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ORIGINAL PAPER

Molecular diversity of 16S rRNA and *gyr*B genes in copper mines

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Abstract The molecular diversities of the microbial communities from four sites impacted by acid mine drainage (AMD) at Dexing Copper Mine in Jiangxi province of China were studied using 16S rRNA sequences and *gyr*B sequences. Of the four sampled sites, each habitat exhibited distinct geochemical characteristics and the sites were linked geographically allowing us to correlate microbial community structure to geochemical characteristics. In the present study, we examined the molecular diversity of 16S rRNA and *gyr*B genes from water at these sites using a PCR-based cloning approach. We found that the microbial community appears to be composed primarily of *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Firmicutes*, *Chlorella* and unknown phylotypes. Of clones affiliated with *Nitrospira*, *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* and *Leptospirillum* group III were all detected. Principal-component analysis (PCA) revealed that the distribution of the microbial communities was influenced greatly by geochemical characteristics. The overall PCA profiles showed that the sites with similar geochemical characteristics had more similar microbial community structures. Moreover, our results also indicated that *gyr*B sequence analysis may be very useful for differentiating very closely related species in the study of microbial communities.

Keywords Molecular diversity · 16S rRNA and *gyr*B gene · Geochemical characteristics · Principal-component analysis

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Introduction

Microbial community structure is strongly controlled by many environmental factors including pH (Paul 1996), nutrient content (Compton et al. 2004; Jonasson et al. 1996), availability of electron acceptors (Kopke et al. 2005), carbon resources (Torsvik and Ovreas 2002; Webster et al. 2001; Zhou et al. 2002) and temperature (Fey and Conrad 2000; Paul 1996). Abiotic and biotic (mostly sulfur and metal oxidation by acidophilic bacteria and archaea)-mediated processes in acid mine drainage (AMD) sites produce an acidic discharge rich in multiple metals that form complex chemical mixtures, creating highly complex habitats that can not be replicated with single metal studies within a laboratory setting (Colmer et al. 1950; Coram and Rawlings 2002; Hallberg and Johnson 2001, 2003; Maeda et al. 1999; Nakagawa et al. 2002; Rawlings 2002; Rohwerder et al. 2003; Knight et al. 1997). Because of these complex interactions, the geochemical characteristics of these sites can be variable (Feris et al. 2004). Therefore, using these variable geochemical characteristics, such as pH, metal composition, will greatly increase our understanding of microbial

distributions and community structure in association with geochemical characteristics in a complex system (Palumbo et al. 2004). In recent years, intensive efforts have been made to investigate microbial communities within these environments (Bond et al. 2000; Baker and Banfield 2003).

From the original culturing studies, it was thought that *Acidithiobacillus ferrooxidans* was the likely candidate for the primary organism responsible for most AMD (Schrenk et al. 1998). With molecular tools, *Leptospirillum ferrooxidans* was determined to be the primary iron oxidizer present in areas of low pH and high temperature close to ore bodies (Vasquez and Espejo 1997; Helle and Onken 1988). So, molecular tools have helped to reveal the diversity and complexity of these communities. In most studies, the 16S rRNA gene has been proven useful for analyzing microbial populations (Bano et al. 2004; Brown and Bowman 2001; Cilia et al. 1996; Fuchs et al. 1996; Hiraishi et al. 1998; Marchesi et al. 1998; Reardon et al. 2004; Wang et al. 1994). The *gyr*B gene encodes the β-subunit of DNA gyrase, a type II DNA topoisomerase, which plays an important role in the DNA replication process (Yamamoto et al. 1999, 2000). The *gyr*B sequence may be a more suitable phylogenetic marker for differentiating very closely related species because of its higher evolutionary rates than 16S rRNA (Kelly and Wood 2000; Watanabe et al. 1999; Yamamoto and Harayama 1996; Yamamoto et al. 2000; Yanez et al. 2003). Moreover, *gyr*B genes are essential and ubiquitous among bacteria; their sequences are long enough for phylogenetic identification and more specific with respect to "statistical considerations". Also compelling is that the *gyr*B sequences are easily obtainable with conserved primers from diverse sources, which make them useful for studies of biodiversity (Huang 1996).

