



Characterization of the microbial acid mine drainage microbial community using culturing and direct sequencing techniques



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ABSTRACT

We characterized the bacterial community from an AMD tailings pond using both classical culturing and modern direct sequencing techniques and compared the two methods. Acid mine drainage (AMD) is produced by the environmental and microbial oxidation of minerals dissolved from mining waste. Surprisingly, we know little about the microbial communities associated with AMD, despite the fundamental ecological roles of these organisms and large-scale economic impact of these waste sites. AMD microbial communities have classically been characterized by laboratory culturing-based techniques and more recently by direct sequencing of marker gene sequences, primarily the 16S rRNA gene. In our comparison of the techniques, we find that their results are complementary, overall indicating very similar community structure with similar dominant species, but with each method identifying some species that were missed by the other. We were able to culture the majority of species that our direct sequencing results indicated were present, primarily species within the *Acidithiobacillus* and *Acidiphilium* genera, although estimates of relative species abundance were only obtained from direct sequencing. Interestingly, our culture-based methods recovered four species that had been overlooked from our sequencing results because of the rarity of the marker gene sequences, likely members of the rare biosphere. Further, direct sequencing indicated that a single genus, completely missed in our culture-based study, *Legionella*, was a dominant member of the microbial community. Our results suggest that while either method does a reasonable job of identifying the dominant members of the AMD microbial community, together the methods combine to give a more complete picture of the true diversity of this environment.

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1. Introduction

Extreme environments, habitats at the boundaries of where life can exist, can still be complex and dynamic systems, inhabited and shaped by an array of microorganisms adapted to the unique conditions. These harsh environments are characterized by extremes, e.g. in pH, salinity, temperature, pressure, or radiation (Vandenburg, 2003; Pikuta et al., 2007). Sites impacted by acid mine drainage (AMD), water acidified through the exposure to iron and/or sulfide mining waste, are considered extreme environments because of the low pH (1.5–3.5) and high metal, such as nickel and copper concentrations (Costa and Duarte, 2005; Denef et al., 2010). AMD sites are self-contained biogeochemical systems based on iron or sulfur oxidation as a microbial energy source

(Tyson et al., 2004). This chemolithotrophic energy system is reflected in generally lower biodiversity than other natural, or less extreme, environments (Denef et al., 2010). The relative microbial simplicity is expected to translate into better success in determining full community diversity than in more complex systems.

AMD is produced by the chemical and microbial oxidation of sulfide mineral waste rock, a by-product of the mining industry (Leduc et al., 2002). Sulfide minerals are initially oxidized to sulfates when exposed to air and moisture (Johnson, 2003). Sulfur-oxidizing neutrophiles such as species in the *Thiobacillus* and *Thiomonas* genera, then further oxidize the remaining sulfides. The initial environmental and bacterial oxidation lowers the pH of the AMD to between 4.0 and 4.5. Sulfur-oxidizing acidophiles, e.g. *Acidithiobacillus* and *Acidiphilium* species, then continue the sulfur-oxidation (Leduc et al., 2002). Ferrous sulfate (FeSO₄) is oxidized to ferric sulfate (Fe₂(SO₄)₃) by acidophilic bacteria such as *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and other iron-oxidizing acidophiles (Leduc and Ferroni, 1994; Hallberg and Johnson, 2003). Further oxidation of FeS₂ and ferric sulfate by iron oxidizing acidophiles results in the production of additional FeSO₄, which is then further oxidized as explained above. Sulfur-oxidizing acidophiles also play a role in lowering the pH by oxidizing the elemental sulfur (S⁰) to sulfuric acid

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(H₂SO₄). AMD is a severe environmental problem; the associated extremes in acidity, heavy metal concentrations, salinity, and ferric iron precipitation lead to pronounced destruction of surrounding biota (Sheoran et al., 2010; Leduc et al., 2002). AMD generation is continually occurring at operational and abandoned mine sites, as well as areas designed to contain mine waste products such as tailing ponds (Sheoran et al., 2010). In fact, AMD is the largest environmental liability affecting Canadian mining with sites covering more than 12,000 ha in Canada alone and clean-up costs estimated at between \$2 and \$5 billion dollars (MEND, 2001).

Although it is estimated that only 1–10% of microbial species can be cultured and isolated (Cardenas and Tiedje, 2008), culturing techniques continue to be used to characterize and study microbial organisms living in a variety of environments (Delavat et al., 2012; Johnson, 1995). Recent studies have successfully identified previously uncultured, and possibly unculturable, microbes from AMD samples using culture independent (i.e. molecular) methods (e.g. Bruneel et al., 2005), indicating that AMD species are likely missed by purely culture dependant methods (Bruneel et al., 2005). Molecular techniques, particularly the direct sequencing of the 16S rRNA gene from environmental samples, have greatly expanded our estimates for species diversity, uncovering members of the “rare-biosphere”, species representing <1% of the community composition as well as provided a greater degree of community characterization. However, the impact of primer bias, sequence errors, and sequencing depth is still not well understood (Kunin et al., 2010; Acinas et al., 2005), suggesting that tandem culture-dependent and culture-independent studies will give us a more complete understanding of the strengths and weaknesses of these approaches, and ultimately of these environmental systems. The ecological and functional roles of the rare biosphere are still unclear. Although the abundant community members are most often studied, the rare biosphere includes a large number of diverse species (Galand et al., 2009) that may contribute important functional roles to the greater community.

