Vol. 47: 141–151, 2007 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published May 16

Bacterial diversity and community structure in acid mine drainage from Dabaoshan Mine, China

Yu Yang[1,2], Min-xi Wan[1], Wu-yang Shi[1], Hong Peng[1], Guan-zhou Qiu[1,2], Ji-zhong Zhou[3], Xue-duan Liu[1,2,*]

[1]School of Minerals Processing and Bioengineering, Central South University, 25 South Lushan Road, Changsha, Hunan 410083, China

[2]Key Laboratory of Biometallurgy, Ministry of Education, 25 South Lushan Road, Changsha, Hunan 410083, China

[3]Department of Botany and Microbiology, 770 Van Vleet Oval, University of Oklahoma, Norman, Oklahoma 73019-4110, USA

ABSTRACT: Three samples of acid mine drainage (AMD) collected from Dabaoshan Mine (Guangdong Province, China) were studied. In addition to physico-chemical analyses, bacterial diversities and community structures of these samples were described at genetic level by amplified ribosomal DNA restriction analysis (ARDRA). A total of 60 different ARDRA patterns were obtained from 377 clones and were studied as operational taxonomic units (OTUs), which were re-amplified and sequenced. The sequence data and phylogenetic analysis showed that *Acidithiobacillus ferrooxidans* represented 88.0% of the bacterial population in Sample LC. However, Samples JX and FS contained more diverse colonies of bacteria, such as *Leptospirillum ferrooxidans* (JX: 16.9%, FS: 39.1%), *Acidiphilum* sp. (JX: 38.7%, FS: 25.8%) and *A. ferrooxidans* (JX: 12.1%, FS: 10.2%). These diversities were characterized by the reciprocal of Simpson's index (1/*D*) and correlated with the concentrations of ferrous iron and toxic ions in AMD.

KEY WORDS: Bacterial diversity · Microbial community · Acid mine drainage · Amplified ribosomal DNA restriction analysis

INTRODUCTION

Recently, some microorganisms that can grow in extreme circumstances have attracted considerable attention because of their unusual physiology, ecological properties and some important biotechnological applications (Russell 2000, Margesin & Schinner 2001, Rawlings 2002). Acid mine drainage (AMD) environments are especially interesting because, in general, the low pH of the habitat is the consequence of microbial metabolism (Gonzalez-Toril et al. 2003a) and not a condition imposed by the system, as is the case in many other extreme environments (e.g. temperature, ionic strength, high pH, radiation, pressure).

AMD is derived from mine dissolution by a series of complex geochemical and microbial reactions when water comes in contact with the mine (Johnson & Hallberg 2003). The functions of some extremophiles that can grow and develop under AMD are commonly considered as important for mine dissolution. An important biotechnological application of these extremophiles is bioleaching. The application is simple and provides effective technology for metal extraction from low-grade ores and mineral concentrates, and is based on the activities of some chemolithotrophic bacteria, which convert insoluble metal sulfides into soluble metal sulfates and are tolerant to metal ions (Suzuki 2001). For decades, chemolithotrophic prokaryotes such as *Acidithiobacillus ferrooxidans* (formerly known as *Thiobacillus ferrooxidans*; Kelly & Wood 2000) and *Leptospirillum ferrooxidans* have been known to enhance acid production in metal-leaching environments by oxidizing ferrous iron and replenishing the oxidant ferric iron (Sand et al. 1995). Furthermore, they have long been regarded as the principal acidophilic sulfur- and iron-oxidizing microbes, and consequently they have been the focus for research on bioleaching for a long time. Several studies on the distribution of these species in leaching tanks, soils and aquatic environments

*Email: xueduanliu@yahoo.com

(e.g. Coram & Rawlings 2002, Kormas et al. 2006) have been conducted.

New studies (since the 90s) on AMD from diverse geographical locations have revealed that many uncultured bacteria might be very important in the generation of acidic environments (Golyshina et al. 2000), and that AMD supports a diverse range of iron- and sulfur-oxidizing chemolithotrophs as well as heterotrophic microorganisms (Johnson 1998). Community structure varies among diverse geographical locations, revealing that AMD niches and changes in dominant bacterial species are affected by geographical conditions.

Studies of bacterial diversity and community structure in diverse AMD can help us to reveal principal bacterial species for mine dissolution in different geographical conditions, and the factors that affect such bacterial communities. To date, although there are many existing reports on bacterial ecology in AMD, to the best of our knowledge such studies in Chinese mines are rare. In this study, we investigated the bacterial and geochemical characteristics of 3 AMD sites in Dabaoshan Mine, Guangdong Province, China. The genomic DNA of the bacterial community at each site was extracted. Subsequently, Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used to analyze the bacterial communities that occur at these sites, and the findings were correlated with the determined geochemistry and mineralogy of each site.

MATERIALS AND METHODS

Study site, sample collection and physicochemical analyses. Dabaoshan Mine (24° 31′ 37″ N, 113° 42′ 49″ E) is located in the mountainous area of the northern part of Guangdong Province, China. It is a large, multi-metallic mineral deposit: the top of the main ore body appears to be a limonite body, while the lower body contains copper sulphur compounds and associated tungsten, bismuth, molybdenum, gold and silver metal ores. As with most metal deposits, the strata contain pyrite (FeS_2) and various other metal sulphide ores.

All AMD samples were collected from different sites in Dabaoshan Mine in August 2005. The first sample (JX) was taken from the mine tunnel located 100 m below the surface and at an elevation of 530 m. The site was dank and abandoned. The second sample (LC) was collected from the open-cast mining area in Dabaoshan Mine, which was in operation at that time. The third sample (FS) was collected from a site where many abandoned ores were deposited. All AMD samples were filtered on site through 0.2 µm nylon filters (Jinjing) with a vacuum pump, and water samples were then immediately transferred into anaerobic jars. Sediment on the filters was collected and kept at –20°C. The filtrate of AMD samples was used for chemical analyses. Physicochemical analyses of the water samples were performed at the Testing Center of Central South University. Flame atomic absorption spectrometry was used for measurements of metal ion, and pH was measured with a pH meter (PHS-25, Leici).

DNA extraction and purification. The microorganisms of AMD samples were isolated in sediment after filtration through 0.2 µm nylon filters. For each site, genomic DNA of the bulk community was extracted from 5 g of sediment following the protocols described by Zhou et al. (1996) and Hurt et al. (2001).

