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ORIGINAL PAPER

Microbial diversity in acid mineral bioleaching systems of dongxiang copper mine and Yinshan lead–zinc mine

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Abstract To understand the composition and structure of microbial communities in acid mineral bioleaching systems, the molecular diversity of 16S rDNA genes was examined using a PCR-based cloning approach. A total of 31 Operational Taxonomic Units (OTUs) were obtained from the four samples taken from four different bioleaching sites in Yinshan lead–zinc mine and Dongxiang copper mine in Jiangxi Province, China. The percentages of overlapping OTUs between sites ranged from 22.2 to 50.0%. Phylogenetic analysis revealed that the bacteria present at the four bioleaching sites fell into six divisions, *α-Proteobacteria* (1.1%), *β-Proteobacteria* (2.3%), *γ-Proteobacteria* (30.8%), *Firmicutes* (15.4%), *Actinobacteria* (0.3%) and *Nitrospira* (50.1%). Organisms of genera *Leptospirillum*, *Acidithiobacillus*, and *Sulfobacillus*, which were in *Nitrospira*, *γ-Proteobacteria*, and *Firmicutes* divisions, respectively, were the most dominant. The results of principal component analysis based on the six phylogenetic divisions and biogeochemical data indicated that the microbial community structure of a site was directly related to the biogeochemical characteristic of that site. It follows therefore that sites with similar biogeochemical characteristics were comprised of similar microbial community structures. The results in our study also suggest that the elements copper and arsenic appear to be the key factors affecting the compositions and structures of microbial community in the four bioleaching sites.

Keywords Microbial diversity · RFLP · Bioleaching · AMD

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Introduction

Acidophilic microorganisms play important roles in environmental and industrial systems, including the environmental problems of acid mine drainage (AMD), acid rock drainage (ARD), and the biotechnological process termed bioleaching (Hallberg and Johnson 2001). Metals are released from sulfide minerals by oxidation of the covalent metal-sulfide bond by Fe^{3+} and the process is catalyzed by the action of acidophilic iron and sulfur oxidizing microorganisms (Olson et al. 2003; Rohwerder et al. 2003).

As the availability of high-grade ores dwindles, it becomes necessary to utilize mineral resources previously deemed uneconomical because of marginal metal content. The use of microorganisms to recover metals from low-grade ores and mineral concentrates has developed into a successful and expanding area of biotechnology (Rawlings 1997). Different engineering approaches have been used to facilitate microbial mineral processing, which include in situ leaching, dump and heap leaching of low-grade ores, and aerated stirred tanks for microbial processing of mineral concentrates (Brierley 1997).

Formerly, phylogenetic analysis of a mixed culture was restricted to plate-based assays. Plating only isolated a

limited number of species and led to the misconception that, at ambient temperatures the microbial populations at AMD, ARD, and bioleaching sites were dominated by a few species, such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. The understanding of the diversity of microorganisms present in acidophilic environments has been accelerated by the application of molecular phylogenetic techniques. The microbial communities are more complex than expected (Brierley 1982; Bond et al. 2000a; González-Toril et al. 2003). The AMD system most intensively studied by culture independent molecular methods is within the Richmond Mine at Iron Mountain in northern California. To date, group III *Leptospirillum* has only been detected via clone library analysis of Iron Mountain microbial communities (Bond et al. 2000a; Druschel et al. 2004). Results at Iron Mountain confirm that a handful of prokaryotic taxa (often less than five groups distinct at the genera level) make up the communities in any specific microenvironment (Bond and Banfield 2001; Druschel et al. 2004). Low diversity has also been noted using cultivation-based approaches (Goebel and Stackebrandt 1994; Johnson et al. 2001).

Compared with AMD and ARD research, there have been relatively few studies on the composition of microbial populations in commercial mineral leaching operations (Norris et al. 2000).

In this paper, four samples were studied from the Yinshan lead–zinc mine and the Dongxiang copper mine in Jiangxi province, China. The four samples were taken from four different sites where heap leaching using microorganisms was being undertaken. The abundance and diversity of microorganisms in four bioleaching sites were determined by a PCR-based cloning approach. In addition, we studied the relationship between the biochemical properties and microbial community structures in the four sites.

Materials and methods

Sites description and samples collection

Samples were collected from the bioleaching sites at the Yinshan lead–zinc mine and the Dongxiang copper mine, both located in Jiangxi province, China. They are both important metal mines and have more than 50 years of exploitation history.

The temperature and pH value in the Dongxiang copper mine while sampling were 20.1°C and 2.0, respectively. There was only one site undertaking bioleaching in Dongxiang copper mine, from which, one sample (sample DX) was collected. The clones isolated containing inserts of 16S rDNA sequences from this sample were given the prefix DX. In the Yinshan lead–zinc mine, there were three sites undertaking bioleaching, one sample was taken from each site, of which temperatures and pH values were all 25.0°C and 1.5, respectively. The samples were labeled D1, DY, and D3 separately, from which the clones isolated containing inserts of 16S rDNA sequences were given the prefix D1, DY, and D3, respectively.