This study used a combination of classical culturing and direct sequencing techniques, comparing these two methods while determining the bacterial diversity of an AMD site in Copper Cliff, Ontario. Our goal was to compare a culture-based technique with direct sequencing, to evaluate their respective effectiveness at capturing the complete range of bacterial species in what was expected to be a less diverse microbial community. Previous culture-based studies suggested that we would identify iron-oxidizing acidophiles, sulfur-oxidizing acidophiles, sulfur-oxidizing neutrophiles, and acidophilic heterotrophs (Leduc et al., 2002). We expected to identify a greater diversity of species using the direct sequencing technique, giving us a more complete understanding of the entire community. The identification of bacterial species in the AMD site is a first step toward understanding the genetic potential and the interaction between all community members, which may lead to the discovery of specialized enzymes or metabolic pathways (Vandenburg, 2003). This work provides a reference for the use of direct/deep sequencing techniques for the mining industry, in the attempt to guide new bioremediation strategies.

2. Methods

2.1. Sample collection

Water samples were collected from a single location at the Vale Copper Cliff Central Tailings facility in October 2010 (UTM 17T 0493 953/5146510). Three sterile 1 L bottles and four sterile 120 mL bottles were filled with water from the tailings pond. The 1 L bottles were processed (DNA extraction and direct sequencing) separately and treated as replicate environment samples (samples A, B and C). Care was taken to ensure that only water, with minimal solid particles, was collected. Each 1 L bottle was individually filtered through a MicronSep membrane (pore size 0.45 µm, diameter 47 mm) immediately after sampling and the membrane stored at –80 °C. Three 120 mL samples were kept at

4 °C until processed for culturing. The fourth 120 mL sample was used to determine the pH.

2.2. Cultivation of AMD bacteria

Three 120 mL AMD samples were 1/10 serially diluted with acidified water (pH 2.5) and 0.2 mL aliquots of each sample dilution were inoculated onto solid selective culture media using the spread plate technique (Buck and Cleverdon, 1960). Plates from the dilution resulting in 30–300 colonies per plate were used to score individual colonies (below) and produce monoclonal cultures. Three separate 120 mL AMD samples were used in the cultivation of bacterial species, along with three types of solid selective media to specifically cultivate AMD bacteria. Iron tryptone soya broth (FeTSB) medium was used to isolate acidophilic heterotrophs as well as iron and sulfur oxidizing bacteria (Johnson et al., 1987). Iron-salts-purified (ISP) agarose medium was used to culture acidophilic iron-oxidizers (Manning, 1975). Washed agarose yeast extract (WAYE) medium was used to isolate heterotrophic acidophiles (Johnson, 1995). After inoculation, all plates were incubated at room temperature (~25 °C). Once colonies were visible, colony morphology including form, elevation, margin, surface, opacity, and chromogenesis (color) of each isolate, was scored and colonies were classified by morphology and media type.

Individual colonies with unique morphology (i.e. visually differentiable), and media type were transferred to liquid media and allowed to grow at room temperature (~25 °C) on a gyratory shaker at 150 rpm. Once the liquid became turbid, a small volume was streaked onto solid plates to obtain pure colonies.

2.3. Colony 16S rRNA amplification and sequencing

We amplified and sequenced the 16S rRNA gene from each cultured isolate. PCR amplification was performed on single colonies re-suspended from solid media into the PCR mixture. PCR was performed using Qiagen TopTaq PCR Master Mix Kit (California, USA) following manufacturer's protocols. Several universal bacterial 16S rRNA gene primers were used: Colonies 1–4, 6–13, and 17 were amplified using primers, 27f 5'-AGAGTTTGATCMTGGCTCAG-3' (Lane, 1991) and reverse primer 1492r 5'-TACGGYTACCTTGTTACG ACTT-3' (Turner et al., 1999). The remaining colonies, 5 and 14–16, were amplified using a combination of primers; 515R 5'-ATG GTA TTA CCG CGG CTG CTG CTG GCA-3' (Randazzo et al., 2002), and 531R 5'-CTN YGT MTT ACC GCG GCT GC-3' (Armougom and Raoult, 2009), 8F 5'-CAC GGA TCC AGA GTT TGA TYM TGG CTC AG 3' (Turner et al., 1999) and 1492r. We were unable to amplify a 16S rRNA fragment from colonies 5, 14–16 using a disrupted colony as DNA source. For these colonies, we instead precipitated the DNA from 500 µL of cultured liquid media with 50 µL of 3 M sodium acetate and 1000 µL of 95% ethanol. The resulting DNA pellet was washed and re-suspended in sterile water and 1 µL was used as template in the PCR reaction. DNA template from a laboratory sample of *Acidithiobacillus thiooxidans* (ATCC # 19377) was amplified as a positive control and a reaction mixture without the addition of template DNA was used as a negative control. PCR reactions of 50 µL were performed using an initial denaturation step of 10 min at 94 °C followed by 30 cycles of, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Several colonies needed a second amplification reaction in which 5 µL of an initial PCR reaction was added to a subsequent PCR mix that used the same protocol. After amplification, reactions were stored at –20 °C and amplification of a single product was confirmed by agarose gel electrophoresis using 1% agarose gels (1.0 g agarose, 100 mL 1× TBE (0.45 M Tris, 0.45 M Boric acid, 10 mM EDTA)) and 1 µL ethidium bromide to expose the DNA under UV light. Gels were run at 100 V constant for 1 h. Colony PCR products were sent to Genome Québec, (Montréal, Canada) for sequencing of the amplified 16S rRNA gene fragment.

