded FLX pyrosequencing method, have enabled this technique to be applied to the analysis of numerous diverse environmental samples. The 'bar-tagging' approach uses sample specific sequence tags incorporated in the amplification primers (Dowd et al. 2008, Youssef et al. 2009).

Recent studies have investigated the overall bacterial and archaeal community structures in streams of East Tennessee contaminated with mercury and other heavy metals (Porat et al. 2010, Vishnivetskaya et al. 2011). The goal of the present study was to further these efforts and concentrate on the known guild of methylating bacteria by characterizing the distribution of *Deltaproteobacteria* in the anthropogenically contaminated surface sediments of streams within the Oak Ridge Reservation. More specifically, these efforts were focused on (1) establishing relationships between the distribution of deltaproteobacterial members and chemical variables measured in the streams and (2) determining if the *Deltaproteobacteria* present could be identified as close relatives of known Hg methylators and thus as candidates for methylating mercury in these streams.

MATERIALS AND METHODS

Stream sediment samples were obtained from 6 sampling sites of 4 streams located in or near the Department of Energy reservation in Oak Ridge, TN. Locations included 5 contaminated sites: 3 sites in East Fork Poplar Creek (EFK [K = river kilometer measured from the mouth of the creek] 6.3, EFK 13.8, and EFK 23.4), 1 site in Bear Creek (BCK 12.3), and 1 site in White Oak Creek (WCK 3.9). Hinds Creek (HCK 20.6) was used as an uncontaminated reference site with similar general chemistry, hydrology and underlying geology to the contaminated sites (Fig. 1).

Samples were collected in May, July and September of 2008. Two samples were collected at each site, one from the middle of the stream and a second adjacent to the stream bank (herein referred to as midchannel and bank samples), by skimming the upper 2 to 3 cm of sediment with a sterile wide-mouthed high-density polyethylene (HDPE) jar and immediately transferring the material to a sterile HDPE 1 l bottle. Water samples were collected at the same locations as the sediment samples for laboratory analysis of dissolved metals and anions, dissolved inorganic and organic carbon (DIC and DOC), soluble reactive phosphorous, dissolved Hg, total Hg in sediment and dissolved MeHg. For detailed methods, see Vishnivetskaya et al. (2011).

All sediment and water samples were placed immediately on ice and returned to the laboratory within 2 h. Immediately upon arrival at the laboratory, sediment samples were centrifuged (3700 × *g*, 4°C, 30 min; Sorvall Legend RT Benchtop Centrifuge, Thermo Fisher Scientific). Gravel and pebbles were removed, while fine sediments (<2 mm grain size) were frozen (–80°C) until analysis and DNA extraction. A total of 36 surface sediment samples were collected and analyzed.

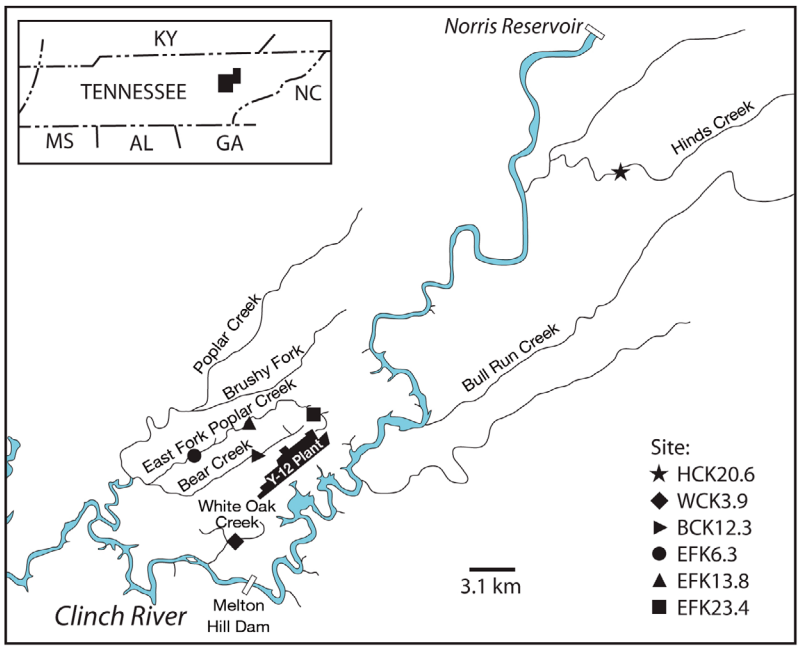

Fig. 1. Sampling sites used in the present study. The background site (HCK20.6) is located ~23 km linear distance upstream from site EFK23.4. The industrial facility (Y-12 Plant) is indicated schematically

