Correlating Microbial Diversity Patterns with Geochemistry in an Extreme and Heterogeneous Environment of Mine Tailings

Jun Liu, Zheng-Shuang Hua, Lin-Xing Chen, Jia-Liang Kuang, Sheng-Jin Li, Wen-Sheng Shu, Li-Nan Huang

State Key Laboratory of Biocontrol and Key Laboratory of Biodiversity Dynamics and Conservation of Guangdong Higher Education Institutes, School of Life Sciences, Sun Yat-sen University, Guangzhou, People's Republic of China

Recent molecular surveys have advanced our understanding of the forces shaping the large-scale ecological distribution of microbes in Earth's extreme habitats, such as hot springs and acid mine drainage. However, few investigations have attempted dense spatial analyses of specific sites to resolve the local diversity of these extraordinary organisms and how communities are shaped by the harsh environmental conditions found there. We have applied a 16S rRNA gene-targeted 454 pyrosequencing approach to explore the phylogenetic differentiation among 90 microbial communities from a massive copper tailing impoundment generating acidic drainage and coupled these variations in community composition with geochemical parameters to reveal ecological interactions in this extreme environment. Our data showed that the overall microbial diversity estimates and relative abundances of most of the dominant lineages were significantly correlated with pH, with the simplest assemblages occurring under extremely acidic conditions and more diverse assemblages associated with neutral pHs. The consistent shifts in community composition along the pH gradient indicated that different taxa were involved in the different acidification stages of the mine tailings. Moreover, the effect of pH in shaping phylogenetic structure within specific lineages was also clearly evident, although the phylogenetic differentiations within the *Alphaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* were attributed to variations in ferric and ferrous iron concentrations. Application of the microbial assemblage prediction model further supported pH as the major factor driving community structure and demonstrated that several of the major lineages are readily predictable. Together, these results suggest that pH is primarily responsible for structuring whole communities in the extreme and heterogeneous mine tailings, although the diverse microbial taxa may respond differently to various environmental conditions.

Present-day extreme environments are considered to be similar to Earth's early conditions (1, 2). Thus, exploring the diversity of microbial life therein and the ecological links between environmental geochemistry and microbial communities will reveal how extraordinary organisms survive and thrive in extreme habitats and provide insights into the development and expansion of life on Earth (3). For many years, much research has attempted to use molecular techniques to uncover the relationships between microbial diversity and geochemical parameters, demonstrating that environmental variables such as conductivity and rainfall (4), pH (5), temperature and sulfide (6), and organic carbon (7) have strong influences on the phylogenetic differentiation among microbial assemblages in extreme environments. Although limited in the number of samples analyzed and the resolution offered by the conventional molecular approaches, these studies have provided initial insights into the physical and chemical factors affecting microbial community structure and increased our understanding of how microbes respond to shifts in the harsh environmental conditions.

High-throughput next-generation sequencing technologies have rapidly become powerful tools for the study of diversity and distribution of microbes in the environment. By large-scale sampling and deep sequencing of microbial communities from different geographic regions, recent investigations have revealed the regional and global microbial diversity patterns and the effects of specific geochemical factors on community composition in extreme environments (8–10). In marked contrast, surprisingly little work has been undertaken to extensively sample (and thus obtain sufficient coverage of) a specific site and to systematically explore the local biodiversity of extremophilic microbes and how different

lineages respond to various environmental conditions. In particular, the differences in the factors and the potential underlying processes driving microbial community composition at local and large geographic scales remain largely unknown, precluding reliable prediction of the relative abundance of taxa as functions of environmental variables. In fact, even for “nonextreme” environments, there is a lack of dense spatial analyses of specific sites to resolve the local distribution of complex microbial populations and how communities are shaped by the prevailing environmental conditions.

