RESEARCH ARTICLE

Identification of sulfate-reducing bacteria in methylmercury-contaminated mine tailings by analysis of SSU rRNA genes

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Received 1 May 2008; revised 23 December 2008; accepted 23 December 2008
First published online February 2009.

DOI:10.1111/j.1574-6941.2009.00658.x

Editor: Alfons Stams

Keywords
sulfate-reducing bacteria; mine tailings; mercury; SSU rRNA; microbial community.

Abstract

Sulfate-reducing bacteria (SRB) are often used in bioremediation of acid mine drainage because microbial sulfate reduction increases pH and produces sulfide that binds with metals. Mercury methylation has also been linked with sulfate reduction. Previous geochemical analysis indicated the occurrence of sulfate reduction in mine tailings, but no molecular characterization of the mine tailings-associated microbial community has determined which SRB are present. This study characterizes the bacterial communities of two geochemically contrasting, high-methylmercury mine tailing environments, with emphasis on SRB, by analyzing small subunit (SSU) rRNA genes present in the tailings sediments and in enrichment cultures inoculated with tailings. Novel *Deltaproteobacteria* and *Firmicutes*-related sequences were detected in both the pH-neutral gold mine tailings and the acidic high-sulfide base-metal tailings. At the subphylum level, the SRB communities differed between sites, suggesting that the community structure was dependent on local geochemistry. Clones obtained from the gold tailings and enrichment cultures were more similar to previously cultured isolates whereas clones from acidic tailings were more closely related to uncultured lineages identified from other acidic sediments worldwide. This study provides new insights into the novelty and diversity of bacteria colonizing mine tailings, and identifies specific organisms that warrant further investigation with regard to their roles in mercury methylation and sulfur cycling in these environments.

Introduction

Recent studies have shown some sulfate reducers to be metabolically active in environments with $pH \geq 2$ (e.g. Praharaj & Fortin, 2004). Indirect, culture-based cell count estimates and sulfate reduction rate (SRR) measurements (e.g. Fortin *et al.*, 1996, 2000, 2002) revealed that sulfate-reducing bacteria (SRB) were present and active in both pH-neutral gold mine tailings and in acidic base-metal tailings. While SRB can aid in the natural bioremediation of mine tailings by precipitating toxic metals and increasing pH, they are also linked to methylmercury (MeHg) production (e.g. Choi & Bartha, 1994; Devereux *et al.*, 1996; King *et al.*, 2001). It is therefore important to understand which groups of SRB predominate in different types of mine tailings environments, but currently, these environments are under-characterized with respect to the composition of the extant SRB populations.

Two contrasting mine tailings sites were selected for SRB community analysis: a low-Hg acidic site [Kidd Metsite (KM)] in Northern Ontario, containing tailings produced from sulfidic, base-metal ores, and a high-Hg pH-neutral site in Nova Scotia, composed of tailings from gold ores extracted by Hg-amalgamation [East Lake Catcha (ELC)]. Previous work at KM (Winch *et al.*, 2008a) determined that despite low Hg_T concentrations ($< 1\,\mu mol\,kg^{-1}$ dry wt sediment), MeHg levels reached 12.1 $nmol\,kg^{-1}$ in tailings and 87.9 pM in porewater. The zone of highest MeHg corresponded to a zone of high SRR (max 2771 $nmol\,cm^{-3}\,day^{-1}$), located in a surficial, acidic (pH 3.5–5), oxidizing layer of tailings.

In contrast with the acidic tailings, Hg_T concentrations and saturation conditions were better predictors of MeHg

levels in the Hg-contaminated gold mine tailings (Winch *et al.*, 2008b). Of six Nova Scotia gold tailings dumps, the ELC site featured high Hg_T levels (10–30 mol kg^{-1}) and MeHg concentrations > 5 nmol kg^{-1} in the tailings. The MeHg : Hg_T ratio was unusually high compared with other high-Hg_T gold tailings. This site also had the highest SRR (64.5 nmol cm^{-3} of sediment day^{-1}) and SRB cell count estimates (2.9×10^7 CFU g^{-1} dry wt of sediment) of all the gold mine tailings dumps studied.

To the best of our knowledge, no studies have examined bacterial nucleic acids extracted from geochemically contrasting gold and base-metal tailings to describe the structure of the SRB fraction of the microbial community. The study presented here identified bacteria in these two tailings environments, focusing on lineages most likely contributing to sulfate reduction and Hg methylation. Specifically, we characterized the *Deltaproteobacteria* class using two overlapping primer sets to clone and sequence small subunit (SSU) rRNA gene (DNA) sequences in nucleic acid extractions from mine tailings sediments and SRB enrichment cultures inoculated with mine tailings. We hypothesized that *Desulfovibrio*-type species, as well as Gram-positive sulfate reducers related to *Desulfotomaculum*, would be detected in the acidic tailings, as these groups were previously observed by microscopy in KM tailings (Fortin *et al.*, 1995). We also hypothesized that *Deltaproteobacteria* would be frequently detected in the SRB community in the pH-circumneutral gold mine tailings from Nova Scotia, as these organisms generally favour neutral pH environments. The results suggested that although the SRB sequences derived from both tailings environments included representatives of the *Deltaproteobacteria* and the *Firmicutes*, at the subphylum level, the microbial community structures featured novel species and reflected the unique properties of each environment.

Materials and methods

Sampling

The two sites chosen for molecular microbiological analysis have high MeHg concentrations and high SRR relative to other samples from similar environments. Frozen field samples from KM obtained in August 2004 and cultures grown from a fresh KM sample of oxidized tailings obtained in June 2005 were used for DNA analysis. A core of gold tailings from ELC was obtained in October 2005. A fraction of the core was used to inoculate SRB enrichment culture medium with the remaining sediment was frozen for Hg_T and MeHg analysis, and DNA extraction. Sampling methods are described elsewhere (Winch *et al.*, 2008a, b). Table 1 presents a summary of the physicochemical and microbiological characteristics of each sample.