Environmental DNA extraction and pyrosequencing of the bacterial 16S rRNA genes

The total community genomic DNA (cgDNA) was extracted from ~1 g (wet weight) of sediment using a PowerSoil™ DNA Isolation kit (MO BIO Laboratories) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Pyrosequencing of the cgDNA isolated from 36 samples was conducted using the method described at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://pyro.cme.msu.edu/index.jsp). Briefly, the hypervariable V4 region (~290 bp) of the 16S rRNA gene was amplified using a primer set identical to that described in the RDP Pyrosequencing Pipeline; primers contained sequences (adaptors) required for GS 454 FLX pyrosequencing, and the forward primer contained an additional short key (tag) sequence, so that 40 samples could be analyzed in 1 sequencing run. PCR reactions (50 µl) consisted of 1.5 µl of each forward and reverse primer (10 µM each), 1 µl template DNA (10 to 80 ng μl^{-1}), and 0.6 µl (2.5 U μl^{-1}) high fidelity AccuPrime™ *Pfx* DNA polymerase (Invitrogen). Samples were initially denaturated at 95°C for 2 min, then amplified using 30 cycles of 95°C for 15 s, 55°C for 30 s and 68°C for 45 s, with a final extension of 3 min at 68°C. Template controls were always included. The PCR amplicons were purified using the Agencourt AMPure solid-phase paramagnetic bead technology (Agencourt Bioscience). The purity, concentration and size of the PCR amplicons were estimated using DNA 1000 chips and an Agilent 2100 Bioanalyzer (Agilent Technologies). Sequencing reactions were performed on a GS 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics). Raw 454 FLX data (~235 670 Mb) were initially processed through the RDP pyrosequencing pipeline (Cole et al. 2009). During this process, sequences were sorted by tag sequence, and the 16S primers and low-quality sequences were removed. From 5580 to 16 706 high quality sequences of 200 to 220 bp were obtained for each sample.

Phylogenetic analyses

Bacterial 16S rRNA sequences were assigned to a set of hierarchical taxa using a naïve Bayesian rRNA classifier version 2.0 with a confidence threshold of 99% (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang et al. 2007). Sequences from the present study were subsequently aligned using the fast, secondary-structure aware Infernal aligner (Nawrocki & Eddy 2007) and clustered by the complete-linkage clustering method available through the RDP Pyrosequencing Pipeline.

Statistical analyses

Constrained ordination techniques were used to identify patterns of variation of the *Deltaproteobacteria* sequences among streams and correlations between the distribution of the sequences and environmental descriptors. Sequence abundances for each genera were converted into weight percentage values by dividing by the total abundance for that sample; weight percentage values were natural log transformed (ln + 1). Detrended correspondence analysis (DCA), an indirect gradient analysis based on segment length, was performed to determine the modality of the sequence data. The analyses resulted in short (<2.0) segment lengths, indicating that the datasets were linear; thus, redundancy analysis (RDA) was performed (CANOCO 4.5, Microcomputer Power). RDA identified patterns of variation and cor-

related those patterns to all environmental descriptors in the 36 samples (see Tables S1 to S3 in the supplement at www.int-res.com/articles/suppl/a066p271_supp.pdf), when complete geochemical measurements were available. Sequence data were used as the response variables, and the predictor variables used were the measured environmental and geochemical parameters. All physical parameters were entered into the analysis, but forward selection of the predictor variables followed by Monte Carlo permutation tests was used to prevent artificial inflation of variation due to autocorrelation in the constrained ordination model (Leps & Smilauer 2003).

Cluster analysis

Phylogenetic analysis was also utilized to compare the sequences detected in the samples from the present study to known methylators found in previous studies to identify potential Hg methylators in the sediments. All *Deltaproteobacteria* sequences were clustered at 97% through the RDP pyrosequencing pipeline, and representative sequences from the clusters containing >10 sequences were selected and aligned with sequences of 33 known *Deltaproteobacteria* including methylators and non-methylators (King et al. 2000, Jay et al. 2002, Ekstrom et al. 2003, Fleming et al. 2006, Kerin et al. 2006, Ekstrom & Morel 2008, Schaefer & Morel 2009). A phylogenetic tree using the neighbor-joining method (Saitou & Nei 1987) was constructed with the combined dataset to examine relationships between the 13 representative environmental sequences from the present study to 33 culturable strains of *Deltaproteobacteria* using MEGA4 software (Tamura et al. 2007). Evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and bootstrap values based on 100 replicates (Felsenstein 1985).

Nucleotide sequence accession number

The 16S rRNA gene pyrosequences identified as *Deltaproteobacteria* at the 99% confidence level from the RDP database were deposited in GenBank under accession numbers GQ458262 to GQ461334.

RESULTS

Stream geochemistry characteristics

The ranges of mercury concentrations and selected chemical and physical parameters for the 6 sampling sites are summarized in Table 1. The complete dataset for dissolved metals and anions, DIC, soluble reactive phosphorous, dissolved Hg and MeHg and total sediment Hg was recently published (Vishnivetskaya et al. 2011) and is presented herein (see Table S1A–C in the supplement).