2.4. 16S rRNA phylogenetic analyses

Sequence files were edited by eye using CodonCode Aligner software (CodonCode Inc.) to manually verify base calls. Where possible, the edited forward and reverse sequences were aligned to generate a single contiguous sequence from each colony. Colonies were identified to species or genera using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) matches to known organisms within the National Center for Biotechnology Information (NCBI) nucleotide database for each isolate. A neighbour-joining phylogenetic tree was generated using pairwise deletion and the Maximum composite likelihood model of sequence evolution with 1000 bootstrap replicates implemented in MEGA 4.0 (Tamura et al., 2007) was generated from the 16S rRNA sequences from both the cultivated isolates, the corresponding direct sequencing members (below), and the top two BLAST results for each isolate. Local BLAST searches were performed using BioEdit (version 7.0.9) against the pyrosequencing reads (see below) to determine if the sequences we found in the cultured colonies were also found by direct sequencing.

2.5. Pyrosequencing analysis

Total DNA for direct sequencing was extracted by membrane filtration of the 1 L AMD samples using 0.45 µm filter and a PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., California, USA) following the manufacturer guidelines. Total DNA was extracted from the three separate 1 L AMD samples. A Nanodrop spectrophotometer (Nanodrop 8000, Thermo Scientific, Ottawa, ON) was used to quantify DNA concentrations and samples were then stored at –80 °C until further processing. 50 µL of the extracted DNA solution was sent to the Research and Testing Laboratory in Lubbock, Texas, for pyrosequencing of a fragment of the 16S rRNA gene using the universal bacterial primers 28F (5'-GA GTT TGA TCM TGG CTC AG-3') and 519R (5'-GWA TTA CCG CGG CKG CTG-3') (Turner et al., 1999) using a Roche 454 FLX platform (454 Life Sciences, Branford, Connecticut).

The sequence reads were processed and analyzed using the Ribosomal Database Project (RDP) pyrosequencing pipeline (Cole et al., 2009) including: alignment, clustering, and dereplication. Clustering was done at distance cutoff values of 0.01 and 0.03 (see Supplemental Figs. 1 and 2). Taxonomical classification of each cluster was confirmed using NCBI BLAST searches. The three samples were analyzed separately and species composition was compared across all three replicates. Finally, rarefaction curves were generated to show the level of diversity captured by each sample.

3. Results

3.1. Isolation of bacteria

We identified 17 distinct colonies, differing in morphology, color, and/or media growth requirements (pH 2.92), from the AMD samples. Individual colonies were isolated for continued culture and molecular identification. Isolates 1–8 and 15–17 were grown on WAYE media, isolates 9–11 on FeTSB media, and isolates 12–14 on ISP media. A summary of colony morphology is shown in Table 1 and photographs of each isolate in Supplemental Fig. 1. Suspected AMD fungal species were found on each type of media but were not considered in this study.

3.2. Amplification of isolate 16S rRNA

The near complete 1400pb 16S rRNA gene sequence was obtained for all isolates except 5, 14, 15, and 16. These last four isolates proved difficult to amplify using the universal 27f and 1492r primers, but we were able to amplify and sequence smaller fragments within the V1–V3 regions of the gene from these colonies using nested primer PCR

reactions (following Lane et al., 1985). All 17 isolates were identified to genus or species by conducting a BLAST search of the NCBI database (Table 2). We identified six genera across the cultured isolates: *Acidiphilium*, *Acidocella*, *Acidithiobacillus*, *Acidobacterium*, *Alicyclobacillus*, and *Halomonas*. Our phylogenetic analysis of the 16S rRNA gene sequences included the isolates, and the two highest scoring BLAST matches of each isolate, depicting the evolutionary relationship and distance between isolates (Fig. 1).

The majority of the isolates (10 out of 17) were determined to belong to *Acidiphilium*, a genus commonly found in AMD sites (Zhang et al., 2007). Isolates 2, 3, 8 and 10 were identified as *Acidiphilium multivorum* with individual isolates differing in morphology (Supplemental Fig. 1). These isolates are likely different strains or morphotypes, within this species (Table 1). Isolate 4 showed high sequence similarity to *Acidiphilium* species with 99% identity (Table 1) while isolate 6 showed a 99% sequence similarity to *Acidiphilium angustum*. Isolates 11, 12 and 15 all were found to have at least 96% identities (Table 2) and were closely related to *Acidiphilium rubrum* while isolate 16 was identified as *Acidiphilium acidophilum*.