Table 1. Physicochemical and microbiological characteristics of samples from the Kidd Metsite (Ontario) and East Lake Catcha (Nova Scotia) (Winch *et al.*, 2008a, b)

	Sample	
Parameter	Kidd (sulfidic Cu–Zn tailings)	ELC (Au tailings, extracted by Hg amalgamation)
pH	3.7	6.3–6.6
E_h (mV)	509	85–280
SO_4^{2-} – porewater (mM)	560	0.9–4
HS^- – porewater (μM)	0.05	$\leq$ 0.39
DOC – porewater (mmol C L^{-1})	1.3	N/A
Organic content – tailings (%)	1.4	0.6–2.0
Hg_T – tailings (μmol kg^{-1})	0.7	16.3–32.6
MeHg – tailings (nmol kg^{-1})	12.1	6.0–10.6
Hg_T – porewater (nM)	0.26	N/A
MeHg – porewater (pM)	88	11–19
SRB (CFU g^{-1} dry wt)	980	3.8×10^5–9.9×10^5
SRR (nmol cm^{-3} day^{-1})	1720	44–65

Culture-based analysis

Enrichment cultures inoculated from gold mine tailings sediment, designated nSRB, were grown in pH-neutral freshwater medium (Widdel & Bak, 1992) containing 28 mM sulfate, 2.5 mM $FeCl_2$ and a final concentration of 10 mM lactate or acetate. The medium was inoculated under anaerobic conditions with 1.7 g tailings, and then serially diluted in 10-fold steps. Cultures were incubated at 30 °C and monitored for indications of SRB activity, i.e., formation of a black FeS precipitate. Cultures from the highest dilutions with observable activity were transferred to fresh medium under anaerobic conditions and incubated at 30 °C.

Base-metal tailings sediments from the oxidized, acidic upper layer of the KM tailings were used to inoculate acidic medium to enrich for acidophilic (or acid tolerant) sulfate reducers (aSRB). Enrichment cultures were inoculated under anaerobic conditions and grown in a basal salts/yeast extract medium according to Sen & Johnson (1999), with glucose as the electron donor, and incubated at 30 °C. (Lactate and acetate were used for the gold mine cultures and not in the acidic cultures because of concerns that these organic acids would be toxic to SRB at low pH.) Once activity was observed, cultures were transferred into fresh medium with glucose, ethanol or glycerol as the electron donor, incubated and monitored until growth was again observed.

DNA extraction

DNA was extracted from both sediment and enrichment culture samples. Sediment samples were collected from 5- to 10 cm depth at KM and from 10 to 15 cm and 30 to 35 cm

depth at ELC. Genomic DNA was then extracted from 2 g (wet weight) of tailings sediments using the Hurt *et al.* (2001) method. The DNA was then purified for PCR using the Qiagen DNA/RNA kit (Midi) (Qiagen) according to the manufacturer's suggested protocols. Enrichment cultures were pooled from two to six replicates of each culture type (aSRB with ethanol or glucose, and nSRB with lactate or acetate, as described above). DNA was extracted using an UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc.) according to the manufacturer's suggested protocols.

PCR amplification with SSU rRNA gene-specific primers

DNA extracted from sediments and cultures was PCR amplified using *Bacteria* domain-specific primers 27F (Lane, 1991) and 1392R (Stahl *et al.*, 1988). Each PCR amplification mixture contained 0.1 U μL^{-1} Taq polymerase (TaKaRa Shuzo) (TaKaRa Bio Inc., Japan), 0.5 μM of each primer, 0.25 mM of each dNTP (TaKaRa Shuzo dNTP mixture), 5 μL of 10 × LA PCR buffer (TaKaRa Shuzo) and 1 μL of DNA template. The mixture was adjusted to a final volume of 50 μL with distilled water. 16S rRNA gene amplification reactions were cycled in an EP Gradient thermocycler (Eppendorf, Germany). PCR products, visualized in a 0.7% SeaKem LE agarose gel stained with ethidium bromide and illuminated with UV, were compared with molecular size standards. For cloning and sequencing, appropriately sized bands were excised from the gel and purified using Qiaex II gel extraction kit (Qiagen), according to the manufacturer's recommendations.

Primers specific to the class *Deltaproteobacteria* (385F – Amann *et al.*, 1990; 1492R – Lane, 1991) were utilized under PCR conditions described in Klepac-Ceraj *et al.* (2004) to increase detection of SRB from tailings and enrichment cultures. *Deltaproteobacteria* gene amplification reactions were cycled in an Eppendorf thermocycler. PCR products were visualized using gel electrophoresis and eluted as described previously.

Amplification of Gram-positive SRB-related SSU rRNA gene sequences derived from tailings and cultures was attempted using primers specific for *Desulfotomaculum* spp. (DFM 140–158, 5′-TAG MCY GGG ATA ACR SYK G-3′, and DFM 842, 5′-ATA CCC SCW WCW CCT AGC AC-3′, respectively – Daly *et al.*, 2000). However, no significant amplification was detected from any samples using these primers.

Clone library construction

Purified pooled *Bacteria* domain-specific and *Deltaproteobacteria*-derived amplicons were cloned using the TOPO TA cloning vector pCR2.1 according to the manufacturer's instructions (Invitrogen). Inserts were subsequently PCR amplified from lysed colonies with primers specific for the vector, M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′), and then digested (2 h, 37 °C) with the restriction enzymes HaeIII and MspI (Promega). Restriction fragment length polymorphism (RFLP) patterns were visualized using 2% SeaKem LE agarose gel electrophoresis, and were then used to group clones into phylotypes. DNA from selected clones was purified using the QIAquick PCR purification kit and then sequenced in both forward and reverse directions (× 2 coverage) at the FSU Sequencing Facility, using an Appied Biosystems 3100 genetic analyzer. In highly diverse samples, a representative from each phylotype containing multiple identical RFLP patterns was sequenced to assure overall phylotype integrity. A random selection of those phylotypes represented by single RFLP patterns was also sequenced. Before comparative sequence analysis, vector sequences flanking the inserted amplicon were removed manually.

Sequence analysis

Contiguous sequences were assembled with the program BIOEDIT v7.0.0 (Hall, 1999). Previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990). Clone sequences were checked for chimeras using the program CHIMERA CHECK from the Ribosomal Database Project II (Cole *et al.*, 2003). All clone sequences and reference sequences were aligned in the ARB software package using the Fast Aligner algorithm, incorporating ribosomal secondary structure data (Strunk & Ludwig, 1997). Neighbor-joining trees incorporating a Jukes–Cantor distance correction were created from the alignments using the ARB software package (Strunk & Ludwig, 1997). Bootstrap data represented 1000 samplings. Fifty nucleotide sequences were submitted to the GenBank database under accession numbers EF464596–EF464645.