Microbial community description

The *Deltaproteobacteria* made up a small portion of the 359 307 high quality sequences of 200 to 250 bp that remained after processing the 36 samples (Table 2). The sequence data were taxonomically assigned using the RDP Classifier (Wang et al. 2007) at the 99% confidence level. The 99% confidence level was chosen to allow some room for sequence errors but to yield high assurance that the sequences used in the analysis were truly associated with *Deltaproteobacteria*. The *Deltaproteobacteria* constituted 0.9 to 2.7% of the total bacterial sequences in each sample (Table 2). The site with the highest concentrations of nitrate, uranium and other metals (BCK 12.3) was equivalent to other sites in the number of *Deltaproteobacteria* sequences as other stations over-

Table 1. Mean stream water and sedimentary concentrations of selected ions from the 6 study sites. A full list of geochemical parameters is located in the supplement at www.int-res.com/articles/suppl/a066p271_supp.pdf. Hg sediment samples were extracted from sediment samples; the rest of the ions were from stream water samples. Data are the averages of duplicate samples taken over 3 sampling dates (May, July and September). Numbers in parentheses are the standard error. RK: river kilometer

	Hg sediment (ng mg^{-1})	Hg dissolved (ng l^{-1})	MeHg (ng l^{-1})	Uranium (µg l^{-1})	Nitrate (mg l^{-1})	Manganese (µg l^{-1})
HCK 20.6	0.06 (0.02)	1.50 (0.31)	0.03 (0.03)	2.49 (1.55)	0.09 (0.03)	43.30 (35.30)
EFK 6.3	13.50 (2.59)	22.30 (7.28)	1.21 (0.53)	6.90 (1.64)	0.64 (0.08)	1580.00 (1070.00)
EFK 13.8	14.90 (1.29)	42.00 (17.20)	0.30 (0.03)	6.70 (0.33)	0.18 (0.06)	62.30 (4.78)
EFK 23.4	39.30 (5.04)	81.10 (16.50)	0.52 (0.12)	7.36 (0.22)	0.27 (0.05)	30.40 (3.74)
BCK 12.3	1.61 (0.06)	4.87 (0.32)	0.05 (0.03)	224.00 (28.90)	15.40 (1.05)	8.75 (4.78)
WCK 3.9	8.37 (3.52)	5.25 (3.52)	0.34 (0.25)	0.37 (0.06)	0.15 (0.09)	20.90 (5.92)

all (Table 2) but was virtually devoid of sequences corresponding to sulfate-reducing bacteria, with up to 4 sequences found at some sampling times and none at others.

The *Deltaproteobacteria* community was represented by sequences from 25 genera within 14 families and 7 orders (Fig. 2). The lowest genetic richness was found at the site with the highest concentration of nitrate and uranium (BCK 12.3), with only 11 of the 25 detected genera present; EFK 13.8 had the highest richness, with 21 genera present. Whether nitrate and uranium were driving factors in the community richness is unknown because several other factors may also contribute, such as higher Ba and Sr or lower Al or Mn levels at BCK 12.3. The reference stream, HCK 20.6, had 19 genera in the samples, and EFK 6.3, EFK 23.4 and WCK 3.9 each contained 17 genera.

The BCK 12.3 samples were the most distinct in *Deltaproteobacteria* community composition. The majority of the sequences in all samples grouped as unclassified *Deltaproteobacteria* (16.9–49.8%), with BCK 12.3 having the majority of unclassified *Deltaproteobacteria* sequences (33.3–49.8%). Five genera were found in all samples: *Geobacter* (0.8–16.03%), *Byssovorax* (0.97–11.1%), *Bdellovibrio* (0.62–7.2%), *Anaeromyxobacter* (0.8–5.7%) and *Bacteriovorax* (0.3–6.2%). Two of the aforementioned genera, *Byssovorax* (8.8–11.12%) and *Bdellovibrio* (4.0–4.4%), were found in highest abundance in the samples taken from BCK 12.3. *Geobacter* spp. were found in highest abundance in the uncontaminated site HCK 20.6 (5.6–16.03%), while *Anaeromyxobacter* appeared to be evenly distributed across sites and sampling dates. *Desulfobulbus* (2.6–26.7%) and *Desulfonema* (1.5–9.6%) were found in all samples, with the exception of BCK 12.3, where no sequences were detected on any of the sampling dates. Of the 5 sampling sites where these 2 genera were present, there were no significant differences in the number of sequences detected amongst the samples for *Desulfobulbus* spp. ($p = 0.475$, $F = 0.905$). Conversely, *Desulfonema* spp. sequences were found in significantly lower abundances at EFK 23.4 than at the other sites where it was found ($p = 0.043$, $F = 2.87$).

Table 2. Distribution and mean number of sequences extracted from stream sediments from 6 sites over 3 sampling dates. Standard deviation (in parentheses) represents the variation between the midchannel and near bank sampling sites

	Bacterial sequences	*Deltaproteobacteria* sequences	%
HCK 20.6			
May	7189 (1294.0)	71 (14.1)	1.0 (0.4)
Jul	9937 (3607.7)	171 (4.2)	1.8 (0.6)
Sep	15292 (1974.2)	226.5 (17.7)	1.5 (0.1)
EFK 6.3			
May	12985.5 (157.7)	129.5 (20.5)	1.0 (0.2)
Jul	9415.5 (3310)	159 (14.1)	1.8 (0.5)
Sep	7722.5 (2305.9)	130 (39.6)	1.7 (0.0)
EFK 13.8			
May	10012.5 (3479.7)	136 (43.8)	1.4 (0.0)
Jul	7817 (2076.8)	196.5 (33.2)	2.7 (1.1)
Sep	13280.5 (40.3)	278 (8.5)	2.1 (.1)
EFK 23.4			
May	9874 (1506.1)	114 (17.0)	1.2 (0.3)
Jul	7170 (2274.1)	70 (28.3)	1.0 (0.1)
Sep	11464 (2180.7)	119.5 (57.3)	1.0 (0.3)
BCK 12.3			
May	9680.5 (1638.4)	105 (25.5)	1.1 (0.4)
Jul	9992.5 (1782.6)	94 (38.2)	0.9 (0.2)
Sep	8389.5 (1448.9)	87 (32.5)	1.0 (0.2)
WCK 3.9			
May	10772.5 (2192.7)	133 (17.0)	1.2 (0.1)
Jul	8994.5 (2445.9)	95 (35.4)	1.0 (0.1)
Sep	9355 (77.8)	137.5 (10.6)	1.5 (0.1)

