



A novel acidophile community populating waste ore deposits at an acid mine drainage site

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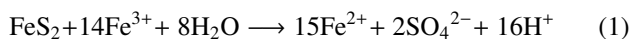
Abstract

Waste ore samples (pH 3.0) were collected at an acid mine drainage (AMD) site in Anhui, China. The present acidophilic microbial community in the waste ore was studied with 16S rRNA gene clone library and denaturing gradient gel electrophoresis (DGGE). Eighteen different clones were identified and affiliated with *Actinobacteria*, low G + C Gram-positives, *Thermomicrobia*, *Acidobacteria*, *Proteobacteria*, candidate division TM7, and *Planctomycetes*. Phylogenetic analysis of 16S rRNA gene sequences revealed a diversity of acidophiles in the samples that were mostly novel. It is unexpected that the moderately thermophilic acidophiles were abundant in the acidic ecosystem and may play a great role in the generation of AMD. The result of DGGE was consistent with that of clone library analysis. These findings help in the better understanding of the generation mechanism of AMD and in developing a more efficient method to control AMD.

Key words: acid mine drainage; acidophile; 16S rRNA; DGGE; waste ore

Introduction

A serious ecological problem caused by the mining industry is the occurrence of acid mine drainage (AMD). The mining wastewaters typically contain high levels of metal ions, such as iron, copper, aluminum, and manganese, as well as metalloids of which arsenic is generally of greatest concern. AMD generates when metal sulfide minerals, particularly the pyrite (FeS_2), comes in contact with oxygen and water. The overall reaction can be written as:



Fe^{3+} is the predominant oxidant at low pH and is usually the limiting reagent. The inorganic oxidation rate of ferrous ion (Fe^{2+}) below pH 3 is slow. Acidophilic organisms, however, can generate energy by converting ferrous iron to ferric iron. Hence, the oxidation of pyrite and acidification of the AMD is greatly increased in the presence of iron-oxidizing species, such as *Acidithiobacillus ferrooxidans* (Johnson, 1998).

A. ferrooxidans is the best-studied organism that catalyzes the generation of AMD. Models have been proposed for its energetic characteristics and the role in pyrite dissolution. Many investigations have studied iron-oxidizing and respiratory enzymes since its isolation in 1947 (Rawlings, 2001; Rojas-Chapana and Tributsch, 2001;

Gonzalez-Toril *et al.*, 2003). *A. ferrooxidans* has long been considered the main organism leading to the extreme conditions of AMD systems. Only recently, molecular ecology studies have shown that other iron-oxidizing microorganisms, such as *Leptospirillum* spp. and *Ferroplasma* spp., might be more important than *A. ferrooxidans* in the generation of acidic environments (Schrenk *et al.*, 1998; Edwards *et al.*, 2000).

Most AMD-related acidophilic microbes are sensitive to organic substances and grow at an extremely low rate on solid medium. These traits make it very difficult to culture these bacteria as pure cultures. Many researchers have used the rRNA approach to detect microbial populations and describe the structures of microbial communities in various environments without isolating the component microorganisms (Godon *et al.*, 1997; Watanabe *et al.*, 2000; Zhang *et al.*, 2004). These studies have shown that most 16S ribosomal RNA gene sequences directly amplified from environmental samples were different from the sequences of comparable laboratory strains. It has been concluded that microbial diversity in the natural environment is much greater than the diversity of the bacteria that have been isolated (Orphan *et al.*, 2001; Watanabe *et al.*, 2002). The introduction of molecular biology techniques, such as denaturing gradient gel electrophoresis (DGGE), and cloning has led to significant advances in the field of microbial ecology (Hugenholtz *et al.*, 1998).

The field site was the Nanshan waste ore deposits at the Mountain Xiang, Anhui, China, located approximately 20 km northwest of Maanshan City. The dominant ore body

of Mountain Xiang is pyrite (more than 90%), which has been mined for more than 50 years. Mining operations cracked the soil and rock that lead to an increase in permeability and large numbers of unprocessed deposit. Most of the waste ores were deposited in the Nanshan refuse dump at Mountain Xiang, where the rotten waste ore was sampled. These activities have increased the sulfide mineral surface area and the total reactive ore that was exposed to oxygen and surface water. The exposed ore was oxidized by acidophiles and released large amounts of acid drainage mine and formed an acid lake, the volume of which was about $3 \times 10^6 \text{ m}^3$.