Statistical analysis

Statistical analyses were used to determine the sampling efficiencies and diversity differences within clone libraries based on RFLP analysis. Rarefaction curves were calculated using ANALYTIC RAREFACTION 1.3 (Heck *et al.*, 1975; Holland, 2003). ESTIMATES (Colwell *et al.*, 2004) was used to estimate species richness nonparametrically with Chao1 and to calculate the Shannon–Wiener and the reciprocal of Simpson's ($1/D$) indices. The percent coverage (C) of the clone libraries was calculated according to the equation $C = [1 - (n_1/N) \times 100]$, where n_1 is the number of unique clones as determined by RFLP analysis and N is the total number of clones in the library (Good, 1953). Clone library

Table 2. Statistical analysis of SSU rRNA gene clone libraries derived from mine tailings sediments using general *Bacteria* primers (bac) and primers specific for *Deltaproteobacteria* (del)

Samples	No. of clones	No. of phylotypes	Species richness	Shannon	1/*D*	Percent coverage	θ(π)	Nucleotide diversity	Gene diversity
Kidd del	94	20	58 (29, 170)*	2.01	5.18	85	51.9 ± 25.1	0.14 ± 0.07	0.74 ± 0.02
Kidd bac	48	24	194 (94, 435)	2.56	8.19	58	242 ± 119	0.17 ± 0.08	0.67 ± 0.07
Gold del	90	83	1123 (423, 3266)	4.38	435	11	78.2 ± 39.0	0.20 ± 0.10	0.96 ± 0.03
Gold bac	86	79	596 (281, 1403)	4.32	446	15	314 ± 160	0.21 ± 0.11	0.91 ± 0.04

*The numbers in parentheses are 95% confidence limits.

sequence data were used to compare phylogenetic diversity between samples. Clone sequence diversity indices for gene and nucleotide diversity (Nei, 1987), and 2(*B*), an estimate of nucleotide diversity (Tajima, 1983) were calculated using ARLEQUIN (Schneider *et al.*, 2000).

X-ray diffraction (XRD)

The precipitates formed by SRB cultures inoculated from acidic mine tailings and grown in acidic cultures under anaerobic conditions (aSRB) were analyzed using XRD. Cultures were freeze-dried under vacuum, and then flushed with N_2 gas to avoid contact with oxygen. XRD analysis was performed using a Philips X'Pert diffractometer, with a CuKα source operating at 45 kV and 40 mA, and a Kevex Si (Li) solid-state detector. The goniometer was outfitted with a gas line so the sample holder could be continuously flooded with N_2 gas during the operation of the instrument. All samples were run in step-scan mode at 0.02 N/60 s from 5 to 75 N (2θ).

Results

RFLP and statistical analysis of clone libraries

Clone libraries were constructed using amplified DNA from SSU rRNA gene targets (Tables 2 and 3). In total, 502 clones were generated with 266 from gold mine tailings sediments and enrichment cultures, and 236 from acidic base-metal mine tailings sediments and cultures. RFLP analysis of the DNA-derived clones indicated that 120 distinct phylotypes were detected using *Deltaproteobacteria*-specific primers (94 from gold sediments and cultures; 26 from acidic sediments and cultures), and 110 phylotypes with *Bacteria* primers (84 from gold sediments and cultures; 26 from acidic sediments and cultures). *Bacteria* primers only detected one *Deltaproteobacteria*-related phylotype (18 clones) in one culture, whereas the *Deltaproteobacteria* primers detected another six phylotypes in acidic and gold tailings.

Microbial communities associated with both acidic tailings and gold mine tailings sediments were biodiverse, as indicated by the species richness and the biodiversity indices (Table 2). Rarefaction curves did not indicate saturation (i.e. the slope was > 0, Heck *et al.*, 1975), and percent coverage was *c.* 15% in the highly diverse gold mine tailings, in contrast with 58–85% in the acidic tailings. Although additional sampling of ELC-derived clones would be necessary to fully describe the overall community diversity, sufficient sampling was carried out to establish the novelty and diversity of this environment, as shown by diversity and species richness indices (Table 2). The focus of this study was to identify SRB, which appeared to constitute a relatively small fraction of the overall microbial community diversity.

Phylogenetic analysis of the clone libraries

Thirty-three percent of the sequences obtained from both primer sets were $< 90\%$ similar to cultured lineages. An additional 39% were 90–94% similar to cultured lineages and only 28% were $> 95\%$ similar to sequences derived from cultured organisms registered in the NCBI database (Table 3). Three of the *Deltaproteobacteria* sequences detected using the *Deltaproteobacteria* primers could only be classified at the class level by ARB (Fig. 2). An additional four sequences were 94–99% similar to clones from unclassified candidate divisions OP8, OP10 and WS1 (Fig. 1). Although similar phyla were detected in both types of tailings sediments and associated cultures, genus- and species-level sequence classifications indicated distinct microbial communities at each site.

Deltaproteobacteria

Many *Deltaproteobacteria* are sulfate reducers, and to date, all known Hg-methylating bacteria belong to this class. With the exception of one clone that could not be classified beyond the class level, *Deltaproteobacteria* sequences derived from gold mine sediments using the *Deltaproteobacteria* primers were related to known lineages of the order *Syntrophobacterales* (*Syntrophobacter sulfatireducens* – 93% similar; *Syntrophus gentianae* – 93% similar; *Desulfobacca acetoxidans* – 90% similar; Fig. 2). However, the most closely related sequences to those detected in the gold mine sediment ($> 95\%$ similarity) were unclassified clone

Table 3a. Frequency of characterized *Deltaproteobacteria*- and *Firmicutes*-related clone types in libraries derived from tailings and cultures