The 5 g of sediment was mixed with 13.5 ml of extraction buffer (0.1 M phosphate [pH 8.0], 0.1 M EDTA, 1.5 M NaCl, 1% CTAB) and added to 50 µl Proteinase K (QIAGEN, 10 mg ml^{-1}) in 50 ml centrifuge tubes, and then incubated at 37°C for 30 min. Next, 1 ml of 20% SDS was added and mixed gently, followed by further incubation at 65°C for 2 h with gentle inversion every 15 to 30 min. The mixture was centrifuged at 4000 × *g* for 5 min at 25°C, and the supernatant then transferred into a new 50 ml centrifuge tube. Following resuspension of the soil pellet by vortexing in 4.5 ml extraction buffer, 0.5 ml 20% SDS was added to the mixture and mixed gently. Samples were then incubated at 65°C for 15 min followed by centrifugation at 4000 × *g* for 5 min at 25°C, and the supernatant was then collected and combined with the previous supernatant. The supernatant was extracted with an equal volume of chloroform, and gently mixed and then centrifuged at 4000 × *g* for 20 min. After the supernatant was collected, 0.6 volumes of 2-isopropanol was added to the supernatant, which was then gently mixed and left to stand at room temperature overnight. Samples were centrifuged at 16 000 × *g* for 30 min at 25°C. The pellet was washed with 70% ethanol, centrifuged at 4000 rpm for 10 min at 25°C, and then dissolved in 200 to 500 µl of sterile water. The quality of genomic DNA was analyzed by agarose gel electrophoresis and purified using Wizard DNA Clean-Up Kit (Promega).

16S rRNA gene amplification and cloning. Two primers were used to amplify approximately 1300 bp of a consensus 16S rRNA gene fragment: forward primer 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer 1387R (5′-GGGCGGWGTGTACAAGGC-3′) (Marchesi et al. 1998).

The PCR mixtures (50 µl) contained 10 pmol of each appropriate primer, 2 µl of genomic DNA (~0.1 µg $µl^{-1}$), 5 µl of 10× dNTP (2 mmol l^{-1} each), 5 µl of 10× PCR buffer, and 0.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer). Thermal cycling was carried out using a Whatman Biometra T1 Thermocycler programmed for an initial step of 5 min at 94°C, followed by 30 cycles of

45 s at 94°C, 45 s at 65°C, 90 s at 72°C, and then 7 min at 72°C. The PCR products were visualized by 1.0% low-melting-point agarose gel electrophoresis, and purified using Wizard DNA Clean-Up Kit (Promega).

The purified 16S rRNA gene fragments were ligated into the vector PCR2.1 TOPO, then transformed into *E. coli* TOP10F′ competent cells according to the manufacturers′ instructions (Invitrogen). Recombinants were identified based on blue-white screening, and grown overnight in Luria-Bertani (LB) agar plates containing appropriate amounts of ampicillin, X-gal and IPTG (Isopropylthio-β-D-galactoside at 37°C. Next, 168 to 176 white colonies from each of these 3 libraries were randomly selected, and the recombinant plasmids containing 16S rRNA gene fragments were re-amplified by PCR using the vector primers M13F (5′-GTAAAACGACGGCCAGTG-3′) and M13R (5′-GGAAACAGCTATGACCATG-3′). The PCR mixtures (50 µl) also contained 10 pmol of each appropriate primer, 2 µl of plasmid DNA (~0.1 µg μl^{-1}), 5 µl of 10× dNTP (2 mmol l^{-1} each), 5 µl of 10× PCR buffer, and 0.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer). Thermal cycling was carried out using a Whatman Biometra T1 Thermocycler programmed for an initial step of 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, and then 7 min at 72°C.

Amplified ribosomal DNA restriction analysis (ARDRA). The amplified rRNA PCR products of the correct size (approximately 1.3 kb) were digested with *Hin*6I and *Msp*I (Fermentas) overnight at 37°C. The resulting ARDRA products were separated by gel electrophoresis in 3.0% agarose. The ARDRA patterns were visualized by UV excitation. Jaccard coefficients were computed for all pairwise comparisons of ARDRA banding patterns, and dendrograms constructed using the unweighted pair group mean average method in Molecular Analyst version 1.1 (Bio-Rad). ARDRA banding patterns that were identified were grouped into an operational taxonomic unit (OTU), and a representative clone was selected for nucleotide sequence determination in each OTU.

Sequencing and phylogenetic analysis. After selecting clones in different ARDRA patterns, a total of 60 clones were sequenced by Sunbiotech (Beijing). Sequence identification was initially estimated at the BLASTN facility of the National Center for Biotechnology Information (www.ncbi. nlm.nih.gov/BLAST). The initial phylogenetic trees were based on all available sequences and were constructed using the DNA distance neighbor-joining program with Felsenstein correction in ARB (Smith et al. 1994). After appropriate subsets of 16S rRNA gene sequences were selected, analyzed, and aligned with CLUSTAL-X (version 1.8) based on the initial phylogenetic results, the final phylogenetic trees were generated.

Statistical analysis. Principal component analysis (PCA) was performed using the SYSTAT version 13.0 (SPSS) and by consulting protocols described by Bagwell et al. (2006) and Palumbo et al. (2004) for each sampling site. PCA simultaneously considers many correlated variables and identifies the lowest number required 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 did or did not differ based on their biogeochemical parameters (pH, temperature, mercury, arsenic, phosphorus, cobalt, magnesium, copper, tungsten, zinc, lead, manganese, silver, sulphur, molybdenum, chromium, iron, aluminium, tin, calcium, potassium). Biogeochemical parameters were entered into the Data Editor of SPSS, and PCA analysis was performed according to the SPSS manual. The whole analysis was run automatically after data were entered, and all data were auto-standardized by SPSS. The conditions of the analysis were correlation matrix, unrotated factor solution, 2 factors, maximum iterations for convergence = 25. Output data were plotted in 2 dimensions. The PCA results were applied to ascertain which biogeochemical parameters contributed to the differences among stations. Similarly, the correlation analysis was applied to the biological parameters, whereby the relative amount of each OTU (unique ARDRA pattern) for each station was used as a variable.

The rarefaction analysis was performed with SigmaPlot version 8.0 software. An exponential model, $y = a[1 - \exp(-bx)]$, was applied in SigmaPlot to fit the clone distribution data. SAS was used primarily for PCA of the clone data.