In the present study, we sampled acidic water from four sites in the Dexing Copper Mine. These sites represented different geochemical characteristics. Our objectives were to determine (1) the microbial community structure along these sites; (2) whether geochemical characteristics affect microbial community alterations along the gradient; and (3) whether *gyr*B sequence analysis was useful for community studies. Analysis of the microbial community was done by a PCR-based cloning approach with 16S rRNA and *gyr*B genes. The majority of the microorganisms were affiliated with the *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Firmicutes*, *Chlorella* and unknown phylotypes. The profiles of principal-component analysis (PCA) showed that the distribution of the microbial communities was influenced greatly by geochemical characteristics. Our results also suggested that the *gyr*B gene may be a useful tool for determining the diversity of the community structure.

Materials and methods

Site description and sample collection

The Dexing Copper Mine, located in Jiangxi Province, China, is the largest open-cut copper mine in China and the world's third largest (He et al. 1997; Wen and Allen 1999). It has been once mined for approximately 800 years before present and the current production rate is over 100,000 ton of ore per day (Wen and Allen 1999). Here, microbial oxidation of metal sulfide waste produce large quantities of acidic drainage which contaminate the surrounding environment.

Water samples were collected from four sites in the area chosen to represent a gradient of disturbance in August of 2006. The sites located in the upper part of the copper district were the Dawutou and the Shuilongshan sites (DWT and SLS) which were formed through oxidative dissolution of sulfide minerals. The DWT site was a hole that was once mined about 800 years ago. The SLS site was an acidic pile filled with acid mine drainage form the oxidative dissolution of sulfide minerals. The Zujia (ZJ) and Yangtaowu (YTW) sites are reservoirs filled with acid mine drainage. From each site, we collected water (about 20 l), which was then filtered through a 0.2-μm nylon filter. The remainder with filter were immediately transferred to a jar and kept at −20°C prior to molecular analysis.

Chemical analysis of water sample

We measured the concentrations of 25 kinds elements, including iron, aluminium, mercury, arsenic, tungsten, zinc, lead, manganese, calcium, copper, and other elements (Table 1) by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Baird Plasma Spectrovac PS-6(N + 1)).

DNA extraction and purification

For all four sites, the bulk community DNA was extracted from 5 g of biomass, including the filter using a protocol described by Zhou et al. (Hurt et al. 2001; Zhou et al. 1996). Combining grinding, freeze and thaw, and treatment with sodium dodecyl sulfate, allowed for various types of bacteria to be effectively lysed. The crude DNA was purified by the minicolumn purification method and quantified by ethidium bromide-UV detection on an agarose gel.

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

16S rRNA genes of the microbial community were amplified in the reaction mixtures containing about 100 ng of DNA per μl, 1 × PCR buffer (10 mM Tris–HCl, pH 8.3;

Table 1 Geochemical elemental properties of the four AMD samples (samples DWT, SLS, YTW and ZJ)

Elements (mg/l)	DWT	SLS	YTW	ZJ
Ag	0	0	0	0.01
Al	94.3	783.7	1013	1645
As	0	0.02	0.02	0.03
B	0.2	0.5	0.6	0.2
Be	0.01	0.04	0.07	0.2
Bi	0	0.02	0.03	0.04
Ca	80.8	183.2	288.7	405.6
Co	0.3	3.3	4.1	8
Cr	0.06	0.4	0.4	0.5
Cu	3.6	18.7	71.5	146
Fe	421.4	1907	1677	431.9
Ga	0.08	0.3	0.4	0.5
K	0.3	0	0	0
La	0.05	0.2	0.3	0.6
Mg	53.6	351.4	1395	3094
Mn	19.8	17.1	46.4	173.1
Mo	0.01	0.07	0.09	0.01
Na	1	0.5	1.1	3.8
Nb	0	0	0	0.08
Ni	0.5	2.7	4	8
P	1.7	11.6	11.1	8.7
Pb	0.05	0.4	0.4	0.7
S	476.9	3554	3379	6845
Sb	0	0.03	0.04	0.06
Sc	0.02	0.2	0.2	0.5
Si	15.8	35	51.4	73.1
Sn	0.03	0.1	0.2	0.2
Ta	0.01	0.05	0.04	0.02
Ti	0	0.01	0.03	0.1
W	0	0.2	0.2	0.4
Zn	1.7	1.3	3.2	8.8
Zr	0	0.05	0.04	0.06
pH	1.5	2	2.2	3

50 mM KCl; 2 mM $MgCl_2$; and 0.001% [wt/vol] gelatin), 2 mM dNTPs, 5 pM each of the forward and reverse primers, and 0.025 U of AmpliTaq Gold (Perkin–Elmer, Norwalk, CT) per µl. The reverse primer was the universal 1387R (5′-GGGCGGWGTGTACAAGGC-3′) and the forward primer was the Bacteria universal 63F (5′-CAGG CCTAA CACATGCAAGTC-3′) (Marchesi et al. 1998). The initial denaturing step of 5 min at 94°C was followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C with a final extension step of 7 min at 72°C.

