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Compositions and Structures of Archaeal Communities in Acid Mineral Bioleaching Systems of Dongxiang Copper Mine and Yinshan Lead–Zinc Mine, China

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Abstract Four samples were studied from four separated sites with heap leaching in the Yinshan Lead–Zinc Mine and the Dongxiang Copper Mine in Jiangxi province, China. The compositions and structures of archaeal communities in four sites were identified by a polymerase chain reaction-based cloning approach. A total of six operational taxonomic units (OTUs) was obtained from four samples. The highest percentage of overlapped OTUs was 88.9% between sites DX and D1. Phylogenetic analysis revealed that archaea in the four acid mineral bioleaching systems fell into two divisions: *Thermoplasma* and *Ferroplasma*. The proportions of *Thermoplasma* and *Ferroplasma* in all four sites were 20.6% and 79.4%, respectively. The proportions of clones clustered with *Ferroplasma* in four sites were 93.8% (D1), 30.5% (D3), 100% (DY), and 93.2% (DX), respectively. The proportions of clones clustered with *Thermoplasma* in the other three sites were 6.2% (D1), 69.5% (D3), and 6.8% (DX), respectively. The results of principal component analysis based on the percentages of six OTUs obtained from four sites and geochemical data from four sites suggested that the concentrations of elements such as lead, cobalt, and sulfur might be the reason causing the different archaeal structure in site D3 than those in the other three sites.

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Introduction

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 [20]. Different engineering approaches have been used to facilitate microbial mineral processing. These approaches include *in situ* leaching, dump and heap leaching of low-grade ores, and aerated stirred tanks for microbial processing of mineral concentrates [4].

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 [12]. 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 [16, 22].

Archaeal organisms are important members of the microbial communities that populate hot, extremely acidic, metal-rich environments [2, 5, 6, 26]. There was less attentions paid to the compositions and constructions of archaeal communities in the bioleaching systems [7], compared with so much attentions paid to the bacterial communities in ARD, AMD, and bioleaching systems; for example Goebel and Stackebrandt [8, 9] cloned 16 S rRNA genes from acidophilic bacteria obtained from enrichment cultures of acidic runoff from a chalcocite overburden heap and a laboratory-scale mineral leaching bioreactor.

In this article, four samples from the Yinshan Lead-Zinc Mine and the Dongxiang Copper Mine in Jiangxi province were studied. The four samples from four separated sites

were undertaken using heap leaching. The abundance and diversity of archaea in these four acid mineral bioleaching systems were determined by a polymerase chain reaction (PCR)-based cloning approach. In addition, we studied the relationship between the geochemical properties and archaeal communities in four sites.

Materials and Methods

Site Description and Sample Collection

Samples were collected from four bioleaching sites in the Yinshan Lead-Zinc Mine and the Dongxiang Copper Mine, both in Jiangxi province, China. The two mines are important metal mines in China and have more than 50 years of exploitation history.

All four samples were water samples. One of them was from the Dongxiang Copper Mine, which is called DX in this article. The clones with 16 S rDNA inserts from this site were given the prefix DXG. The three samples were from the Yinshan Lead-Zinc Mine. They were called D1, DY, and D3, from which the clones with 16 S rDNA inserts were given the prefix D1G, DYG, and D3G, respectively. The details of the four samples are shown in Table 1.

A 10-L water sample was collected from each position. Samples were processed within 24 h after collection. Four water samples were filtered through a 0.22-μm hyperfiltration membrane (Bio Basic Inc., Canada) with a vacuum pump. The sediments were washed twice using sterile deionized water. Then the sediments were stored at −70°C until analysis. The filtered water samples were prepared for chemical analysis.

Chemical Analysis of Water Samples

The element analysis of filtered water samples was carried out by inductively coupled plasma–atomic emission spectrometry (ICP-AES). Twenty-eight elements were tested in each water sample: Hg, As, Ni, Co, Cr, Be, Ti, W, Zn, In, Mg, Mn, Ca, S, Mo, Bi, Au, Fe, Pb, Cu, Sn, Sb, Cd, P, Ga, Pt, Al, and Ag.