Because the reciprocal of Simpson's index ($1/D$) has a good to moderate discriminating ability, and is used widely in ecological studies (Magurran 1988), it was chosen to characterize the microbial communities in our AMD samples. The use of $1/D$ instead of the original formulation of Simpson's index ensures that an increase in the reciprocal index reflects an increase in diversity (Magurran 1988, Zhou et al. 2002).

RESULTS

Biogeochemical properties of stations

The biogeochemical properties of the AMD samples from Dabaoshan Mine are summarized in Table 1. The temperature at Sites LC and FS stabilized at around 22°C in the daytime. Because Site JX was underground, temperature here was maintained at 20°C all year round. The pH values were very similar to each other, ranging from 1.9 to 2.3 (Table 1).

Table 1. Biogeochemical properties of AMD samples

Parameter (all elements in mg l^{-1})	Site JX	LC	FS
pH	2.3	2.0	1.9
Temperature (°C)	20	22	22
Hg	0.83	0.89	2.51
As	1.95	1.78	5.23
P	3.95	4.22	13.28
Co	0.65	2.66	1.43
Mg	643	344	733
Cu	13.9	1120	2762
W	2.89	4.15	9.71
Zn	55.86	59.98	287
Pb	3.13	4.19	7.39
Mn	50.55	151	150
Si	30.15	59.34	63.79
Ag	0.3	0.37	0.39
S	2719	2881	10266
Mo	0.47	0.51	1.26
Cd	0.59	0.5	3.35
Fe^{3+}	378.9	120.6	6331.5
Fe^{2+}	6.0	136.5	6.5
Al	429.1	391.5	1506
Ti	0.16	0.15	0.22
Sn	0.87	0.92	2.6
Sb	2.4	2.61	6.03
Ni	0.85	2.78	2.8
Cr	0.27	0.49	0.66
Ca	392	511	285
K	10.84	6.08	3.08

ARDRA analysis of 16S rRNA clone libraries

In this study, clones containing partial 16S rRNA gene inserts were obtained from the direct cloning of PCR products, which were copied from the genomic DNA isolated from each of the different AMD samples. From each sample, 124 to 128 clones were chosen randomly, and the PCR products of 16S rRNA gene fragments inserted into these clones were hydrolyzed by the restriction enzymes *Hin*6I and *Msp*I. The ARDRA analysis revealed extensive phylogenetic diversity in 16S rRNA for all 3 samples examined (Fig. 1). Nine OTUs (Nos. 4, 5, 6, 10, 23, 25, 31, 38 and 46) were common to all 3 samples, and dominant 16S rRNA clones were detected for each sample.

In Sample JX, 42 OTUs were detected and 2 OTUs (Nos. 5 and 23) were frequently recovered, accounting for 14.5 and 13.7% of the clone library respectively. Moreover, 20 OTUs had a single clone.

In Sample LC, OTU No. 6 was the most dominant OTU and represented 56% of the clone library. OTU No. 46 was also dominant in the sample, representing 29.6% of the clone library. The other 15 OTUs in Sample LC jointly represented 14.4% of all clones.

In Sample FS, OTU No. 5 accounted for 19.5% of the clone library; 3 other OTUs (Nos. 9, 57, 37) were also dominant, respectively representing 11.7, 10.9, and 10.2% of all FS clones.

Among these samples, the greatest number of unique clones was found in Sample JX (42 clones). Sample FS had 34 unique clones, and Sample LC contained the lowest number of unique 16S rRNA gene sequences (only 17 clones).

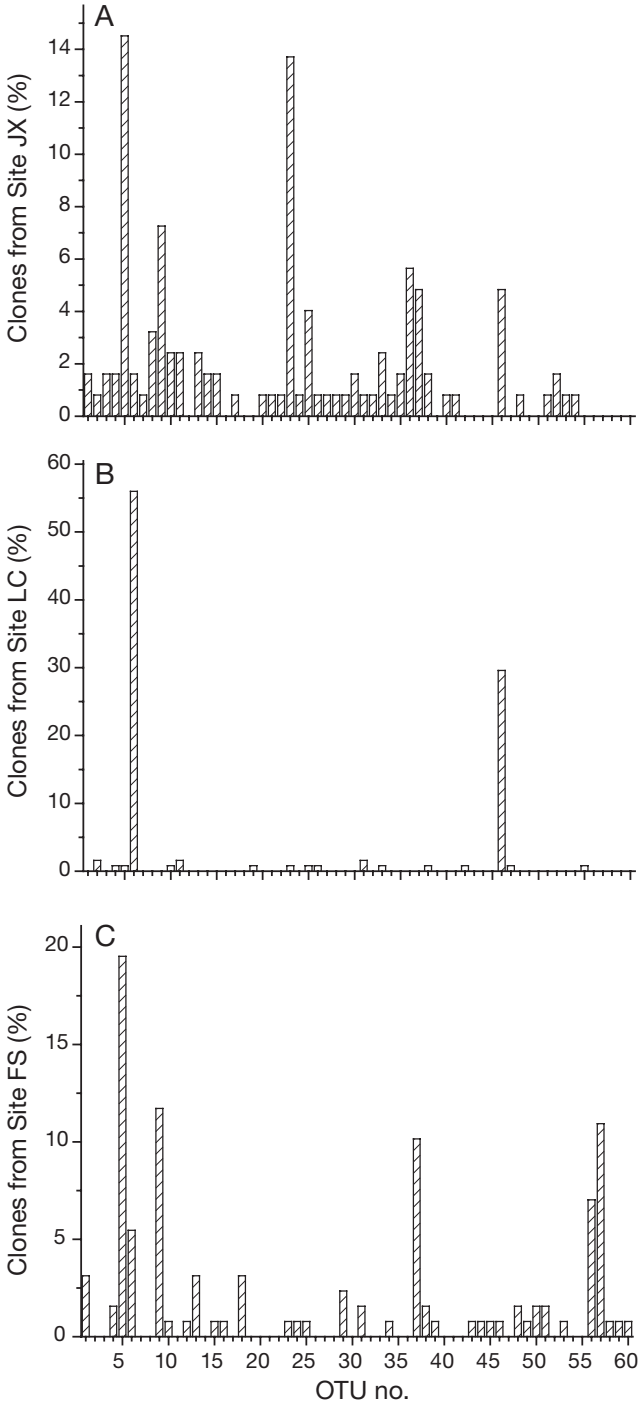

Fig. 1. Percentage of clones in (A) Sample JX, (B) Sample LC, and (C) Sample FS

Rarefaction curves (plots of the cumulative number of OTUs as a function of a clone number) indicated that the majority of the OTUs in the samples were detected (Fig. 2). While more than 90% of the OTUs were detected within the first twenty 16S rRNA clones for Sample LC, less than 10% of the total OTUs were detected among the remaining 80% of clones. Moreover, while more than 80% of the OTUs were detected within the first 60 clones of the remaining samples, less than 20% of the total OTUs were detected among the remaining ~50% of clones. This suggests that the level of analysis was sufficient to detect the community diversity and infer the distribution within these communities.