The *gyr*B genes of the microbial community were amplified by PCR in mixtures containing about 100 ng of DNA per µl, 1 × PCR buffer (Perkin–Elmer), a 2 mM concentration of each of the four deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 5 pM each of the forward and reverse primers, and 0.025 U of AmpliTaq Gold (Perkin–Elmer) per µl. In the reaction, the reverse primer was the universal UP1 (5′-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AAR TTY GA-3′) (Yamamoto and Harayama 1995), and the forward primer was the bacteria universal UP 2r (5′-AGC AGG GTA CGG ATG TGC GAG CCR TCN ACR TCN GCR TCN GTC AT-3′) (Yamamoto and Harayama 1995). Optimum temperature and cycling parameters were determined to be an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C (1 min), 57°C (1 min), and 72°C (2 min), with a final extension step of 72°C for 7 min. Amplicons of the expected size (approximately 1.26 kb) were excised from 1.0% low-melting-point agarose gels and purified with the Promega purification columns 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 vector PCR 2.1 TOPO and *Escherichia coli* TOP10F'competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmid clones were identified based on blue–white screening and grown overnight on plates with ampicillin (100 mg/ml) and X-gal (15 mg/ml). White colonies from each of those 8 libraries were randomly selected and the cloned inserts were reamplified with the vector primers M13F and M13R. Aliquots of amplified rDNA and *gyr*B PCR product were digested with 1 U each of the restriction endonucleases *Hin*PI and *Msp*I in 1 × NEB buffer (New England Biolabs, Beverly, MA) overnight at 37°C. The resulting RFLP products were separated by gel electrophoresis in 3.0% agarose. Bands were visualized by staining with ethidium bromide and UV illumination. Jaccard coefficients were calculated for all pairwise comparisons of RFLP banding patterns and dendrograms constructed with the unweighted pair group mean average method in Molecular Analyst (version 1.1; Bio-Rad, Hercules, CA). Each unique RFLP banding patterns were identified for nucleotide sequence determination.

DNA sequencing and phylogenetic analysis

Altogether, 68 unique *gyr*B clones and 49 unique 16S rRNA clones were sequenced. The phylogenetic trees were based on all 16S rRNA and *gyr*B sequences, and were constructed using the DNA distance program Neighbor-Jointing with Felsenstein correction in ARB software package (version 2.5b; Strunk et al. 2004, Technische Universität München, http://www.arb-home.de).

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 identifies the lowest number to accurately represent the structure of the data. These variables are then linearly combined with the eigenvectors of the correlation matrix to generate a principal component axis. In the present study, PCA was used to group or separate stations, which were similar or different, based on the biogeochemical parameters.

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

All sequences that we acquired were submitted to NCBI database, their accession numbers were EF409822–EF409938.

Results

Geochemical characteristics of station

The pH values of the four sites ranged from 1.5 to 3.0. At DWT site, the pH value was the lowest at 1.5. The highest pH was found at ZJ at 3.0.

The highest levels of magnesium, sulfur, calcium and aluminum were found at ZJ. DWT had the lowest concentration of these ions. The iron content ranged from a low of 421.4 mg/l at DWT site to a high of 1907 mg/l at SLS site (Table 1).

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

16S rRNA (1.3 kb) and *gyr*B gene (1.26 kb) products of the expected size were successfully amplified from community genomic DNA from the four samples. A total of 369 16S rRNA and 322 *gyr*B positive colonies were recovered from all samples and these clones were compared by RFLP analysis. RFLP analysis revealed 9 to 28 unique patterns of 16S rRNA and 19 to 29 unique patterns of *gyr*B gene depending on these samples. As far as for the clones similarity, 86.8% of the 16S rRNA clones detected from the SLS sample were also present in the YTW sample and 28.4% of the 16S rRNA clones detected from the SLS sample were also found in the ZJ sample. As far as *gyr*B similarity, 58.4% of *gyr*B clones were common at both SLS and YTW sites and only 17.8% of *gyr*B clones were shared by both SLS and ZJ sites (Table 2). The diversity indexes calculated from the clone data showed that the 16S rRNA had the lower diversity, while *gyr*B genes diversity was higher than what was found in the 16S rRNA (Table 2). Moreover, the rarefaction analysis suggested that the amount of the 16S rRNA and *gyr*B clones analyzed was sufficient to represent the microbial community (Fig. 1).