Clone	Source	Primer set	# related clones	Phylogenetic group	Nearest relatives	Nearest environment	% Similarity	Accession #	Nearest cultured relative	% Similarity	Accession #
K5_62	Acidic tailings	385F/1492R	27	*Deltaproteobacteria*	Clone H74	Acidic mine sediment, China	99	DQ328626	None		
					RCP1-24	Forested wetland impacted with reject coal	99	AF523882			
					RCP1-85	Forested wetland impacted with reject coal	99	AF523883			
					RCP2-16	Forested wetland impacted with reject coal	99	AF523884			
					BA71	Iron Mountain, CA	98	AF225447			
K5_114	Acidic tailings	385F/1492R	17	*Deltaproteobacteria*	Clone AS6	Iron Mountain, CA	99	AF543496	None		
					BA18	Iron Mountain, CA	99	AF225446			
ELC_10_13	Gold tailings	385F/1492R	1	*Deltaproteobacteria*	Clone RB344	Reed bed reactor biofilm	97	AB240344	None		
					Delta clone WCHB1-27	Hydrocarbon- and chlorinated solvent-contaminated aquifer	96	AF050538			
ELC_10_36	Gold tailings	385F/1492R	2	*Deltaproteobacteria*	FW clone pLW-45	Freshwater lake sediments	99	DQ067029	*Desulfobacca acetoxidans*	90	AF002671
					Delta clone Sh765B-TzT-29	U mine wastes	95	AJ519630			
ELC_30_18	Gold tailings	385F/1492R	1	*Deltaproteobacteria*	Soil clone HS9-74	Pb-, Cr- and organic-contaminated soils	95	AY221614	*Syntrophus gentianae*	93	X85132
ELC_30_41	Gold tailings	385F/1492R	1	*Deltaproteobacteria* *Syntrophobacter sulfatireducens* str. TB8106	Clone c5LKS15 93	Lake Kinneret sediments AY651787	98		AM086112		
A37	Gold cult/ acetate	16S	18	*Deltaproteobacteria*	*Desulfovibrio ferrireducens* str. CY2	Freshwater lake sediments	99	AJ582758	Same		
K23	Acidic tailings	16S	15	*Firmicutes*	*Alicyclobacillaceae* clone SLC66	Mineral leaching cultures	92	AY040739	*Alicyclobacillus pomoram*	92	AB089840
K5_128	Acidic tailings	385F/1492R	26	*Firmicutes*	Clone G26	Gold ore bio-oxidation	93	DQ364429	*Alicyclobacillus pomoram*	92	AB089840
G9	Acidic cult/ glucose	385F/1492R	6	*Firmicutes*	*Alicyclobacillus pomorum*		94	AB089840	Same		
G13	Acidic cult/ glucose	16S	24	*Firmicutes*	*Alicyclobacillus cycloheptanicus*		93	AB042059	Same		
G23	Acidic cult/ glucose	385F/1492R	19	*Firmicutes*	*Alicyclobacillus cycloheptanicus*		93	AB042059	Same		
E17	Acidic cult/ EtOH	385F/1492R	2	*Firmicutes*	*Alicyclobacillus cycloheptanicus*		93	AB042059	Same		
E43	Acidic cult/ EtOH	385F/1492R	1	*Firmicutes*	*Alicyclobacillus cycloheptanicus*		93	AB042059	Same		

K22	Acidic tailings	16S	1	*Firmicutes*	*Desulfosporosinus orientis*		93	AJ493052	Same		
E25	Acidic cult/ EtOH	16S	6	*Firmicutes*	*Desulfosporosinus orientis*		93	AJ493052	Same		
G15	Acidic cult/ glucose	385F/1492R	1	*Firmicutes*	*Desulfosporosinus* sp. 5apy		97	AF159120	Same		
A51	Gold cult/ acetate	385F/1492R	1	*Firmicutes*	*Desulfosporosinus* sp. 5apy		98	AF159121	Same		
A27	Gold cult/ acetate	385F/1492R	19	*Firmicutes*	*Desulfosporosinus* sp. Y5		97	AY233860	Same		
A11	Gold cult/ acetate	16S	3	*Firmicutes*	*Desulfosporosinus* sp. Y5		96	AY233860	Same		
E11	Acidic cult/ EtOH	385F/1492R	20	*Firmicutes*		*Desulfitobacterium hafniense* Y51		94	AP008230	Same	
E15	Acidic cult/ EtOH	16S	7	*Firmicutes*	*Desulfitobacterium* sp. Viet-1 93	*Desulfitobacterium hafniense* clone 3174 AF357919		94	AF403181		
E27	Acidic cult/ EtOH	16S	2	*Firmicutes*		*Desulfitobacterium* sp. Viet-1	river sediment, Vietnam	94	AF357919	Same	
E41	Acidic cult/ EtOH	16S	2	*Firmicutes*		*Desulfitobacterium* sp. RPf35Ei	Reactor treating AMD	94	AY548779	Same	
ELC_10-6	Gold tailings	385F/1492R	1	*Firmicutes*	Clone AKAU3868	Uranium-contaminated soil	94	DQ125755	*Clostridium papyrosolvens*	94	X71852
ELC_30_37	Gold tailings	385F/1492R	2	*Firmicutes*	*Bacillus* sp. IDA4917		98	AY289504	Same		
ELC_30_21	Gold tailings	16S	2	*Firmicutes*	Anoxic bulk soil clone BSV82	Anoxic soil of a flooded rice microcosm	99	AJ229226	*Clostridium* sp. FA2/18	98	AY188849
ELC_30_34	Gold tailings	16S	2	*Firmicutes*	Clone AKAU4085	Uranium-contaminated soil	98	DQ125854	*Bacillus* sp. IDA4921	98	AY289500
L45	Gold cult/ lactate	385F/1492R	14	*Firmicutes*	*Sedimentibacter* sp. C7		96	AY766466	Same		

Table 3b. Frequency of characterized clone types related to *Beta-* and *Gammaproteobacteria*, *Nitrospirae*, *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Actinobacteria*, Candidate Divisions WS-1, OP8 and OP10 in libraries derived from tailings and cultures

