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Bacterial diversity based on 16S rRNA and *gyr*B genes at Yinshan mine, China

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Abstract

The diversity of bacterial communities at three sites impacted by acid mine drainage (AMD) from the Yinshan Mine in China was studied using comparative sequence analysis of two molecular markers, the 16S rRNA and *gyr*B genes. The phylogenetic analyses retrieved sequences from six classes of bacteria, *Nitrospira*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, and *Actinobacteria*, as well as sequences related to the plastid of the cyanobacterium *Cyanidium acidocaldarium* and also some unknown bacteria. The results of phylogenetic analyses based on *gyr*B and 16S rRNA were compared. This confirmed that *gyr*B gene analysis may be a useful tool, in addition to the comparative sequence analysis of the 16S rRNA gene, for the analysis of microbial community compositions. Moreover, the Mantel test showed that the geochemical characteristics, especially the pH value and the concentration of iron, strongly influenced the composition of the microbial communities.

Keywords: Acidic mine drainage (AMD); Bacterial diversity; 16S rRNA; *gyr*B gene; Phylogenetic analysis; Mantel test

Introduction

The occurrence of acid mine drainage (AMD) has become a serious ecological problem due to its high concentrations of metal ions such as iron, copper, aluminum, and manganese. Despite their metal toxicity, many microorganisms, including Bacteria, Eukaryota, and Archaea inhabit such environments. These microorganisms are able to alleviate the pollution and have the potential to be utilized in bioleaching systems [2,3,34]. Some of these microorganisms found in AMD are responsible for the solubilization of metals from ores and they have been used as effective tools for extracting metals from low-grade or difficult-to-process sulfide ores or mineral concentrates in the bioleaching industry [22,24]. Thus, investigation of the microbial community compositions of the AMD environments and the distinctive functions of their microorganisms will benefit the bioleaching and bioremediation industry.

Restriction fragment length polymorphism (RFLP) is a useful tool in microbial diversity studies. The PCR

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product of a target sequence is digested with restriction enzymes and then the fragments are separated and detected with electrophoresis. The resulting banding patterns can be used to detect microbial community structure and this method is useful for detecting any structural changes and diversity in such communities [14,15,19,21]. Currently, 16S rRNA-based RFLP methods are accepted as rapid and accurate methods for the study of bacterial phylogenetic relationships and the diversity of microbial communities because of the high conservation of the molecule [6,7,14,19]. However, since it was found that the 16S rRNA gene did not distinguish between some closely related species, the *gyr*B gene, which encodes the β-subunit of DNA gyrase, has been shown to be a suitable molecular marker for the higher nucleic base substitutions [10,13,27,30,31]. Moreover, the *gyr*B genes are essential and ubiquitous throughout microorganisms and are sufficiently large in size for use in analysis of microbial communities [11].

In the present study, both the 16S rRNA and *gyr*B genes were used as the molecular markers to evaluate the diversity of the microbial community in acid environments of the Yinshan lead-zinc mine, Jiangxi province, China. Therefore, this work aimed to understand the microbial community of Yinshan Mine and the possible influence of the environment on changes in the community compositions.

Materials and methods

Site description and sample collection

Yinshan Mine is located in the Dexing mining district in Jiangxi Province, China. This site has been mined for extraction of copper, silver, zinc, and lead. Mining operations were conducted both underground and in open pits. Underground mining has resulted in over 20 miles of interconnected tunnels. Mining had increased the sulfide mineral surface area and the reactive ore that is exposed to oxygen and water.

Water samples for molecular analysis of microbial populations were collected in August 2006 from three different sites: YSK1 (29°0′19.98″N, 117°39′43.77″E), YSK2 (28°59′ 38.43″N, 117°39′55.65″E) and YSK3 (29°0′3.76″N, 117°40′45.40″E). YSK1 was an abandoned pool with a depth of 5 m that stored the AMDs. The surroundings of this site were covered with large quantities of sulfide minerals and the AMD produced by the oxidation of these minerals continually flowed into the pool, which led to the lower pH at the site. YSK2 was a reservoir filled with acidic mine drainage at an opencast site. The process of mining exposed the large mineral surface to the air and so greatly accelerated the formation of AMD. The reservoir was constructed to prevent pollution from AMDs. The YSK3 sample was collected from the AMDs resulting from the process of underground mining. In comparison with the first two sites, this site had the lowest temperature.