Phylogenetic analysis

To determine the phylogenetic diversity, representative 16S rRNA clones were sequenced. Comparative sequence analysis was conducted on nucleotide sequences (on average, 1310 comparable positions for 16S rRNA gene). The major sequences of the clones differed by less than 5% from those in current databases. The phylogenetic relationships of samples from all 3 sites was established with a bootstrap neighbor-joining method using the sequences from all known and candidate divisions (Fig. 3). These sequences fell into 5 main putative phylogenetic divisions, *Acidobacteria*, *Acitinobacteria*, *Nitrospira*, *Alphaproteobacteria* and *Gammaproteobacteria*, which amounted to OTUs of 2, 2, 16, 22 and 18, respectively. Clones were affiliated with *Gammaproteobacteria* (47.5%), *Alphaproteobacteria* (29.4%) or *Nitrospira* (19.5%), except a few clones that were affiliated with *Acidobacteria* (2.5%) and *Acitinobacteria* (1.1%). No clones were affiliated with *Firmicutes* or *Betaproteobacteria*.

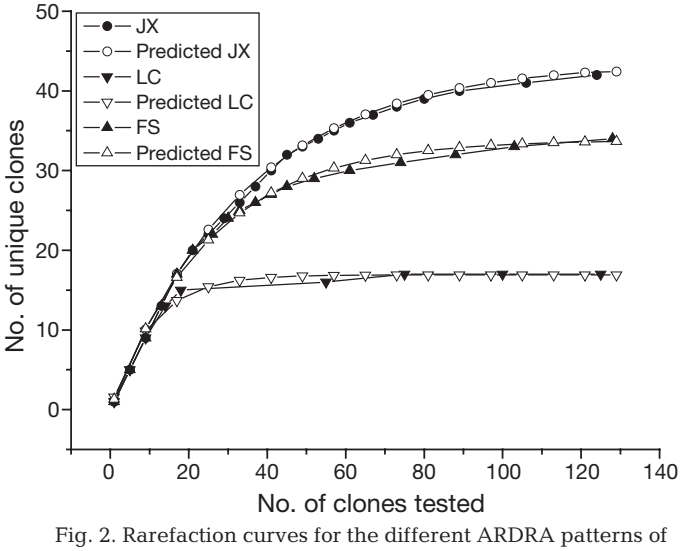

Fig. 2. Rarefaction curves for the different ARDRA patterns of all clones used in this study. The expected number of ARDRA patterns is plotted versus the actual number of patterns

Acidobacteria

Phylogenetic analysis indicated that clone Nos. 14 and 25 fell into the *Acidobacteria* group. DBS(Dabaoshan mine)-Clone 14 exhibited 95% identity with IM-AMD Clone BA2 (Druschel et al. 2004), and DBS-Clone 25 showed 98% identity with *Acidobacteriaceae* strain WJ7, isolated from remediate mine drainage (Hallberg & Johnson 2003). Each sample in this group contained DBS-Clone 25.

Actinobacteria

Actinobacteria was detected in LC and FS samples. The clones (Nos. 21 and 29) associated with *Actinobacteria* fell into 1 group. DBS-Clone 21 shared 93% identity with *Actinobacterium* WJ25, and DBS-Clone 29 shared 99% identity with *Ferrimicrobium acidiphilum* strain T23, a heterotrophic iron-oxidizing acidophile from acid mine waters.

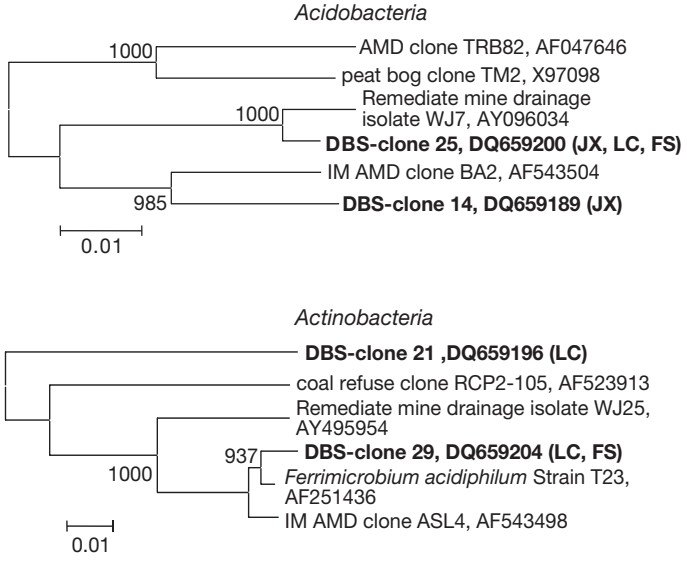

Fig. 3. (above and following 3 pages) Phylogenetic relationship of partial 16S rDNA sequences generated in this study. Scale bar represents the number of changes per base position. Numbers at tree nodes represent the number of times the topology to the right of the node was recovered in 1000 bootstrap resamplings. Clone names in **bold** correspond to sequences determined in the present study. JX: Site JX; LC: Site LC; FS: Site FS; IM: Iron Mountain