Table 1 Temperature and pH in four samples

Sample	Site name	PH	Temperature (°C)	Mine
1	DX	2.0	20.1	Dongxiang Copper Mine
2	D1	1.5	25.5	Yinshan Lead–Zinc mine
3	DY	1.5	25.2	Yinshan Lead–Zinc mine
4	D3	1.5	25.6	Yinshan Lead–Zinc mine

DNA Extraction and Purification

Extraction of nucleic acids was according to procedure described by Zhou et al. [28]. Five grams of sediment was mixed with 13.5 mL of extraction buffer [0.1 M phosphate (pH 8.0), 0.1 M EDTA, 0.1 M EDTA, 1.5 M NaCl, 1% cetyl trimethyl ammonium bromide (CTAB)] and 50 μL protenase K (10 mg/mL) in a 50-mL centrifuge tube and then incubated at 37°C for 30 min. Then 1.5 mL of 20% sodium dodecyl sulfate (SDS) was added, mixed gently, and then incubated at 65°C for 2 h. The mixture was centrifuged and the supernatant was transferred into a new 50-mL centrifuge tube. The soil pellet was resuspended with extraction buffer, and 0.5 mL of 20% SDS was added. The mixture was incubated at 65°C for 15 min and 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 1 h or overnight and then centrifuged. The pellet was washed with 70% ethanol and dissolved with 200–500 μL sterile water. By using the combined method that included grinding, freezing, and thawing and treatment with SDS, various types of bacteria could be effectively lysed. The crude DNA was purified by using Wizard plus sv Minipreps DNA purification system (Promega Corp., USA) and quantified by ethidium bromide–UV (ultraviolet) detection on an agarose gel.

PCR and Fractionation of 16 S rDNA Genes

Polymerase chain reaction amplification of archaeal 16 S rDNA genes were carried out following the PCR reactions described as follows: an initial denaturation at 94°C for 4 min, followed by 34 cycles of 94°C for 40 s, 50°C for 30 s, and 72°C for 1 min, and ending with an extension period of 10 min at 72°C. Primers used to selectively amplify archaeal 16 S rDNA genes were as follows: S-D-Arch-0025-a-S-17 (5′-CTGGTTGATCCTGCCAG-3′) [21] or S-D-Arch-0344-a-S-20 (5′-ACGGGGCGCAGCAGGC GCGA-3′) [27] with S-*-Univ-1517-a-A-21 (5′-ACGG CTACCTTGTTACGACTT-3′) [19] to yield 1500-bp or 1120-bp PCR products, respectively. Products from the amplification reactions of expected size were pooled and purified and then quantified by ethidium bromide-UV detection on an agarose gel before ligation later.

Cloning, RFLP, and Sequencing

The purified PCR products were ligated to the vector PGEM-T (Promega Corp., USA) and used to transform DH5α competent host cells. An optimal insert vector ratio of 3:1 was used. The plasmid clones were identified based on blue-white screening. At least 64 white colonies were

Table 2 Concentration of 28 elements in 4 sites

Site	As (mg/L)	P (mg/L)	Zn (mg/L)	Pb (mg/L)	Mg (mg/L)	Co (mg/L)	Hg (mg/L)
D1	29.0	21.1	24.0	0.1	33.2	2.2	3.7
DY	28.0	29.2	34.6	0.1	13.8	2.6	3.9
D3	26.0	30.6	70.0	0.1	72.6	1.6	3.0
DX	9.7	90.0	408.4	21.6	2512.4	19.3	18.0
Site	Ca (mg/L)	S (g/L)	Fe (g/L)	Cu (mg/L)	Al (mg/L)	Mo (mg/L)	Ni (mg/L)
D1	45.6	6.55	7.47	182.4	629.3	1.7	4.9
DY	20.3	6.08	8.04	188.3	341.5	1.7	5.1
D3	51.0	4.53	5.16	220.5	324.7	1.3	2.2
DX	593.7	27.90	46.89	245.2	3471.7	7.9	17.9
Site	Cr (mg/L)	Be (mg/L)	Ti (mg/L)	W (mg/L)	In (mg/L)	Mn (mg/L)	Au (mg/L)
D1	1.3	0.1	0.9	4.5	11.9	11.5	4.4
DY	1.1	0	0.4	4.6	12.8	4.3	4.8
D3	0.9	0.1	0.6	4.5	9.6	43.1	4.0
DX	5.0	0.4	0.5	19.6	48.1	1565.0	39.9
Site	Sn (mg/L)	Sb (mg/L)	Cd (mg/L)	Ga (mg/L)	Pt (mg/L)	Bi (mg/L)	Ag (mg/L)
D1	3.3	7.1	1.1	11.7	8.8	12.2	0.6
DY	3.4	7.1	1.1	13.0	9.7	13.5	0.7
D3	2.6	5.6	0.8	9.8	7.6	11.4	0.6
DX	15.8	27.4	8.8	63.8	36.0	39.9	0.7