Clone	Source	Primer set	# related clones	Phylogenetic group	Nearest relatives	Nearest environment	% similarity	Accession #	Nearest cultured relative	% Similarity	Accession #
L13	Gold cult/lactate	16S	24	*Betaproteobacteria*	*Microvirgula aerodenitrificans* str. LMG 4329		99	AJ487013	Same		
K41	Acidic tailings	16S	3	*Gammaproteobacteria*	Clone fg11	Tinto River, Spain (extreme acidic environment)	99	DQ303276	*Acidithiobacillus ferrooxidans* str. QXS-1	99	DQ168465
E29	Acidic cult/EtOH	16S	1	*Gammaproteobacteria*	AF clone KF/GS-JG36-22	U mine waste pile sediment	99	AJ295655	*Acidithiobacillus ferrooxidans* str. QXS-1	99	DQ168465
ELC_30_27	Gold tailings	16S	2	*Gammaproteobacteria*	*Acinetobacter johnsonii* str. S35		99	AB099655	Same		
K5_130	Acidic tailings	385F/ 1492R	5	*Nitrospirae*	Clone fe12	Mine waste rock	99	DQ303277	*Leptospirillum ferrooxidans* str. P3a	99	AF356837
ELC_10_8	Gold tailings	16S	2	*Nitrospirae*	Clone HSM-SS-003	Peat soil, Japan	99	AB238766			
				Thermodesulfovibrio islandicus	89	X96726					
ELC_10_10	Gold tailings	385F/ 1492R	2	*Nitrospirae*	FW clone IRD18B03	River sediments, USA	99	AY947906	None		
ELC_10_47	Gold tailings	385F/ 1492R	1	*Nitrospirae*	Clone FW19	Forested wetland	99	AF524005	None		
ELC_10_21	Gold tailings	385F/ 1492R	1	Cand. Div. OP8	Clone WFeA1-59	Hydrocarbon and chlorinated solvent contaminated aquifer	95	AF050555	None		
ELC_30_43	Gold tailings	385F/ 1492R	1	Cand. Div. OP8	Clone SHA-124	Mixed culture with 1,2-dichlorpropan (bioremed study)	97	AJ306781	None		
K30	Acidic tailings	16S	3	*Acidobacteria*	Clone: Takashi AB-B21	Fe-oxidizing nodules in reducing sediments	97	AB254782	None		
K5_76	Acidic tailings	385F/ 1492R	3	*Acidobacteria*	Clone: Takashi AB-B21	Fe-oxidizing nodules in reducing sediments	99	AB254782	None		
ELC_10_49	Gold tailings	385F/ 1492R	1	*Acidobacteria*	Clone BS073	Reed bed reactor soil	99	AB240251	None		
ELC_30_8	Gold tailings		1	*Acidobacteria*	Clone AT-s3-28		92	AY225646	None		

		385F/1492R				Mid-Atlantic ridge hydrothermal sediment					
ELC_30_13	Gold tailings	385F/1492R	1	Cand. Div. WS-1	Clone SJA-43	Anaerobic, trichlorobenzene-transforming microbial consortium	94	AJ009463	None		
ELC_30_46	Gold tailings	385F/1492R	1	Cand. Div. OP10	Clone ZZ12AC2	Benzene-contaminated groundwater	99	AY214187	None		
ELC_30_3	Gold tailings	385F/1492R	1	*Planctomycetes*	Clone 655077	Nitrate and heavy metal contaminated soils	96	DQ404737	None		
ELC_10_12	Gold tailings	385F/1492R	1	*Chloroflexi*	Clone WCHB1-44	Hydrocarbon and chlorinated solvent contaminated aquifer	99	AF050565	None		
ELC_30_7	Gold tailings	16S	2	*Chloroflexi*	Clone SHD-71	Anaerobic bioreactor that dechlorinates 1,2-dichloropropane	93	AJ278167	*Dehalococcoides* sp. BHI80-15	90	AJ431246
K15	Acidic tailings	16S	7	*Actinobacteria*	Clone D3-7	Acid mine drainage, China	99	DQ464144	*Ferrimicrobium acidiphilum*	99	AF251436
ELC_10_21	Gold tailings	16S	1	*Actinobacteria*	Clone D3-7	Acid mine drainage, China	99	DQ464144	*Ferrimicrobium acidiphilum*	99	AF251436
ELC_10_31	Gold tailings	16S	3	*Actinobacteria*	*Actinobacterium* clone ARFS-2	Flooded anoxic rice paddy soil	97	AJ277687	*Acidimicrobium ferrooxidans*	87	U75647
ELC_30_45	Gold tailings	385F/1492R	4	*Actinobacteria*	Clone 122	Anoxic lake water	92	AJ536836	None		
L17	Gold cult/lactate	385F/1492R	1	*Actinobacteria*	Uncultured *Actinobacterium* clone						

DR546BH1103001SAD23Subsurface water of Kalahari shield, S. Africa
95DQ234644*Eggerthella sinensis* str. HKU14
91AY321958K5_72Acidic tailings385F/1492R
2UnclassifiedClone DUNssu007 (+1B)Acidic forest soil, Germany
94AY724094None

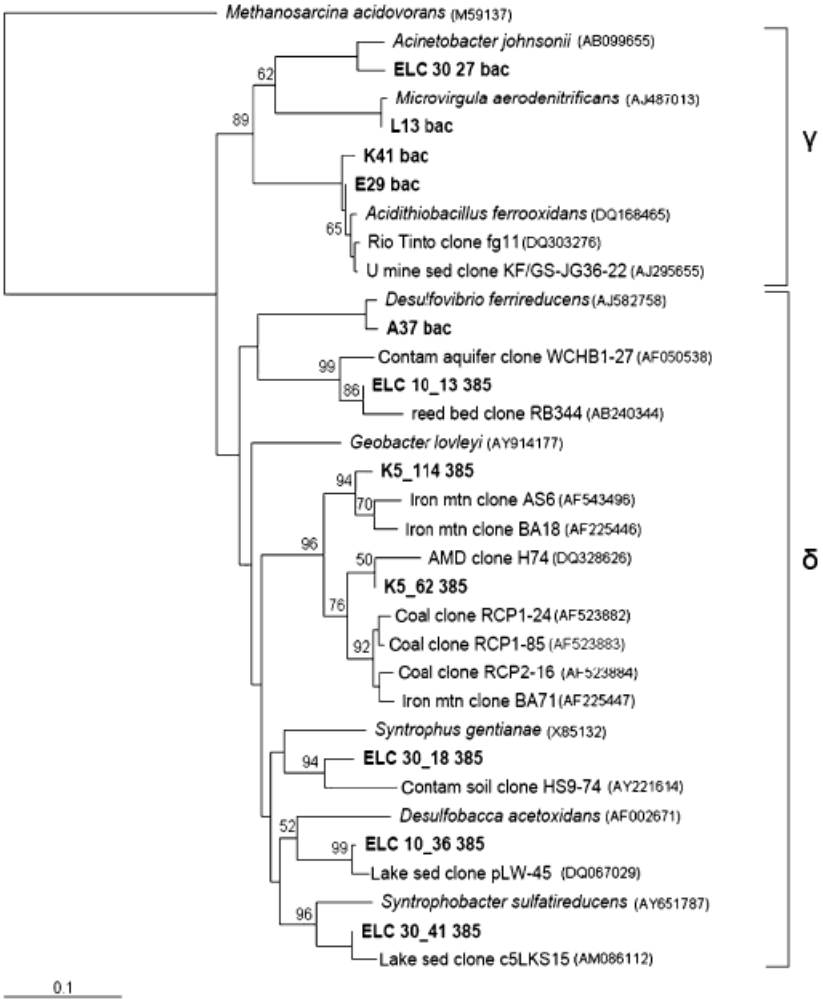

Fig. 1. Neighbor-joining phylogenetic tree of *Proteobacteria*-related sequences, incorporating a Jukes–Cantor distance correction, based on SSU rRNA genes from tailings and cultures from the KM (denoted K5), ELC and ELC cultures amended with acetate (A). Sequences from this study and close relatives were aligned using the Fast Aligner algorithm, verified by hand, and compared with the *Escherichia coli* SSU rRNA secondary structure using the ARB software package. Bootstrap analyses were conducted on 1000 samples. *Methanosarcina acidovorans* was used as the outgroup. Scale bar = 0.10 change per nucleotide position.