Approximately, 20 l of water was collected from each site, which was then filtered through a sterile 0.22 μm Nucleopore filter. These filters were then immediately transferred to a tube, and stored at −20 °C until they were required for molecular analysis.

Chemical analysis of the water samples

The concentrations of 36 elements, such as iron, aluminum, mercury, and arsenic, were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Baird Plasma Spectrovac PS-6(N + 1)). All the elements measured are shown in Table 1.

DNA extraction and purification

For all three sites, the bulk community DNA was extracted from 5 g of filtered sediment using a protocol described by Zhou et al. [35]. The crude DNA was further purified by agarose gel electrophoresis (3% agarose) and the Wizard DNA Clean-Up Kit (Promega, Madison, Wis.). All nucleic acids were stored at −20 °C until used.

PCR and fractionation of 16S rRNA and *gyr*B genes

The 16S rRNA and *gyr*B genes from the microbial community were amplified in reaction mixtures that contained 100 ng of DNA template, 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM $MgCl_2$, and 0.001% [w/v] gelatin), 2 mM dNTPs, 5 pM each of the forward and reverse primers, and 2.5 U of AmpliTaq Gold (Perkin Elmer). A final volume of 50 μl was adjusted with distilled water.

For 16S rRNA genes, the reverse primer was the universal 1387R (5′-GGGCGGWGTGTACAAGGC-3′) and the forward primer was the universal 63F (5′-CAGGCCTAACACATGCAAGTC-3′) [17]. The thermal cycling protocol used included initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s. A final extension step of 72 °C for 7 min was also used.

For the *gyr*B gene amplifying reaction, the reverse primer was the universal UP1 (5′-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3′) [29], and the forward primer was the universal UP2r (5′-AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3′). Optimum temperature and cycling parameters were determined to be initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 57 °C

Table 1. Geochemical element properties, pH, and temperature of the AMD samples YSK1, YSK2, and YSK3

Elements (mg/l)	YSK1	YSK2	YSK3
Ag	0.01	0	0
Al	307.8	136.1	159.6
As	0.7	0.01	0.02
B	0.2	0.1	0.3
Be	0.02	0.02	0.02
Bi	0.09	0.02	0.01
Ca	60.9	150.7	146
Cd	0.01	0	0.01
Ce	8.5	0.7	0.2
Co	1.0	0.4	0.7
Cr	0.5	0.1	0.2
Cu	264.6	84.7	33.5
Fe	5626	235.5	635.3
Ga	0.7	0.1	0.4
K	5.4	1.5	0.7
La	0.6	0.3	0.2
Mg	82.8	57.6	249.1
Mn	55.2	65.4	207
pH	1.0	2.0	2.5
Mo	0.1	0.01	0.04
Na	2.4	4.5	72.2
Nb	0.05	0.04	0
Ni	1.7	0.8	1.7
P	18.4	0.9	2.7
Pb	0.4	0.09	0.1
S	5574	552.3	1219
Sb	0.04	0.01	0.02
Sc	0.2	0.05	0.05
Si	46.1	36	19.2
Sn	0.2	0.03	0.05
Ta	0.2	0.07	0.2
Ti	0.3	0.01	0
V	1.2	0.05	0.1
W	0.4	0.2	1.2
Y	0.3	0.2	0.2
Zn	95.5	93.4	517.5
Zr	0.2	0.02	0.01
Temperature (°C)	28	25	20

for 1 min, and 72 °C for 2 min, then a final extension step of 72 °C for 7 min. Amplimers of the expected size for the 16S rRNA (approximately 1.3 kbp) and *gyr*B (approximately 1.26 kbp) genes were excised from 0.8% low melting point agarose gels and purified with the Wizard PCR Clean-Up system (Promega) in accordance with the manufacturer's instructions.