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. These unpurified PCR products were digested with two restriction endonucleases *Afa*I and *Msp*I (TaKaRa Biotechnology Corp., 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 on UV illumination. RFLP patterns were identified and grouped, and representative clones were selected for nucleotide sequencing.

Phylogenetic Analysis

Phylogenetic affiliations of the partial sequences were initially estimated using the program BLAST (basic alignment search tool) [3]. Similarity of partial sequences was determined using ARB (a software environment for sequence data) [25]. The initial phylogenetic trees were based on all available sequences and were constructed by using the DNA distance program Neighbor-Joining with Felsenstein correction in ARB [24]. Based on the initial phylogenetic results, appropriate subsets of 16 S rDNA sequences were selected and subjected to a final phylogenetic analysis with CLUSTAL X.

Statistical Methods

Principal component analysis (PCA) was performed by using the SYSTAT statistical computing package (version 13.0; SPSS, Inc., Chicago, IL) for each sampling site. PCA simultaneously considers many correlated variables and then identifies the lowest number to accurately represent the structure of the data [15, 23].

Nucleotide Sequence Accession Numbers

Sequences have been submitted to GenBank with accession numbers as followS: *DQ464157* (DXG1), *DQ464158* (DXG44), *DQ464159* (DXG51), *DQ464160* (DXG4), *DQ464161* (DXG36), and *DQ464162* (D3G12).

Results and Discussion

Geochemical Properties of Four Samples

Among the four sites, site DX was the most different, with a higher pH value (2.0) and lower temperature (20.1°C). Site DX also had higher concentrations of elements than the other three sites, except arsenic (shown in Table. 2).

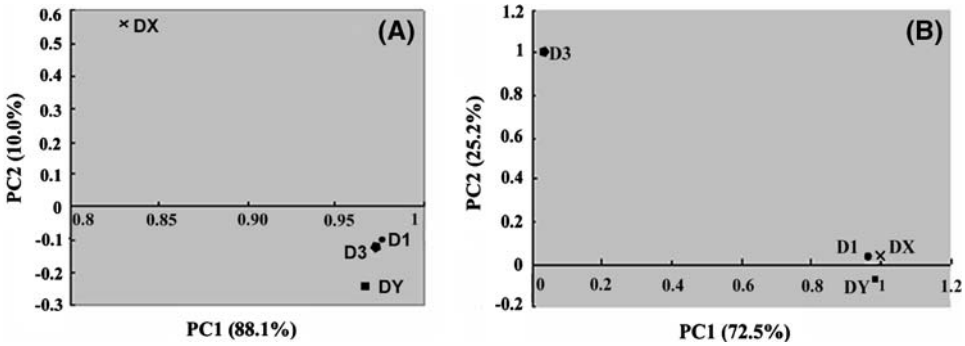

Fig. 1 Ordinate plots from PCA in four sites. (**A**) based on the data of 28 elements' concentrations; (**B**) based on six OTUs obtained from four samples. DX: a sampling position in Dongxiang copper mine; D1, DY and D3: three different sampling positions in Yinshan Lead-Zinc mine

The geochemical parameters detected by ICP-AES were analyzed by PCA to reveal geochemical variability among the four sites .The results in Fig. 1A show that sites D1 and D3 were grouped together based on the data of the 28 elements' concentrations. Sites DY and DX were different from each other and distant from sites D1 and D3. It suggested that sites D1 and D3 had similar geochemical properties, whereas sites DX and DY were different from the other sites.

Analysis of 16 S rDNA Clone Libraries by RFLP

A total of six operational taxonomic units (OTUs) (unique RFLP patterns) were obtained from four samples. The number of OTUs in sites D1, DY, D3, and DX was 4, 3, 4, and 5, respectively. The distribution of OTUs in four sites are represented in Fig. 2, in which the same OTU among four sites was marked with the same color.