In this study, a molecular characterization of the microbial diversity and composition of the rotten waste ore taken from the site beside the acid lake are presented. The results considerably extend the knowledge of the diversity of microorganisms in AMD environments.

1 Materials and methods

1.1 Sample collection

Two parallel waste ore samples were collected from the Nanshan deposits on Mountain Xiang (Anhui, China) on August 6, 2005. The samples were all representative rotten waste ore that generated AMD when the acidophilic microorganisms were present. The sites were situated on the banks of the AMD lake that was 5 m above water level. The waste ore samples were collected directly into a sterile container and stored below 4°C until processing within 2 d. Field measurement of pH was carried out using a pH meter (Thermo Orion, USA). The element analysis was conducted by Analytical and Testing Center, Beijing Normal University, using an inductively coupled plasma emission spectrometer (JYI-ULTIMA, France).

1.2 DNA extraction and purification

The method used for extraction of nucleic acids from waste ore was based on the protocol described previously (Zhou *et al.*, 1996) and included initial wash steps to exclude iron and raise the pH before cell lysis. Extractions were performed on 1.5 g samples that were pelleted by centrifugation, washed twice with 5 ml 0.02 mol/L H_2SO_4 and then with 5 ml STE buffer (sucrose 100 mg/ml, Tris-HCl 50 mmol/L, EDTA 10 mmol/L, NaCl 100 mmol/L, pH 8.0). Subsequently, the samples were resuspended in 3 ml DNA extraction buffer (sucrose 100 g/L, Tris-HCl 50 mmol/L, EDTA 10 mmol/L, NaCl 100 mmol/L, 10 g/L CTAB, pH 8.0), and 50 μl lysosyme (10 mg/L) was added to the suspension that was then incubated for 40 min at 37°C. After 50 μl Proteinase K (10 mg/ml) and 1 ml 10 g/L SDS were added, the mixture was further incubated for 45 min at 50°C. The supernatants were collected after centrifugation at $6000 \times g$ for 10 min at room temperature and transferred into new centrifuge tubes. The cell lysates were extracted twice with an equal volume of chloroform:isoamylalcohol (24:1, v/v). The nucleic acids were precipitated from the aqueous phase with 2 volumes of ethanol at -20°C for 2 h. The pellet of nucleic acids was

obtained by centrifugation at $12000 \times g$ for 20 min at 4°C, washed with ice cold 70% ethanol, and dissolved in 50 μl sterile deionized water.

1.3 PCR and fractionation of 16S rRNA genes

The 16S rRNA genes of the microbial community were amplified from the genomic DNA of the waste ore with the primer pair 27f (5'-AGA GTT TGA TCM TGG CTC AG-3', M=C or A) and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3', Y=C or T) complementing positions 8 to 27 and 1510 to 1492 of *Escherichia coli* 16S rDNA (Kellner, 2002). PCR cycling was performed with Applied Biosystem Gene Amp PCR system 2700. PCR amplification reaction conditions were as previously described (Sambrook and Russell, 2001). PCR products were purified using EZ-10 spin column DNA Gel Extraction kit (BBI, Canada), quantified by ethidium bromide-UV detection on an agarose gel, ligated into the pGEM-T easy vector (Promega, USA). Then the resulting plasmids were transformed into *E. coli* DH5 α following the manufacturer's instructions.

1.4 ARDRA analysis and sequencing of inserted 16S rRNA genes

For amplified rDNA restriction analysis (ARDRA) and sequencing, the inserted fragment was amplified by PCR and template DNA was provided by contacting a small pipette tip with a colony of cloned host cells which were immersed in the PCR mixture. Primers for the PCR were the vector-specific T7 (5'-TAA TAC GAC TCA CTA TAG GGC-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG AAT ACT C-3'). The PCR products were digested with two four-base-specific restriction enzymes (Rsa I and Msp I) (Toyobo, Japan). The procedure of restriction enzyme digestion of the unpurified PCR products and observation of restricted fragments were as previously described (Picard *et al.*, 2000). ARDRA patterns were grouped and representative cloned fragments were sequenced by the BGI LifeTech Co., Ltd., China.