Large amounts of tailings are produced from mining activities and waste tailing dumps from either inactive or abandoned mine sites are prevalent in many parts of the world. These mining residues represent an important source of acid mine drainage (AMD), a very widespread environmental problem, because of the microbially mediated oxidative dissolution of sulfide minerals (11). Because of their biological and geochemical simplicity and the steep environmental gradients associated with different oxidation stages, the extreme tailing environments are ideal targets for the study of local microbial diversity and how communities respond

Received 26 January 2014 Accepted 2 April 2014

Published ahead of print 11 April 2014

Editor: S.-J. Liu

Address correspondence to Li-Nan Huang, eseshln@mail.sysu.edu.cn.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00294-14>.

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doi:10.1128/AEM.00294-14

to environmental changes. The microbial community diversity in mine tailings has recently been investigated by comparative 16S rRNA sequencing (12–16). While these studies have significantly expanded our knowledge of the biodiversity of extreme tailing environments (including the detection of less-well-known or novel taxa), systematic exploration of the driving factors in local community composition and how microbial populations interact with changing environmental conditions has thus far been impossible. This is due primarily to the limited number of samples analyzed at a single mine tailing site and, in most cases, the insufficient sequencing depth provided by a standard clone library analysis. A recent report has conducted pyrosequencing-based comparative phylogenetic and metagenomic profiling of microbial communities at a Pb/Zn mine tailing site (15), but replicate tailing samples from only a few typical oxidation stages were analyzed. To address this knowledge gap, we have generated the largest mine tailing sequence data set to date, from 90 environmental samples collected from a massive copper tailing impoundment (see Fig. S1 in the supplemental material), by using barcoded pyrosequencing. We examined how microbial assemblages are shaped by the geochemical characteristics within the tailings and how the phylogenetically divergent lineages respond to various environmental conditions. Furthermore, since the extensive and representative 16S rRNA sequences achieved a reasonable coverage of our field site, we attempted microbial composition prediction for these mine tailings with relatively low species richness and high geochemical heterogeneity.

MATERIALS AND METHODS

Site description and sample collection. The Shuimuchong (SMC) copper tailing impoundment (30°55′53.90″N, 117°50′42.27″E) is located approximately 3 km northeast of Tongling City, Anhui Province, China, and covers a total surface area of 44 ha (see Fig. S1 in the supplemental material). The climate in this region is humid subtropical with an annual precipitation of around 1,360 mm and an annual mean temperature of 16.2°C. Approximately 9 million tons of waste materials (40% coarse grained and 60% fine grained) from the processing of copper sulfide ores were deposited in the SMC dump, which was operated continuously for about 16 years and then abandoned in 2006. In September 2011, 90 samples were randomly collected within an area of approximately 10,000 m² located in the flat plateau (400 by 400 m without vegetation) of the tailing impoundment (see Fig. S1 in the supplemental material). This area was selected on the basis of our continuous field monitoring and preliminary physiochemical analysis, which identified diverse tailing zones with distinct geochemistry associated with different stages of the oxidation process. For each sample, five tailing cores were collected at a depth of 0 to 5 cm by driving five metallic tubes side by side (in a circular pattern that was 4 cm in diameter) into the impoundment and then mixed. All samples were kept in cooler boxes during sampling and then transported to the laboratory, where they were stored at 4°C prior to subsequent analyses.

Physicochemical analysis. Moisture content was measured by determining the difference between the weights of a subsample before and after drying at 105°C for 12 h. The tailing samples were air dried, and total organic carbon (TOC) (TOC-VCPH; Shimadzu, Columbia, MD), total nitrogen (TN) (Kjeltec TM 2300; Foss, Hilleroed, Denmark), and total phosphorus (TP) (SmartChem; Westco Scientific Instruments Inc., Brookfield, CT) were analyzed according to standard methods. Electrical conductivity (EC) and pH were measured at a 1:2.5 (wt/vol) sample-to-distilled-water ratio (17). Total heavy metals (including Fe, Cu, Zn, Pb, and As) and HCl-extractable Cu were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 2100DV; Perkin-Elmer, Wellesley, MA). HCl-extractable ferric and ferrous iron was determined

by the 1,10-phenanthroline method at 530 nm (18), and sulfate (SO₄^{2−}) was measured by a BaSO₄-based turbidimetric method (19).