Isolates 9 and 13 were identified as *A. ferrooxidans*, while isolates 17, 7 and 1 were each identified to different genera. The sequence from isolate 17 was identified with a 99% sequence similarity to the *Acidobacteriaceae* family, and isolates 7 and 1 were identified as *Acidocella facilis* and *Granulicella paludicola* with 99% and 97% sequence similarities respectively. We were only able to amplify and sequence smaller 16S rRNA fragments from isolates 5 and 14: 738 continuous bases for colony 5 within the V5–V9 regions, and a continuous 588 base pair fragment of isolate 14 within the V1–V3 regions. BLAST search of the sequence obtained from isolate 14 identified its closest match as *Alicyclobacillus pohliae*, a gram positive, aerobic, acidophilic bacterium not previously identified in AMD. Our BLAST search of isolate 5 found the closest sequence similarity to *Halomonas ventosae*, a gram negative, high salt tolerant, halophilic proteobacteria, also not previously identified in AMD.

3.3. Pyrosequencing data

We obtained over 5000 reads of the V1–V3 regions of the 16S rRNA gene for each replicate water sample: 5935 for sample A, 5579 for sample B, and 7920 for sample C. These sequences were classified to taxonomic levels according to sequence similarity. The majority of

Table 1
Colony and cell morphology for each bacterial isolate.

Isolate #	Isolation media	Colony morphology
1	WAYE	Circular, raised, entire, smooth, opaque, pink
2	WAYE	Circular, raised, entire, wrinkled, opaque, beige
3	WAYE	Circular, umbonate, lobate, smooth, opaque, beige with darker center
4	WAYE	Circular, raised, entire, wrinkled, opaque, beige with darker center
5	WAYE	Circular, raised, entire, wrinkled, opaque, peach
6	WAYE	Circular, umbonate, entire, smooth, opaque, brown
7	WAYE	Circular, umbonate, lobate, smooth, opaque, beige
8	WAYE	Circular, raised, entire, smooth, opaque, beige
9	FeTSB	Circular, flat, undulate, wrinkled, opaque, orange with brown center
10	FeTSB	Circular, raised, lobate, smooth, opaque, beige
11	FeTSB	Irregular, raised, undulate, smooth, opaque, light brown
12	ISP	Circular, raised, entire, smooth, opaque, white/beige
13	ISP	Circular, raised, lobate, smooth, opaque, orange with brown center
14	ISP	Filiforme, yellow
15	WAYE	Circular, raised, entire, smooth, opaque, red/brown
16	WAYE	Circular, raised, entire, smooth, opaque, grey/green
17	WAYE	Circular, raised, entire, smooth, opaque, red

Table 2

Partial 16S rRNA sequence alignment results from NCBI BLAST searches for each isolate. Direct sequencing representatives show the number of direct sequence reads obtained per cultured isolate within the triplicate samples.

Isolate #	Sequence length (bp)	BLAST ID (closest cultured representative)	Accession #	E Value	Sequence coverage (%)	Max identity (%)	Direct sequencing representatives (A) (B) (C)		
1	1365	<i>Granulicella paludicola</i>	FR716684	0.0	99	97	3	4	1
2	1358	<i>Acidiphilium multivorum</i>	AP012035	0.0	100	99	77	91	94
3	1345	<i>Acidiphilium multivorum</i>	AP012035	0.0	99	99	77	91	94
4	1355	<i>Acidiphilium sp.</i>	DQ355186	0.0	99	99	77	91	94
5	738	<i>Halomonas ventosae</i>	NR_042812	0.0	100	97	NA	NA	NA
6	1343	<i>Acidiphilium angustum</i>	NR_025850	0.0	97	99	77	91	94
7	1340	<i>Acidocella facilis</i>	NR_025852	0.0	99	99	1	2	1
8	1347	<i>Acidiphilium multivorum</i>	AP012035	0.0	99	99	77	91	94
9	1392	<i>Acidithiobacillus ferrooxidans</i>	DQ676506	0.0	100	99	199	189	278
10	1342	<i>Acidiphilium multivorum</i>	AP012035	0.0	100	99	77	91	94
11	1348	<i>Acidiphilium rubrum</i>	NR_025854	0.0	99	99	77	91	94
12	1296	<i>Acidiphilium rubrum</i>	NR_025854	0.0	99	96	77	91	94
13	1383	<i>Acidithiobacillus ferrooxidans</i>	EU839491	0.0	100	99	808	352	1014
14	588	<i>Alicyclobacillus pohliae</i>	NR_042184	0.0	92	90	NA	2	NA
15	837	<i>Acidiphilium rubrum</i>	NR_025854	0.0	100	99	77	91	94
16	844	<i>Acidiphilium acidophilum</i>	NR_036837	0.0	100	98	77	91	94
17	1349	<i>Acidobacteriaceae bacterium</i>	FN870338	0.0	97	99	3	4	1

*Direct sequencing of *Acidiphilium* species could only be confidently (80%) assigned to the genus level.