Cloning and restriction digestion of 16S rRNA and *gyr*B genes

The PCR products from each microbial community were cloned into the pCR 2.1-TOPO vector and *Escherichia coli* Top10F' competent cells, according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). Plasmid clones were identified based on blue-white screening and they were grown overnight on plates with ampicillin (100 mg ml^{-1}) and X-gal (15 mg ml^{-1}). White colonies from each of these six libraries were randomly selected and the cloned inserts were reamplified with the vector primers M13F and M13R. The PCR products of the 16S rRNA and *gyr*B genes were digested with 1 U each of the restriction endonucleases *Hin*PI and *Msp*I in 1 × NEB buffer (New England Biolabs, Beverly, Mass.) overnight at 37 °C. The digestion products were detected by gel electrophoresis in 3.0% agarose with ethidium bromide staining and were observed under UV illumination. RFLP banding patterns were identified, and a representative clone was selected for nucleotide sequence determination.

DNA sequencing and phylogenetic analysis

To understand phylogenetic diversity, each unique RFLP pattern of the 16S rRNA and *gyr*B unique clones were partially sequenced. Altogether, 68 unique *gyr*B patterns and 52 unique 16S rRNA patterns were sequenced. The sequencing was performed with an ABI PRISM Big Dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems, Foster City, California) and an ABI PRISM 3700 DNA analyzer (Applied Biosystems). Sequence identification was estimated initially by the BLASTN facility from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Before the 16S rRNA tree was constructed, the sequences with a similarity $\geqslant$97% to the researched sequences in the NCBI database were chosen as reference sequences. At the same time, some *gyr*B sequences affiliated with the acidophiles in the NCBI or ICB databases were also chosen as references and these acidophiles were often shown in the AMDs when constructing the *gyr*B tree. Then, these 16S rRNA and *gyr*B sequences (both the reference sequences and researched sequences) were aligned using CLUSTAL W (with the following parameters: gap opening penalty = 5.00; gap extension penalty = 0.05). The final 16S rRNA and *gyr*B phylogenetic trees were constructed with the MEGA III software. Pairwise distances were calculated with the distance only option and the phylogeny trees were constructed using the neighbour-joining method (bootstrap phylogeny test: 500 replicates, seed = 70,189).

Statistical methods

The Mantel test [16] was used for inferring the relationship between site geochemistry and the microbial community. All the matrices required for the test

were constructed based on Euclidean distance measurements, and both the R package v. 4.0 [4] and the R vegan package were used for comparison. The majority of analyses were carried out by the functions in the vegan package with some additional code utilizing the package functions.

Nucleotide sequence accession numbers

All of the sequences described in this study have been submitted to GenBank under accession numbers EF612964 to EF613023 (16S rRNA) and EF613024 to EF613109 (*gyr*B genes).

Results

Geochemical characteristics of the sampling stations

The pH values of the three sites were 1.0 (YSK1), 2.0 (YSK2), and 2.5 (YSK3). The results from the analysis of ICP-AES indicated that the concentration of each element was very different. YSK1 had the highest concentrations of iron, copper, aluminum, and sulfur, whereas YSK2 and YSK3 had similar iron concentration levels. Moreover, the temperature of the three sites was different, since YSK3 had the lowest temperature (20 °C) and YSK1 had the highest temperature (28 °C). The data for the pH values and concentrations of each element are shown in Table 1.

RFLP analysis of 16S rRNA and *gyr*B clone libraries

The 16S rRNA (1.3 kb) and *gyr*B gene (1.26 kb) products of the expected size were successfully amplified from the community genomic DNA in the three samples. After T-A cloning, a total of 372 16S rRNA and 383 *gyr*B positive colonies were recovered from all samples and then these clones were screened by RFLP analysis. The RFLP analysis revealed extensive diversity of the 16S rRNA and *gyr*B genes for the three AMD samples. There were 22, 33, and 27 16S rRNA gene operational taxonomic units (OTUs) and 24, 37, and 32 *gyr*B gene OTUs in YSK1, YSK2, YSK3, respectively. Regarding the clone similarities, YSK2 and YSK3 had higher similarity than YSK1, both in the 16S rRNA and *gyr*B gene analyses (Table 2). Moreover, the results also showed that all the similarity values for 16S rRNA genes were higher than those of the *gyr*B genes (Table 2). The diversity indexes calculated from the clone data showed that the *gyr*B gene diversity was higher than the 16S rRNA diversity in all three samples (Table 2). In addition, the rarefaction analysis suggested that the number of 16S rRNA and *gyr*B clones analyzed was sufficient to detect the level of microbial community diversity and infer the level of distribution within the communities of the three samples (data not shown).