Table 2 Percent similarity and diversity index (*H*) in 16S rRNA and *gyr*B community structure among three samples determined by analysis of RFLP patterns

Sites	16S rRNA				*gyr*B gene			
	Similarity (%)			H	Similarity (%)			H
	SLS	YTW	ZJ		SLS	YTW	ZJ	
DWT	1.2	3	20.6	1.07	10	11.1	22.1	3.87
SLS		86.8	28.4	4.08		58.4	17.8	4.18
YTW			20.6	3.83			15.7	3.84
ZJ				3.18				4.3

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

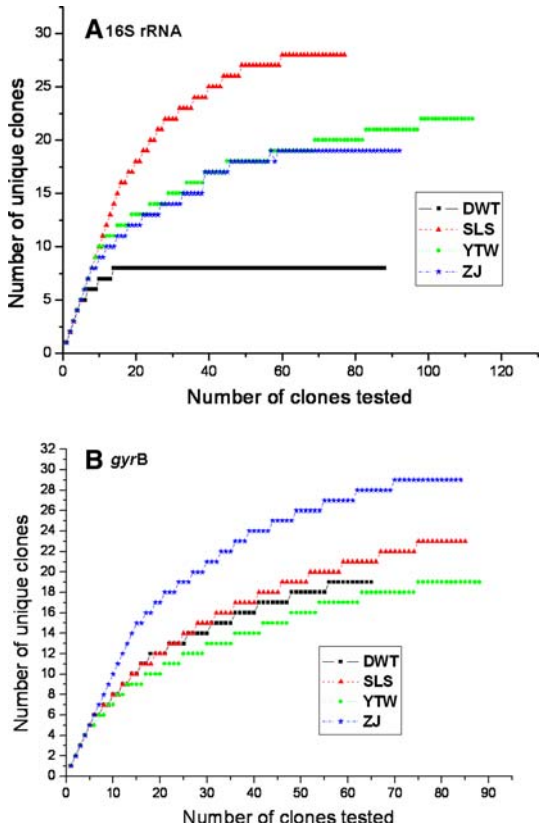

Fig. 1 Evaluation of the representation of the clones obtained from the four samples by rarefaction analysis

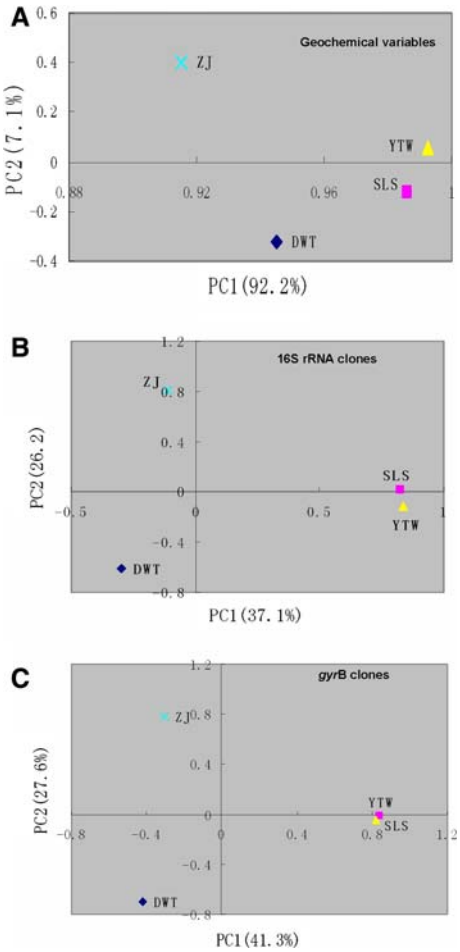

Fig. 2 Ordinate plots from PCA of geochemical variables, 16s rRNA clones and *gyrB* clones

Principal component analysis (PCA)

PCAs of the 16S rRNA data (Fig. 2B) and the *gyr*B data (Fig. 2C), which represent 63.3 and 68.9% of the total variance of the clone distributions, respectively, revealed some similarity among the four sites examined. 16S rRNA and *gyr*B had somewhat similar distribution patterns at these sites, as revealed by PCA. For example, the SLS and YTW sites grouped together, and the DWT and ZJ sites were respectively separated from other two sites (Fig. 2).

The results of the PCA of geochemical characteristics, which represented 99.3% of the total variance, revealed a profile similar to that obtained by PCA of 16S rRNA and *gyr*B. YTW and SLS sites were close together and were distant from DWT and ZJ sites. Thus, the biogeochemical properties at SLS and YTW sites were similar but different from those at DWT and ZJ sites. The overall profiles (Fig. 2) showed that the microbial community and geochemical characteristics were more similar at the sites that were closer together.