the sequences fall within one phylum, Proteobacteria, within which we identified three dominant genera: *Acidithiobacillus*, *Legionella* and *Acidiphilium*. *Acidithiobacillus* was the most abundant genus, representing ~90% of the sequences (88.9% in A, 89.3% in B, and 91.3% in C), followed by *Legionella* at ~6% (6.5% in A, 5.6% in B, and 4.7% in C) and then *Acidiphilium* at ~3% (2.6% in A, 3.2% in B, and 2.4% in C). We examined the reproducibility of our community structure by comparing our results across the three replicates. For each sample, we identified representative sequences (sequence chosen from many identical replicate sequences with the highest quality and length to serve as operational taxonomic units (OTUs)) using the RDP pyrosequencing pipeline. These OTUs are matched taxonomically to genus and species to allow comparison of sequences across the water samples. Six representative sequences were identified for sample A, eight for sample B and seven for sample C. Clustering of the sequences groups the OTUs, or phylotypes, based on the amount of sequence similarity. The sequence alignment for all three replicate sample OTUs, as well as by their clustering (97% similarity), indicates similar composition between the three samples at the genus/species level. The sequences were clustered into three groups at the 0.03 distance cutoff (Supplemental Table 1); consistent with the three dominant genera previously identified (*Acidithiobacillus*, *Acidiphilium*, and *Legionella*). However, at a more stringent level of sequence similarity (>99% similarity) the representative sequences are divided into 5 clusters (same genera) (Supplemental Table 2), possibly indicating the presence of multiple sub-species or strains within each replicate.

Rarefaction curves, plots indicating the degree of species richness based on sequencing depth (Heck et al., 1975), were generated for a compiled replicate dataset and each sample replicate (Supplemental Fig. 1a and b). These plots are often used to demonstrate the species

richness based on the number of samples between replicates, environments, or habitats for example (Hughes et al., 2001). Rarefaction curves are used to indicate the percentage of species sampled by a number of reads, where as more reads will identify additional OTUs until all species are found. Samples A and B captured 120 OTUs from 4968 sequences and sample C captured 160 OTUs from 6720 sequences (Supplemental Fig. 2B). While none of the samplings reached saturation, a large number of OTUs were captured overall and rarefaction curves began to reach the plateau (Supplemental Fig. 2B) suggesting the large majority of species were sampled.

Local BLAST searches of the colony 16S rRNA sequences against our pyrosequencing data identified matches for all colony sequences, except colony 5. The 16S rRNA fragment that we amplified from this isolate did not overlap with the pyrosequencing fragment.

4. Discussion

We used traditional culturing methods and direct pyrosequencing techniques to characterize the bacterial species found at an AMD site in Copper Cliff, Ontario. The techniques were complementary and we found that culture techniques captured the majority of dominant species found by direct sequencing. The direct sequencing results provide us an idea of relative species abundance not present in the culture-based technique and the culture methods do underestimate diversity, although not to the extreme extent we had predicted. Interestingly, culture techniques did identify “rare” (low abundance) species that would have been commonly disregarded in the analysis of the direct sequencing data. With the use of both techniques we were able to identify the presence of several species that would have been missed with the use of one characterization technique alone.

4.1. Cultured AMD bacteria and species definitions

Each colony was identified to genus or species level by comparison of a fragment of the 16S rRNA gene to sequences deposited in international databases. Fifteen of the 17 isolates were inferred to be species previously associated with AMD. Two isolates, 5 and 14, were inferred to be from the *Halomonas* and *Alicyclobacillus* genera, respectively. Although these genera have not been previously described in AMD sites, they have been found in similar environmental conditions of extremely low pH and high metal concentrations (Lopez-Archilla et al., 2004; Johnson and Hallberg, 2007; Guo et al., 2009). Ten of the isolates were inferred to be species from the genus *Acidiphilium*, a genus that includes acidophilic mixotrophs with an optimal pH of 3.0 (Johnson, 1995; Hao et al., 2010). As mixotrophs, these species can switch between chemoheterotrophy and chemoautotrophy depending on environmental conditions, an advantage in oligotrophic environments such as AMD (Hao et al., 2010). Further, many species of *Acidiphilium* have also shown a resistance to heavy metals such as copper, nickel and zinc commonly found in AMD (Mahapatra and Banerjee, 1996).

Our BLAST similarity and phylogenetic analysis of the 16S rRNA fragment from isolates 2, 3, 8 and 10 identified all four as *A. multivorum*, and all with 99% sequence identity, even though these isolates each have distinct phenotypes, such as colony shape, color, or media requirements. Similar cases of distinct biology, but similar or identical 16S rRNA gene sequence, have previously been reported (Karavaiko et al., 2003; Pace, 2009). Such cases of dissimilar biology associated with identical marker sequence make interpreting different colonies based on traits difficult; it is unclear how phenotypes should be prioritized, weighted, or discounted. Further physiological chemotaxonomic tests, and molecular phylogenetic analysis using additional molecular sequences, could be used to determine if these are different *Acidiphilium* species or morphotypes, but are beyond the scope of this project. Additional *Acidiphilium* species were also identified; isolates 11, 12, and 15 are similar to *A. rubrum*, a species that has been shown to share similar characteristics, such as nutrient growth requirements and physiology,