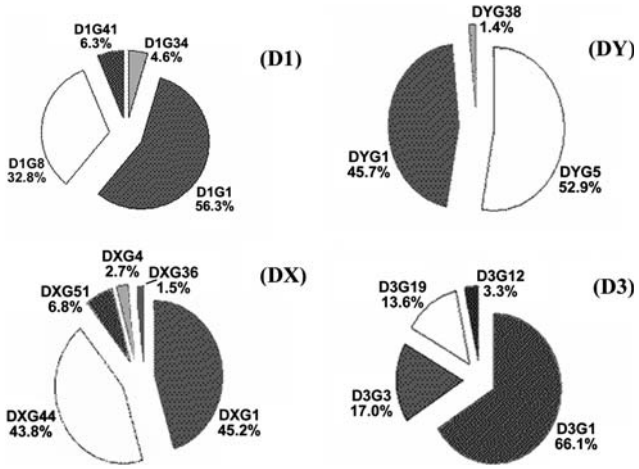

Fig. 2 Distributions of OTUs in clone libraries in four sites. DX: a sampling position in Dongxiang copper mine; D1, DY and D3: three different sampling positions in Yinshan Lead-Zinc mine. Clones DXG1, D1G1, DYG1 and D3G3: the RFLP patterns were same, with 99% similarity to uncultured archaeon clone ant h4; clones DXG44, D1G8, DYG5 and D3G19: the RFLP patterns were same, with 99% similarity to *Ferroplasma cyprexacervatum*; clones DXG51, D1G41 and D3G1: the RFLP patterns were same, with 99% similarity to uncultured archaeon clone ant d5; clones DXG4, D1G34 and DYG38: the RFLP patterns were same, with 99% similarity to *Ferroplasma acidiphilum*; clones DXG36: the RFLP patterns were same, with 100% similarity to *Ferroplasma acidiphilum* strain DR1; clones D3G12: the RFLP patterns were same, with 95% similarity to uncultured archaeon clone ant g10

Five of the six OTUs were detected in site DX. This might be due to the higher concentration of elements in site DX. In site DX, the elements' concentrations were ~10-fold higher than those in the other three sites, except for arsenic. It was in agreement with the previous report that showed that a comparison of physical–chemical characteristics with microbiological characteristics of AMD sites worldwide revealed a correlation between the presence of *Ferroplasma* or related archaea and the highest amounts of total iron, ferrous iron, and other metals (Al, Mn, Cu, Zn, etc.) [13].

Principle component analysis based on six OTUs obtained from four sites captured virtually most of the variability in the clone data. The results in Fig. 1B show that sites DX and D1 were the closest. Site DY was a little farther from sites DX and D1. Site D3 was distant from the other three sites. It suggested that sites DX and D1 had similar archaeal community structure, whereas sites DY and D3 were much different than the other sites.

Phylogenetic Analysis

Representative 16 S rDNA clones of OTUs were sequenced. There were six sequences obtained from four sites, the similarities among which varied from 79% to 96%. The archaeal phylogenetic tree (shown in Fig. 3) was established using a bootstrap neighbor-joining method with the six sequences.

The six OTUs fell into two phylogenetic divisions, *Thermoplasma* and *Ferroplasma*. The proportions of *Thermoplasma* and *Ferroplasma* in all four sites were 20.6% and 79.4%, respectively. There were four OTUs (DXG36, DXG1, DXG4, and DXG44) belonging to *Ferroplasma*. *Ferroplasma* was found to be dominant in the four samples we studied. The proportions of clones clustered with *Ferroplasma* in four sites were 93.8% (D1), 30.5% (D3), 100% (DY), and 93.2% (DX) respectively.