Microbial community composition in relation to geochemical parameters

The microbial community composition was analyzed using all 36 samples (from the 3 sampling dates) (see Fig. S1 in the supplement at www.int-res.com/articles/suppl/a066p271_supp.pdf), and because MeHg data were only available from the May and July 2008 sampling dates, an RDA analysis was performed including only the 24-sample subset to determine if MeHg concentrations in the sediments were associated with microbial community composition (Fig. 3A). The RDA triplot of the subset of samples that included MeHg data described 40.7% of the variation ($p = 0.002$, $F = 6.39$). The majority of the variation (36.2%) was described by RDA Axis 1, and the BCK 12.3 samples grouped away from the other samples with good replication for each sampling date. Because many of the geochemical parameters measured in the present study were autocorrelated, isolation of the individual influences from each geochemical parameter was not always possible. Forward selection of the independent variables followed by Monte Carlo permutations during the RDA selected the most significant ($p < 0.05$) environmental variable. The BCK 12.3 samples had higher abundances of *Byssovorax* and unclassified members of *Deltaproteobacteria* and significant correlation with

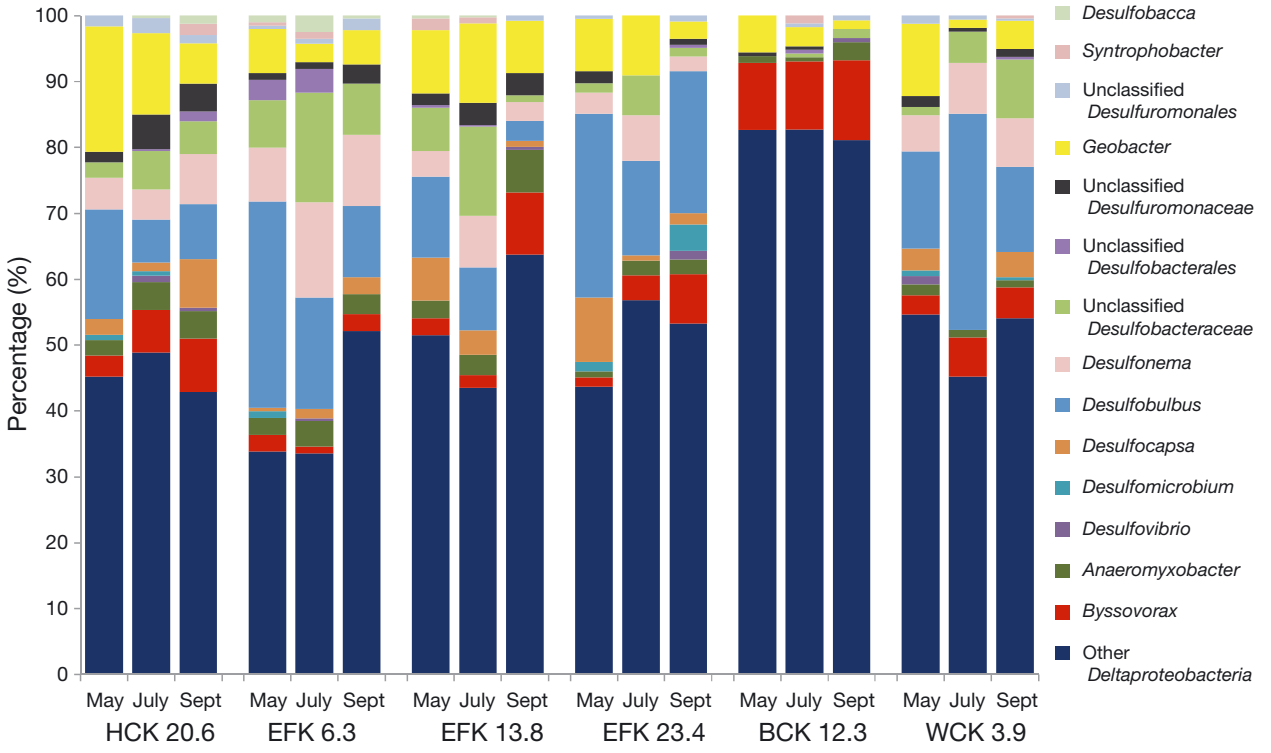

Fig. 2. Percent contribution of identified members of *Deltaproteobacteria* extracted from stream sediments from 6 sites over 3 sampling dates

uranium concentrations (R = 0.7671) as well as the autocorrelated variables: NO_3^-, Ca, Ba, Cl^-, Mg, Sr, DIC and conductivity values (r > 0.75). In this instance, uranium was the most significant of the correlated variables. Three samples, EFK 6.3 (bank and mid-channel) and the WCK 3.9 bank sample, taken in July were associated with the increased MeHg concentrations in the streams. These samples had higher relative abundances of *Desulfobacca* and *Desulfonema*. The remaining samples in the analysis of the 24-sample subset had RDA scores near zero, and there was not enough evidence to draw conclusions on the relationships between the samples and geochemical parameters.