1.5 Phylogenetic affiliation of isolates

The sequences obtained in this study were inspected for chimeric artifacts by the check-chimera program of the Ribosomal Database Project (RDP). These sequences were also compared with the 16S rDNA sequences deposited in public databases using the BLASTTM search program. The 16S rRNA gene sequences of various bacteria (including those closely related to the unknown sequences, as indicated by the BLAST search) obtained from the GenBank database were aligned with the new sequences using ClustalXTM. The phylogenetic tree was constructed by the neighbor-joining with robustness of 1000 bootstrapping using PHYLIP 3.65. Phylogenetic trees were viewed by TreeviewTM.

1.6 PCR amplification of 16S rRNA gene fragments and DGGE analysis

The primers 968f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA

GAA CCT TAC-3') and 1401r (5'- GGT GTG TAC AAG ACC C-3') were used for amplification of 16S rRNA gene fragments for DGGE. The fragment comprises of variable regions V6 to V8 of 16S rRNA gene positioned from nucleotide 968 to nucleotide 1401 (*E. coli* numbering). PCR conditions were as previously described (Watanabe *et al.*, 2001).

DGGE of PCR products were performed using the Dcode™ System (BioRad, USA). Polyacrylamide gels (80 g/L of a acrylamide:bisacrylamide (37:1) mixture in 1×TAE buffer), with a gradient of 40%–60% denaturant, were made with a gradient maker according to the manufacturer's guidelines. Gels were run for 12 h at 100 V in 1×TAE buffer at a constant temperature of 60°C. The gels were stained for 20 min with EB. Gel images were obtained using the Gel Doc 2000™ system.

1.7 Nucleotide sequence accession numbers

Sequences reported in this study have been submitted to GenBank with accession numbers DQ453119-DQ453121 and DQ468056-DQ468070.

2 Results

2.1 Waste ore sample

The khaki waste ore was predominantly derived from a pyrite-dominated ore body. The moisture content of the waste ore was 15% (w/w), and its pH was 3.0. The pH of the nearby AMD is 2.2, and metal ion concentrations lie above 10 g/L.

The element composition analysis of the waste is shown in Table 1.

Table 1 Elemental composition of the waste ore (% , w/w)

Sample	Fe	Mn	Ca	Al	Na	K	S
Waste ore	8.30	0.02	0.09	1.25	1.51	0.98	0.34

2.2 Cloning and sequencing

The bacterial population in the waste ore was characterized by ligating the PCR product of an almost full-length 16S rDNA into a cloning vector. 200 clones were selected from the generated clone library. The results of the *in-situ*-PCR indicated that 152 of the 200 chosen clones had inserted fragments of the correct size.

Cloned inserts were amplified by PCR and digested with restriction enzymes. ARDRA banding patterns were clearly distinguishable. The 152 clones could be grouped into 22 distinct ARDRA patterns. Representatives of each group were sequenced. Sequence similarity then reduced the ARDRA groups to 18 representative sequences (Table 2). None of the sequences were judged to be a chimera. Most of the closest relatives of the sequences were from environment clones, which indicated that the biodiversity of the acidic ecosystem was very novel.

2.3 Phylogenetic analysis of 16S rDNA

Clone S5, S7, S11, S57, and S101 were related to *Actinobacteria*, representing 34.2% of total clones, which was the most abundant sequence group. These sequences cannot be affiliated with the sequences of cultured bacteria and were only related closely to certain clones from the acidic environment (Fig.1). Clone S11 and S57 could not cluster with any existing sequences in the database and instead formed a monophyletic group, and the phylogenetic relationships of them were different from other groups. Furthermore, clone S11 and S57 had only 95% similarity to known sequences, hence they seemed to be novel microorganisms belonging to *Actinobacteria*. Clone S7 was affiliated with environment clones originating from an extreme acidic river and a forested wetland impacted by reject coal, having 96% similarity to its closest relative. Clone S101 and a number of environmental clones formed another phylogenetic group. The sequence similarity between S101 and the closest relative (clone YNPFFP40) was 96%. Clone YNPFFP40 was found in an extreme thermal soil, which indicated that S101 played a role in