A water sample was taken from each site and processed within 24 h after collection. The four water samples were filtered through separate 0.22 μm hyper filtration membranes (Biobasic Inc.). The sediments on the membranes were washed twice with sterile distilled water, and stored at −70°C for later analysis. Chemical analysis was subsequently carried out on the filtered water samples.

Chemical analysis of samples

Elemental analysis on the filtered water samples was carried out using Inductively Coupled Plasma-Atomic Emission Spectgrometry (ICP-AES). Each sample was tested for the presence of 29 elements, Hg, As, P, Ni, Co, Cr, Be, Ti, W, Zn, In, Mg, Mn, Ca, S, Mo, Bi, Au, Fe, Si, Cu, Sn, Sb, Cd, Ga, Pt, Al, Ag, Pb.

DNA extraction and purification

The extraction of nucleic acids was carried out according to the procedure described by Zhou et al. (1996). Five gram of sediment was mixed with 13.5 mL extraction buffer [0.1 M phosphate (pH 8.0), 0.1 M EDTA, 0.1 M EDTA, 1.5 M NaCl, 1% CTAB] and 50 μL protenase K (10 mg/mL) in 50 mL centrifuge tube, then incubated at 37°C for 30 min. Twenty percent SDS (1.5 mL) was added and mixed gently, then incubated at 65°C for 2 h. The mixture was centrifuged and the supernatant was transferred into a new 50 mL of centrifuge tube. The pellet was resuspended with extraction buffer, and 0.5 mL 20% SDS was added. The mixture was incubated at 65°C for 15 min, then centrifuged and the supernatant was collected and combined with the previous supernatant. The combined supernatant was extracted with chloroform. 2-Isopropanol was added to the supernatant collected and then mixed gently. The mixture was kept at the room temperature for an hour or overnight, then centrifuged. The pellet was washed with 70% ethanol and dissolved with 200–500 μL sterile water. By using combined methods that included grinding, freezing and thawing, and treatment with sodium dodecyl sulfate, various types of bacterial could been effectively lysed. The crude DNA was purified by using Wizard plus sv Minipreps DNA purification system (Promega Corporation, USA) and quantified by ethidium bromide-UV detection on an agarose gel.

PCR, RFLP and sequencing

Community 16S rDNA genes were amplified with the primer set, 1492R (5′-CGGCTACCTTGTTACGACTT-3′) and 27F (5′-AGAGTTTGATCCTGGCTC AG-3′) (Lane 1991). A gene amp (Biometra, T-Grandient, Genman) was used to incubate reactions through an initial denaturation at 94°C for 3 min, followed by 32 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 1 min, and completed with an extension period of 10 min at 72°C. Products from the amplification reactions of expected size (about 1,500 bp) were pooled and purified before subsequent ligation.

The purified PCR products were ligated to the vector PGEM-T (Promega Corporation, USA), and used to transform *E. coli* DH5α competent host cells. About 120 white clones were randomly selected from each library. For restriction fragment length polymorphism (RFLP) and sequencing, the inserted fragments were amplified with the vector-specific T7 and SP6 primers. The unpurified PCR products were digested with two restriction endonucleases *Afa* I and *Msp* I (TaKaRa Biotechnology Corporation, Japan), incubated at 37°C for 3 h. The restricted fragments were separated by gel electrophoresis in 3.0% agarose with ethidium bromide staining and observed with UV illumination. RFLP patterns were identified and grouped, and representative cloned fragments were selected for sequencing.

Phylogenetic analysis

Phylogenetic affiliations of the partial sequences were initially estimated using the program BLAST (Basic alignment search tool) (Bond et al. 2000b). Similarity of partial sequences was determined using ARB (a software environment for sequence data) (Strunk and Ludwig 1995). The initial phylogenetic trees were based on all available sequences and constructed using the DNA distance program Neighbor-Joining with Felsenstein correction in ARB (Smith et al. 1994). Based on the initial phylogenetic results, appropriate subsets of 16S rDNA sequences were selected and subjected to a final phylogenetic analysis using CLUSTAL X.

Statistical methods

Principal-component analysis (PCA) was performed using the SYSTAT statistical computing package (version 13.0; SPSS Inc., Chicago, IL, USA) for each sampling site. PCA simultaneously considers many correlated variables and then identifies the lowest number to accurately represent the structure of the data (Sharma 1996; Liu et al. 2003).

In the present study, PCA was used to group or separate stations, which were similar or different, based on the biogeochemical parameters for each station. Similarly, the correlation analysis was applied to the biological parameters. The relative amounts of operational taxonomic units (OTUs) (unique RFLP patterns) for each station were also used as variables.

The rarefaction analysis was performed with SigmaPlot software. An exponential model, $y = a \times [1 - \exp(-b \times x)]$, was used with SigmaPlot 8.0 nonlinear regression software to fit the clone distribution data.

Nucleotide sequence accession numbers

Accession numbers of the partial 16S rDNA gene sequences for the OTUs in this study are given in Table 1. The nucleotide sequences are available through the DDBJ/EMBL/GenBank nucleotide sequence databases.