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

When we assess genetic diversity and ecological distribution of 16S rRNA and *gyr*B genes, each unique RFLP banding pattern was sequenced and a total of 117 sequences (49 of 16S rRNA sequences and 68 of *gyr*B sequences) were obtained. Phylogenetic reconstruction was based on all recovered sequences from the present study and selected sequences recovered from different environmental sources in the gene bank and ICB database (Figs. 3 and 4). According to the phylogenetic analysis of 16S rRNA, all 16S rRNA sequences fell into seven of the main phylogenetic divisions (*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Firmicutes*, *Chlorella* and unknown; Fig. 3). These main clusters can also be found using the *gyrB* gene except for *Chlorella* (Fig. 4).

In the DWT site, *Nitrospira* was the most dominant microorganism. In the phylogenetic tree of 16S rRNA gene, three 16S rRNA sequences were affiliated with the *Nitrospira* (including *Leptospirillum ferrooxidans*, *Leptospirillum ferriphilum* and *Leptospirillum* group III; Fig. 3), but these 16S rRNA gene clones associated with *Nitrospira* represented 87.5% of the total of 16S rRNA gene clones (Table 3). Interestingly, only one 16S rRNA sequence but 84.09% of the 16S rRNA gene clone library were associated with *Leptospirillum ferrooxidans* (exhibiting 99% similarity). In the phylogenetic tree of *gyr*B gene, *Nitrospira* was still the dominant group (67.7% the total of *gyr*B clones) in the DWT site. However, nine *gyr*B sequences (Fig. 4) were related to *Nitrospira* and eight of the nine sequences (66.2% of the *gyr*B clones) were clustered with *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* (Table 3).

In the SLS site, the *Leptospirillum* group III was the dominant group based on both 16S rRNA gene phylogenetic tree (35.06% of all 16S rRNA clones) and *gyr*B gene phylogenetic tree(28.24% of all *gyr*B clones).

In the YTW site, most of the clones fell into *Acidithiobacillus ferrooxidans*(34.8% of 16S rRNA clone library and 48.86% of *gyr*B clone library (Table 3). However, only two 16S rRNA sequences affiliated with *Acidithiobacillus ferrooxidans* (>98% similarity) by contrast with seven *gyr*B sequences related to the species (>80% similarity) (Figs. 3 and 4).

In the ZJ site, the most abundant clones were related to *Acidobacteria*. Three 16S rRNA sequences were associated with the group and were represented 45.7% of the total of 16S rRNA clones. According to the phylogenetic tree of *gyr*B, five sequences (35.7% of *gyr*B clones) clustered together and affiliated with *Acidobacteria* (Fig. 4).