Table 2 BLAST analysis of 16S rDNA sequences of acidophiles in the waste ore

Representative sequence	No. of clones (frequency)	Microbial group affiliation	Closest relative (Genbank accession number)	Similarity (%)
S5	4 (2.6%)	<i>Actinobacteria</i>	Uncultured bacterium clone ASL8 (AF543499)	96
S7	22 (14.5%)	<i>Actinobacteria</i>	Uncultured bacterium clone fb11 (DQ303258)	96
S11	8 (5.3%)	<i>Actinobacteria</i>	Uncultured bacterium clone 1969a-05 (AY917713)	95
S15	12 (7.9%)	Low G + C Gram-positives	Uncultured bacterium clone 1894a-21 (AY917551)	92
S23	1 (0.7%)	<i>Thermomicrobia</i>	Uncultured bacterium clone 1921-1 (AY425777)	94
S25	13 (8.6%)	<i>Acidobacteria</i>	Uncultured bacterium clone DUNssu021 (AY913242)	97
S26	4 (2.6%)	<i>Thermomicrobia</i>	Uncultured bacterium clone AKAU4060 (DQ125834)	93
S29	6 (3.9%)	candidate division TM7	Uncultured bacterium SBR1071 (AF268996)	91
S31	32 (21.1%)	Low G + C Gram-positives	Uncultured bacterium clone RCP2-66 (AF523921)	98
S35	3 (2.0%)	<i>Thermomicrobia</i>	Uncultured bacterium clone 1959b-19 (AY917683)	94
S57	16 (10.5%)	<i>Actinobacteria</i>	Uncultured bacterium clone fb11 (DQ303258)	95
S72	2 (1.3%)	α - <i>Proteobacteria</i>	Uncultured bacterium clone JE89 (DQ125834)	94
S74	6 (3.9%)	Low G + C Gram-positives	Uncultured bacterium clone 1973c-07 (AY917790)	94
S76	2 (1.3%)	<i>Planctomycetes</i>	<i>Isophaera</i> sp. (X81958)	95
S77	6 (3.9%)	δ - <i>Proteobacteria</i>	Uncultured bacterium clone RCP2-54 (AF523886)	96
S80	10 (6.6%)	TM7	Uncultured bacterium clone EV221H2111601SAH66 (DQ223221)	92
S91	3 (2.0%)	<i>Thermomicrobia</i>	Uncultured bacterium clone 1969a-23 (AY917723)	87
S101	2 (1.3%)	<i>Actinobacteria</i>	Uncultured thermal soil bacterium clone YNPFFP40 (AF391981)	96

the oxidation of Fe and S because the extreme thermal soil usually contained high Fe and S. Clone S5 was clustered with the closest relative clone ASL8 recovered from AMD at Iron Mountain in USA (Druschel *et al.*, 2004), having 96% similarity to this.

After the *Actinobacteria*-affiliated sequences, clone types clustering with low G + C Gram-positives were the next most abundant of the clone library, representing 32.9% of total clones. The most abundant sequence type, representing 32 clones, was S31. This sequence was positioned firmly in the *Sulfobacillus* group, having 98% similarity to the closest relative, and formed a monophyletic group with bacteria of *Sulfobacillus* and certain acidic environmental clones (Fig.2). Clone S15 and S74 formed another cluster in the phylogenetic tree with certain environmental clones. The closest relatives of the two clones were all from Hawaiian Volcanic Deposits. Besides, S15 and S74 had high similarity to the sequences detected in the acid-impacted subalpine stream sediments. These implicated that S15 and S74 had relevance to the oxidation of Fe and S in acidic environment.