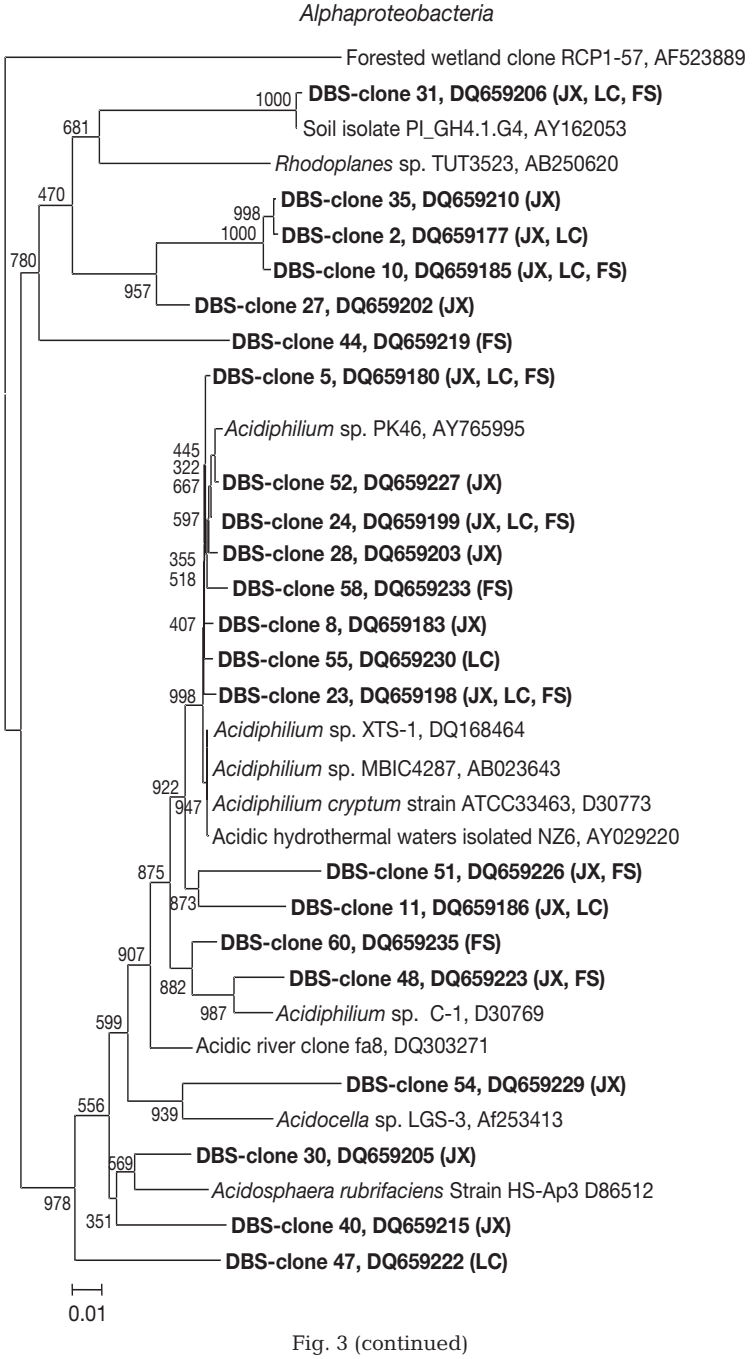

Fig. 3 (continued)

Leptospirillum

Within the *Leptospirillum*, clones fell into 3 major groups. The most abundant group was affiliated with *Leptospirillum ferriphilum* (17.2% in the total clone library and 90% of all *Nitrospira* clones). Within *Leptospirillum* Group I, clones (Nos. 1, 12, 56, 13, 36, 45, 49, 39, 57, 9, 19, 43 and 50) all showed ~95 to 99% identity with known *L. ferrooxidans*. However, within *Leptospirillum* Group II, only DBS-Clone 59 was detected. Two clones (Nos. 17 and 18) were associated with *Leptospirillum* Group III, but only represented a small branch of the phylogenetic tree.

Alphaproteobacteria

Alphaproteobacteria was another common group found in acid drainage waters. Kusel et al. (1999) isolated 6 species of heterotrophic *Alphaproteobacteria* of the genus *Acidiphilum*, which was originally isolated from acidic iron-rich sediment from a lake associated with a coal mine in eastern Germany. Within the *Alphaproteobacteria*, the major clones were affiliated with *Acidiphilium* sp. (20.1% in the total clone library and 68.3% of all *Alphaproteobacteria* clones). The *Alphaproteobacteria* could be divided into 3 groups. The first group was closely related to *Acidiphilium* spp. The major clones clustered well into this group and were very similar (~97 to 99%) to known *Acidiphilium* sp. Only 4 clones were affiliated with the second group: three shared ~90 to 95% identity with *Acidosphaera rubrifaciens*, while 1 clone was affiliated with *Acidocella* sp. LGS-3 (95% identity). In the third group, 6 clones were associated with *Rhodoplanes* sp. (~90% identity), and DBS-Clone 31 shared 99% identity with *Alphaproteobacteria* PI_GH4.1.G4, which was isolated from a soil sample (Zengler et al. 2002).

Gammaproteobacteria

Gammaproteobacteria have been found to be the dominant species in many AMD environments (Johnson et al. 2002, Johnson & Hallberg 2003) Within the *Gammaproteobacteria*, the major clones were affiliated with *Acidithiobacillus ferrooxidans* (37.9% in the total clone library and 79.8% of all *Gammaproteobacteria* clones). All clones related to the *Gammaproteobacteria* fell into 4 major groups. The first group was affiliated with *Acidithiobacillus* species. In this group, all clones exhibited ~98 to 99% identity with *A. ferrooxidans*. Four clones (Nos. 37, 53, 34, 22) were clustered with the second group, and were closely related to the Gammaproteobacterium WJ2 with identities of ~95 to 99%. Four clones (Nos. 41, 3, 20, 16) were clustered with the third group, and were related to *Legionella* species but to a lesser