Results

Biogeochemical properties of four sites

The same temperature (25.0°C) and pH (1.5) were detected at sites D1, D3 and DY. But highest pH (2.0) and lowest temperature (20.1°C) were detected at site DX. The elemental concentrations at site DX were also higher (except arsenic) than those at the other three sites. The data are shown in Table 2.

The parameters detected by ICP-AES were analyzed by PCA to determine the geochemical relationships among the four sites. The results are shown in Fig. 1a. Sites D1 and D3 were grouped together by PCA based on the concentration data of the 29 elements. The greatest variation in geochemical properties was observed at site DX, however, site DY also showed great variation from the other sites.

The variation of elemental concentrations at the four sites was also analyzed and the results suggested that the elements copper and arsenic might be the key factors causing the diversity among the sites.

Analysis of 16S rDNA cloning libraries by RFLP

Diverse patterns of 16S rDNA fragments were observed within the four samples screened by RFLP analysis; 120 white clones containing inserted 16S rDNA genes were obtained from each sample. A range of 7–19 different unique 16S rDNA fragments were detected and isolated from each sample. A total of 31 OTUs (unique RFLP patterns) were obtained from all samples. The greatest

Table 1 Inventory of bacterial 16S rDNA cloned fragments arranged into groups according to RFLP patterns and sequence similarity

Clones	Accession number	Closest relative (accession number)	Similarity (%)	Plylogenetic division
D3-8	DQ464127	*Acidithiobacillus albertensis* DSM14366 (AJ459804)	99	*γ-Proteobacteria*
D3-93	DQ464128	Uncultured bacterium clone fppg9 (DQ303294)	99	*γ-Proteobacteria*
D3-77	DQ464129	Uncultured bacterium clone fa8 (DQ303271)	95	*α-Proteobacteria*
D3-15	DQ464130	*Acidovorax* sp. (AJ012071)	99	*β-Proteobacteria*
D3-1	DQ464131	Uncultured bacterium BA29 (AF225448)	99	*Nitrospira*
D3-6	DQ464132	*Leptospirillum* sp. (AJ237902)	99	*Nitrospira*
D3-22	DQ464133	*Leptospirillum ferrooxidans* strain CF12 (AF356834)	99	*Nitrospira*
D3-21	DQ464134	*Leptospirillum ferriphilum* strain Fairview (AF356830)	99	*Nitrospira*
D3-25	DQ464135	Uncultured soil bacterium clone PYR10d2 (DQ123667)	99	*β-Proteobacteria*
D3-31	DQ464136	*Sulfobacillus thermotolerans* strain KR-1 (DQ124681)	99	*Firmicutes*
D3-32	DQ464137	Uncultured bacterium Tui3-12 (AF353297)	98	*β-Proteobacteria*
D3-33	DQ464138	*Cenibacterium arsenoxidans* (AY728038)	99	*β-Proteobacteria*
D3-43	DQ464139	*Leptospirillum ferrooxidans* strain Sy (AF356839)	99	*Nitrospira*
D3-69	DQ464140	*Acidithiobacillus thiooxidans* ATCC19377 (Y11596)	99	*γ-Proteobacteria*
D3-34	DQ464141	Uncultured soil bacterium clone NAP7d51 (AY699603)	99	*γ-Proteobacteria*
D3-5	DQ464142	Uncultured bacterium clone BA8 (AF543505)	97	*Firmicutes*
D3-28	DQ464143	Uncultured bacterium clone JTC05 (AY805540)	94	*Firmicutes*
D3-7	DQ464144	*Ferrimicrobium acidiphilum* (AF251436)	99	*Actinobacteria*
D3-44	DQ464145	*Captivus acidiprotistae* cloneASL45 (AF533506)	93	*α-Proteobacteria*
D1-95	DQ464151	Uncultured bacterium clone SAH95 (DQ223234)	99	*Nitrospira*
D1-48	DQ464150	*Acinetobacter* sp. An9 (AJ551148)	97	*γ-Proteobacteria*
D1-90	DQ464149	Uncultured bacterium clone AW11 (AF543503)	96	*Nitrospira*
D1-93	DQ464148	Uncultured bacterium clone SAH79 (DQ223229)	99	*Nitrospira*
D1-23	DQ464146	Gram-positive iron-oxidizing acidophile G1 (AY529492)	96	*Firmicutes*
D1-55	DQ464147	Uncultured bacterium clone G2-4 (DQ364427)	99	*Nitrospira*
DY-12	DQ464153	*Acidithiobacillus ferrooxidans* strain YTW (DQ062116)	99	*γ-Proteobacteria*
DY-60	DQ464152	*Acidithiobacillus ferrooxidans* (AJ278722)	99	*γ-Proteobacteria*
DY-5	DQ464154	Uncultured bacterium clone ff2 (DQ303293)	99	*Nitrospira*
DY-65	DQ464126	Uncultured bacterium clone RB7C10 (AF407387)	92	*β-Proteobacteria*
DY-93	DQ464155	Uncultured bacterium clone RCP1-70 (AF523924)	99	*Nitrospira*
DX48-40	DQ464156	*Acidiphilium* sp. (D30769)	99	*α-Proteobacteria*

