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ORIGINAL PAPER

Application of clone library analysis and real-time PCR for comparison of microbial communities in a low-grade copper sulfide ore bioheap leachate

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Abstract The microbial communities of leachate from a bioleaching heap located in China were analyzed using the 16S rRNA gene clone library and real-time quantitative PCR. Both methods showed that *Leptospirillum* spp. were the dominant bacteria, and *Ferroplasma acidiphilum* were the only archaea detected in the leachate. Clone library results indicated that nine operational taxonomic units (OTUs) were obtained, which fell into four divisions, the *Nitrospirae* (74%), the *γ-Proteobacteria* (14%), the *Actinobacteria* (6%) and the *Euryarchaeota* (6%). The results obtained by real-time PCR in some ways were the same as clone library analysis. Furthermore, *Sulfobacillus* spp., detected only by real-time PCR, suggests that real-time PCR was a reliable technology to study the microbial communities in bioleaching environments. It is a useful tool to assist clone library analysis, to further understand microbial consortia and to have comprehensive and exact microbiological information about bioleaching environments. Finally, the interactions among the microorganisms detected in the leachate were summarized according to the characteristics of these species.

Keywords Real-time PCR · Clone library · Heap bioleaching · 16S rDNA

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Introduction

In recent years biohydrometallurgy has been a hot field for researchers and engineers. Using this technology to extract copper and gold has been commissioned in many countries. Copper produced by bioleaching accounts for one fourth of the world's copper production [1]. Nowadays it is believed that biohydrometalurgy is mainly a chemical process. The role of bacteria in this process, which cannot be negligible, is to oxidize reduced sulfur compounds and Fe^{2+} and provide extracellular polymeric substances—the space for the action taking place [2, 3]. Microorganisms in biomining environments can be divided into two kinds: (1) iron and/or sulfur oxidizers, such as *Acidithiobacillus ferrooxidans*, whose oxidation rate of Fe^{2+} is 5×10^5 to 1×10^6 as high as that of dissolved oxygen, and the produced Fe^{3+} in this process oxidizes sulfide ores [4]; (2) heterotrophic acidophiles, such as *Acidiphilium* spp., which use the organic substances produced by autotrophs to eliminate the inhibitory effect, reduce Fe^{3+} to Fe^{2+} in low oxygen concentration environments and supply the iron oxidizers [5].

Bioleaching is a complex process, involving interactions among biological, chemical and physical factors. Microbial communities will change with temperature, pH, the ratio of Fe^{3+} to Fe^{2+} and concentrations of dissolved oxygen, carbon dioxide, sulfate and metal ions. On the contrary the changes of microbial communities will influence the leaching efficiency. Due to the complexity of the process, it is difficult to control the microbial communities of bioleaching process. The study of microbial diversity during this process is to unravel the dominant microorganisms of different phases and the rules that microbial communities change with environmental conditions. From these rules the optimal biological and physiochemical conditions for

bioleaching processes can be found, which can be very important to control and optimize this process.

Nowadays, molecular microbiological methods are used to study the microbial populations during bioleaching, such as clone library, FISH (fluorescence in situ hybridization), DGGE (denaturing gradient gel electrophoresis) and real-time PCR [6–9]. The methods of clone library and DGGE are end-point testing, which use PCR products of bulk environmental DNA and cannot acknowledge the initial composition of microorganisms in environments. However, this can be obtained by real-time PCR through monitoring fluorescent signals during amplification, which is more sensitive, but this method cannot detect unknown microorganisms.

In this work clone library and real-time PCR were both used to study the microbial communities of leachate from a low-grade copper sulfide ore bioleaching heap. This paper combined endpoint and initial testing and may provide more comprehensive and exact information about microbial populations in bioleaching environments; thus, the application of biohydrometallurgy may be improved.

Methods

Site description and DNA extraction

Zijinshan copper mine, located in Shanghang County, Fujian Province, was the first commercial plant of bio-heap leaching with a capacity of 10,000 t Cu/a in China [10]. The leachate used for study was collected from the collecting pool of this plant.