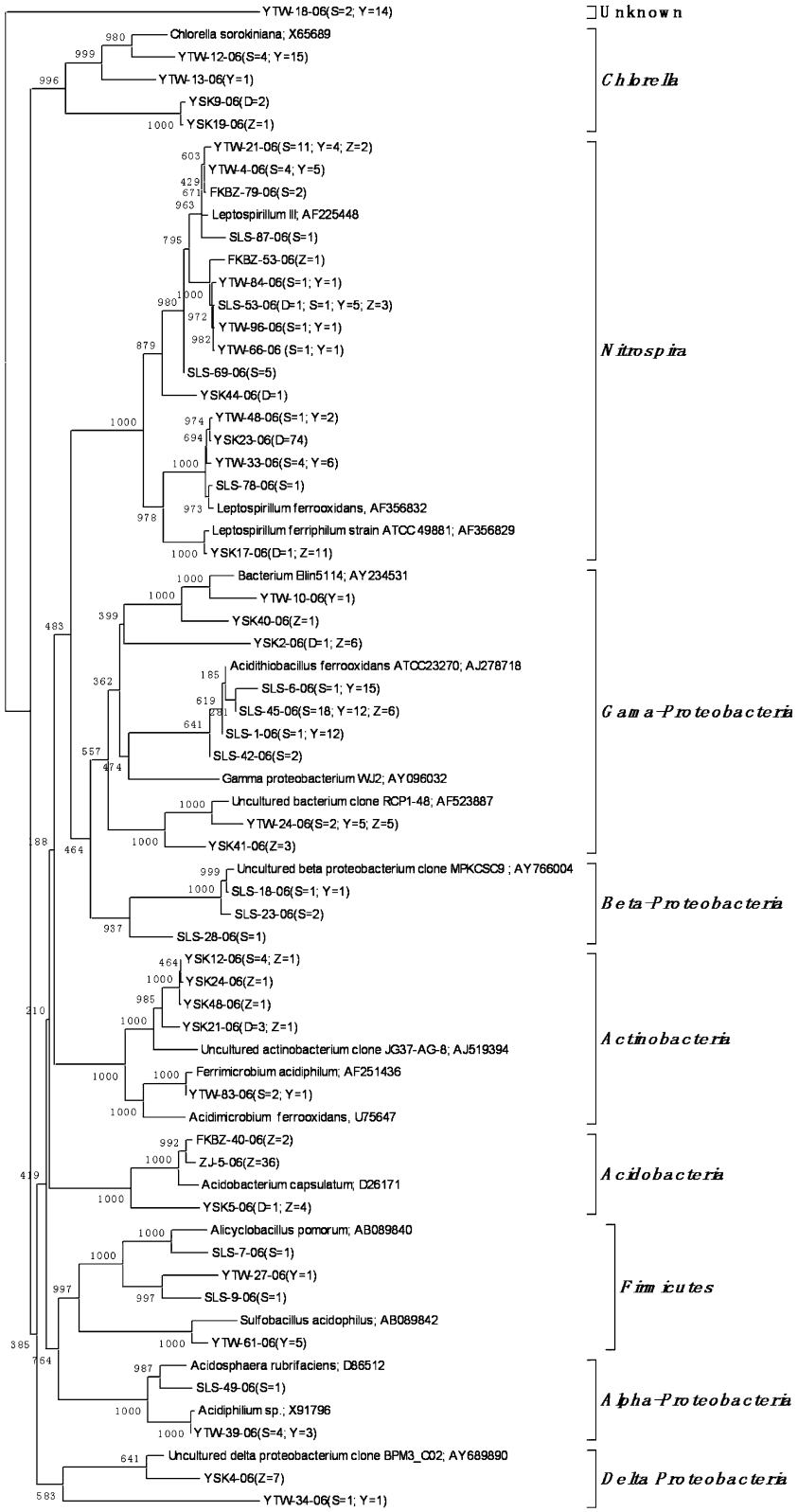

Fig. 3 Phylogenetic analysis of recovered 16s rRNA gene sequences from samples at Dexing Copper Mine and selected sequences from other different environmental sources. The clone composition of each station is provided. Cloned sequences from four stations are labeled by margin (*D* station DWT; *S* station SLS; *Y* station YTW; *Z* station ZJ)

Discussion

Acid mine drainage (AMD) and the associated sites are important from a number of viewpoints, including bioremediation, basic research, and biotechnology (Baker and Banfield 2003; Bond et al.2000; Bridge and Johnson 1998; Dopson et al. 2003; Edwards et al. 1999a, b; Gonzalez-Toril et al. 2003; Hallberg and Johnson 2003; Lopez-Archilla