Because a large proportion of the variation in the microbial communities in the stream sediments could be attributed to the BCK 12.3 samples that contained U(VI), an additional RDA was performed excluding the BCK 12.3 samples. The RDA Axes 1 and 2 of the 20-sample subset analysis described 24.8% of the variation of the microbial composition between the samples (p = 0.04, *F* = 2.08) (Fig. 3B). Samples taken from EFK 6.3 in July 2008 were most highly correlated to MeHg concentrations (R = 0.7785). These samples had high abundances of *Desulfobulbus*, *Desulfonema* and unclassified *Desulfobacterales*. The May EFK 6.3, the May EFK 23.4 bank sample and both WCK 3.9 samples all had positive RDA 1 scores but could not be significantly correlated to any geochemical factor or group of *Deltaproteobacteria*. July samples from EFK 13.8 and HCK 20.6 were significantly correlated with stream water turbidity (R = 0.7047) and had higher abundances of *Desulfuromonas*. Samples from WCK 3.9 taken in May were characterized by higher abundances of unclassified *Deltaproteobacteria* and tended to have higher concentrations of dissolved oxygen (R = 0.5128).

Further examination of the Hg and MeHg concentrations in the EFK stream indicated decreasing Hg (both sedimentary and dissolved) in the sites traveling downstream of the point source and increasing MeHg concentrations moving downstream (Fig. 4A). While only 3 points of data are shown, more comprehensive analyses showing the same decreasing trend of Hg(II) and increasing MeHg has been performed (Brooks & Southworth 2011). While there was a positive correlation of increasing MeHg concentrations with the abundances of *Desulfonema* spp. (r = 0.617, p = 0.054) and *Desulfobulbus* spp. (r = 0.403, p = 0.069) going downstream, these relationships were not statistically significant (Fig. 4B).