Table 2 The concentrations of the 12 main elements detected in the four sites

Element	Site D1	Site DY	Site D3	Site DX
As (mg/L)	29	28	26	9.7
P (mg/L)	21.1	29.2	30.6	90
Zn (mg/L)	24	34.6	70	408.4
Pb (mg/L)	0.14	0.12	0.05	21.6
Mg (mg/L)	33.2	13.8	72.6	2,512.4
Co (mg/L)	2.2	2.6	1.6	19.3
Ca (mg/L)	45.6	20.3	51	593.7
S (g/L)	6.55	6.08	4.53	27.9
Fe (g/L)	7.47	8.04	5.16	46.89
Cu (mg/L)	182.4	188.3	220.5	245.2
Al (mg/L)	629.3	341.5	324.7	3,471.7
Mo (mg/L)	1.7	1.7	1.3	7.9

number of OTUs (19) was found in the sample from site D3. The numbers of OTUs in the other three sites DY, D1, and DX were 13, 11 and 7, respectively.

Rarefaction analysis was used in the RFLP analysis. The results are shown in Fig. 2. Nonlinear regression suggested that 70, 60, 40 and 30 white clones were enough detected for constructing cloning libraries of samples from sites D3, DY, D1 and DX, respectively. These results also suggested that the clones tested in the experiment were sufficient to detect the level of community diversity within the four bioleaching sites.

The RFLP analysis revealed extensive diversity among the 16S rDNA genes found in the four sites. The distribution of OTUs, which were ranked in order of abundance, is represented in Fig. 3. Three to five dominant RFLP patterns were detected in each site. The RFLP patterns of clones D1-24, D1-26, D1-39 and D1-45 represented 66.7,

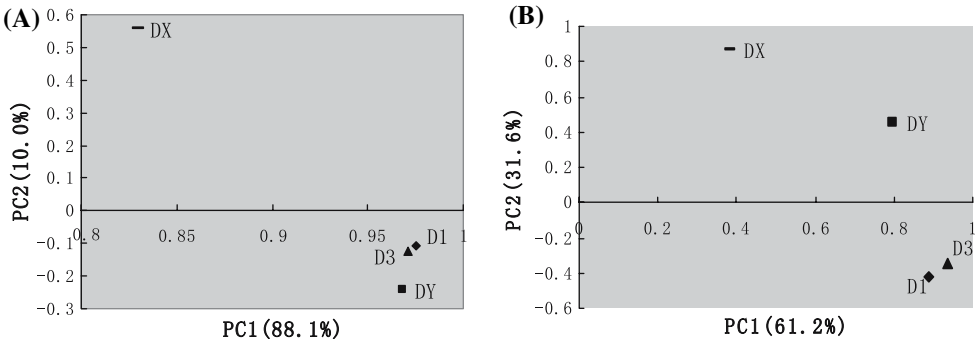

Fig. 1 Ordinate plots from PCA based on the concentration data of 29 elements (**a**), and on the six phylogenetic divisions derived from 16S rDNA phylogenetic analysis (**b**). DX: a sampling position at the Dongxiang copper mine; D1, DY and D3: three different sampling positions at the Yinshan lead–zinc mine

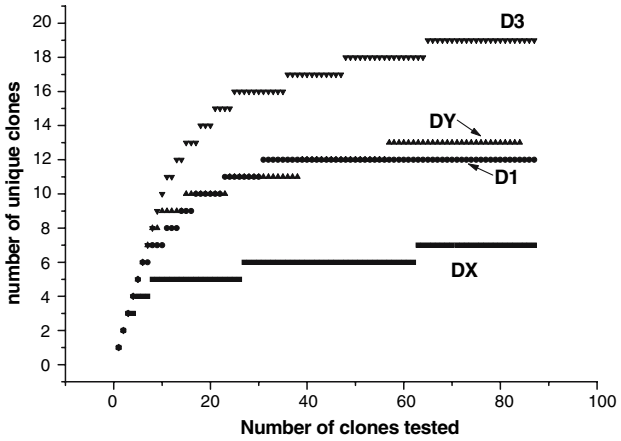

Fig. 2 Evaluation of the representative clones obtained from four sites by rarefaction analysis. DX: a sampling position at the Dongxiang copper mine; D1, DY and D3: three different sampling positions at the Yinshan lead–zinc mine

8.9, 6.7 and 4.4%, respectively, of the total clone populations in site D1. Five dominant clones, DY-6 (33.3%), DY-121 (21.4%), DY-21 (17.9%), DY-12 (10.7%) and DY-90 (6.0%) were found in site DY. Five dominant clones, D3-21 (26.4%), D3-6 (19.5%), D3-22 (13.8%), D3-5 (12.6%) and D3-8 (4.8%) were found in site D3. Lastly, three dominant clones, DX48-3 (47.5%), DX48-27 (30.5%) and DX88-2 (16.1%) were detected in site DX. The RFLP patterns in the four sites frequently overlapped. The percentages of overlapping OTUs between sites ranged from 22.2 to 50.0%. However, most of the RFLP patterns detected in sites D1, DY and DX also appeared in site D3. The RFLP patterns representing all of the clones (except DX48-40) detected in site DX were also found at the other three sites.