Table 2. Percentage similarity and diversity index (*H*) for 16S rRNA and *gyr*B community structure between three samples determined by analysis of RFLP patterns

Sites	16S rRNA			*gyr*B gene		
	Similarity (%)			Similarity (%)		
	YSK2	YSK3	*H*	YSK2	YSK3	*H*
YSK1	18.3	15.2	3.91	13.5	7.8	4.28
YSK2		60.4	4.48		55.9	4.74
YSK3			4.27			4.48

16S rRNA and *gyr*B similarity were based on RFLP patterns. *H* was calculated as follows: $H = -\Sigma(pi)(\log_2 pi)$, where *pi* is the proportion of the RFLP banding pattern.

Phylogenetic analysis of 16S rRNA and *gyr*B genes

The phylogenetic trees of the 16S rRNA and *gyr*B genes were inferred based on all sequences obtained from the present study and some sequences selected from different environmental sources in the GenBank and ICB databases with a bootstrap neighbour-joining method (Figs. 1 and 2). According to the phylogenetic analysis of 16S rRNA, all 16S rRNA sequences fell into six classes of bacteria (*Nitrospira*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Acidobacteria*, and *Actinobacteria*), an algal species (*Cyanobacteria*) and some unknown bacteria. (Fig. 1). Interestingly, sequences of *Cyanobacteria* were related to the 16S rRNA gene from *Cyanidium acidocaldarium* plastid. *C. acidocaldarium* is a eukaryote which has been reported in many studies, and it was only found at the YSK1 site during this study. These main clusters could also be found using the *gyr*B gene phylogenetic tree, except for *C. caldarium* plastid, *Actinobacteria* and *Deltaproteobacteria*, but there were many unknown sequences in this tree due to the incompleteness of the *gyr*B gene database (Fig. 2).

At the YSK1 site, *C. caldarium* (31.9% of the clones), *Actinobacteria* (24.5% of the clones), *Nitrospira* (21.3% of the clones), and *Gammaproteobacteria* (16.0% of the clones) were the dominant microorganisms in the 16S rRNA gene phylogenetic tree. The microorganisms affiliated to *Nitrospira* were mainly composed of *Leptospirillum ferrooxidans* and *Leptospirillum ferriphium* (Fig. 1). In the *gyr*B gene phylogenetic tree, *Nitrospira* was still the dominant group (28.3% of the total *gyr*B clones) at the YSK1 site. Interestingly, all *gyr*B sequences (Fig. 2) related to *Nitrospira* clustered with *L. ferriphium*. The *C. caldarium* plastid and the