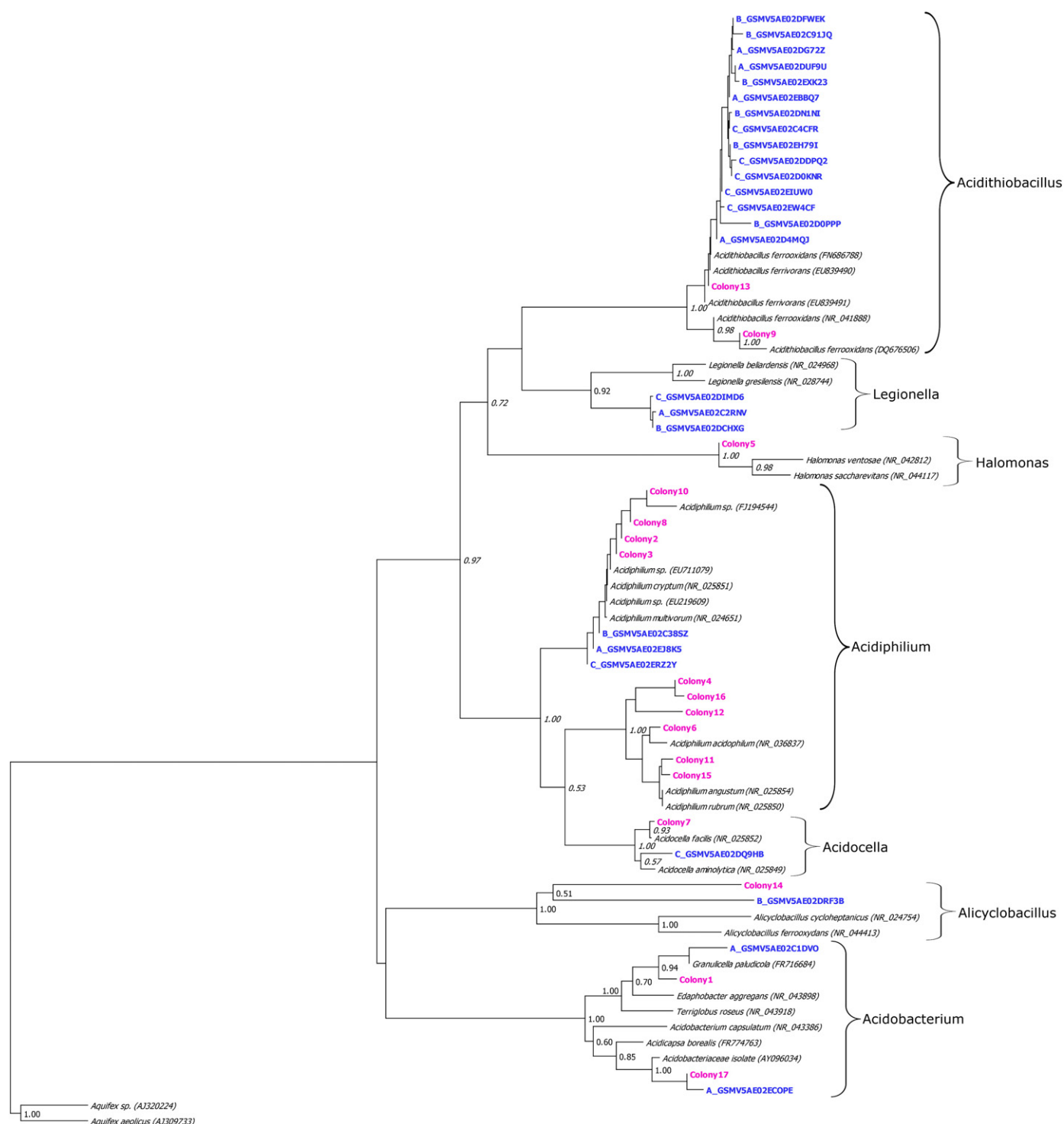


Fig. 1. Phylogeny depicting evolutionary relatedness of 16S rRNA gene of cultured isolates (pink), direct sequence representatives (blue), and reference sequences (italicized). Phylogeny created using neighbour joining and maximum composite likelihood model with pairwise deletion and 1000 bootstrap replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to other species within the *Acidiphilium* genus (Wichlacz et al., 1986). Finally, isolate 16 is similar to *A. acidophilum*, also commonly found in AMD sites (Johnson et al., 2001; Zhang et al., 2007).

Isolates 9 and 13 were identified as *A. ferrooxidans*, another bacterial species commonly found in AMD (Leduc et al., 2002; Duquesne et al., 2003) and previously cultured in the laboratory (Deneff et al., 2010). This species is an acidophilic chemolithotroph that is resistant to high

heavy metal concentrations (Duquesne et al., 2003) and oxidizes ferrous iron, or reduced sulfur compounds, as a source of energy (Mahmoud et al., 2005). *A. ferrooxidans* has been regarded as the most important species accelerating the dissolution of metals and the oxidation of iron species within AMD; the rate at which AMD is produced is largely determined by *A. ferrooxidans* and *L. ferrooxidans* (Long et al., 2003; Nordstrom and Southam, 1997).

Isolates 17, 7, and 1 were each assigned to different genera. Isolate 17 was found to be related to the *Acidobacteriaceae* family, a group previously isolated from other AMD sites (Hallberg and Johnson, 2003; Yang et al., 2007). Isolate 7 was identified as *A. facilis*, an acidophilic Proteobacteria that is resistant to metals (Ghoch et al., 1997; Wakao et al., 2002) and has also been detected in similar AMD environments (Johnson et al., 2001). Isolate 1 was identified as *G. paludicola*, an unusual genus and species only recently proposed (Pankratov and Dedysh, 2010) to be part of the Acidobacteria phylum. *Acidobacteria* are acidophilic, psychrotolerant, heterotrophs able to grow in low pH conditions (3.0–7.5) and a wide range of temperatures (2–33 °C), conditions consistent with our study site.