DNA extraction, PCR amplification, bar-coded pyrosequencing, and data processing. Genomic DNA was extracted from the tailing subsamples as described previously (20), except that a FastDNA Spin kit for soil (Qbiogene, Carlsbad, CA) was used instead. The V4 region of bacterial and archaeal 16S rRNA genes was amplified with prokaryotic universal primers F515 and R806 (21) and a sample-specific 8-bp barcode on F515. Each DNA sample was amplified in triplicate in 50-μl reaction mixtures, and replicate amplicons were pooled for purification with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA). A single composite sample was prepared by combining approximately equimolar amounts of purified PCR products from each sample and then sequenced on a 454 GS-FLX Titanium pyrosequencer (Roche 454 Life Sciences, Branford, CT).

Raw pyrosequencing data were processed with the pipeline coupling mothur (22) and QIIME (23) software. The commands shhh.flows (24), pre.cluster (25), and chimera.uchime (26) were used to denoise the sequence data, reduce sequencing error, and identify and remove chimeras, respectively. Operational taxonomic units (OTUs) were identified with uclust at the 97% sequence similarity level (27). Representative sequences were aligned and used to build the phylogenetic tree with FastTree (28). Taxonomic assignment of the OTUs was determined at the 80% threshold with the RDP Classifier (version 2.6) (29). As the minimum number of quality sequences per sample was 2,235 across all of the samples, alpha diversity (phylogroup richness and Faith's phylogenetic diversity [PD]) and beta diversity (unweighted and weighted UniFrac distances) were calculated on the basis of a subset of 2,235 randomly selected sequences per community with 100 iterations. UniFrac-based principal-coordinate analysis (PCoA) was used to find clusters and the most important axes of variation among samples.

For the analyses of diversity within specific lineages (phylogroup richness, Faith's PD, and Bray-Curtis distance), the number of sequences per sample was limited to 195, 80, 80, 160, 130, 25, 375, and 25 for *Euryarchaeota*, *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Nitrospirae*, respectively. Sequences for the target lineages were selected randomly from each community. Normalization of the number of sequences per sample allowed us to control for the effects of survey effort in comparing lineage-specific diversity metrics across the samples (30). The lineage-specific diversity analyses were conducted on 32 to 71 of the 90 samples (Table 1), excluding those that did not have the required number of sequences set for each lineage.

Prediction of microbial assemblages in tailings. A recent study has reported a microbial assemblage prediction (MAP) model predicting microbial taxon abundance as a function of environmental factors and predicted microbial interactions (31). It demonstrated that the model could generate more accurate predictions than other single-species models that do not incorporate microbial interactions. In the present study, a predictive model of microbial assemblages was constructed by the MAP approach as described previously (31). All environmental data were normalized (the normalized values ranged between 1 and 100), and the normalized environmental variables and OTUs were merged into a single input matrix. Relationships between all parameters were estimated by Bayesian network inference with the software BANJO v2.2.0 (32). The generated consensus EIN (environmental interaction network) would show the relationships that were expressed as a set of formulas such that the value of every node was a function of the value of its parent nodes by using the software Eureqa II (33). For details of the methods used, see the supplemental material.