Eleven clones (8.3% of the total clones), represented by S23, S26, S35, and S91, were affiliated with the phylum *Thermomicrobia*. Similar to S15 and S74, clone S23 and S35 were affiliated firmly to the sequences originating from the Hawaiian Volcanic Deposits and acid-impacted subalpine stream sediments (Fig.3a), which indicated that S23 and S35 may play similar roles as S15 and S74. S26 and S91 positioned in a different cluster from the one including S23 and S35. S91 was affiliated with the sequence found in soil samples of uranium wastes. Furthermore, S91 had only 87% similarity to existing sequences. Thus, S91 may be a new bacterium that can oxidize metals in an acidic habitat. S26, having 93% similarity to the closest relative, was clustered with the sequences from the coastal marine sediment with accelerated sulfur cycle and uranium reduction and reoxidation system (Asami *et al.*, 2005). The phylogenetic analysis revealed that S26 may be a sulfur-oxidizing acidophile.

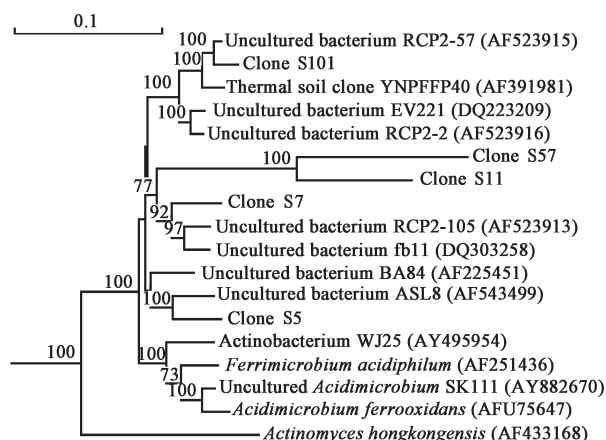


Fig. 1 Neighbor-joining phylogenetic tree from analysis of 16S rRNA gene sequences of *Actinobacteria*. Numbers at the node indicate bootstrap values of 1000 resampling. *Coriobacterium glomerans* (X79048) was used as outgroup (not shown). The scale bar represents changes per nucleotide.

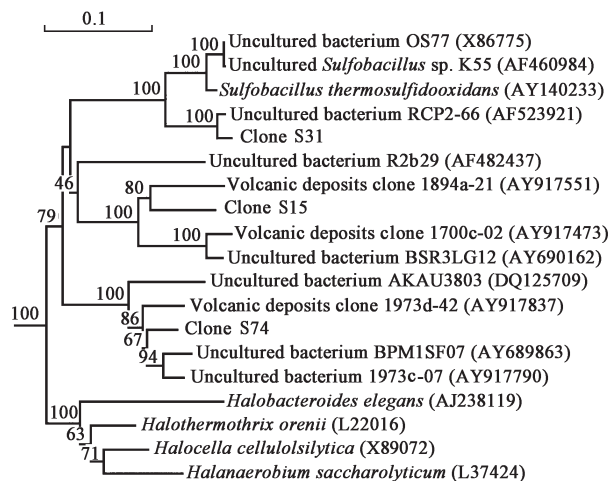


Fig. 2 Neighbor-joining phylogenetic tree from analysis of 16S rRNA gene sequences of low G + C Gram-positives. Numbers at the node indicate bootstrap values of 1000 resampling. *Schwartzia succinivorans* (AY09434) was used as outgroup. The scale bar represents changes per nucleotide (not shown).

Clone S29 and S80, representing 16 clones (10.5% of total clones), were affiliated with candidate division TM7 (Fig.3b), which was one of several described bacterial divisions exclusively characterized by environmental sequence data. TM7 was originally proposed based on partial 16S rDNA sequences obtained from PCR clonal studies of a peat bog (Rheims *et al.*, 1996). Their sequences have since been detected in diverse habitats, such as soils, rhizosphere, groundwater, seawater, deep-sea sediments, and human oral cavity (Hugenholtz *et al.*, 2001). But as yet, they have not been found in acidic environment in previous researches. Furthermore, clone S29 and S80 show only 91% and 92% similarity, respectively to their closest relatives, so they may be new members of Candidate division TM7.

Clone S25, representing 13 clones (8.6% of total clones), was related to the *Acidobacteria* and had 97% similarity to the known sequences, which were mostly from uncultured bacteria (Fig.3b). *Acidobacteria* are heterotrophic acidophiles and are usually present in an acidic environment. They utilize organic compounds, such as cell exudates and lysates originating from the autotrophic primary producers. They form communal associations with autotrophic acidophiles by "detoxifying" the environment for the latter group, which is more sensitive to organic substances (Johnson, 1998).