Phylogenetic analysis

To determine the phylogenetic diversity, representative 16S rDNA clones of OTUs that occurred more than once in the cloning libraries, as well as representatives of the unique OTUs, were fully sequenced. The details of representative clones sequenced are shown in Table 1.

The phylogenetic analysis of the four samples was established with a bootstrap neighbor-joining method. The results are shown in Fig. 4. The sequences detected were very similar to the 16S rDNA sequences of bacteria from the six phylogenetic divisions, *α-Proteobacteria* (1.1%), *β-Proteobacteria* (2.3%), *γ-Proteobacteria* (30.8%), *Firmicutes* (15.4%), *Actinobacteria* (0.3%) and *Nitrospira* (50.1%).

Among the 31 OTUs found in the four sites, three OTUs were identified as members of the *α-Proteobacteria*. A total of seven OTUs were identified as members of the genera *Acinetobacteria* and *Acidithiobacillus* (both of the division *γ-Proteobacteria*). Organisms belonging to genus *Acinetobacteria* were only detected at site D3 and constituted 1.1% of the total clone population. The proportions of members affiliated with *Acidithiobacillus* at each site were 79.7% (DX), 33.3% (DY), 9.2% (D3), and 1.1% (D1). Five OTUs were identified as members of the *β-Proteobacteria*. Four OTUs were identified as members of the *Firmicutes*. The organisms identified as members of *Firmicutes* were mostly affiliated with genus *Sulfobacillus*. The proportion of the clones affiliated with *Sulfobacillus* was 30.0, 10.0, and 18.4% at sites D1, DY, and D3, respectively. One OTU was affiliated with the *Actinobacteria*. The 11 remaining OTUs were identified as members of the *Nitrospira* division. The proportion of clones affiliated with this division in each site was 19.5% (DX), 29.8% (DY), 62.1% (D3), and 88.9% (D1). The organisms identified as members of *Nitrospira* in all sites were affiliated with the genus *Leptospirillum*.

The profiles of the six phylogenetic divisions in each site are shown in Fig. 5. All six divisions found in this study were detected at site D3. Five of the six divisions except *Actinobacteria* were detected at site DY. Members belonging to the *Nitrospira*, *Firmicutes* and *γ-Proteobacteria* divisions were detected at site D1. Three divisions, *γ-Proteobacteria*, *Nitrospira* and *α-Proteobacteria* were detected at site DX.