Statistical analyses. All statistical analyses were implemented with SPSS 19.0 software and various packages within the statistical program R (<http://www.r-project.org>). We used aggregated boosted tree (ABT) analysis (34) to quantitatively evaluate the relative influence of environmental variables on the patterns of overall microbial diversity and relative abundance of specific lineages with the gbmplus package. Results of ABT anal-

TABLE 1 Correlations between the diversity metrics of dominant lineages and primary environmental parameters^a

Phylum/class (no. of samples)	Correlation with:			
	Relative abundance ^b	Phylotype richness ^c	PD ^c	Bray-Curtis distance ^c
<i>Euryarchaeota</i> (38)	pH (0.697, <0.001) ^d	pH (0.209, 0.004) ^d	pH (0.324, <0.001) ^d	pH (<i>r</i> , 0.422, 0.001) ^e
<i>Actinobacteria</i> (71)	Fe ³⁺ (0.429, <0.001)	Fe ³⁺ (0.157, 0.001)	As (0.070, 0.025)	pH (<i>r</i> , 0.520, 0.001)
<i>Firmicutes</i> (69)	pH (0.367, <0.001)	Zn (0.152, 0.001)	Zn (0.198, <0.001)	Fe ²⁺ (<i>r</i> , 0.365, 0.001)
<i>Alphaproteobacteria</i> (41)	pH (0.424, <0.001)	Fe ³⁺ (0.157, 0.010)	Pb (0.143, 0.015)	Fe ³⁺ (<i>r</i> , 0.254, 0.001)
<i>Betaproteobacteria</i> (60)	pH (0.457, <0.001)	pH (0.145, 0.003)	Pb (0.175, 0.001)	pH (<i>r</i> , 0.251, 0.002)
<i>Deltaproteobacteria</i> (32)	TOC (0.198, <0.001)	Moisture content (0.195, 0.011)	Moisture content (0.213, 0.008)	Fe ³⁺ (<i>r</i> , 0.281, 0.002)
<i>Gammaproteobacteria</i> (64)	Fe ³⁺ (0.111, <0.001)	pH (0.642, <0.001)	pH (0.741, <0.001)	pH (<i>r</i> , 0.298, 0.001)
<i>Nitrospirae</i> (41)	pH (0.433, <0.001)		Fe (0.109, 0.035)	pH (<i>r</i> , 0.203, 0.014)

^a Linear regression relationships between the primary environmental parameters and the diversity metrics (such as relative abundance, phylotype richness, and PD) were analyzed. The BIO-ENV procedure was used to find the correlations (Spearman rank correlations were estimated by using Mantel tests) between Bray-Curtis distances within dominant lineages and euclidean distances of the primary environmental parameters.

^b Ninety tailing samples were analyzed.

^c The number of tailing samples analyzed is shown in parentheses in the leftmost column.

^d *R*² and *P* values are in parentheses.

^e *r* and *P* values are in parentheses.

ysis for specific lineages were clustered and visualized with the heatmap2 function in the gplots package. Multiple linear regression (MLR) analysis by SPSS 19.0 was employed to test the significance of differences between diversity indices and microbial taxon abundance and tailing properties. The BioEnv procedure within the vegan package was applied to determine the best environmental parameters, so that the euclidean distances of the scaled environmental parameters have the maximum (rank) correlations with community dissimilarities (Bray-Curtis distances). The significance of these correlations was then tested by using the Mantel test within the vegan package (35). Additionally, the least significant difference test was used to identify the differences in the relative abundance of each dominant genus across various pH gradients by using SPSS 19.0.

Nucleotide sequence accession number. The pyrosequences determined in this study have been deposited in the NCBI Sequence Read Archive under accession number [SRR1040478](https://www.ncbi.nlm.nih.gov/sra/SRR1040478).

RESULTS

Geochemical characteristics of mine tailings. For detailed physicochemical parameters of the tailing samples studied, see Table S1 in the supplemental material. The pH values and concentrations of dissolved solids (measured as EC) ranged from 2.2 to 7.3 and from 462 to 1,987 $\mu\text{S cm}^{-1}$, respectively. In general, the tailings had relatively high levels of residual heavy metals, with concentrations of copper ion and ferric and ferrous iron fluctuating widely across the samples (averaging at 215 mg kg^{-1} and 22 and 3.5 g kg^{-1} , respectively). TOC contents were typically low and varied broadly from 0.99 to 9.9 g kg^{-1} .