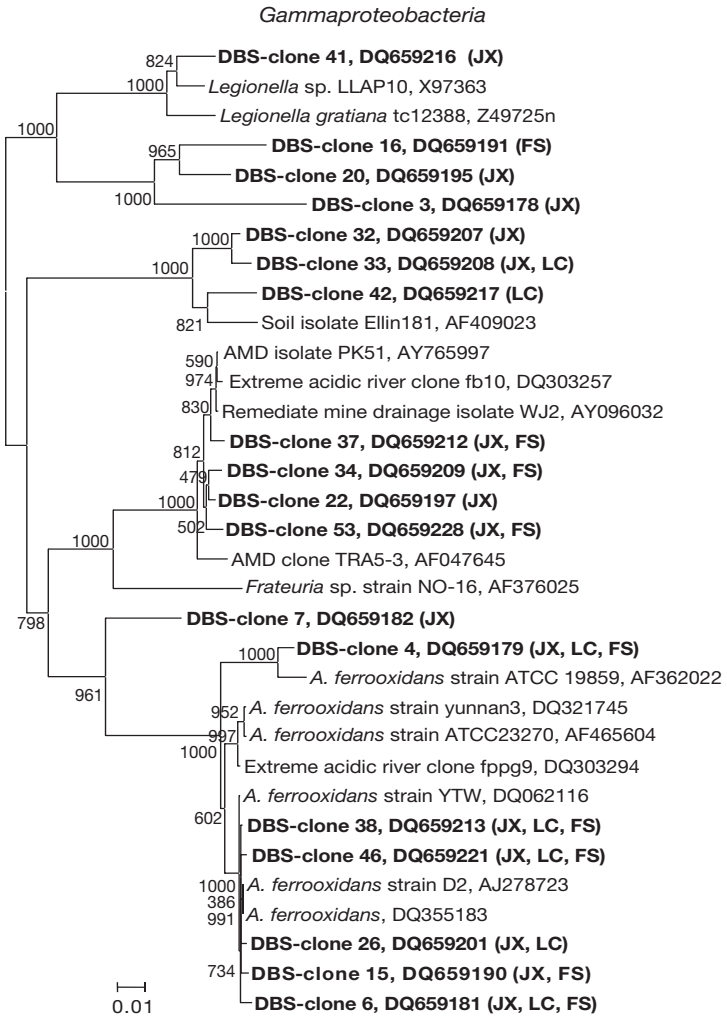

Fig. 3 (continued, and overleaf)

extent (~89 to 97% identity). The last 3 sequences were clustered with the fourth group and affiliated with soil isolate Ellin181 (~95 to 97% identity).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of DBS-Clone 1 to DBS-Clone 60 described in this study were submitted to GenBank under accession numbers DQ659176 to DQ659235.

Statistical analysis

PCAs based on the 60 bacterial units captured virtually all of the variability in the clones' data. Approximately 88.21% of the variability in the 31 bacterial units used in the analysis was represented in the PCs. The first PC (PC1) captured 54.02% of the variability, and PC2 captured 34.19% of the variability. In the PCA (Fig. 4), the biogeochemical properties of Samples JX and LC were similar yet significantly different from those of Sample FS. According to the PCA (Fig. 5), the overall bacterial community structures of Samples JX and FS were more similar to each other than to those of Sample LC. Calculations of diversity revealed that the diversity of Sample LC ($1/D = 2.51$) was the lowest, the diversity of Sample FS ($1/D = 12.35$) intermediate, and the diversity of Sample JX ($1/D = 18.42$) the highest.

Structure of bacterial communities

We identified major bacterial components in 3 AMD samples. Previous studies of AMD identified major bacterial lines of descent as divisions within the *Proteobacteria*, *Nitrospira*, *Firmicutes*, *Actinobacteria* and *Acidobacteria* (Baker & Banfield 2003, Johnson & Hallberg 2003). In our study, *Proteobacteria*, *Nitrospira*, *Actinobacteria* and *Acidobacteria* were found, but *Firmicutes* was absent. However, the diversity of bacterial communities clearly differed among the 3 samples.

In Sample JX, the dominant bacterial communities were 38.7% *Acidiphilium* sp. (38.7%), *Leptospirillum ferriphilum* (16.9%) and *Acidithiobacillus ferrooxidans* (12.1%). The percentage contributions of other *Gammaproteobacteria*, *Alphaproteobacteria*, *Acidobacteria*, *Actino- bacteria* and *Leptospirillum* were 14.5, 8.9, 5.6, 1.6 and 0.8%, respectively.

In Sample LC, the majority of these clones (88.0%) belonged to *Acidithiobacillus ferrooxidans*. The percentage contriubtions of *Acidiphilium* sp., other *Alphaproteobacteria*, *Gammaproteobacteria*, *Leptospirillum ferriphilum* and *Acidobacteria* were 4, 4.8, 1.6, 0.8 and 0.8%, respectively.

In Sample FS, the dominant bacterial community was composed of 39.1% *Leptospirillum ferriphilum*, 25.8% *Acidiphilium* sp. and 10.2% *Acidithiobacillus ferrooxidans*. The percentage contributions of *Gammaproteobacteria*, *Leptospirillum*, *Alphaproteobacteria*, *Actinobacteria* and *Acidobacteria* were 12.5, 6.3, 3.1, 2.3 and 0.8%, respectively.

DISCUSSION

To quantitatively measure diversity in the samples, we used the inverse of Simpson's index ($1/D$), which is

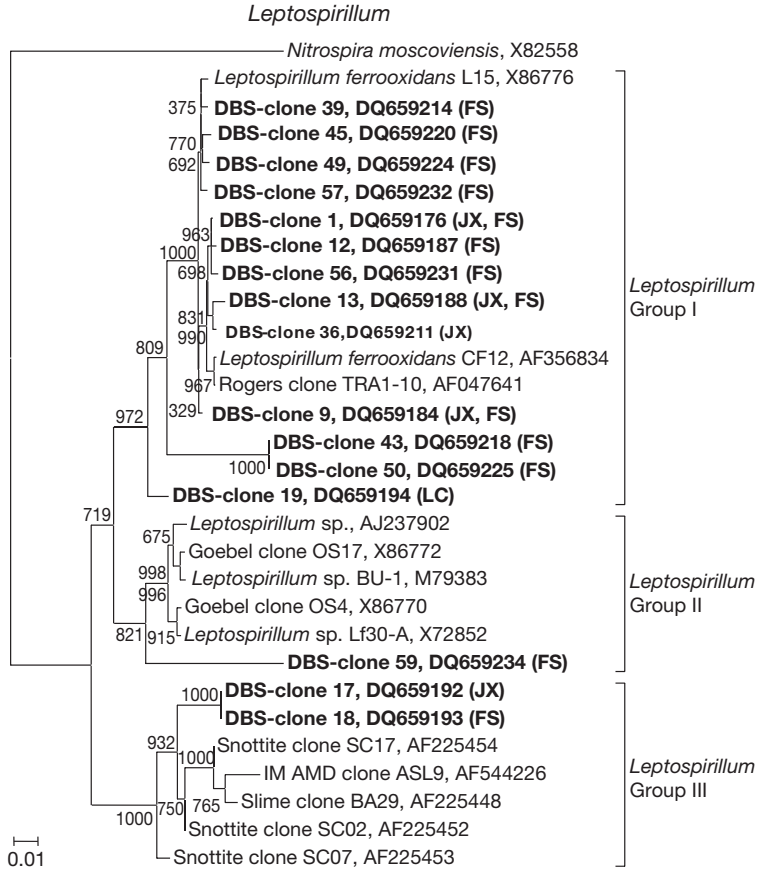

Fig. 3 (continued)