Leachate samples were stored at 4°C and delivered to our laboratory immediately. The chemical properties of the leachate are shown in Table 1. For molecular analysis, 1,000 ml leachate was filtered through a 0.2-μm polycarbonate membrane (Nuclepore, USA) and stored at −20°C in TE (10 mM Tris–HCl, 1 mM EDTA, pH = 8.0). The filter was cut and washed twice with deionized water. Then DNA was extracted using a modified method by Oved [11]. The filter was suspended in 1 ml of extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, pH = 8.0), then 100 μl 10% PVP, 100 μl 20% SDS and 0.2 g 106 μm beating bead (Sigma, USA) were added, and the tube was vortexed for 5 min at 3,000 rpm. The mixture was centrifuged for 1 min at 12,000 rpm, and then the supernatant was transferred into a new tube; 1/10 volume of ice incubated 3 M NaAc was added and incubated for 10 min. After centrifugation for 5 min at 12,000 rpm, the supernatant was transferred and mixed with two volumes of isopropanol and incubated on ice for 30 min. The pellet of crude nucleic acids was obtained by centrifugation at 12,000 rpm for 10 min, washed with cold 70% ethanol and resuspended in sterile deionized water to give a final volume of 50 μl. The extracted DNA was further purified using the DNA Clean-up Kit (Promega, USA) and finally resuspended in 50 μl TE.

Table 1 Chemical properties of the leachate

pH	SHE (mV)	Fe^{2+} (g/l)	Fe^{3+} (g/l)	Cu^{2+} (g/l)	Cell number (ml^{-1})
1.84	678	2.77	3.44	7.53	1×10^6

DNA extraction from clones

DNA used for the construction of standard curves was extracted from clones of our previous studies. Clones with 99% similarity to *Acidithiobacillus* sp., *Leptospirillum* sp., *Sulfobacillus* sp., *Ferroplasma* sp. and a blue clone were picked and resuspended in 200 μl 6% Chelex 100 (Biorad, USA). The mixtures were incubated at 56°C for 30 min. Then tubes were vortexed at high speed for 10 s. The tubes were inserted in a 100°C heat block for 8 min and vortexed at high speed for 10 s. Then tubes were centrifugated at 12,000 rpm for 2 min, and 10 μl of the supernatant was used in a 50-μl PCR reaction.

16S rRNA gene cloning, sequencing and analysis

16S rRNA gene from leachate was amplified with a GeneAmp PCR System 2700 (Applied Biosystems, UK), using 30 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min. Universal primers 530F and 1490R [12, 13] were used to amplify the bacteria and archaea 16S rDNA. The amplified 16S rRNA genes were gel purified, inserted into pGEM-T easy vectors (Promega, USA) and transformed into *E. coli* DH5α. Fifty white clones were randomly selected from the 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 restriction endonuclease *Rsa* I (Promega, USA) and then 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. Chimera sequences were detected by the chimera function at the RDP site (http://rdp.cme.msu.edu/), and all chimera sequences were eliminated. Sequences were analyzed using BLAST at the NCBI database (http://ncbi.nlm.nih.gov/BLAST). Alignments of 16S rRNA gene sequences were performed with the CLUSTAL_X program, version 1.64b [14]. A neighbor-joining

phylogenetic tree was constructed based on evolutionary distances that were calculated with the Kimura two-parameter model using MEGA 4 [15]. GenBank accession numbers for rDNA sequences determined in this work are given in Table 2.

Real-time PCR

Real-time PCR based on SybrGreen I was performed with Rotor-Gene 6000 (Corbett Research, Australia). The reaction mixture contained 12.5 μl of SybrGreen PCR Master Mix (Applied Biosystems, UK), 1 μl of template DNA, 6.25 pmole of each primer and H_2O added to a total of 25 μl. The PCR program consisted of one cycle of 95°C for 10 min, and then 45 cycles of 95°C for 15 s and 60°C for 1 min. After each run, melting curves for the amplicons were measured by raising the temperature slowly from 65 to 95°C while monitoring fluorescence. The specificity of the PCR amplification was checked by examining the derivatised melting curve for Tm, its symmetry and the lack of non-specific peaks. All tests were conducted in triplicate.

For the quantification of *Acidithiobacillus* sp., *Leptospirillum* sp., *Sulfobacillus* sp., *Ferroplasma* sp. and prokaryotes, primer pairs were referenced in previous study [16]. To convert DNA copy numbers to cell numbers, the following conversion factors were used: 4.1 for bacteria, 1.5 for archaea [17], 12 for *Acidithiobacillus* sp., 10 for *Leptospirillum* sp., 6 for *Sulfobacillus* sp., and 3 for *Ferroplasma* sp. [16].