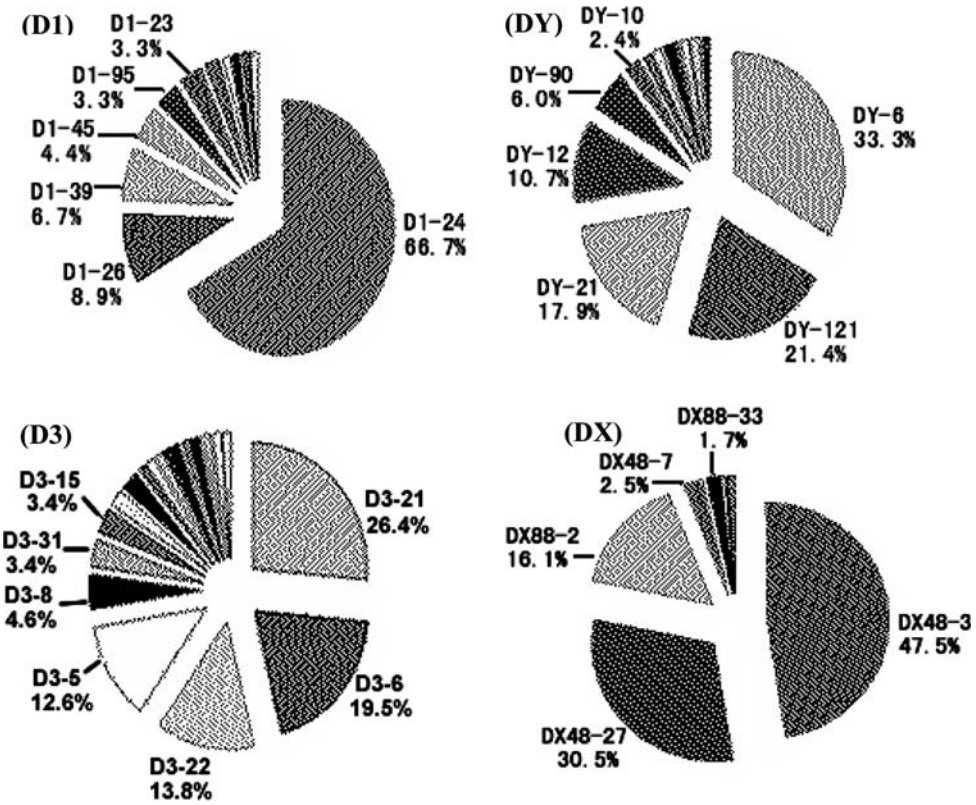

Fig. 3 Distributions of OTUs in the four cloning libraries. Clones having the same OTU are represented by the *same color*. DX: a sampling position at the Dongxiang copper mine; D1, DY and D3: three different sampling positions at the Yinshan lead–zinc mine. Clones D3-21, DY-21, D1-39 and DX88-2: the RFLP patterns were the same, with 99% similarity to the *Leptospirillum ferriphilum* strain Fairview; clones D3-6, D1-26, DY-10 and DX48-7: the RFLP patterns were the same, with 99% similarity to *Leptospirillum* sp.; clones D3-31, D1-45 and DY-6: the RFLP patterns were the same, with 99% similarity to the *Sulfobacillus thermotolerans* strain KR-1; clones D3-8 and DX88-33: the RFLP patterns were the same, with 99% similarity to *Acidithiobacillus albertensis* strain DSM 14366; clones DY-121and DX48-3: the RFLP patterns were the same, with 99% similarity to Uncultured bacterium clone fppg9; clones DY12 and DX-48-27: the RFLP patterns were the same, with 99% similarity to *Acidithiobacillus ferrooxidans* strain YTW; clones D1-95and DY-90: the RFLP patterns were the same, with 99% similarity to Uncultured *Proteobacterium* clone; clone D3-22: 99% similarity to the *Leptospirillum ferrooxidans* strain CF12; clone D3-5: 97% similarity to uncultured bacterium clone BA8; clone D3-15: 99% similarity to *Acidovorax* sp.; clone D1-23: 96% similarity to Gram-positive iron-oxidizing acidophile G1; clone D1-24: 99% similarity to uncultured bacterium BA29

PCA based on the six phylogenetic divisions was used to reveal the differences in the clone distributions among the four sites examined. The results are shown in Fig. 1b. Approximately 92.8% of the total variance of clone distributions was represented by PCA. Principal Component 1 (PC1) captured 61.2% of the variation, and Principal Component 2 (PC2) captured 31.6% of the variation. The results of PCA suggested that sites D3 and D1 were closely related in terms of microbial community structure. Site DX was the least similar of the four sites, but site DY also exhibited great variation from the other sites.

Discussion

In this work, the bacterial community structures in four bioleaching sites were identified. The results indicated that the bacteria detected fell into six phylogenetic divisions, *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Nitrospira*. Organisms of genera *Leptospirillum*, *Acidithiobacillus*, and *Sulfobacillus*, of the divisions *Nitrospira*, *γ-Proteobacteria*, and *Firmicutes*, respectively, were the most dominant at four sites examined.

The organisms identified as members of *Nitrospira* were affiliated with the genus *Leptospirillum*. To date, all isolated *Leptospirillum* spp. and environmentally derived clones were affiliated with one of three phylogenetically distinct groups [*L. ferrooxidans* (group I), *L. ferriphilum* (group II) and group III which has only been detected via clone library analysis of Iron Mountain microbial communities] (Bond et al. 2000a; Hippe 2000; Coram and Rawlings 2002). These three phylogenetically distinct groups were all detected in the four sites studied.