sensitive to the level of dominance in a community (Magurran 1988). The reciprocal of Simpson's index ($1/D$ = 2.51 to 18.42) effectively proved that bacterial community diversities of AMD were much lower than those of other environments, such as surface soils and marine sediments. Zhou et al. (2002) showed that $1/D$ values in surface soils ranged from 507 to 27331, while Ravenschlag et al. (1999) demonstrated that bacterial diversity in permanently cold marine sediments was high. The $1/D$ value of actinobacterial diversity was 51, even 5 to 12 cm below the sea floor at a depth of 3814 m (Stach et al. 2003). It is noteworthy that AMD environments examined in the present study exhibited very low microbial diversity indices because of high ion concentrations and low pH. However, bacterial diversities were obviously distinct among different AMD sites: $1/D$ values were 2.51 and 18.42 in Samples LC and JX respectively. This phenomenon — variable diversities among samples — has also been observed in some other studies. For example, the community of Sample LC exhibited low OTU diversity and greater dominance by fewer OTUs, which is similar to the pattern observed in low-carbon vadose zone sediments: some $1/D$ values of the saturated subsurface soil communities at low-carbon sites ranged from just 13 to 25, while others ranged from 70 to 27 331 (Zhou et al. 2002).

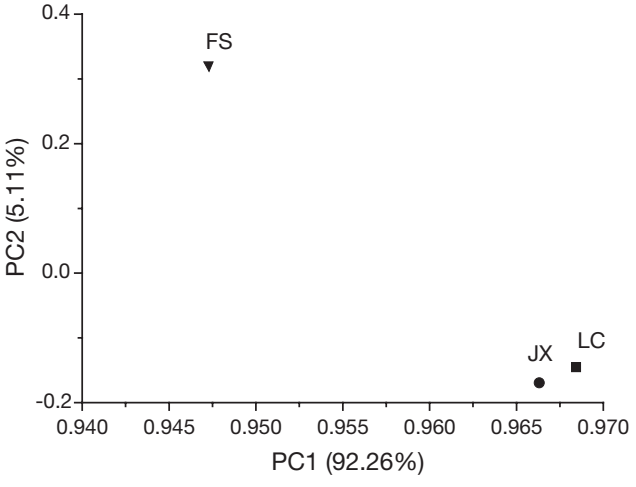

Fig. 4. Ordination plots from PCA of biogeochemical properties of Samples JX, LC and FS. Values in parentheses indicate variance

Some species of *Actinobacteria*, which were isolated from a forested wetland impacted by reject coal (Brofft et al. 2002) and the AMD environments of Wheal Jane (Johnson et al. 2002) and Iron Mountain (Nordstrom et al. 2000), were also detected in samples from Sites LC and FS in Dabaoshan Mine, China. *Acidobacteria* is a genus of very important microbes in AMD ecology, which performs an indispensable function in the formation of AMD. Moreover, *Acidobacteria* exists in relatively moderate AMD environments (20 to 37°C and pH of 3.0 to 6.0), and some analyses suggested that this group is limited to environments of high pH (pH > 1.4) (Druschel et al. 2004). Druschel et al.'s (2004) study is in accordance with our results: *Acidobacteria* was found in samples from all 3 sites.

A heterotrophic bacterial species, *Acidiphilium* sp., was found in samples from all sites. *Acidiphilium* sp. is considered able to adapt to temperatures ranging from 17 to 45°C, and to pH values ranging from 1.5 to 6.0 (Baker & Banfield 2003). Peccia et al. (2000) suggested that there might be a mutualistic relationship between *Thiobacillus* sp. and *Acidiphilium* sp. *Acidiphilium* sp. was reported along with iron- and sulfur-oxidizing chemolithoautotrophs and in extreme acidic environments (Harrison 1984, Gonzalez-Toril et al. 2003b). These microorganisms may play a critical role in such ecosystems. For example, some studies proposed that

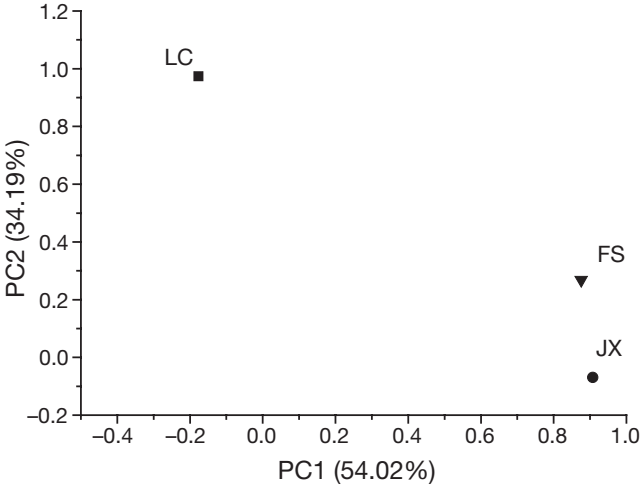

Fig. 5. Ordination plots from PCA of bacterial community structure of Samples JX, LC and FS. Values in parentheses indicate variance

Acidiphilium sp. could remove organic toxic compounds for *Leptospirillum* and *Acidithiobacillus* (Johnson 1995). Meanwhile, the physiological and ecological capabilities of *Acidiphilium* sp., which can utilize a variety of substrates and reduce ferric iron both in the presence and absence of oxygen, also indicates its important ecological roles in AMD, especially in the turnover of iron at oxic-anoxic interfaces (Kusel et al. 1999) .