Composition and diversity of microbial assemblages. A total of 9,148 phylotypes were identified in the 548,242 quality sequences generated for the 90 tailing samples, of which 58% were represented by a single sequence and 68% were detected in only a single sample. Rarefaction analysis indicated that even at this depth of sequencing, we did not capture the full extent of PD within each individual sample. Phylotype richness and Faith's PD, calculated on the basis of 2,235 sequences randomly selected from each sample, ranged from 46 to 318 and from 5.5 to 37, respectively (Fig. 1).

A total of 18 phyla were detected in the complete data set, with *Proteobacteria*, *Actinobacteria*, *Euryarchaeota*, *Firmicutes*, and *Nitrospirae* accounting for 40, 19, 12, 9.5, and 3.7% of the total quality sequences, respectively. The remaining phyla were largely rare in most of the tailing samples, representing <0.5% of the se-

quences. At the genus level, members of the genera *Rubrobacter*, *Acidithiobacillus*, *Sulfobacillus*, and *Leptospirillum* were most frequently detected (17, 13, 5.7, and 3.7% of the sequences, respectively). More specifically, the *Acidithiobacillus* sequences were predominantly affiliated with *Acidithiobacillus ferrooxidans* (~50%) and *A. caldus* (~48%), the *Leptospirillum* reads were affiliated with "*Leptospirillum ferrodiazotrophum*" (~79%) and *L. ferriphi-*

FIG 1 Linear regression relationships between tailing pH and overall microbial diversity measured by using the number of phylotypes (A) and PD (B).

FIG 2 Relative abundances (percent) of dominant lineages in all tailings combined and in tailings separated into pH categories. The value above each column is the number of tailing samples in each category.

lum (~20%), and the *Sulfobacillus* sequences were affiliated with *Sulfobacillus benefaciens* (~37%), *S. thermotolerans* (~27%), and *S. acidophilus* (~26%). For the *Archaea*, the genera *Thermogymnomonas* and *Ferroplasma* were most frequently encountered, representing 30 and 11% of the archaeal sequences, respectively. The dominant *Ferroplasma* species (~85%) detected was most closely related to *Ferroplasma acidiphilum*, with the remaining 15% related to the environmental clones. In general, notable shifts of microbial composition along the pH gradient were clearly evidenced and relative abundances of different lineages varied greatly across the tailing communities (Fig. 2; see Fig. S5 in the supplemental material).

Influence of environmental variables on overall microbial diversity. Both overall phylotype richness and PD varied considerably across the tailing communities (Fig. 1). ABT models were applied to interpret the relative influence of different environmental parameters on the overall diversity patterns. The results demonstrated that pH was the primary factor driving patterns of both phylotype and PD, explaining 43 and 24% of the relative importance, respectively (see Fig. S2 in the supplemental material). In supporting this, MLR analysis revealed significant positive correlations between tailing pH and overall microbial diversity (Fig. 1). Furthermore, MLR analysis with multivariate models did not lead to a significant improvement of correlation values (phylotypes, $R^2 = 0.578$, $P < 0.001$; Faith's PD, $R^2 = 0.687$, $P < 0.001$) over those for pH alone. Additionally, the statistical analyses (ABT and MLR) also detected moderate effects of other environmental variables (e.g., Fe^{2+} , Cu^{2+} , and EC) on both diversity estimates (see Fig. S2).

We further used a phylogeny-based metric, UniFrac, to assess the differences among microbial communities. UniFrac-based PCoA again revealed strong primary clustering of tailing assemblages by pH, which explained 67% of the total variance in the weighted UniFrac distances (Fig. 3). The ABT analyses also identified pH as the primary factor shaping microbial assemblages (see

Fig. S3 in the supplemental material). Moreover, of all of the environmental parameters examined, the variation in tailing pH calculated by euclidean distance was most strongly correlated with overall weighted and unweighted UniFrac distances (Spearman's $r = 0.738$ and 0.689 , $P = 0.001$) between microbial communities by Mantel test.