Standard curves

For the quantification of individual 16S rDNA species, each PCR product that was amplified from the clones with specific genes by using the specific primers was purified and quantitated spectrophotometrically on an Optizen 2120 UV spectrophotometer (Mecasys Co., Ltd., Korea). The PCR products were diluted serially and amplified by real-time PCR to construct specific standard curves by plotting the known concentration of input DNA versus the threshold cycle (Tc). The standard curve for prokaryotes was constructed from the partial 16S rRNA gene of *E. coli*. To convert the mass of DNA to copy number, the following formula was used [18]:

$$\frac{\text{Mass (in grams)} \times \text{Avogadro's Number}}{\text{Average mol. wt. of a base} \times \text{template length}} = \text{copy numbers of DNA}$$

For double-stranded templates, 660 gm/mole per base was used. One nanogram of a 400 bp PCR product was equivalent to 2.51×10^9 copies.

Results

RFLP analysis of 16S rDNA clone libraries

A pair of universal primers was used to amplify bacterial and archaeal 16S rRNA gene from the leachate sample of Zijinshan copper mine. A length of 1,000 bp PCR product was amplified. After T-A cloning, 50 clones containing the inserted 16S rDNA were obtained. A partial profile of RFLP is shown in Fig. 1.

Rarefraction analysis was used in RFLP analysis. The result is shown in Fig. 2. Nonlinear regression suggested that 37 white clones were saturant for constructing the library. The result suggested that clones tested in the experiment were sufficient to detect the level of microbial community diversity in the leachate.

The RFLP analysis revealed extensive diversity of 16S rDNA. The distribution of operational taxonomic units (OTUs), which were ranked in the order of abundance, is shown in Fig. 3. The RFLP patterns of clones clone-1, clone-2, clone-6, clone-17, clone-24, clone-33, clone-35, clone-36 and clone-37 represented 34, 24, 10, 4, 6, 4, 2, 6

Table 2 Inventory of prokaryotic 16S rDNA cloned fragments arranged into groups according to RFLP patterns and sequence similarity

Clones	Number of clones	Accession number	Phylogenetic division	Closest relative (accession number)	Similarity
Clone-1	17	EU383018	*Nitrospirae*	*Leptospirillum ferrooxidans* strain Sy (AF356837)	99% (1,007 bp)
Clone-2	12	EU383019	*Nitrospirae*	*Leptospirillum ferriphilum* strain UBK03 (DQ534052)	99% (1,001 bp)
Clone-6	5	EU383020	*γ-Proteobacteria*	*Acidithiobacillus ferrooxidans* strain ATCC23270 (AF465604)	99% (995 bp)
Clone-17	2	EU383021	*Nitrospirae*	*Leptospirillum ferriphilum* strain FTH (EF025342)	90% (1,012 bp)
Clone-24	3	EU383022	*Actinobacteria*	*Ferrimicrobium acidiphilum* (AF251436)	97% (983 bp)
Clone-33	2	EU383023	*γ-Proteobacteria*	*Acidithiobacillus ferrooxidans* strain 2 (DQ909077)	99% (1,000 bp)
Clone-35	1	EU383024	*Nitrospirae*	*Leptospirillum ferriphilum* strain FTH (EF025342)	99% (1,001 bp)
Clone-36	3	EU383025	*Euryarchaeota*	*Ferroplasma acidiphilum* (AJ224936)	99% (991 bp)
Clone-37	5	EU383026	*Nitrospirae*	*Leptospirillum ferrooxidans* strain P3a (AF356837)	99% (996 bp)

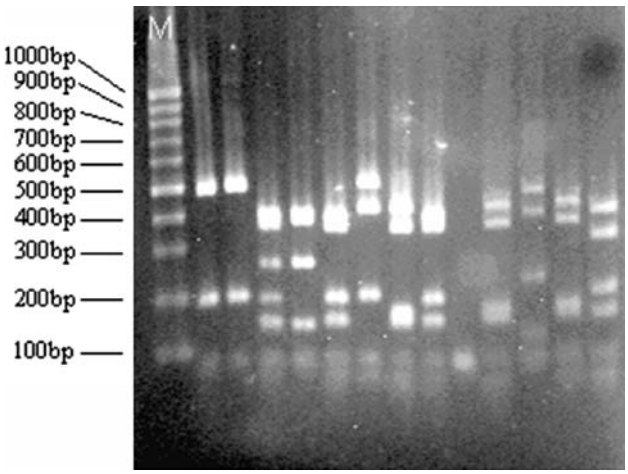

Fig. 1 Restriction fragment length profiles of 16 rDNA fragments amplified from the leachate sample in Zijinshan Copper mine. The 16S rDNA fragments were amplified using the primer set 530F and 1490R, digested with the restriction endonucleases *Rsa*I, and then analyzed by 3.0% agarose and electrophoresis. M: 100 bp DNA ladder plus