Six clones (3.9% of total clones), represented by S77, were positioned within δ -subdivision of *Proteobacteria* (Fig.3b). The sequence similarity between S77 and its closest relative, clone RCP2-54, was 96%. Clone RCP2-54 was previously found in the bacterial community of a forested wetland impacted by reject coal (Brofft *et al.*, 2002). S77 could represent anaerobic sulfate-reducing heterotrophs, as sulfate reduction is the dominant physiological trait of this group. Within the microbial consortium, sulfate-reducing heterotrophs could have a similar synergistic effect as mentioned for *Acidobacteria*. Clone S72 was affiliated with α -*Proteobacteria* and formed a cluster with several

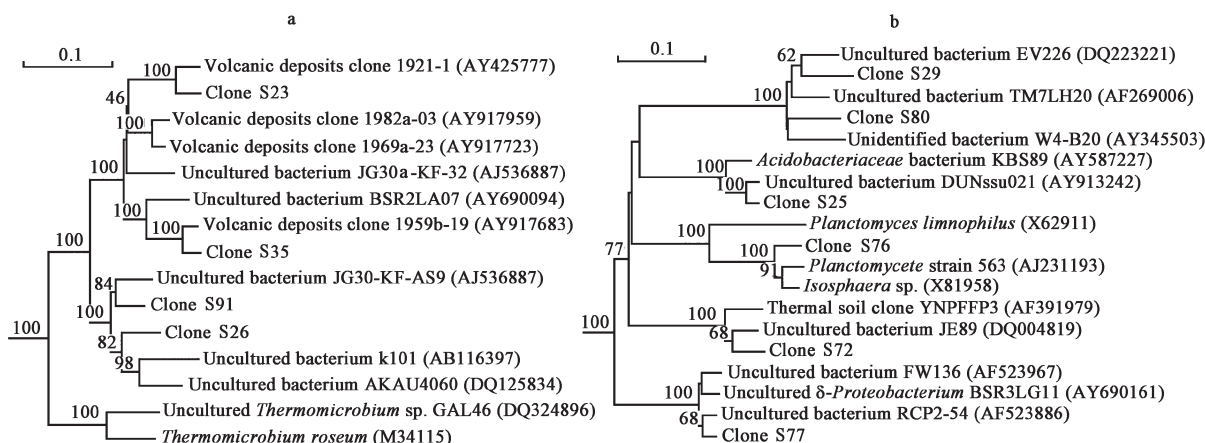


Fig. 3 Neighbor-joining phylogenetic tree from analysis of 16S rRNA gene sequences of *Thermomicrobia* (a) and several clones and their close relatives (b). Numbers at the node indicate bootstrap values of 1000 resampling. *Herpetosiphon geysericola* (AF039293) was used as outgroup (not shown). The scale bar represents changes per nucleotide.

environmental clones. S72 represented 2 clones (1.3% of total clones) and had 95% similarity to the closest relative.

Clone S76, representing two clones, was affiliated with the phylum of *Planctomycetes*. The sequence similarity between S76 and its closest relative was 95%. To date, this is the first occasion that microorganisms from *Planctomycetes* have been detected in such acidic environments. The function of S76 in the microorganism community is unknown.

2.4 Denaturing gradient gel electrophoresis

The DGGE profiles revealed the composition of the acidophiles community in the waste ore (Fig.4). The two lanes represent two samples taken from the banks of the AMD lake. The dominant bands were excised from the DGGE fingerprints, reamplified, purified, and sequenced. The obtained sequences were identified using the BLAST™ program and DNAMAN 6.0™. The dominant bands 1–6 were identified as clone S15, S7, S31, S25, S57, and S80, respectively. The result of DGGE was consistent with that

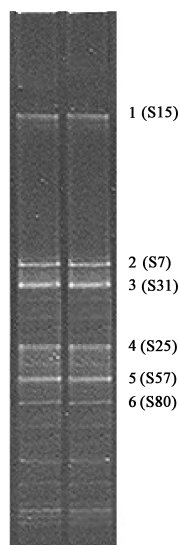


Fig. 4 DGGE profiles of acidophiles in waste ore.

of clone library analysis.