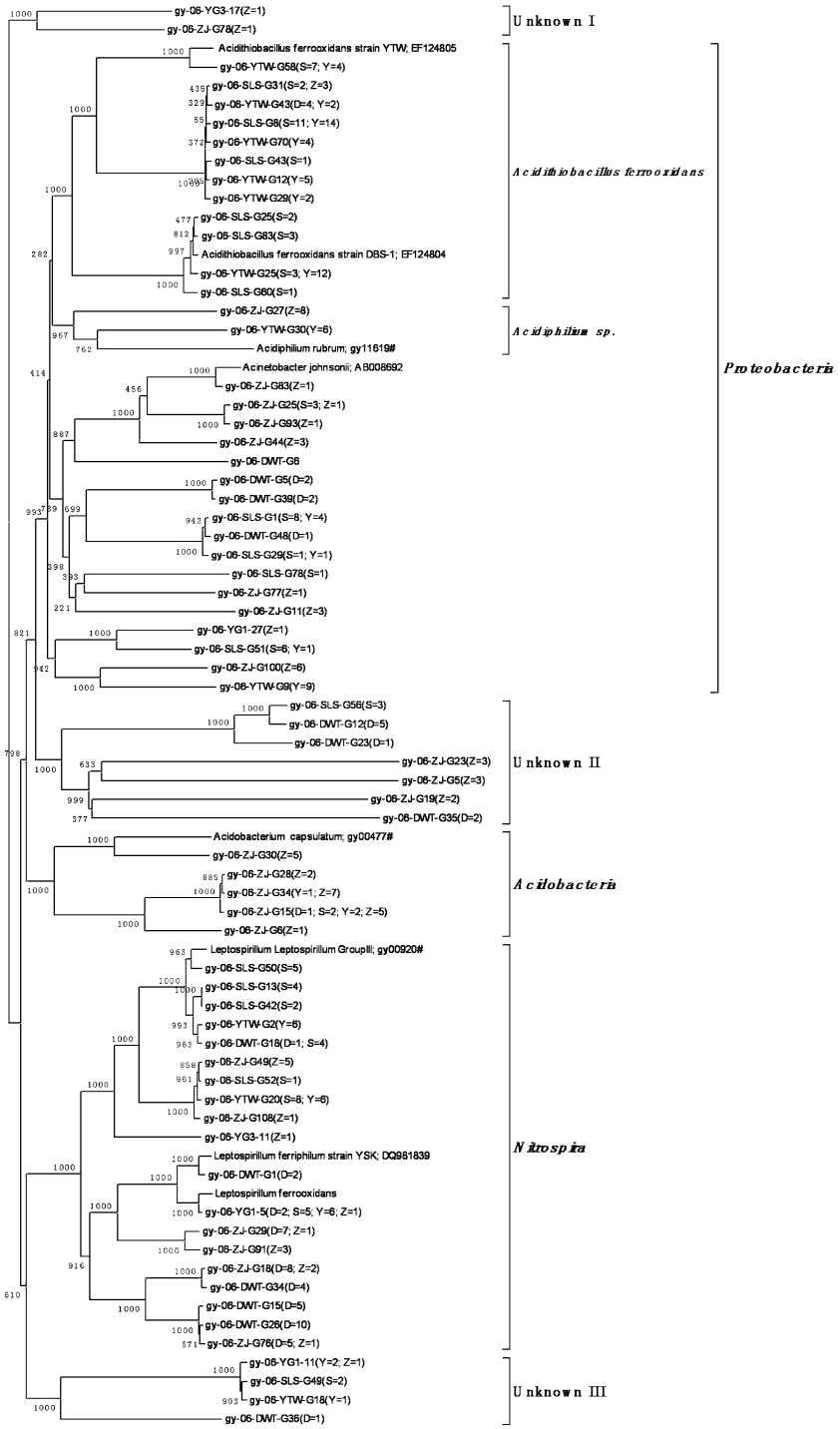

Fig. 4 Phylogentic analysis of recovered *gyr*B gene sequences from four samples at Dexing Copper Mine and selected sequences from four stations are labeled by margin (*D* station DWT; *S* station SLS; *Y* station YTW; *Z* station ZJ). These sequences with # were acquired from the ICB database (The website is http://www.mbio.jp/icb/)

et al. 2004, 2001). The complexity of the impacts of AMD not only creates numerous environmental problems such as watershed degradation and reduction of food web complexity (Gray 1998), but also creates numerous opportunities such as examination of microbial community structure and ecology along some sites with different geochemical characteristics. Aside from the insights gained regarding microbial ecology, elucidating the microbial communities that develop in AMD will enable development of microbial strains that can be utilized to extract metals from their ores potentially prohibiting the creation of large amounts of pollution from metals and acidity.

Using the *gyr*B gene and 16S rRNA gene, we found that the microbial community appears to be composed primarily of the *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Nitrospira*, *Firmicutes*, *Chlorella* and unknown phylotypes. The PCA profiles showed that the distribution of the microbial communities was influenced greatly by geochemical

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

Clustered	DWT		SLS		YTW		ZJ	
	16S	*gyr*B	16S	*gyr*B	16S	*gyr*B	16S	*gyr*B
Chlorella	2.273	N[a]	5.195	N	14.3	N	1.09	N
Nitrospira	87.5	67.7	42.85	34.1	22.3	20.46	18.5	17.9
Acidithiobacillus ferrooxidans	0	6.15	28.57	35.3	34.8	48.86	6.52	3.57
Other Proteobacteria	1.136	10.8	15.59	22.4	9.82	23.87	23.9	29.7
Actinobacteria	7.955	N	2.597	N	0.89	N	4.35	N
Acidobacteria	1.136	1.54	0	2.35	0	3.409	45.7	35.7
Firmicutes	0	N	2.597	N	5.36	N	0	N
Unknown	0	13.8	2.597	5.88	12.5	3.409	0	13.1

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

parameters, though the sites differed in which geochemical characteristics seemed to control the distribution. This leads us to believe that the myriad factors which control the structure of a microbial community involves highly complex interactions among the organisms and between the organisms and their environment that can not be easily simplified. For instance, Feris et al. (2004) has shown that metal deposition differentially impacts microorganisms from different families. Additionally, these organisms recover and acclimate to increased metal deposition at different rates as well (Feris et al. 2004). Of course, other studies have assumed that ferrous iron or pH were the primary factor structuring the microbial communities of AMD (Edwards et al. 1999b). Altogether, it has been suggested that the microbial community was influenced greatly by geochemical parameters (Southam and Saunders 2005). In the present study, the *gyr*B sequences analysis combined with that of 16S rRNA seems to support this trend.