sequences previously detected in either freshwater sediments (Nercessian *et al.*, 2005; and an unpublished study from the NCBI database) or contaminated soils and mine wastes (Dojka *et al.*, 1998; Joynt *et al.*, 2006; unpublished studies from NCBI database).

The one *Deltaproteobacteria*-related phylotype detected by *Bacteria*-specific primers represented 86% of clones derived from acetate-amended cultures enriched for SRB and inoculated with gold mine tailings. It was 99% related to *Desulfovibrio ferrireducens* (str. CY2) (Table 3), a newly cultured species (Vandieken *et al.*, 2006).

In contrast with the gold mine sediments, the two *Deltaproteobacteria* phylotypes (K5_62 and K5_114) that jointly comprised 55% of clones derived from acidic mine sediments with *Deltaproteobacteria*-specific primers were not closely related to any cultured lineages. However, phylotype K5_62 was 99% similar to a clone detected in an acidic sediment at an extreme acid mine drainage site in China (unpublished study in NCBI database), 99% similar to three clones isolated from an acidic (pH 2.5–3) reject coal pile (Brofft *et al.*, 2002) and 98% similar to a clone extracted from Iron Mountain, CA, a well-known extreme acid mine drainage environment (Bond *et al.*, 2000). K5_114 was 99% similar to clones detected in two separate studies on Iron Mountain (Bond *et al.*, 2000; Druschel *et al.*, 2004). Representative clone sequences from these two phylotypes were 95% similar to each other.

Potential sulfate reducers of the *Firmicutes* phylum

All of the *Firmicutes*-related phylotypes detected by both primer sets in acetate-amended SRB enrichment cultures inoculated with gold mine tailings were at least 96% similar to *Desulfosporosinus* spp., which are endospore-forming sulfate reducers (Robertson *et al.*, 2001). The majority of these clones (96%) were most closely related to *Desulfosporosinus* sp. Y5 (Liu *et al.*, 2004). One *Firmicutes*-related phylotype detected in the acidic tailings sediments was 93% similar to *Desulfosporosinus orientis* (Fig. 3, Table 3), a

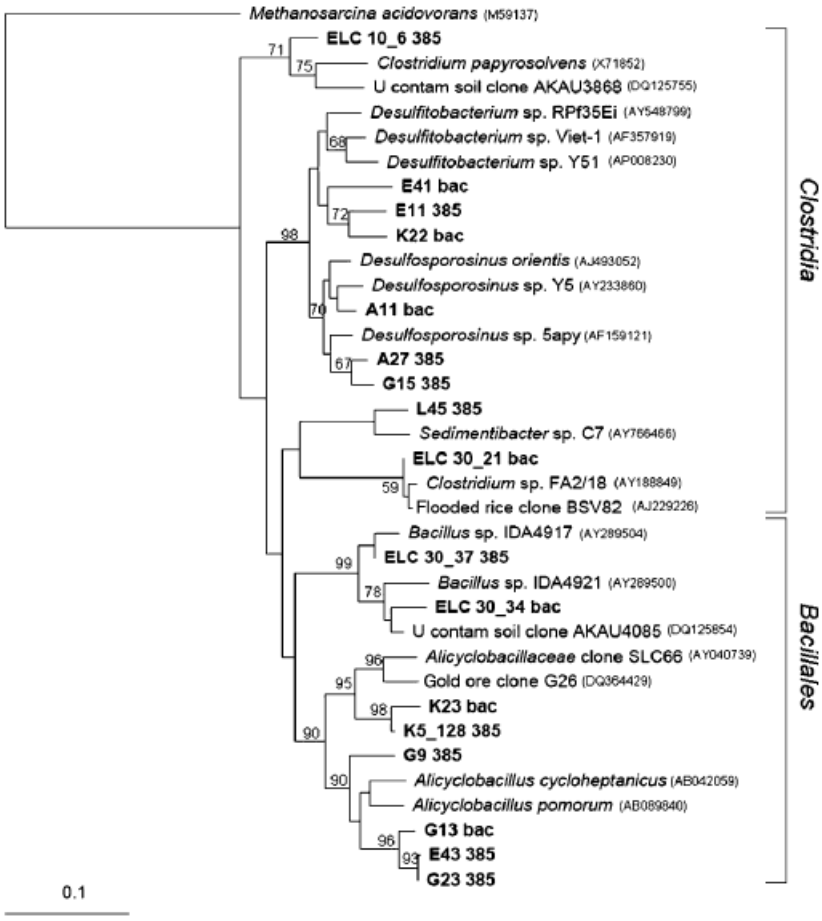

Fig. 2. Neighbor-joining phylogenetic tree of *Firmicutes*-related sequences, incorporating a Jukes–Cantor distance correction, based on SSU rRNA genes from tailings and cultures from the KM (denoted K5, K), ELC, ELC cultures amended with acetate (A) or lactate (L), and KM cultures amended with ethanol (E) or glucose (G). Sequences from this study and close relatives were aligned using the Fast Aligner algorithm, verified by hand, and compared with the *Escherichia coli* SSU rRNA secondary structure using the ARB software package. Bootstrap analyses were conducted on 1000 samples. *Methanosarcina acidovorans* was used as the outgroup. Scale bar = 0.10 change per nucleotide position.

Gram-positive, endospore-forming sulfate reducer previously detected in acidic lakes created by coal-mining activity in Germany (Kusel *et al.*, 2001) with a pH range of 4.9–6.1 and optimum pH 5.5.