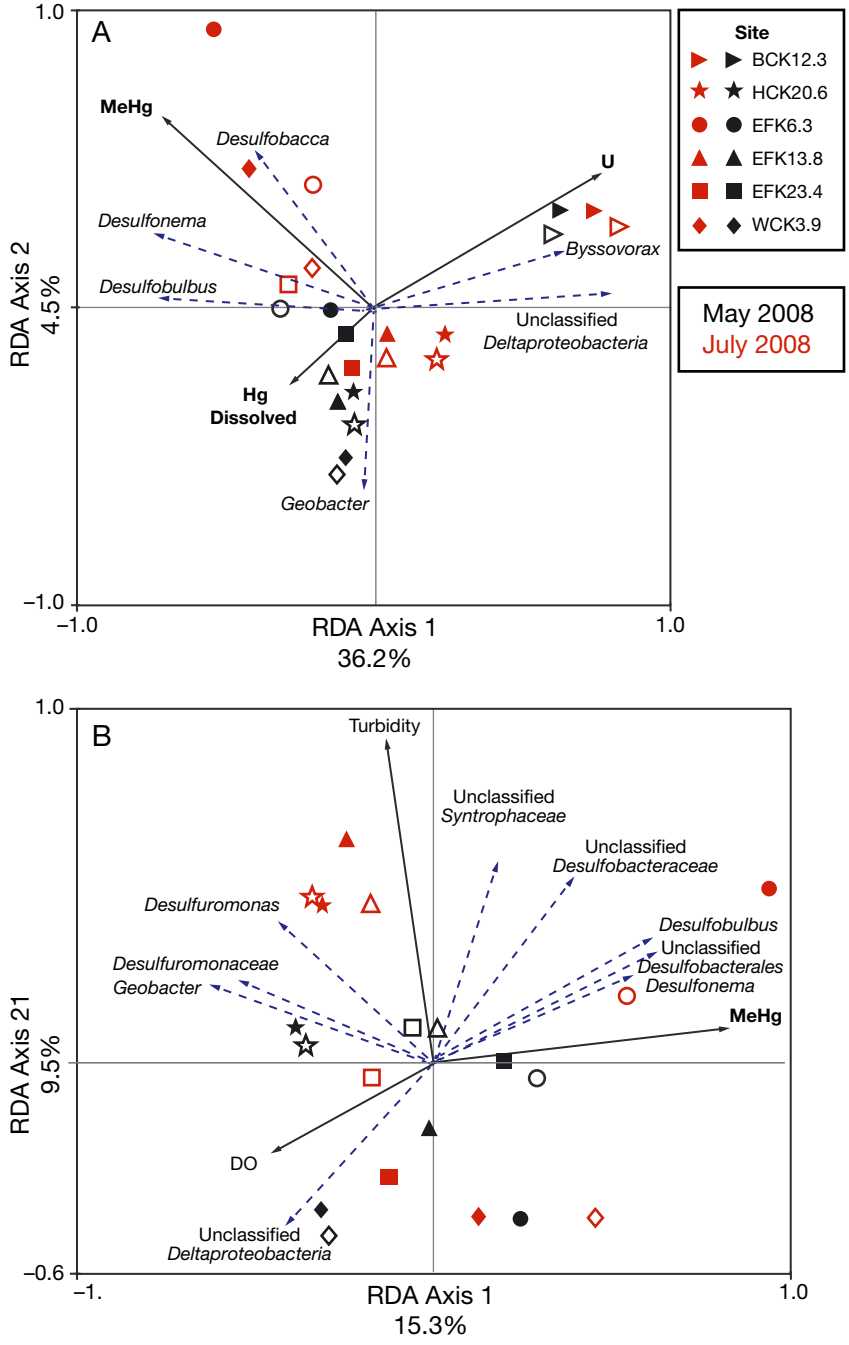

Fig. 3. Triplot of the redundancy analysis (RDA) for bacterial taxa from (A) the 24 stream sediments samples at 6 sites and (B) the 20 stream sediments samples at 5 sites (BCK samples excluded), all sites located on or near the Oak Ridge Reservation, with forward selection of predictor variables followed by Monte Carlo permutations. Solid arrows represent predictor (geochemical) variables significantly associated ($p < 0.05$) with the variation in the bacterial community structure. Dashed arrows represent individual taxa ($|r| > 0.6$) significantly associated with the variation among samples. The length of the arrow is correlated with the degree of relation between the response variables. The arrows point in the direction of the maximum change for the associated variable. Open symbols: midchannel, closed: near bank, DO: dissolved oxygen. Keys apply to both (A) and (B)

Phylogenetic comparison with cultured sequences

Several of the sequences detected in the present study grouped within the clusters of known methylators and within 0.02 changes per nucleotide position, suggesting that these bacteria may be responsible for Hg methylation in the sediments. These results were observed from a cluster analysis at 97% similarity level that resulted in 34 clusters. Clusters containing ≤10 sequences were eliminated from further analysis, leaving 13 clusters. The phylogenetic relationship between representative sequences from each of the 13 clusters and sequences from 36 cultured *Deltaproteobacteria* is presented (Fig. 5). Most notable was the predominant *Deltaproteobacteria* cluster of 329 sequences (3-fold larger than any other found) from EFK 6.3 being closely related to *Desulfobulbus propionicus* strain 1pr3, an organism capable of Hg methylation (Benoit et al. 2001). The other known Hg methylating strain of *Desulfobulbus propionicus* (MUD10) did not have 16S sequences available for comparison (Rodríguez-González al. 2009). There were also 2 clusters (93 sequences total) that were closely related to *Desulfomonas palmitatis* from EFK 13.8 and 6.3, and 1 cluster (31 sequences) from EFK 13.8 that grouped closely with *Geobacter sulfurreducens*; both of those related species are known to methylate Hg. An additional 107 sequences were found from EFK 6.3 that are most closely related to *G. chapelli*. While this organism has not been tested for methylation, 3 other members of this genus tested to date are capable of methylation (Kerin et al. 2006, Schaefer & Morel 2009). Two clusters (152 sequences total) were closely related to *Byssovorax cruenta*, while another cluster (52 sequences) was more similar to members of the class *Myxococcales*. No clusters were identified that contained sequences of the known methylator *Desulfovibrio desulfuricans* ND132 (Jay et al. 2002).

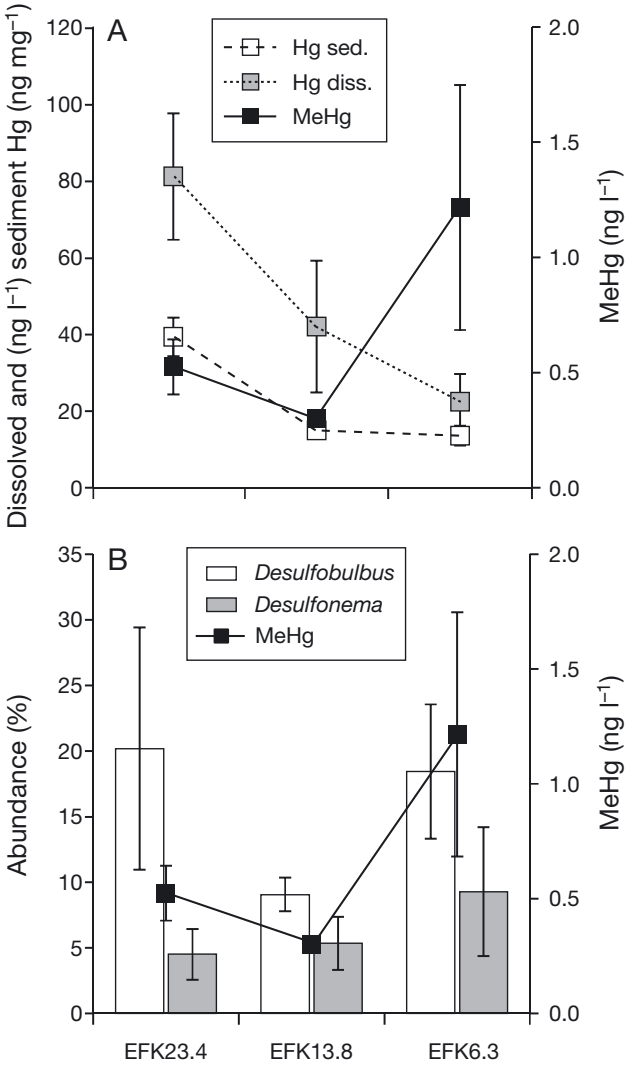

Fig. 4. (A) Concentrations of dissolved (diss.) Hg, sediment (sed.) Hg and MeHg and (B) percent abundance of *Desulfobulbus* spp., *Desulfonema* spp. and MeHg concentrations in EFK sites traveling downstream of a point source