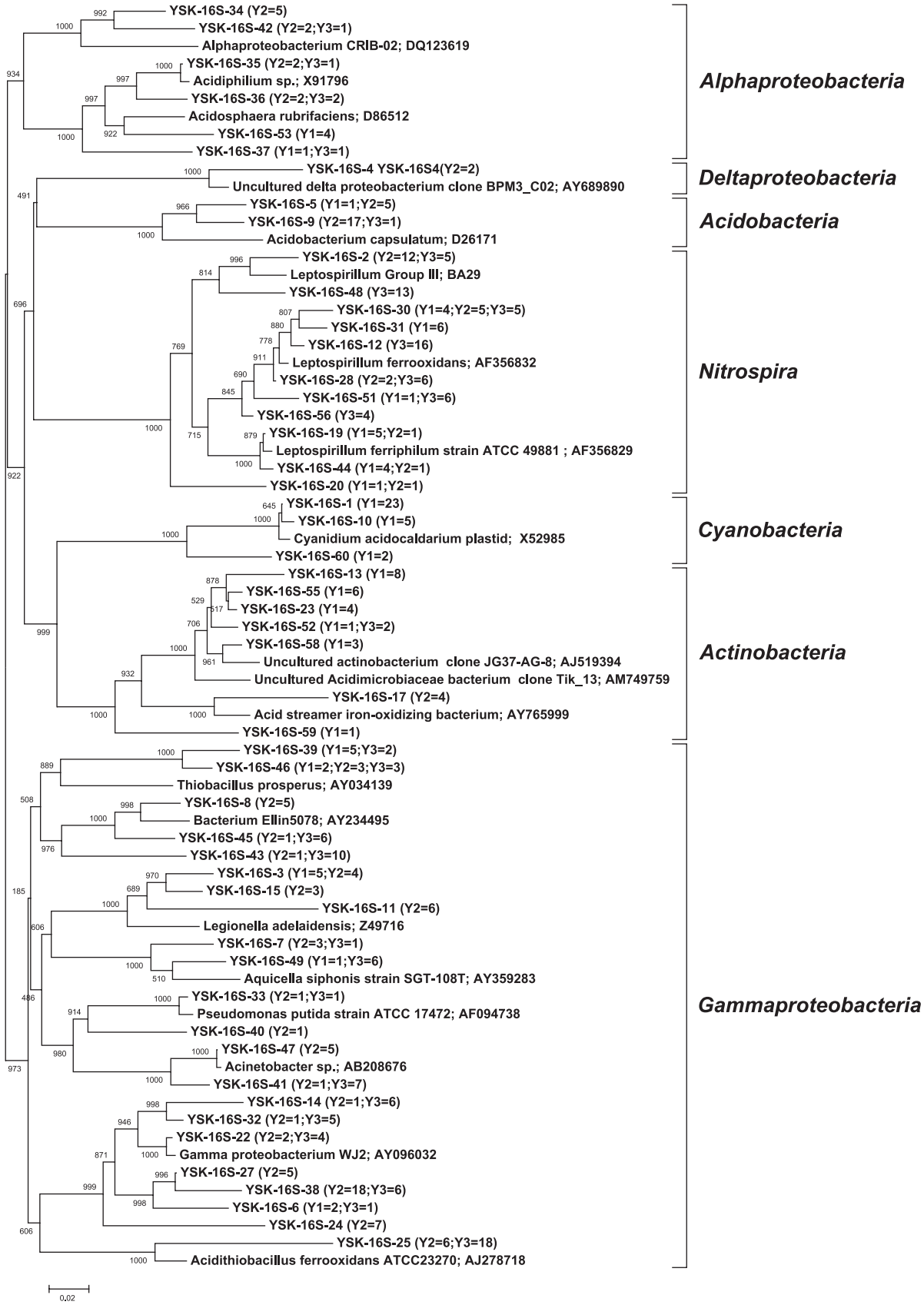

Fig. 1. Phylogenetic analysis of 16S rRNA gene sequences recovered from Yinshan Mine and from other different environmental sources. The clone composition of the three sites is provided. The numbers of clones retrieved from the sites are coded as Y1 representing site YSK1, Y2 representing site YSK2, and Y3 representing site YSK3.