Influence of environmental variables on specific lineages.

The phylogenetically divergent lineages coexisting in the tailing community may give diverse responses to the local environmental characteristics. To test this, additional statistical analyses were conducted to explore the relationships between relative abun-

FIG 3 PCoA results colored by pH, with a weighted UniFrac distance matrix comparing the 90 tailing samples. The percentages of variation in the samples described by the plotted principal coordinates are indicated on the axes.

FIG 4 Relative influence (percent) of environmental factors on the relative abundances of specific lineages (A) and dominant genera (B) evaluated by ABT models. The results are clustered and visualized with heatmap2 in R software. Moi, moisture content.

dances and diversity metrics of specific lineages and environmental variables. For the relative abundance, specific tailing lineages were divided into three groups by ABT analysis (Fig. 4A). The first group was mostly affected by pH and consisted of *Euryarchaeota*, *Firmicutes*, *Nitrospirae*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Deltaproteobacteria*. The relative abundances of *Euryarchaeota*, *Firmicutes*, and *Nitrospirae* decreased with increasing pH, while a positive effect of pH on the abundances of other lineages was detected (Fig. 4A). In addition, EC seemed to have a negative

effect similar to that of pH on the *Euryarchaeota* and *Betaproteobacteria*, whereas Fe^{2+} exhibited an influence similar to that of pH on *Deltaproteobacteria*. The physiological features of the second group, which included members of the class *Actinobacteria*, were probably different from those of the first group since Fe^{3+} had a stronger relative influence than pH. The third group, including the *Gammaproteobacteria*, was strongly influenced by the total copper concentration. These findings were largely supported by the MLR analyses (Table 1), except for the less clear relationship between *Gammaproteobacteria* and total copper.

We then determined the relationships between the diversity metrics of the specific lineages and the measured environmental parameters. MLR analysis indicated that patterns of phylotype richness and PD for *Euryarchaeota* and *Gammaproteobacteria* were apparently shaped by tailing pH, while these metrics for the *Deltaproteobacteria* and *Firmicutes* were significantly associated with moisture content and the total amount of zinc, respectively. In contrast, no environmental factors showed strong correlations with the phylotype richness of *Nitrospirae*, although PD within *Nitrospirae* was affected by the total amount of iron (Table 1). Additionally, a Mantel test revealed significant correlations ($P < 0.05$) between tailing pH and Bray-Curtis distances within the dominant lineages *Euryarchaeota*, *Actinobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Nitrospirae* ($r = 0.203$ to ~ 0.520) (Table 1). Additional significant associations were found between the ferric iron concentration and Bray-Curtis distances within the *Alphaproteobacteria* and *Deltaproteobacteria* and between the ferrous iron concentration and Bray-Curtis distances within the *Firmicutes* (Table 1).

At the genus level, the diverse dominant taxa in the tailings also displayed different responses to the environmental characteristics. ABT analysis revealed that the relative abundances of *Ferroplasma* and *Leptospirillum* were largely influenced by pH, while EC and Fe^{3+} showed the strongest effects on the abundance of *Thermogymnomonas* and *Rubrobacter*, respectively (Fig. 4B). These findings were in good agreement with the results of MLR analysis (see Fig. S4 in the supplemental material). In addition, the relative abundances of *Sulfobacillus* and *Acidithiobacillus* were strongly affected by Fe^{2+} and Cu, respectively. However, MLR analysis revealed a significant correlation between pH and *Sulfobacillus* abundance and that changes in the ferric iron concentration largely explained the variation of *Acidithiobacillus* abundance (see Fig. S4).