Isolate 14 showed greatest similarity to the genus *Alicyclobacillus*; *A. pohliae*, and *Alicyclobacillus ferrooxidans*, a known sulfur and iron oxidizing species (Jiang et al., 2008), were the most similar cultured species (90% and 89% sequence identities respectively). Other members of *Alicyclobacillus* are thermo-acidophilic, spore forming, heterotrophic organisms, growing at a pH range of 2 to 6 (Jiang et al., 2008; Guo et al., 2009). Although most members of the genus *Alicyclobacillus* have been found in acidic geothermal vents and soils (Guo et al., 2009), several species such as *Alicyclobacillus tolerans*, *Alicyclobacillus disulfidooxidans*, and *A. ferrooxidans* have been found to oxidize not only ferrous iron, but also elemental sulfur and sulfides (Kovalenko and Malakhova, 1983; Dufresne et al., 1996; Jiang et al., 2008). *Alicyclobacillus* species have also been reported in other acidic copper mine drainage sites (Johnson and Hallberg, 2007; Guo et al., 2009).

Isolate 5 shows similarity to the *Halomonas* genus according to our BLAST analysis using the smaller, 738 bp, 16S rRNA fragment we were able to amplify from this isolate. *Halomonas* species have not previously been associated with AMD sites, but have been associated with AMD-like environments. *Halomonas* species are halophiles; extremophile organisms that can survive in areas of high concentration of salt (Franzmann and Tindall, 1990), and most commonly found in water surrounding deep-sea vents, and are capable of utilizing a variety of carbon and nitrogen sources (Franzmann and Tindall, 1990; Mata et al., 2002). A study of ocean microbes identified a novel iron oxidizing bacteria related to the *Halomonas* genus and not closely related to any previously known iron oxidizers or autotrophs (Edwards et al., 2004). Similarly, a bacterial strain related to the *Halomonas* genus was found to oxidize ferrous iron and reduce nitrate within the seafloor crust of the Juan de Fuca Ridge (Smith et al., 2011). Perhaps most directly relevant to this system, *Halomonas* related phylotypes have been reported in the acidic Tinto River, Spain (Lopez-Archilla et al., 2004) a natural environment similar to our AMD environment.

4.2. Pyrosequencing

Our direct sequencing analysis identified three dominant genera: *Acidithiobacillus*, *Legionella*, and *Acidiphilium*. All three have been previously associated with AMD environments, although *Legionella* much less so. Ninety percent of the 16S rRNA sequences included the genus *Acidithiobacillus* mainly two species *A. ferrooxidans* and *Acidithiobacillus ferrivorans*, typically found in AMD environments (Leduc et al., 2002). *Acidiphilium*, the third most abundant genus (3% of the 16S rRNA reads), are also commonly found in AMD environments (Hao et al., 2010). *Legionella* was the second most abundant genus (6% of the 16S rRNA reads). *Legionella* species are generally neutrophilic chemoorganotrophs typically found in damp, naturally occurring habitats, such as soils, ponds, and streams as well as humid man-made environments like air conditioning cooling towers and evaporative condensers (Hao et al., 2010). This genus is not typically found in AMD environments and generally grows best under neutral conditions (pH 6.8–7.0). There has, however, been an earlier reported case of *Legionella* in AMD from a sulfide mine in China (Hao et al., 2010). These authors hypothesized that the presence of *Legionella* may be linked to the ciliated protozoans and amoeba populations also found in their sample. *Legionella* is known

to resist digestion and replicate within vacuoles of 13 species of amoeba and two species of ciliated protozoa, living in an endosymbiotic relationship within these eukaryotic cells in the community (Murga et al., 2001). The AMD *Legionella* could be surviving in these extreme environments by essentially “hiding” within a eukaryotic host (Hao et al., 2010). We did not screen for eukaryotes in our AMD microbe sample, but future research will pursue this hypothesis. Why *Legionella* has not been found more commonly at AMD sites is more difficult to explain, but could reflect the large variability in community structure between AMD sites (Kock and Schippers, 2008). Further analyses examining the eukaryotic populations at a variety of AMD sites are necessary to determine the presence or absence of *Legionella* between sites. Eukaryotic species are present in AMD but are generally less studied than bacteria, although they are thought to play an important role in the cycling of carbon molecules and overall community metabolism (Baker et al., 2009).

Rarefaction curves can be used to compare the observed species abundance with sampling depth in order to compare sites or samples, or as a general method to determine the number of species present in an environment (Heck et al., 1975; Gotelli and Colwell, 2001; Hughes et al., 2001; Sogin et al., 2006). Our compiled rarefaction curve (Supplemental Fig. 2A) shows a slight levelling off suggesting that we have sampled the majority of species richness with only 5000 sequences/sample, in contrast to aquatic and soil environments where greater species richness requires greater sequencing depth (Vasileiadis et al., 2012). The rarefaction curves were generated using the Ribosomal Database Projects: a curve for each of the individual replicates (Supplemental Fig. 2B), and a complete compiled curve including all three sequence clusters (Supplemental Fig. 2A). Overall, the direct sequence analysis suggests that we have characterized the majority of, but possibly not all, species. This conclusion is consistent with our culture results in which we identified 16 of 17 colonies in the direct sequence results, but did fail to find a match to isolate 5, likely *H. ventosae*, that was not found by our direct sequencing.