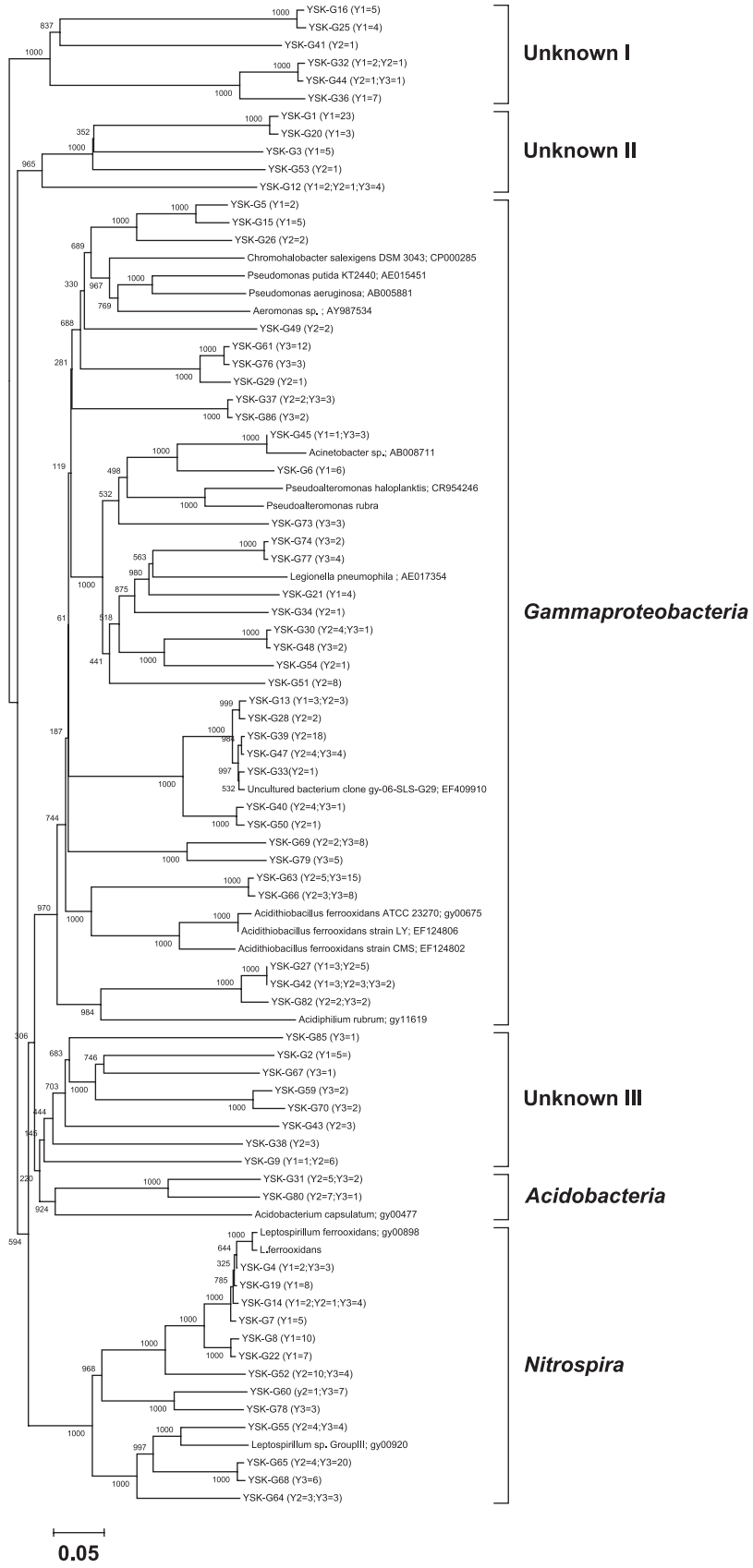

Fig. 2. Phylogenetic analysis of *gyr*B gene sequences recovered from sites at Yinshan Mine and selected sequences from other different environmental sources. The numbers of clones retrieved at each site is provided (Y1 represents site YSK1, Y2 represents site YSK2, and Y3 represents site YSK3).

Actinobacteria sequences were not found in the *gyr*B phylogenetic tree but, again, there were many unknown sequences (48.3% of the total *gyr*B clones) in this phylogenetic tree due to the incompleteness of the *gyr*B database.

In the YSK2 sample, *Gammaproteobacteria* was the dominant group based on both the 16S rRNA phylogenetic tree (54.8% of all 16S rRNA clones) and the *gyr*B phylogenetic tree (51.2% of all *gyr*B clones). Of the clones that affiliated with *Gammaproteobacteria*, most of the microorganisms were related to *Legionella adelaidensi* and *Gammaproteobacterium* WJ2 (an iron-oxidizing bacterium). *Nitrospira* (16.3% of the 16S rRNA clone library and 18.4% of the *gyr*B clone library) and *Acidobacteria* (16.3% of the 16S rRNA clone library, and 14.4% of the *gyr*B clone library) were the other two major groups at this site. All sequences were affiliated with *Nitrospira*, including *L. ferrooxidans*, *L. ferriphium*, and *Leptospirillum* group III in both the 16S rRNA and *gyr*B phylogenetic trees (Figs. 1 and 2). Moreover, the clones affiliated with *A. ferrooxidans* only occupied a low proportion of both trees (4.4% of the 16S rRNA clone library and 6.4% of the *gyr*B clone library) (Table 3).

At the YSK3 site, the most abundant clones were related to *Gammaproteobacteria* (53.1% of the 16S rRNA clone library and 50.7% of the *gyr*B clone library) (Table 3), and the prevailing clones affiliated with *A. ferrooxidans* (12.6% of the 16S rRNA clone library and 16.7% of the *gyr*B clone library). *Nitrospira* was the second most important group (41.3% of the 16S rRNA clone library and 39.1% of the *gyr*B clone library) at this site and it was mainly composed of *Leptospirillum* group III, both in the 16S rRNA and *gyr*B phylogenetic trees. *L. ferrooxidans* and *L. ferriphium* were only detected using the *gyr*B gene.