Prediction of microbial assemblages. The selected EIN generated in the MAP model had a total of 13 interactions, of which 7 were exclusive between microbial taxa (see Fig. S6 in the supplemental material). The significance of these correlations between microbial taxa was checked with SparCC (87) with 20 iterations and 100 shuffled data sets, and all pseudo- P values were less than 0.05 (data not shown). Meanwhile, Mantel test analysis revealed significant correlations ($P = 0.001$) between microbial community structure and three environmental factors (pH, $r = 0.6733$; Fe^{2+} , $r = 0.4581$; Fe^{3+} , $r = 0.4362$) and the relative abundances of those lineages ($r = 0.2161$ to ~ 0.5641). Among the predictive equations, the equation for *Euryarchaeota* was the best ($R^2 = 0.97$), followed by those for *Nitrospirae* and *Alphaproteobacteria* ($R^2 = 0.88$ and 0.85) and other lineages. In contrast, the predictive equation was not well applied for *Betaproteobacteria* ($R^2 = 0.56$). Notably, in the set of predictive equations for the relative abundances of taxa, pH was the most frequently occurring parameter,

followed by relative abundances of *Alphaproteobacteria* and *Betaproteobacteria*. Overall, the consensus network generated revealed that pH was the major factor structuring tailing communities by directly or indirectly affecting microbial taxon abundance.

DISCUSSION

Geochemical parameters, including heavy metal (such as copper iron and ferric and ferrous iron) levels, TOC contents, EC, and especially pH values, fluctuated broadly across the 90 tailing samples, indicating a high environmental heterogeneity in the SMC mine tailing environment. This heterogeneity was likely attributable to the differences in the time of tailing disposal, local availability of air and water, and the levels and nature of microbial metabolic activities of the different sampling sites which apparently cover a wide range of acidification stages. As previous investigations have suggested that phylogenetic clustering is most often due to habitat filtering (38, 39), such heterogeneity in the tailing environment may have a significant influence on the indigenous microbial communities. Indeed, our extensive sampling and pyrosequencing have revealed a higher genetic diversity in the mine tailings than previous estimates based on clone library analysis (13, 14). Numerous rare taxa were detected by deep sequencing, and they collectively accounted for most of the observed PD in the mine tailings. While these low-abundance taxa may be important for the stability and function of communities (36, 37), they may constitute a significant species pool whose members are recruited during progressive acidification of tailings and thus are important contributors to AMD. Indeed, our results have demonstrated that microbial community composition varied greatly within the massive tailing site, likely reflecting the responses of diverse taxa to the highly heterogeneous geochemical conditions. Shifts in the relative abundance of specific lineages along the pH gradient suggest a consistent alteration of microbial community structure and function in the different acidification stages of mine tailings (reference 15; see below). Notably, variations in phylotype richness and PD among samples at pH <4 were apparently smaller than those of samples at pH >6 (Fig. 1), indicating more homogeneous environments associated with the extremely acidic tailings.

We explored the phylogenetic differentiation among microbial communities along environmental gradients, demonstrating that pH was the primary factor regulating microbial populations in the mine tailings. Recent molecular surveys have repeatedly documented pH-dependent large-scale distribution patterns of microbes in a wide array of habitats, such as soils (30, 40), sediments (41, 42), and even extreme AMD environments (5, 10). However, the mechanisms shaping these patterns remain largely unknown. One common explanation is that microbial community structure is influenced by both direct and indirect effects of pH (30, 43). Specifically, pH could directly impose a physiological constraint on individual taxa to inhibit their growth when pH falls outside a certain range, resulting in altered competitive outcomes (44, 45). The significant correlations between pH and the relative abundances of specific lineages observed in the present study may imply a direct effect. On the other hand, shifts in pH may alter other environmental characteristics, such as carbon and nutrient availability (46) and the solubility of metals, especially iron (47, 48), which is the most important substrate for the microbial populations in mine tailings (e.g., *Acidithiobacillus*, *Leptospirillum*, and *Ferroplasma*) (47). Such environmental changes may affect different taxa driving the observed shifts in community composition in

our study and thus supporting pH as an integrating variable indirectly influencing microbial community structure (46, 47).