DISCUSSION

The present study revealed positive trends among the distribution of members of the *Deltaproteobacteria* in stream sediments with varying levels of Hg, MeHg and other metal contamination, some of which have close relatives that are able to methylate mercury. While these trends revealed statistical significance, several other geochemical parameters may contribute, either in a positive or negative manner, to these observed trends. It has been noted that the trends in mercury and methyl mercury are more pronounced in the summer months (Brooks & Southworth 2011), and the detailed analyses presented were concentrated on the warm season.

In the RDA analysis performed with samples from all 3 months (36 samples), positive relationships were observed between the Hg(II) concentrations (both dissolved and sedimentary) and relative abundances of *Desulfobulbus* spp. These correlations were observed in the sites with the highest concentrations of inorganic Hg. When MeHg concentrations were added to the analysis based on 2 sampling dates (24 samples), there was a shift in the significance of the *Desulfobulbus* spp. toward MeHg concentrations (as well as *Desulfobacca* spp. and *Desulfonema* spp.). *Desulfobulbus propionicus*, the closest match to the representative sequence from the cluster analysis, has been shown to methylate Hg under sulfate-reducing and/or fermentative conditions (King et al. 2000, Benoit et al. 2001, Kerin et al. 2006, Rodríguez-González et al. 2009), is often found in high abundances where MeHg is present (Achá et al. 2005) and has been shown to have a positive net MeHg production (Bridou et al. 2011).

The *Desulfonema* spp. sequences in the present study are phylogenetically similar to isolated strains of *D. ishimotonii* and *D. limicola*. *Desulfonema* are filamentous sulfate-reducing bacteria that are capable of oxidizing acetate and other small aliphatic acids completely to CO_2 (Widdel et al. 1983, Fukui et al. 1999) in marine and freshwater environments (Elshahed et al. 2003, Kondo & Butani 2007, Wang et al. 2008, Teske et al. 2009). *Desulfobacca* is a monospecific genus represented by *D. acetoxidans*, a mesophilic sulfate-reducer that oxidizes acetate (Oude Elferink et al. 1999), was isolated from granular sludge and has been reported in metal- and sulfate-contaminated wastewater and acidic fens (Kaksonen et al. 2004, Loy et al. 2004, Shelobolina et al. 2007). Although both of these organisms are known dissimilatory sulfate-reducers, no available studies have been conducted on the ability of either to methylate Hg.

The *Deltaproteobacteria* community in BCK 12.3 appeared to be distinct from the other 5 sites in the study and was predominately composed of *Byssovorax* and unclassified *Deltaproteobacteria* that significantly correlated with U(VI) concentrations in the stream water. However, several other parameters equally auto-correlated with U(VI), such as NO_3^-, Ca, Ba, Cl^-, Mg, Sr, DIC and conductivity. The *Byssovorax* sequences identified in the present study clustered with the only known species of *Byssovorax, B. cruenta*, a cellulolytic myxobacterium (Treude et al. 2003, Reichenbach et al. 2006). Little is known about the metabolism of this bacterium in natural environments, but it has been found in high nitrate environments (Sanford et al. 2002, Noll et al. 2005), which