Acidithiobacillus ferrooxidans and *Leptospirillum ferrooxidans* are widely considered to be the microorganisms that control the rate of generation of AMD, and have been used as model species of leaching environments (Fowler et al. 1999, Rohwerder et al. 2003). To date, *Leptospirillum* isolates and environmentally-derived clones cluster within 1 of 3 phylogenetic distinct groups (Bond et al. 2000): *L. ferrooxidans* (Group I) (Hippe 2000); *L. ferriphilum* (Group II) (Coram & Rawlings 2002); and *Leptospirillum* Group III, which was only detected via clone library analysis of Iron Mountain bacterial communities (Bond et al. 2000, Druschel et al. 2004). In our study, *Leptospirillum* Group I sequences were discovered at all sites, and were dominant in 3 *Leptospirillum* groups. Only 1 sequence was affiliated with Group II in Sample FS, while only 1 and 2 sequences were affiliated with Group III in Samples JX and FS respectively. *L. ferrooxidans* (Group I) is reported to grow within an optimal pH range of 1.6 to 2.0 (compared with 1.4 to 1.8 for *L. ferriphilum*) (Coram & Rawlings 2002). *L. ferriphilum* and *Leptospirillum* Group III primarily reside in lower pH microenvironments within mines, while *L. ferrooxidans* occurs in higher pH environments (pH > 1) (Druschel et al. 2004). Therefore, the high pH values (pH > 1.8) in the 3 samples were likely the reason why *Leptospirillum* Groups II and III were rarely represented.

Acidithiobacillus ferrooxidans-like bacteria were also found to be the dominant iron-oxidizers in a stream draining the former King's copper mine in Roeros, Norway (Johnson et al. 2001). By using FISH (fluorescence *in situ* hybridization) probes, Schrenk et al. (1998) showed that *A. ferrooxidans* is abundant in environments with temperatures < 30°C and pH > 1.3 at Richmond Mine. Edwards et al. (1999) also found *A. ferrooxidans* at greatest abundance (>30%) at moderate temperature (2.5 to 20°C) and pH (1.5 to 2.3) at sites that were peripheral to primary acid-generating sites. In our study, Sample LC (~22°C, pH 2.0) was so favorable for *A. ferrooxidans* that it became the most dominant microbe (88.0% of the bacterial population) — a result that is in good accordance with the report of Edwards et al. (1999). However, in Sample JX (~20°C, pH 2.3) and Sample FS (~22°C, pH 1.9), pH values and temperatures were similar to those of Sample LC, but *A. ferrooxidans* represented only 12.1 and 10.2% of the bacterial population, respectively, and *L. ferrooxidans* was instead more dominant (representing 16.9 and 39.1%, respectively). These results differ from those of Schrenk et al. (1998) and Edwards et al. (1999). Thus, factors other than temperature and pH are likely to have important effects on bacterial community structure in AMD.

In samples from all 3 sites, the correlation between bacterial communities and physiochemical characteristics seemed to be indistinct. Samples JX and LC had similar biogeochemical properties (see Fig. 5), but bacterial communities obviously differed (Fig. 6). In contrast, Samples JX and FS were dissimilar in biogeochemical properties (Fig. 5) but similar in bacterial community composition (Fig. 6). However, it is notable that the concentration of ferrous iron in Sample LC greatly exceeded those in Samples JX and FS (fresh minerals were being mined continuously at Site LC). As is well known, ferrous iron is a key energy substance for many bacterial species. However, with the exception of *Acidithiobacillus ferrooxidans*, other bacterial species capable of making use of ferrous iron were little-represented in Sample LC (e.g. *Leptospirillum ferrooxidans* only represented 0.8% of the total clones). We suggest that *A. ferrooxidans* grows first under conditions of high ferrous iron concentrations. Some previous studies support our inference: McGinness & Johnson (1993) found that *A. ferrooxidans* was the most numerous iron-oxidizer present in AMD draining the abandoned Cae Coch pyrite mine in north Wales, where ferrous iron concentrations were often >500 mg l^{-1}. *L. ferrooxidans* has a higher affinity for Fe^{2+}, and this species was particularly numerous when ferrous iron concentrations were <10 mg l^{-1}. In our

study, *A. ferrooxidans* was particularly numerous in Sample LC (Fe^{2+} concentration = 136.5 mg l^{-1}), while *L. ferrooxidans* was more abundant than *A. ferrooxidans* in Samples JX and FS (Fe^{2+} concentration = 6.0 and 6.5 mg l^{-1}, respectively). Thus, in our study, ferrous iron might be a factor that affected bacterial community structure. Sand et al. (1992) noted that in their bioreactors, high ratios of Fe^{3+} to Fe^{2+} appeared to be less inhibitory to *L. ferrooxidans* than to *A. ferrooxidans*. This may also have been the case in our study.

Furthermore, the amounts of many elements (e.g. Hg, Mg, Cu, As, Pb, S, Fe, Al) in Sample FS were much higher than the other 2 samples. The primary reason for this was that Site FS was used to deposit many abandoned ores for a long period of time, and many ores were dissolved into water by chemical- and microbe-mediated dissolution. Thus, the concentration of toxic ions in AMD at Site FS may have been correlated with time. In addition, the percentage of *Leptospirillum* spp. in Sample FS was the highest, but the percentage of *Acidithiobacillus ferrooxidans* the lowest. Dopson et al. (2003) showed that the growth of *A. ferrooxidans* will be limited when metal concentrations exceeded certain level, and some reports (Edwards et al. 1999, Bond et al. 2000, Olson et al. 2003) have noted that *Leptospirillum ferrooxidans* has a stronger tolerance to toxic ions within a certain concentration range. Thus, toxic ions might represent another potential factor that affects bacterial community structure.

AMD is generated from mine solution and, in the process, a variety of geochemical properties lead to variable microbial ecology. Two factors that affect bacterial communities — the concentrations of ferrous iron and toxic ions — could be correlated with time. Therefore, AMD could be regarded as a dynamic environment, and the different bacterial community structures of 3 AMD samples could simply reflect different periods of an evolution process. Generally, changes in the bacterial community in AMD of a metal mine that was still in use were irregular, because the extant environmental conditions were affected by mining. However, after sites are left abandoned for a long time, the concentration of metal ions will increase slowly and regularly owing to the dissolution process of minerals, and the subsequent evolution of bacterial communities should progress slowly and regularly too. In the early stages, the bacterial species capable of rapidly using ferrous iron will become dominant in bacterial communities. However, with the accumulation of toxic ions, the bacterial species with strong resistance to toxic ions will gradually predominate. Of course, other factors (such as seasonal variations and drought) may also have important effects on this evolution. In the case of bioleaching, the effect of this factor on the bacterial community must be considered.

Acknowledgements. Thanks to Weiliang Chao, Department of Microbiology, Soochow University, Taipei, for invaluable suggestions. This work was supported by 973 Program of China (No. 2004CB619201) and the National Natural Science Foundation of China (NSFC No. 50621063).

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Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA

Submitted: August 27, 2006; Accepted: March 7, 2007
Proofs received from author(s): April 16, 2007