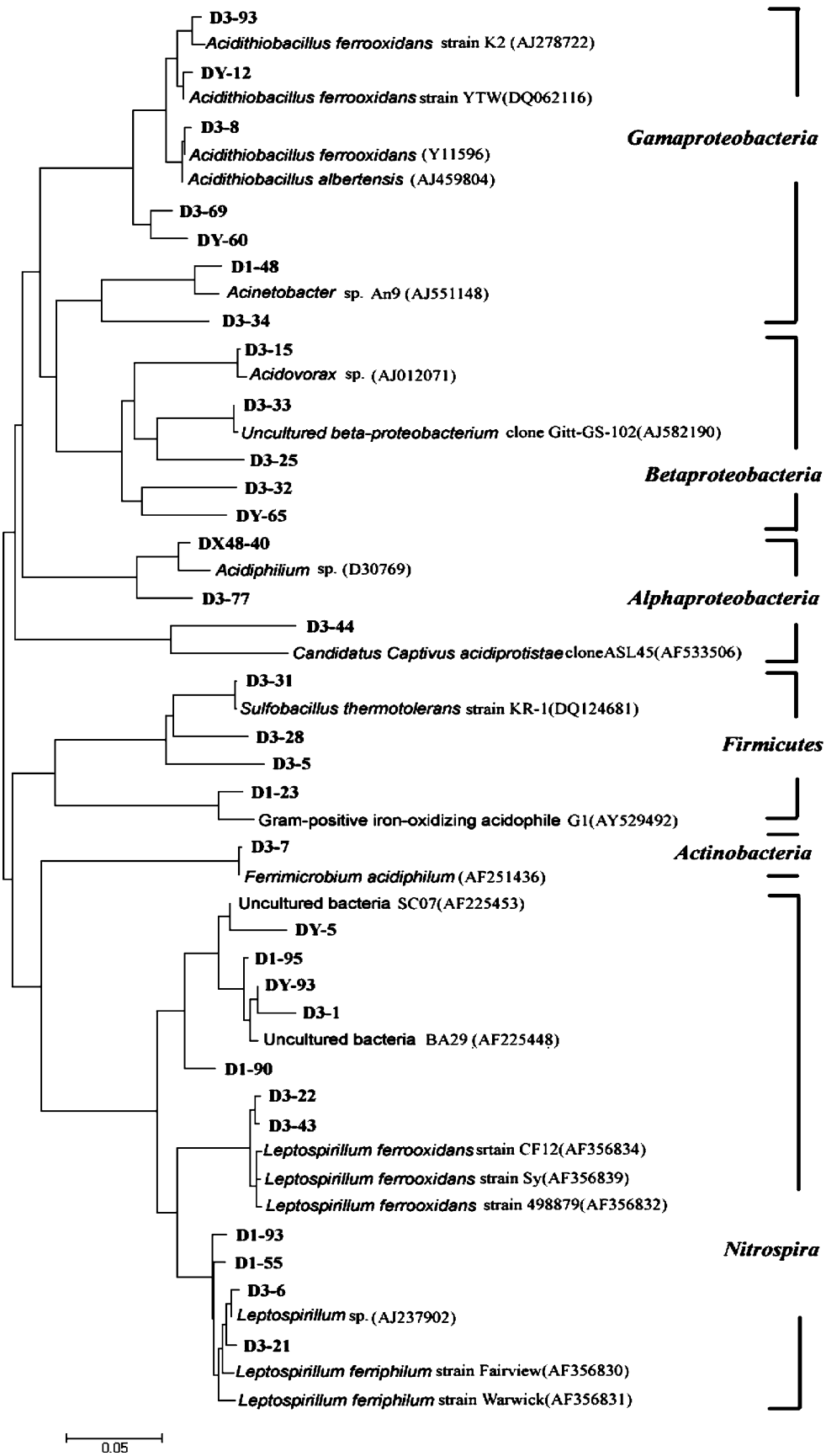

Fig. 4 Phylogenetic tree based on comparative analysis of 16S rDNA sequence data from 31 OTUs, and their close relatives. The sequences obtained in this study are indicated in *bold*

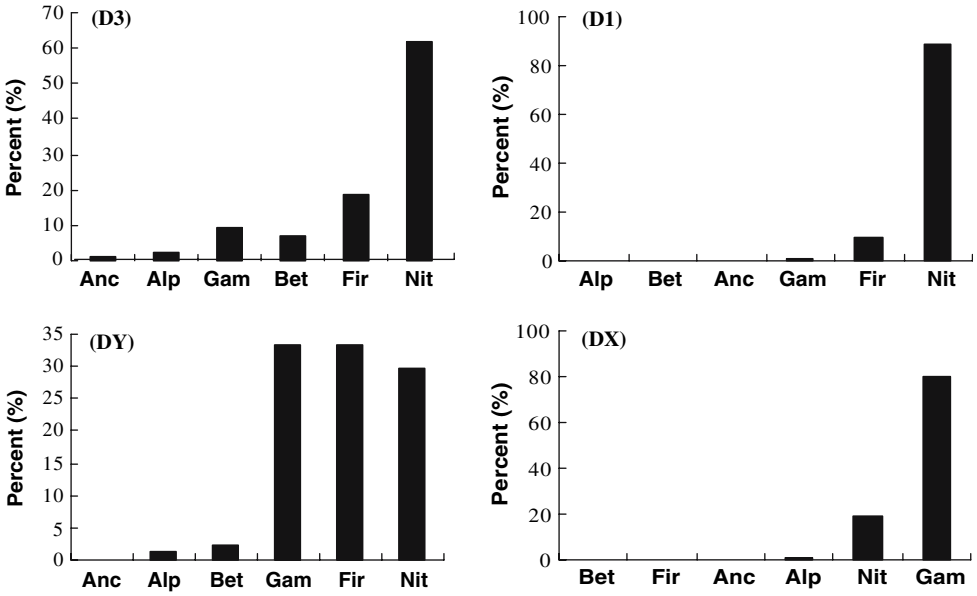

Fig. 5 Profiles of six phylogenetic divisions derived from phylogenetic analysis. *Nit Nitrospira*, *Anc Actinobacteria*, *Fir Firmicutes*, *Alp α-Proteobacteria*, *Bet β-Proteobacteria*, *Gam γ-Proteobacteria*. DX: a sampling position at the Dongxiang copper mine; D1, DY and D3: three different sampling positions at the Yinshan lead–zinc mine

Leptospirillum spp. are commonly considered to be some of the microorganisms that control the rate of generation of AMD environments (Coram and Rawlings 2002). Previous studies with respect to *Leptospirillum* spp. suggest that these organisms use reduced iron as an energy resource and occupy a greater percentage of the community structure at low pH value (Hippe 2000; Tyson et al. 2004). The iron concentrations in the four sites studied ranged from 5.16 to 46.89 g/L, which is high enough to support *Leptospirillum* growth. The pH values at the four sites were very low. The highest value (pH 2.0) was recorded at site DX, while the same value (pH 1.5) was recorded at the other three sites. These results suggest that the environments at the four sites were suitable for the growth of *Leptospirillum* spp.