At all three sites, clones affiliated with *Alphaproteobacteria* and *Deltaproteobacteria* were found by the analysis of the 16S rRNA phylogenetic tree but not from that of the *gyr*B phylogenetic tree. In the 16S rRNA phylogenetic tree, most of the clones affiliated with *Alphaproteobacteria* were related to *Acidiphilium* sp. and *Acidosphaera rubrifaciens*, which have been reported in other studies of AMD microbial communities. As far as *Deltaproteobacteria* was concerned, only one sequence was associated with the bacterium class and it was similar to an uncultured delta-proteobacterium clone BPM3_C02 found in AMD effluent stream sediment.

Links between community structure and environmental variables

The Mantel test was used for detecting the relationship between the microbial community and environmental variables. In the analysis, the proportion of each unique RFLP pattern (16S or *gyr*B genes) was used for the main matrix and the environmental variables were used for the second matrix. Overall, the results of the Mantel test indicated that the environmental variables had a strong impact on the community structure based on either 16S rRNA ($r = 0.9999$, $p < 0.1$) or *gyr*B data ($r = 0.9893$, $p < 0.05$), and that among all geochemical variables the concentration of Fe was a key factor in determining the composition of the microbial community. The Mantel test indicated that 4 out of 11 (based on the 16S rRNA gene), and 4 of 12 (based on the *gyr*B gene) *Leptospirillum* strains had a strong relationship with the concentration of Fe ($p > 0.95$), indicating that these strains may be responsible for the changes of ferrous iron or ferric iron. In addition, the pH may be another crucial factor for some *Gammaproteobacteria* microorganisms, since the Mantel test showed that 8 out of 21 strains (based on the 16S rRNA gene) or 11 out of 28 strains (based on the *gyr*B gene) affiliated with *Gammaproteobacteria* showed a strong correlation with pH ($p > 0.95$).

Table 3. The affiliation of the sequenced 16S rRNA and *gyr*B clones and percentage (%) found at various sites

Clustered	YSK1		YSK2		YSK3	
	16S rRNA	*gyr*B	16S rRNA	*gyr*B	16S rRNA	*gyr*B
C. caldarium plastid	31.9	N[a]	0.0	N[a]	0.0	N[a]
Nitrospira	21.3	28.3	16.3	18.4	41.3	39.1
Alphaproteobacteria	5.3	5.1	8.1	8.0	3.5	2.9
Deltaproteobacteria	0.0	0.0	1.5	0.0	0.0	0.0
Other *Gammaproteobacteria*	15.9	17.5	50.4	44.8	40.5	34.1
Acidithiobacillus ferrooxidans	0.0	0.0	4.4	6.4	12.6	16.7
Acidobacteria	1.1	0.8	16.3	14.4	0.7	2.1
Actinobacteria	24.5	N[a]	3.0	N[a]	1.4	N[a]
Unknown	0.0	48.3	0.0	8.0	0.0	5.1

N[a] means unknown due to the incompleteness of the *gyr*B database.

Discussion

AMDs are characterized with high concentrations of various metal cations, which are toxic to most life forms. However, most acidophilic microorganisms can tolerate and thrive in these acidic waters [9]. More importantly, these microorganisms can be utilized for the extraction of various metals from low-grade ores, such as copper and gold. Knowledge of how acidophiles survive in AMD environments and their community compositions may provide insights into the bioremediation of AMDs and offer useful information for the bioleaching industry. In this paper, PCR amplification of 16S rRNA and *gyr*B genes, and RFLP were performed to analyze the bacterial diversity in the Yinshan lead-zinc mine, Jiangxi Province, China, The relationships between the environmental factors and the community compositions was also studied using the Mantel test.