3 Discussion

Previous culture-based analyses and certain molecular analyses have suggested that microbial communities from acidic environments tend to have low diversity and be dominated by culturable organisms (Walton and Johnson, 1992; Goebel and Stackebrandt, 1994). Consequently, organisms implicated in AMD generation are typically the chemolithoautotrophs, such as *A. ferrooxidans*, *L. ferrooxidans*, and a variety of acidophilic heterotrophs or facultative heterotrophs. However, the results indicated that high microorganism diversity may exist in the acidic environment. Blast search indicated that most of the sequences from this study were novel and their related sequences were mostly from uncultured organisms and environmental samples. After phylogenetic analysis, many of the sequences detected in this research did not cluster with recognized, cultured acidophiles and hence may represent organisms that constitute new taxa.

Clone S31, the most abundant clone type, was affiliated with *Sulfobacillus* group, which represented 21.1% of the total clones. *Sulfobacillus* is moderately thermophilic acidophile which differs from the known acidophilic mesophilic iron oxidizers (Gram-negative, nonsporulating chemolithotrophic bacteria) and the extremely thermophilic iron oxidizers in several fundamental ways, including cellular morphology (they are Gram-positive rods that often form endospores) and range of growth temperature, which are typically 30 to 55°C. Furthermore, *Sulfobacillus* can oxidize iron as well as reduced sulfur and have a highly versatile metabolism and may grow as autotrophs, heterotrophs, mixotrophs (e.g., in media containing both ferrous iron and glucose, in which both CO₂ and glucose are used as carbon sources), or chemolithoheterotrophs (e.g., in ferrous iron-yeast extract medium, in which iron acts as the energy source and yeast extract is the carbon source) (Johnson, 1998). In

addition, S5, S7, and S101, representing 18.4% of total clones, were found to affiliate with *Acidimicrobium* group using Classifier in RDP. *Acidimicrobium* is another type of moderately thermophilic acidophile. Since moderately thermophilic acidophiles accounts for such a large proportion (39.5% of total clones), they play a great role in the generation of AMD. Bond *et al.* (2000) also obtained similar results when they studied the microbial community in the AMD at the Iron Mountain Mine with fluorescent *in situ* hybridization (FISH).

Many clones in this study were related closely to the sequences from Hawaiian Volcanic Deposits. As products of volcanic activity generally contain large amounts of Fe and S, the iron- and sulfur-oxidizers readily colonize the volcanic deposits. Consequently, the microbial populations in the volcanic deposits had high similarity to those in the waste ore deposits.

It is surprising that the common mesophilic acidophiles, such as *A. ferrooxidans* which has been considered principally responsible for the extreme conditions of AMD systems were not detected in this study. This may be caused by the fact that the acidophile community composition had a strong correspondence with the ambient environment. The unique physical and chemical conditions of the Mountain Xiang resulted in the special acidophile biodiversity. Since the waste ore samples were not extremely acidic (pH 3.0), the results obtained from 16S rDNA clone library and DGGE may reflect the microbial community in moderately acidic environments. In previous reports of acidophile biodiversity in similar AMD ecosystem, *A. ferrooxidans* was not detected and only another species of *Acidithiobacillus* was found in very small proportions (Okabayashi *et al.*, 2005). In addition, the waste ore was collected in hot midsummer when the temperature was about 40°C, which may be another reason, why the moderately thermophilic acidophiles were abundant and scarce mesophilic acidophiles.

4 Conclusions

This study indicated that the acidophiles in the acidic waste ore were novel and were related to *Actinobacteria*, low G + C Gram-positives, *Thermomicrobia*, *Acidobacteria*, α -*Proteobacteria*, δ -*Proteobacteria*, candidate division TM7, and *Planctomycetes*. The moderately thermophilic acidophiles were abundant in the acidic ecosystem and may play a great role in the generation of AMD. The unique physical and chemical conditions of the Mountain Xiang may lead to the special acidophile community construction in the waste ore deposits. These results provide useful information to develop more economical and efficient methods to control AMD.

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