Among sequences extracted from ethanol-amended acid mine tailings cultures, 87% were 94% similar to *Desulfitobacterium hafniense* sp. Y51. Sequences from the same cultures detected with *Bacteria*-specific primers were similarly dominated (61% of clones) by sequences from *Desulfitobacterium* species, with an additional 33% of clones being 93% similar to *D. orientis* (Fig. 3, Table 3). In cultures amended with glucose and inoculated with acidic mine tailings, one clone detected by *Deltaproteobacteria* primers was 97% related to *Desulfosporosinus* sp. 5apy.

XRD results

All SRB enrichment cultures inoculated with acidic mine tailings developed a gold-colored precipitate that adhered to the inside of the culture tubes. XRD analysis of these precipitates identified a mixture of pyrite, marcasite, mackinawite and griegite, all Fe-sulfide minerals.

Discussion

Gold mine tailings and acidic tailings hosted *Deltaproteobacteria* and known sulfate reducers of the *Firmicutes* phylum, but these groups differed at the subphylum/class level. Representatives of both lineages reflected the geochemical characteristics of their locations. For example, the only *Deltaproteobacteria* detected in the acidic tailings were the novel phylotypes K5_62 and K5_114. This finding supported the hypothesis derived from the geochemical and microbiological assessment of the KM tailings (Winch *et al.*, 2008a) regarding the possible presence of an acidophilic SRB.

Deltaproteobacteria sequences extracted from the gold tailings and associated cultures were more diverse than those detected in the acidic tailings, and were more closely related to cultured species. This was expected, as the pH-neutral conditions present in the gold tailings would have provided a suitable habitat for more types of SRB (most of which are neutrophilic) than the acidic tailings. Four novel species of *Deltaproteobacteria* were derived from the gold mine sediments while an organism closely related to *D. ferrireducens*

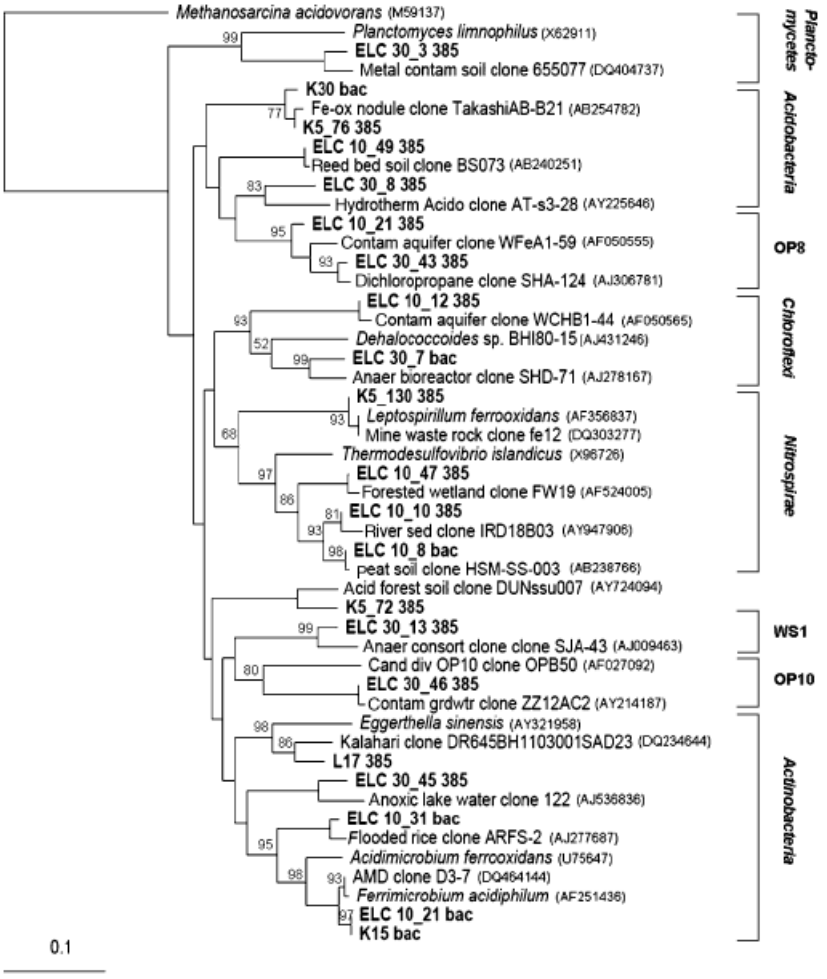

Fig. 3. Neighbor-joining phylogenetic tree of sequences related to all phyla other than *Proteobacteria* and *Firmicutes*, incorporating a Jukes–Cantor distance correction, based on SSU rRNA genes from tailings and cultures from the KM (denoted K5, K), ELC, ELC cultures amended with acetate (A) or lactate (L), and KM cultures amended with ethanol (E) or glucose (G). Sequences from this study and close relatives were aligned using the Fast Aligner algorithm, verified by hand, and compared with the *Escherichia coli* SSU rRNA secondary structure using the ARB software package. Bootstrap analyses were conducted on 1000 samples. *Methanosarcina acidovorans* was used as the outgroup. Scale bar = 0.10 change per nucleotide position.

was detected in the acetate cultures. Although *D. ferrireducens* reportedly tolerated high concentrations of heavy metals, which might allow it to thrive in mine tailings, it has been shown to use only lactate, formate, hydrogen, ethanol, propanol, fumarate and succinate as electron donors (Ramamoorthy, 2005). To our knowledge, no evidence has previously been found of its ability to use acetate; therefore, it is surprising to find a closely related organism in the acetate cultures.

Other *Desulfovibrios* are able to methylate Hg: two of the six strains of *D. desulfuricans* tested to date and one *Desulfovibrio* species (*Desulfovibrio africanus*) was previously reported to have produced detectable MeHg (Gilmour *et al.*, 2006). To date, no Hg methylation studies have been conducted on the newly cultured *D. ferrireducens*, but because a closely related clone was derived from the MeHg-contaminated, high-Hg mine tailings at ELC, and because other *Desulfovibrios* methylate Hg, its capacity to methylate Hg warrants investigation.

Hg methylation has been observed to coincide with iron reduction (Warner *et al.*, 2003; Fleming *et al.*, 2006), but to date, the only iron-reducing bacterium demonstrated to methylate Hg is *Geobacter*, a member of the *Deltaproteobacteria* (Fleming *et al.*, 2006). Although iron reduction has been observed in enrichment cultures inoculated with acidic KM tailings and inhibited for sulfate reduction (Rioux, 2004), *Geobacter* did not likely contribute to Hg methylation in these sediments.