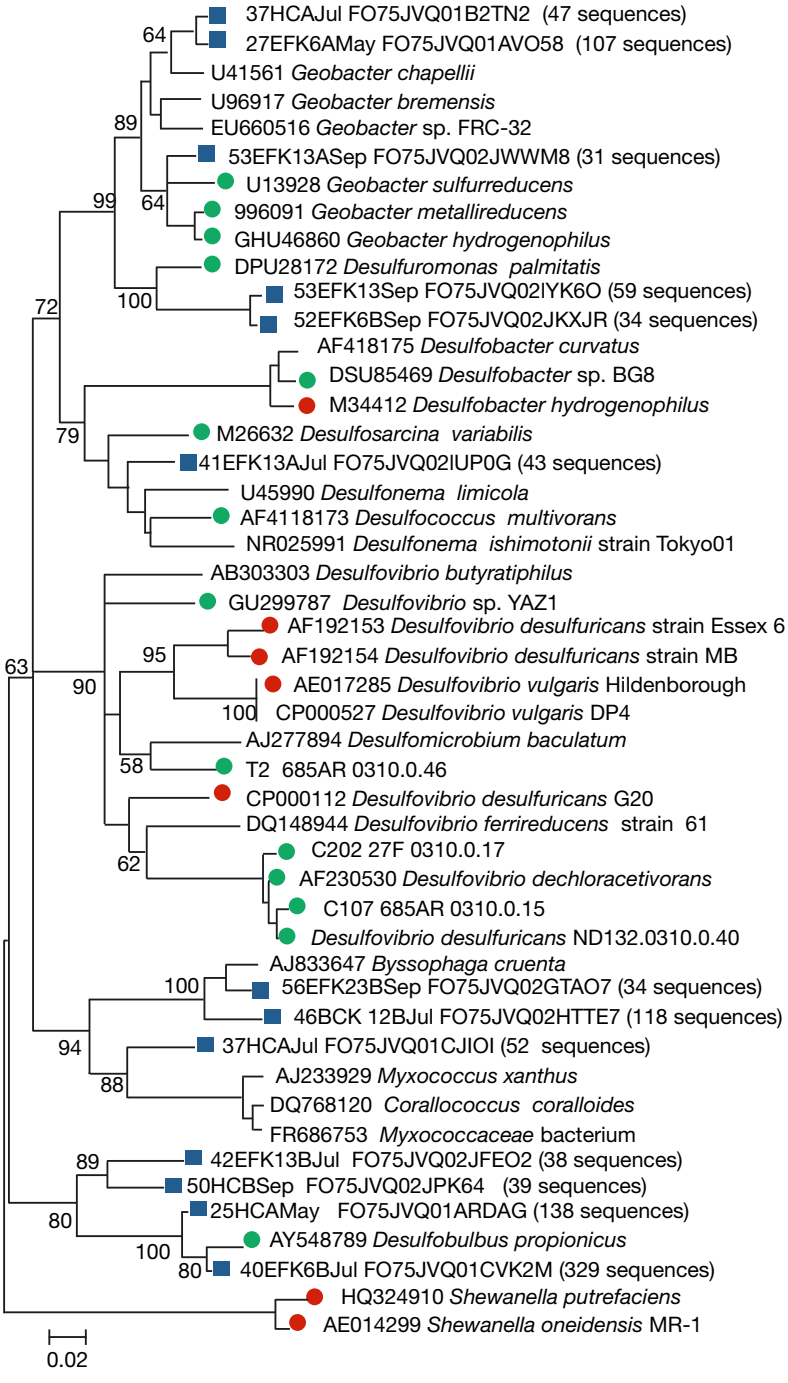

Fig. 5. Phylogenetic relationships of 13 representative environmental sequences (■) and 36 culturable strains of *Deltaproteobacteria*. (●): Hg methylators; (●): non Hg methylators. Representative sequences came from clusters that grouped with known *Deltaproteobacteria* by the complete-linkage clustering method in the RDP pyrosequencing pipeline. The scale bar represents 0.02 changes per nucleotide position. Phylogenetic analyses were conducted in MEGA4. T: type strain

may explain the high abundance in BCK 12.3. It is unknown if these organisms display metal tolerance or reduction, and this may warrant further study.

Higher abundances of *Geobacter* spp. sequences were observed in the uncontaminated (HCK 20.6) site, with the majority clustering with cultured *Geobacter* spp. that have not been tested for Hg methylation (i.e. *G. chapelleii* and *G. bremensis*). However, *Geobacter* sequence clusters from the contaminated sites were related to a combination of methylators and those not yet tested for methylation (i.e. *G. hydrogenophilus*, *G. metallireducens* and *Geobacter* sp. FRC-32). While several members of the genus produce MeHg (i.e. G. *hydrogenophilus, G. metallireducens* strain GS-15 and *G. sulfurreducens*) (Kerin et al. 2006), the abundance of the *Geobacter* spp. present in the samples were not statistically correlated to any measured geochemical or physical factors (i.e. Hg or MeHg concentrations).

It is evident from the data and historical knowledge (Brooks & Southworth 2011) that the source of Hg(II) is in the upstream waters of EFK (near EFK 23.4) and that MeHg concentrations increase with distance downstream to EFK 6.3. This suggests that methylation actively occurs in these systems. Although at this time there is no direct evidence to pinpoint the exact location of methylation (e.g. surface sediments, subsurface sediments and riparian soils) and which organisms are responsible for Hg methylation, the presence and abundance of organisms that are closely related to known Hg methylating *Deltaproteobacteria* in the stream sediments, specifically *Desulfobulbus* spp., were statistically correlated to concentrations of MeHg in the stream water. Functional Hg methylation assays of isolates within and outside the *Deltaproteobacteria* are required to examine these correlations in more detail. Additional sampling in deeper sediments from the stream channel and the riparian zones are planned to help delineate these relationships and to determine rates of Hg methylation throughout the watershed.

CONCLUSIONS

The results of the present study allowed for the identification of bacteria that may contribute to Hg methylation in stream sediments. Strong correlations were demonstrated between Hg and MeHg concentrations and close relatives of known Hg methylators, specifically *Desulfobulbus propionicus.* The phylogenetic relationship of the sequences detected in the current study to cultured strains of *D. propionicus* gave further support to the notion that bacteria similar to *D. propionicus* may play a role in Hg methylation in surface stream sediments at sites near Oak Ridge, TN; however, further physiological studies are required to examine these relationships more closely. These findings shed light on possible site-specific methylators and form the basis for the future isolation of these site-specific methylators.

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