Compared to 16S rRNA, our results from the *gyr*B sequence data and diversity index confirmed that there was more significant richness across these four sites with clones representing *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Nitrospira* detected than that of 16S rRNA. At the genus level, clones affiliated with *Leptospirillum* and *Acidithiobacillus* were almost ubiquitous across the four sites and the phylotype of the *gyr*B gene clones associated with these two genus were also clearly higher than that of 16S rRNA. Microbial diversity in AMD sites has been shown to be higher than suspected (Edwards et al. 1999a, b; Dunbar et al. 1999; Karavaiko et al. 2003), although there are exceptions to the slime community from Iron Mountain, CA (Bond et al. 2000), which could also be due to the use of the *gyr*B gene. Because of the greater rate of substitution, sequence similarity is likely to be, on average, lower than with using a more conserved sequence like 16S rRNA. Moreover, a direct comparison of the genetic distance and the phylogenetic tree determined by the 16S rRNA genes sequence of pure cultures (*A. ferrooxidans*) with those determined by the *gyr*B sequence was conducted (Data not shown). When the 16S rRNA sequence analysis was used, it appeared that some strains shared the same branch of the phylogenetic tree. In contrast, when using the *gyr*B genes, it was clear that these strains were affiliated with separate branches. These results suggested that *gyr*B may be useful for differentiating very closely related species in the study of microbial community.

AMD production derives largely from the biotic dissolution of metal sulfides, leading to soluble sulfate. *Acidithiobacillus ferrooxidans*, a sulfur oxidizer, was once assumed to be the primary species responsible for AMD because it was easily cultured from AMD impacted sites (Harrison 1984; Maeda et al. 1999). However, *Leptospirillum* was found to represent a much larger percentage of the community in many AMD environments with lower pH and was likely responsible for much of the AMD runoff because of its metabolic ability to oxidize geochemically bound sulfur compounds, such as pyrite (Vasquez and Espejo 1997; Helle and Onken 1988). To date, *Leptospirillum* isolates and environmentally-derived clones cluster within one of three phylogenetically distinct groups (*L. ferrooxidans* I, *L. ferriphilum* II, and Group III, Hippe 2000; Baker and Banfield 2003; Coram and Rawlings 2002). From our sites, sequenced clones affiliated with the genus *Leptospirillum* were dominant and divided into three groups. Interestingly, we also found *Leptospirillum* III, which occurred at the Iron Mountain, CA (Bond et al. 2000), using the 16S rRNA sequence and *gyr*B gene sequences. Moreover, some studies (Bond et al. 2000; Baker and Banfield 2003) reported that *L. ferriphilum* and *Leptospirillum group* III primarily reside in lower pH microenvironments in the mine compared to *L. ferrooxidans*. However, our results, based on 16S rRNA and *gyr*B gene, showed that the distribution of the genus *Leptospirillum* was greatly influenced by the complex geochemical characteristics and was not only limited by pH value.

From the *gyrB* phylogenetic tree, we did not find any sequences affiliated with *Chlorella*, which was detected using 16S rRNA. In addition to this, there were some other unknown sequences which were not affiliated with any

known clusters in the *gyrB* phylogenetic tree. These were possibly due to the incompleteness of *gyr*B database. As the gene bank increases and more sequences become available to make comparisons, it might be possible to classify these unknown *gyr*B sequences as *Chlorella* or an existing cluster. It is also possible that if *gyr*B analysis is carried out on these unknowns we may be able to classify them into to a common group.

According to previous studies and our results, the microbial communities among these sites are influenced by a number of variables, including geochemistry and competitive interactions. Because AMD-impacted sites vary greatly in origin, geochemistry of the underlying rock, and many other factors, these results provide important insights into a previously unexamined but major AMD region. Additionally, although the complexities of integrating geochemical characteristics with microbial community structure are difficult to overcome, using less conserved sequences such as *gyr*B and statistical tools may lead to a more detailed representation of the microbial community and greater accuracy in determining the combination of geochemical characteristics structuring these communities.

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