Members of the genus *Leptospirillum* were detected at all sites, but the percentage of the community that they occupied at each site was very different. The percentage of the community occupied by *Leptospirillum* was smallest in site DX. Research on metal ion resistance showed that *Leptospirillum* spp. exhibit lower tolerance to Cu and Co, when compared to some other acidophilic microbes (Norris et al. 1986; Sand et al.1993). The concentrations of elements Cu and Co were highest at site DX, but lower than the maximum tolerable concentration (MTC) for *Leptospirillum* spp. These elements may have therefore contributed to a decrease in the *Leptospirillum* spp. proportion at site DX without eliminating the organisms from the community.

The organisms of the *γ-Proteobacteria* division detected in the four sites were mostly affiliated with the genus *Acidithiobacillus*. *Acidithiobacillus* spp. are frequently detected and usually dominant in AMD environments and acidic bioleaching systems (Johnson 1998; Fowler et al.1999). *Acidithiobacillus* spp. are also commonly considered to be the microorganisms that control the rate of AMD generation and *A. ferrooxidans* has been used as model microbe in bioleaching systems (Boon and Heijnen 1993; Fowler et al. 1999;Baker and Banfield 2003). The largest proportion of clones affiliated with *Acidithiobacillus* (79.7%) was detected in site DX, which showed that this site may be the most suitable for the growth of the organisms among the four sites. The lowest arsenic concentration and pH 2.0 were recorded at site DX. *Acidithiobacillus* spp. are very sensitive to arsenic ions (Dew 1999). ICP-AES analysis revealed that the highest concentrations of the 29 elements except arsenic were presented at site DX. The concentrations of some of the elements found in site DX were ten times greater than that in the other sites but the arsenic concentration was lower by one third. *Acidithiobacillus* occupied a higher community proportion at this site, which might have been facilitated by the low arsenic concentration.

Organisms from the division *Firmicutes* were found in all sites except site DX. The organisms identified as members of this division were mostly affiliated with the genus *Sulfobacillus*. *Sulfobacillus* is usually capable of autotrophic and heterotrophic growth and the optimum temperature and pH are typically about 50 and 2.0°C, respectively (Dufresne et al. 1996). *Sulfobacillus thermotolerans* has recently been isolated from the bioleaching system for gold ores (Tat'yana et al. 2006).

Interestingly, organisms affiliated with the genus *Sulfobacillus*, moderate thermophiles, were dominant at sites D1, DY and D3 where temperatures were all around 25°C. Similar results were obtained during research carried out by Bond et al. Analysis of the microbial community in the drainage waters of the inactive Richmond mine at Irion

Mountain showed that *sulfobacillus* spp. were the dominant organisms in the summer (Bond et al. 2000a). The literature reported that temperatures can reach more than 60°C at some positions in a bioleaching heap (Bosecker 1997). Our results suggest that moderate thermophiles may have grown within hot spots in the heap, and were able to adapt and survive under comparatively lower temperature conditions. *Sulfobacillus* was not detected at site DX. This may have been due to the presence of high concentrations of toxic metal ions.

Moreover, many other microorganisms associated with bioleaching were also detected in the four samples. Although they were present in low proportions, they were still important to the composition of the community structures. For example, *Acidiphilium* spp. which have been found in some other AMD environments and bioleaching systems, were found in all sites examined in this study except site D1. Recent research suggests that these microorganisms remove organic toxins, which are harmful to *Leptospirillum* and *Acidithiobacillus* (Harrison 1985; González-Toril et al. 2003). Previous research also indicated that they might be of ecological significance in AMD, especially in the turnover of iron at oxic–anoxic interfaces (Johnson 1995; Küsel et al. 1999).

In our study, we also analyzed the relationship between microbial communities and geochemical properties in four bioleaching sites by using principal component analysis. The results of PCA based on the geochemical properties showed that sites D1 and D3 had similar geochemical properties, while sites DY and DX were a greater distance away from these two sites. The results also suggested that copper and arsenic appear to be the key factors causing the diversity in the four bioleaching sites. PCA based on the six phylogenetic divisions derived from 16S rDNA phylogenetic analysis, showed that sites D1 and D3 had similar microbial community structures, while sites DY and DX were much different from the other sites. These results suggested that similar geochemical properties resulted in the formation of similar microbial communities within the bioleaching systems at the Yinshan lead–zinc mine and the Dongxiang copper mine. However, to understand the ecology in highly acidic bioleaching systems, further studies are needed to reveal the variations in microbial community compositions and structures in response to environment and seasonal changes. Metabolic pathways and survival strategies of dominant microorganisms should also be studied.

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