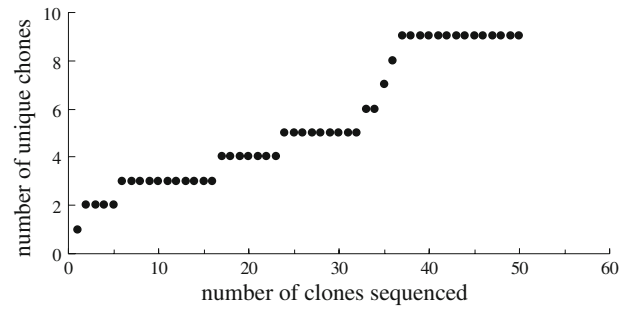

Fig. 2 Evaluation of the representative clones obtained from leachate from Zijinshan copper mine by rarefaction analysis

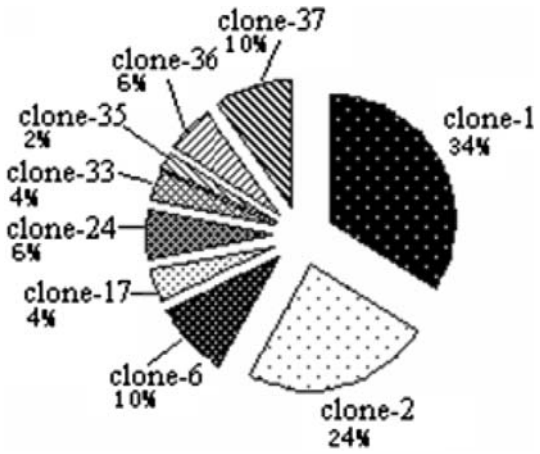

Fig. 3 Distribution of OTUs in clone library of the leachate. Clone 1: 99% similarity to the *Leptospirillum ferrooxidans* strain Sy; clone 2: 99% similarity to the *Leptospirillum ferriphilum* strain UBK03; clone 6: 99% similarity to the *Acidithiobacillus ferrooxidans* strain ATCC23270; clone 17: 90% similarity to the *Leptospirillum ferriphilum* strain FTH; clone 24: 97% similarity to the *Ferrimicrobium acidiphilum*; clone 33: 99% similarity to the *Acidithiobacillus ferrooxidans* strain 2; clone 35: 99% similarity to the *Leptospirillum ferriphilum* strain FTH; clone 36: 99% similarity to the *Ferroplasma acidiphilum*; clone 37: 99% similarity to the *Leptospirillum ferrooxidans* strain P3a

and 10% of the total clone populations in the leachate, respectively.

To determine the phylogenetic diversity, representative 16S rDNA clones of OTUs that occurred more than once in the cloning library, as well as representatives of the unique OTUs, were fully sequenced. The details of representative clones sequenced were as shown in Table 2.

The phylogenetic tree was constructed with the bootstrap neighbor-joining method. The result is shown in Fig. 4. All 50 clones were divided into four major clusters: the *Nitrospirae* (occupying 74% of the total 50 clones), the *γ-Proteobacteria* (14%), the *Actinobacteria* (6%) and the *Euryarchaeota* (6%).

The *Nitrospirae* family was predominant in the leachate. In this family *Leptospirillum* was the only genus detected. OTU clone-1 and clone-37 had a similarity of 99% with *Leptospirillum ferrooxidans*. Sequences of clone 2 and clone 35 were clustered with *Leptospirillum ferriphilum*. Clone 17 only had a similarity of 90% with *Leptospirillum ferriphilum* and did not cluster with the known *Leptospirillum* group I, II and III. The *γ-Proteobacteria* family was another family in the leachate. All the OTUs in this family were clustered with *Acidithiobacillus ferrooxidans*. There was also one OTU belonging to *Actinobacteria*, which was affiliated with *Ferrimicrobium acidiphilum*. The *Euryarchaeota* was the only archaeal family in the leachate. Only one OTU, clone 37, was detected in this family, which had 99% similarity to *Ferroplasma acidiphilum*.