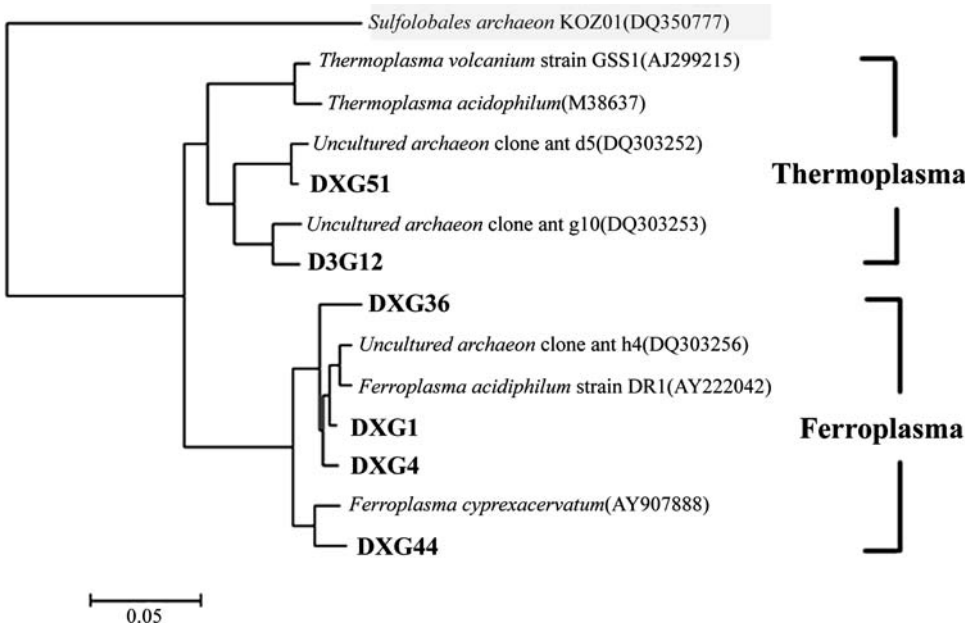

Fig. 3 Phylogenetic tree based on comparative analysis of 16S rDNA sequence data from six OTUs and their relatives. The sequences obtained in this study are in bold. The tree was rooted with *Sulfolobales archaeon* KOZ01 as the out group

The reason might be that the environments in the four sites were suitable for the growth of *Ferroplasma*. Despite of the high concentrations of elements in four sites especially in site DX, temperatures and pH values were also suitable for the growth of *Ferroplasma*. The first isolation in a pure culture of this type of organism, designated *Ferroplasma acidiphilum* strainY (DSM 12658^{T}), was obtained by extinct dilution of a microbial consortium from a pyrite-leaching bioreactor fed with pyrite ores from Bakyrtchik, Kazakhstan [10]. This organism usually grew at pH 1.3–2.2, with an optimum of pH 1.7, and at temperatures ranging from 15°C to 45°C, with an optimum of 37°C [17, 18]. In our study, the pH and temperature in sites D1, DY, and D3 were all around 1.5 and 25°C, respectively, whereas they were 2.0 and 20°C, respectively, in site DX.

The remaining two OTUs (DXG51 and D3G12) belonged to *Thermoplasma*. They exhibited 99% and 95% similarity, respectively, of nucleotide identity with the known database. *Thermoplasma* is usually considered thermophile, growing at 55–60°C and pH 0.5–4 [14]. Clones within the *Thermoplasma* have been detected in clone libraries created from samples collected from many geothermal hot spring environments and AMD waters [5, 11] but are rarely detected in bioleaching systems. Interestingly, in our study, *Thermoplasma* have been detected in bioleaching systems around 25°C except at site DY and, especially, was one of the dominant archaea in site D3 (69.5%). The results in this work showed that *Thermoplasma* were also important organisms in bioleaching systems. Researchers of bioleaching reported that the temperature could reach higher than 60°C at some parts in the heap of bioleaching [1]. The reason why *Thermoplasma* have been detected in bioleaching systems around 25°C might be that the moderate thermophiles occurred and grew in those hot parts and still survived at a lower temperature.

In addition, the relationship between archaeal communities and geochemical properties in the four bioleaching sites was considered. Totally, archaea,especially *Ferroplasma*, seems to prefer the environments with high concentrations of elements. In the PCA based on the geochemical data of four sites, we found that sites D1 and D3 had similar geochemical properties, whereas sites DY and DX were different than the other sites. In the other PCA based on six OTUs obtained from four sites, we found that sites D1 and DX had close microbial community structure, whereas both sites DY and D3 were different than the other sites. It suggested that the concentrations of elements such as lead, cobalt, and sulfur might be the key factors causing the different archaeal structure in site D3 than those in the other three sites. There is still some work needed to be done.

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