The *Firmicutes* species present in each studied environment reflected local geochemical conditions. For example, it has been demonstrated previously that *Desulfosporosinus* sp. Y5, detected in cultures inoculated with gold tailings, is capable of dissimilatory arsenate reduction (Liu *et al.*, 2004; Perez-Jimenez *et al.*, 2005). Arsenic concentrations in the ELC tailings can be expected to approximate those in geochemically similar gold mine tailings from Nova Scotia, i.e. up to 3% w/w As (Wong *et al.*, 1999, 2002). Such an As-rich environment would likely select for *Desulfosporosinus* sp. Y5.

The dominant SRB in the KM tailings were expected to be spore-forming, Gram-positive SRB, such as *Desulfotomaculum* spp., as their ability to sporulate could be expected to make them more tolerant to acidic conditions. When

Desulfotomaculum-specific primers were used to amplify nucleic acids from this sample, insufficient product was obtained to construct clone libraries. Organisms related to *Desulfosporosinus* spp. were, however, detected in the acidic tailings with the 385F/1492R primers and the *Bacteria* primers. The majority of these clone sequences were 93% similar to *D. orientis*, an organism that has previously been detected in other acidic environments, including acidic mining-impacted lake sediments (Kusel *et al.*, 2001), a metal-contaminated, acidic aquifer (Saunders *et al.*, 2005) and a mixed culture containing an SRB originally derived from acidic sediment in a geothermal area of Montserrat (Kimura *et al.*, 2006).

The appearance of iron-sulfide precipitates identified as pyrite, marcasite, mackinawaite and greigite in the acidic cultures inoculated with acidic tailings demonstrates a potentially important natural remediation process in acidic mine tailings. Because these precipitates formed in both the ethanol- and glucose-amended acidic cultures, their formation may be associated with *Desulfosporosinus* spp., which were detected in both of these cultures. This is consistent with results of a previous study, indicating that nanocrystalline metal sulfide minerals were formed in an aerobic, metal-contaminated, acidic (pH 3.1) groundwater aquifer (Saunders *et al.*, 2005), in which the principal sulfate reducers were closely related to *D. orientis*.

Several species that were closely related to *Desulfitobacterium* spp. were detected in the acidic tailings and cultures. The *Desulfitobacterium* group is known to be comprised of metabolically versatile anaerobic species, often found in contaminated environments or in sediments undergoing contaminant bioremediation at sites contaminated with high concentrations of toxic metals (Villemur *et al.*, 2006). *Desulfitobacterium* spp. utilize a variety of terminal electron acceptors including a wide range of metals, such as Mn(IV), Fe(III), U(VI) as well as Se(VI), As(V), hydrogen, sulfite, thiosulfate and sometimes sulfur, and pH for growth of *Desulfitobacterium* spp. reportedly ranged from 6.5 to 7.8 (Bouchard *et al.*, 1996; Christiansen & Ahring, 1996; Sanford *et al.*, 1996; Finneran *et al.*, 2002; Villemur *et al.*, 2006). *Desulfitobacterium hafniense* strain Y51 is the only *Desulfitobacterium* shown to reduce both sulfate and sulfite (Suyama *et al.*, 2001). Sequences related to strain Y51 were found in the cultures inoculated with acidic mine tailings; therefore, *Desulfitobacterium*-related species may contribute to sulfate reduction in these tailings. However, the pH range previously determined for this class is considerably higher than the range observed in the acidic mine tailings (pH 3.7–4.4); therefore, the species detected in this study may be unrelated to previously identified species or the pH tolerances should be adjusted for this genus. The role of *Desulfitobacterium*-type organisms in sulfur cycling in acidic tailings should be further investigated.

Desulfosporosinus spp. and *Desulfitobacterium* spp. are *c.* 95% similar to each other (Stackebrandt *et al.*, 1997). Because a number of sequences detected in the acidic tailings indicate 93–98% similarity to one or the other of these two genera, we can conclude that the acidic mine tailings examined in this study hosted several novel types of organisms related to the *Desulfosporosinus–Desulfitobacterium* branch of the *Clostridium–Bacillus* subphylum. This finding is very similar to results of a previous study, in which acidic water and sediments from inside mine workings also hosted bacteria belonging to the genera *Desulfosporosinus* and *Desulfitobacterium* (Church *et al.*, 2007).

Fortin *et al.* (1995) reported the presence of populations of sulfate-reducing *Desulfovibrio* spp. in acidic KM tailings. Because *Deltaproteobacteria*-specific primers did not detect *Desulfovibrio* spp. even when they were clearly present (as in the acetate cultures inoculated with gold mine tailings), *Desulfovibrio* spp. cannot be ruled out as possible sulfate reducers in the acidic mine tailings. In the study presented here, 47% of clones detected with the 385F 1492R primers were related to *Deltaproteobacteria*, but other groups were also detected. This may be due to the broad specificity of the 385F primer, which was designed to cover all known SRB groups within the *Deltaproteobacteria* (Klepac-Ceraj *et al.*, 2004). The 385F/1492R primer set, however, remains the most specific for detection of *Deltaproteobacteria*-related sequences.

Conclusions

DNA sequences extracted from MeHg-contaminated sediments from historic gold mines and acidic base-metal tailings dumps represented common phyla, but at the subphylum level, microbial communities reflected the distinct geochemical properties of these two environments. This study confirmed that representatives of the *Deltaproteobacteria* class are present in mine tailings, including acidic tailings. Novel and cultured lineages identified herein should be tested for Hg methylation in future research. In particular, the novel *Deltaproteobacteria* represented by clones K5_62 and K5_114, derived from MeHg-contaminated acidic tailings and closely related to other clones detected in other acid mine drainage situations, warrant further investigation.

Gram-positive, *Firmicutes*-related sulfate reducers, including *Desulfosporosinus* spp., were detected in both types of mine tailings. However, *Desulfosporosinus*-type sequences extracted from gold mine tailings were 96–98% related to cultured lineages, while acidic tailings hosted several novel phylotypes that were related to the *Desulfosporosinus–Desulfitobacterium* branch of the *Clostridium–Bacillus* subphylum of Gram-positive bacteria. Precipitates that developed in cultures inoculated with acidic tailings contained several iron-sulfide minerals, demonstrating how some of these

Gram-positive SRB may contribute to the immobilization of metals under anoxic and low pH conditions.

Acknowledgements

This research was supported by the Office of Science (BER), US DOE, Grant No. DE-FG02-07ER64373 to J.E.K. This work was performed at Florida State University and University of Ottawa.

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