Verification of real-time PCR assay

Amplified specific PCR products were used as templates and serially diluted to construct standard curves. The correlation coefficients of all the obtained standard curves were over 0.99, and the amplification efficiencies were between 85 and 118%. The standard curves were constructed using template copies from 10^7 to 10^1. All the amplified products could be detected at a magnitude of 10^1, except for *Acidithiobacillus* sp. and *Leptospirillum* sp., whose detection limit was found to be a magnitude of 10^2.

To test the competition of mixed 16S rDNA samples for specific primers, PCR amplification of 16S rDNA was tested in the presence and absence of non-specific, competing 16S rDNA samples; 2.3×10^5 copies of specific 16S rDNA in the presence and absence of 2.3×10^5 copies of all the other species were carried out in this study. No apparent difference was observed in the presence and absence of the competing non-specific DNA with all

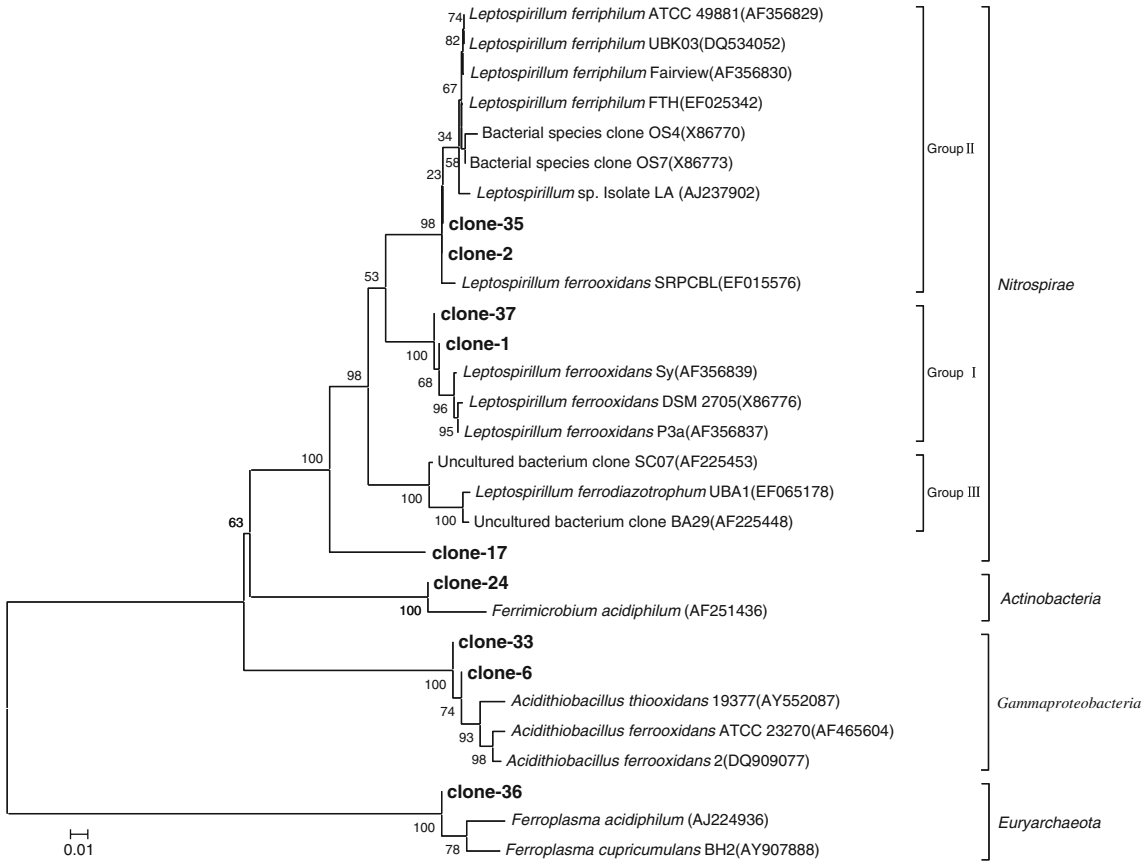

Fig. 4 Rooted neighbor-joining tree based on 16S rDNA sequences that were calculated with the Kimura two-parameter model of MEGA4 showing the relationship of nine unique clones obtained and their close relatives. Sequences belonging to the same division or group were tagged. Bootstrap values were 500. *Scale bar* represents the number of inferred nucleotide substitutions per site. The sequences obtained in this study are indicated in *bold*

primers tested based on the patterns of exponential fluorogenic amplifications (Table 3). The results demonstrated that the assay could be used to detect and quantify target DNA extracted from samples containing mixed microbial species. It also could be seen that total prokaryotes 16S rDNA copies in mixed DNA samples could be quantitated.