For the majority of our analyses, we have followed convention (Huse et al., 2010; Sogin et al., 2006) and disregarded those sequences that account for less than 1% of the total reads from our primary analyses. The filtered 1% includes errors in sequencing, but potentially also an unknown number of rare species (Huse et al., 2010). Direct sequencing techniques cannot distinguish between these two groups (errors and rare species). To avoid misrepresenting these errors as species in a sample, sequences that occur only infrequently are often discarded; but rare species almost certainly exist and have been found in similar AMD environments. The numbers and biological relevance of these species, however, remain controversial (Delavat et al., 2012; Pester et al., 2010). Interestingly, we found that 4 of the 17 unique isolates, cultured isolates 1, 7, 14 and 17, are part of this 1% of the sequence data. In the case of isolate 14, only two sequences were found within one of the direct sequencing triplicates (Table 2) — while this species was still cultured. Although the most abundant community members are assumed to carry out the majority of the biological processes within an AMD system, the importance of rare species cannot be ignored (Denef et al., 2010; Delavat et al., 2012). These rare species are likely integral parts of the community as a whole with roles in biochemical cycles and forming mutualistic interactions with other species (Galand et al., 2009). Further, with changes to the environment such as changes in temperature, pH, or nutrient availability, these rare species could potentially thrive and become increasingly dominant giving an overall environmental plasticity to the microbial community. It is likely that there are additional rare species that we were unable to culture and confirm, but our ability to culture some of these species, as well as the dominant species, not only indicates that members of the AMD rare biosphere are in fact viable but will allow us to test the biological roles and interactions of these species individually and as a group. We do not mean to suggest that all sequences within the rare 1% represent true rare species, sequencing errors and artefacts likely make up the majority of these sequences (Kunin et al., 2010; Huse et al., 2010; Galand et al., 2009).

5. Conclusion

We find that culture-dependent and culture-independent methods for characterization of AMD microbial diversity give largely congruent results, but that each method sheds light on specific, important, limitations of the other. Worldwide, acid mine drainage is the largest source of mining-related pollution (Tsukamoto and Miller, 1999). As a result, many prevention and/or control methods have been attempted including chemical addition to neutralize the water and precipitate metals, control of water flow, modification and storage of wastes, and final processing methods to prevent oxidation (Akciil and Koldas, 2006; Johnson and Hallberg, 2005). The fundamental contribution of bacteria to the development of AMD is well established (e.g. Leduc and Ferroni, 1994; Hallberg and Johnson, 2003); however the diversity within the microbial community at AMD sites is still not well characterized. Traditionally, microbial diversity was assayed through cultivation and species identification through morphology, selective media requirements, and physiological and biochemical traits (Amann et al., 1995). Currently, culture independent methods are more commonly used than culture dependant methods, but few comparisons of their strengths and weaknesses have been documented on AMD sites. We compared culture-based characterization with direct sequencing of the 16S rRNA gene and find that the two methods produced very similar results, i.e. we are able to culture the majority of the abundant species identified by direct sequencing. Our direct sequencing results did, however, identify a greater number of species and give us a much more accurate idea of relative species abundance than our culture method, consistent with earlier studies (Cardenas and Tiedje, 2008; Galand et al., 2009). As an extreme example, direct sequencing identified an entire genus, *Legionella*, which was missed in our culturing survey. *Legionella* is a relatively recent addition to our understanding of the AMD microbial community and was not cultivated likely because it is a neutrophilic heterotroph. This finding highlights the importance of appropriate, and diverse, culture conditions in culture-based studies. Culture techniques are continuing to improve, increasing the diversity of microbes than can be identified from AMD sites using culture based methods (Delavat et al. 2012). Had we known less about the unique environmental conditions of the AMD environment, the number of species missed in our culture analysis would almost certainly be higher. Interestingly, our culture-based survey identified 4 species that we initially missed in our analysis of our direct sequence data. Direct sequencing, by its very nature, produces large volumes of data making it difficult to reliably separate rare species, which are represented by few sequence reads, from sequencing errors, which are also represented by few sequence reads. The four species identified in our culture-based survey and initially missed in our analysis of our direct sequence data are likely members of the “rare biosphere”. Verification of these four rare biosphere species through culturing highlights the need for better algorithms and/or sequencing technologies to accurately characterize viable and low abundance microbial community members. Although the predominant species are thought to drive most biological processes in this (or any) system, testing this assumption requires culturing these species for laboratory experimentation. The inclusion of additional culturing media and techniques would almost certainly identify additional rare species similar to the four identified here. Similarly the use of additional primer sets and greater sequencing depth could also provide a better overall characterization of the AMD community. Our results, similar to previous findings, indicate that the overall community may be more diverse than previously thought and that additional work needs to be done on less abundant genera such as *Alicyclobacillus* and *Halomonas* to determine their in situ AMD roles.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2013.01.023>.

Conflict of interest

The authors declare that they have no conflict of interest.

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