It is still generally accepted that the universal phylogenetic tree based on the 16S rRNA gene sequence reflects the phylogeny of all organisms [28]. Moreover, other molecular markers such as *gyr*B, *dsrAB*, and *apsA* genes have been used for the characterization of physiologically coherent groups of microorganisms in a variety of habitats [5,20,32]. In the present study, the 16S rRNA and *gyr*B genes were used to analyze the diversity of the bacterial community. Both markers suggested dominance of the same bacterial classes, namely *Nitrospira* and *Gammaproteobacteria*. Moreover, the clone libraries of 16S rRNA and *gyr*B genes both indicated that the microbial community structure at sites YSK2 and YSK3 were most similar to each other and different to the one found at site YSK1. This indicates that the *gyr*B gene may be suitable for the analysis of microbial community composition. However, the *gyr*B sequence database is much less complete than that of the 16S rRNA gene sequence in terms of its coverage of bacterial species and isolates. This incompleteness has inevitably resulted in the problem that some sequences can not be affiliated with any microorganisms. Although the *gyr*B database needs to be improved, *gyr*B libraries could still be a good enhancement for molecular ecology. Moreover, compared with the 16S rRNA gene, it seems that the diversity indices based on the *gyr*B gene were clearly higher than those based on 16S rRNA. The potentially higher resolution of the *gyr*B gene may be very useful for the elucidation of microbial community composition, especially for the differentiation of bacterial strains affiliated to the same species.

It had been repeatedly reported in previous studies that geochemical characteristics affect the composition of microbial communities, and this conclusion was also supported by environmental surveys using both 16S rRNA gene sequences and phenotypic characters [12,18,32]. In the present study, the relationship between microbial community composition (based on the clone libraries of 16S rRNA and *gyr*B genes) and geochemical properties was analyzed. The results from the Mantel test indicated that the composition of the bacterial community was influenced by geochemical parameters, especially by the concentration of iron and the pH. The AMDs are well known for their extreme acidity (low pH), and high concentrations of sulfate and iron [12]. However, various microorganisms have still managed to populate these environments. Many of these microorganisms in AMDs have been shown to be chemolithoautotrophic and able to use iron as an electron donor. Moreover, these microorganisms are acidophilic and can grow within the pH range 1.5–3.0 [23], although the capability of acidophiles to tolerate low pH and to utilize iron differs [24,25]. For instance, *A. ferrooxidans* often thrives in AMD environments with a moderately acidophilic nature (pH 1.3–3.0) and a lower redox potential (<690 mV). In comparison with *A. ferrooxidans*, the acidophiles affiliated with *Leptospirillum* are abundant in environments with pH <1.3 and higher redox potential (>690 mV) [2]. Our results confirmed this point. For example, at the YSK1 site with pH $= 1.0$, no clone was associated with *A. ferrooxidans*. Nevertheless, the main iron-oxidizing bacteria at the three sites studied showed great discrepancy. This discrepancy suggested that the three samples were very different with respect to iron oxidation. In the present study, some microorganisms which are not often observed in acidic mine drainage, such as *Pesudomonas*, *Aquicella*, and *Acinetobacter*, were also detected at the sites analyzed. These microorganisms might play a role in removing certain organic matter that exists in acidic environments.

To determine the correlation of bacterial diversity with the environmental variables, the Mantel test was used. Our results indicated that the concentration of iron and the pH strongly influenced microbial community structure. The sites with similar pH and iron concentration had similar microbial community composition, such as at YSK2 and YSK3. Our previous study on the Dexing copper mine in Jiangxi province, China, also suggested that the pH value and iron concentration were key factors contributing to the microbial community compositions [33]. Moreover, similar results from the Mantel test on 16S rRNA and *gyr*B genes further supported the argument that the *gyr*B gene may be useful for the analysis of microbial community diversity.

At our study sites, PCR-RFLP methods have been used for microbial community analysis [33]. However, these methods are compromised by PCR artifacts which also influence the composition of environmental clone libraries [26]. To achieve more quantitative analyses of community composition, methods should be used in future studies that have no PCR bias, such as fluorescence in situ hybridization (FISH) [1,8]. In

conclusion, this study examined the bacterial communities of three AMDs using the 16S rRNA gene and the *gyr*B gene. The results suggested that the *gyr*B gene may be suitable for the analysis of microbial community diversity. Moreover, the Mantel test indicated that geochemical characteristics, especially the pH value and iron concentration have a strong influence on microbial community composition.

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