Real-time PCR quantification of microbial community in the leachate

The real-time PCR assay was used to quantify selected microorganisms in the leachate. The analysis results indicated that copies of *Leptospirillum* sp. were predominant in the leachate (Table 4). *Sulfobacillus* spp. could be detected by real-time PCR that were not found in the clone library.

Table 3 Quantitation of individual and total prokaryotes 16S rDNA species in a mixed DNA sample

Genus	16S rDNA copies		Discrepancy
	Copies added	Copies obtained	Copies obtained/ copies added
Acidithiobacillus	2.3×10^5	2.43×10^5	1.06
Leptospirillum	2.3×10^5	5.06×10^5	2.2
Sulfobacillus	2.3×10^5	2.58×10^5	1.12
Ferroplasma	2.3×10^5	2.24×10^5	0.97
Universal	2.3×10^5	1.86×10^5	0.81

Discussion

The use of biohydrometallurgy technology for the extraction of metals from ores has developed a great deal in recent years, especially for heap bioleaching. The number of plants using this biotechnology in the world is increasing, and most of the plants are scaling or have scaled up [19]. Several research teams have studied the microbial diversity and succession during heap leaching [7, 20–22]. In this study, clone library and real-time PCR were used to study the microbial community of bioheap leachate.

At present there is not much microbiological research related to industrial heap bioleaching processes. In a previous pioneering work [7], microbial community

Table 4 Comparison of results obtained by real-time PCR and clone library

Genus	*Acidithiobacillus* sp.	*Leptospirillum* sp.	*Sulfobacillus* sp.	*Ferroplasma* sp.	Prokaryotes
Copy numbers/ml	1.60E + 03	1.37E + 06	2.54E + 02	7.05E + 02	1.93E + 07
Cell numbers/ml	1.33E + 02	1.37E + 05	4.23E + 01	2.35E + 02	–
Clone library	14%	74%	ND	6%	100%

ND not detected, – unknown

succession in a low-grade copper bioleaching heap was monitored for more than 2 years. From days 225 to 338, the dominant species in the leachate were *Leptospirillum* and *Ferroplasma* group. The sample collected in this paper was in the operation period of about half a year. Due to the mineralogy of the ore, which is mainly chalcocite and can be easily leached, the time is a little shorter than that of previous research. We found similar results that *Leptospirillum* are dominant in the leachate, and *Ferroplasma* is the only detected archaea that accounts for 6% of the whole prokaryotes. *Nitrospirae* accounts for 74% of the community, and all belong to the *Leptospirillum* genus. The chemical properties of the leachate used are similiar to Demergasso's results (Table 1), which indicate that heap bioleaching is in its second stage, and this can be confirmed in the microbiological results. *Leptospirillum* has a high affinity for Fe^{2+} and high resistance to Fe^{3+}, which could be the major reason for the dominance of *Leptospirillum* in the leachate [23]. It is interesting to find that clone 17 in the *Nitrospirae* has only 90% similarity to *L. ferriphilum* and belongs to none of the *Leptospirillum* I, II and III groups (Fig. 4). There was once an isolate *Leptospirillum thermoferrooxidans* in the genus *Leptospirillum*, but the pure culture was lost during subcultivation, and there are not any 16S rDNA sequences related to *L. thermoferrooxidans* [24]. Thus, further study is needed to determine to which linkage clone 17 belongs.

Acidithiobacillus ferrooxidans is the only detected species in the *γ-Proteobacteria* family. *At. ferrooxidans* has been used as a model microorganism in the study of bioleaching processes [25]. *At. ferrooxidans* is the only sulfur-oxidizer detected in the leachate by clone library, and the widely distributed *At. thiooxidans* and *At. caldus* are not found. *At. ferrooxidans* is usually the dominant group in environments at low temperature (<30°C) and high pH value (>2.0) [26]. The sample time was taken close to summer, and the heap located in southern China, so the temperature was not proper for *At. ferrooxidans*; however, *Leptospirillum* instead of *At. ferrooxidans* became dominant.

Ferrimicrobium acidiphilum is a gram-positive heterotrophic bacteria initially submitted as a new 16S rDNA sequence that now has been published as a new genus of acidophilic bacteria [27]. This bacteria clone was once found in the Tinto river, Spain and also in an acid mine drainage in China, but none of the research isolated the pure culture [28, 29]. The main role of *Ferrimicrobium acidiphilum* in the environment was to utilize the organic matter produced by autotrophic bacteria like *Leptospirillum* and *Acidithiobacillus* and eliminate the toxicity of organic matters to autotroph. Furthermore, *Ferrimicrobium acidiphilum* could reduce Fe^{3+} to Fe^{2+} and provide an electron donor for autotroph.

Ferroplasma acidiphilum is the only archaea detected in the leachate. It is a mixotrophic bacteria that lacks a cell wall. This organism usually grows between pH 1.3 and 2.2 (optimum pH 1.7) and at temperatures ranging from 15 to 45°C (optimum 37°C). It can tolerate high concentrations of metal ions [30, 31]. They are widely indentified in bioleaching processes and acid mine drainage because of their mesophilic characteristics and tolerance ability.

Sulfobacillus spp. is not detected by clone library analysis but found by real-time PCR in small amounts (Table 4). Sequences related to *Sulfobacillus* were found in most bioleaching processes and played an important role [8]. Our research was consistent with Demergasso's results of the second stage of heap bioleaching.

Except the clone library analysis, microbial communities in the leachate were determined by real-time PCR. In this work, we targeted specifically four genuses and the total bacteria and archaea that are commonly found in industrial bioleaching applications. According to previous research and the rrndb (The Ribosomal RNA Operon Copy Number Database) [17, 32], most of the bioleaching bacteria and archaea had one to two rRNA gene copies. But southern blot hybridization of the four strains (*At. ferrooxidans*, *L. ferrooxidans*, *S. thermosulfidooxidans* and *F. acidiphilum*) indicated that numbers of copies of the 16S rRNA genes per genome were 12, 10, 6 and 3 separately [16]. The 16S rRNA gene of *At. ferrooxidans* was sequenced and was the same as the strain in rrndb. The phenomenon is ubiquitous that the same strain has different 16S rRNA gene copies. Whole genome sequencing can dismiss multiple copies of repetitive sequences as a single sequence, under-quantifying the copies of the gene per genome, and sometimes different strains of the same microorganisms may have different copies of 16S rRNA genes [33], so our research used the conversion coefficient

used previously to convert 16S rRNA gene copies to cell numbers, and the result is show in Table 4 [16]. In real-time PCR analysis, the dominant microorganisms are *Leptospirillum* sp., which is consistent with the results of clone library analysis. But due to the low number of *Sulfobacillus* sp., they are not detected by the clone library. This indicates that real-time PCR is more accurate and the detection limit can be less than 100 cells/ml. The results obtained by real-time PCR and clone library in some ways are the same, and real-time PCR is a reliable technology to study the microbial communities in bioleaching environments. The results obtained by real-time PCR can be useful to assist clone library analysis.

The interactions among the microorganisms found in the leachate can be summarized according to their characteristics. In aerobic environments, *Leptospirillum* spp., *At. ferrooxidans* and *Sulfobacillus* spp. autotrophically oxidize Fe^{2+} and produce organic compounds that are harmful to autotroph and can be used by heterotrophic *Ferrimicrobium acidiphilum*, *Ferroplasma acidiphilum* and *Sulfobacillus* spp. Moreover, *Ferrimicrobium acidiphilum*, *Ferroplasma acidiphilum* and *Sulfobacillus* spp. can anaerobically reduce Fe^{3+} to Fe^{2+}, which is utilized by iron oxidizers. *At. ferrooxidans* and *Sulfobacillus* spp. can anaerobically reduce Fe^{3+} and oxidize sulfur and reduced sulfur compounds.

Conclusions

Clone library and real-time PCR were used to study microbial communities of leachate from a low-grade copper bioleaching heap. The dominant bacteria are *Leptospirillum* sp. by both methods, and *Ferroplasma acidiphilum* is the only detected archaea. The results demonstrate that real-time PCR is a reliable technology to study the microbial communities in bioleaching environments, and the results obtained by real-time PCR can be useful to assist clone library analysis to gain better understanding of the consortia and comprehensive and exact microbiological information in bioleaching environments.

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