Our analysis has also identified EC and Cu^{2+} as important factors next to pH for explaining community differences. Salinity (as reflected by the EC values) has recently been identified as an important environmental determinant of microbial community composition across diverse habitat types (3, 49). Salinity in soils is typically driven by ionic Na, K, and Cl; thus, variations in environmental salinity can lead to changes in osmotic pressure on microorganisms, and high salinity can reduce their growth rate (50, 51) and so change microbial community composition because of differential tolerance to salinity among microbial genotypes (50, 52, 53). In contrast, correlation analyses indicated that salinity in the mine tailings is likely driven by ionic Fe, Cu, Zn, and S. Therefore, variations in community composition caused by salinity could be attributed largely to oxidative stress and heavy metal toxicity (see below). On the other hand, significant correlations between Cu^{2+} and microbial community composition have been found at abandoned copper tailing dumps (54) and soils near a copper smelter (55). For microorganisms, copper has dual roles, depending on the concentration of copper ion (56–58). While low concentrations of copper ion are required for cell metabolism, high copper concentrations above specific thresholds are toxic to microbial cells because of its ability to interact with radicals (59, 60). In the meantime, microorganisms with high resistance and tolerance to copper have been widely reported (61–63). It is possible that such microorganisms may become dominant in tailings with high levels of Cu^{2+} that inhibit “normal” microbial life. In addition, the absence of any significant relationships between bulk metals and overall microbial diversity is somewhat expected, since a large fraction of the total metals in mine tailings may exist as silicates (64, 65) to which microbes have limited access.

Previous fluorescence *in situ* hybridization-based investigations have demonstrated that microbial populations, including *A. ferrooxidans* and *L. ferrooxidans*, vary spatially and seasonally and correlate with geochemical and physical conditions (pH, temperature, conductivity, and rainfall) in extreme AMD environments (4, 66). Close relationships between acidic pH values plus high heavy metal concentrations and the abundance of chemolithotrophic bacteria were also documented in the Rio Tinto, Spain (67). Correlating microbial lineage abundance with geochemical data has demonstrated that diverse microorganisms exhibited different responses to the different environmental conditions at our tailing site. Specifically, our analyses revealed that the relative abundances of all of the major phyla were influenced mainly by pH of tailings, except for the abundance of *Actinobacteria*, which was affected largely by Fe^{3+} concentrations. While acidophiles may possess a variety of mechanisms for pH homeostasis in acidic environments (68), *Actinobacteria*, as a natural part of the microbiota of metal-rich, acidic ecosystems (11, 13), may have evolved different kinds of resistance mechanisms for dealing with heavy metals (69). Frequently detected within this phylum are orders such as *Acidimicrobiales* and *Rubrobacterales*, which can oxidize iron and sulfides (70), indicating a role for *Actinobacteria* in the biogeochemical processes at this tailing site.

Our data also revealed the various influences of different geochemical parameters on the relative abundances of dominant genera in the tailings. First, the *Acidithiobacillus* and *Rubrobacter* abundances varied consistently with changing ferric ion concentrations (see Fig. S4 in the supplemental material), with *Acidithio-*

bacillus species displaying Fe^{3+} sensitivity and *Rubrobacter* species having an affinity for ferric iron. Although high concentrations of ferric iron can lead to oxidative stress on microbes (71), several strategies, including the synthesis of trehalose (72) and radioresistance mechanisms (73–75), have been found in *Rubrobacter* species to resist oxidative stress. Second, patterns of *Ferroplasma* and *Leptospirillum* abundance were apparently shaped by tailing pH. These acidophiles may use different types of pH homeostatic mechanisms to adapt to extremely acidic habitats, including a highly impermeable cell membrane to restrict proton influx into the cytoplasm (68, 77) and a chemiosmotic gradient generated by a Donnan potential to inhibit proton influx into the cell and pump excess protons out of the cell (68). Copper cations may represent an important environmental stress at the SMC site. Our results indicated that high levels of copper in the tailings may inhibit the growth of microorganisms, including *Ferroplasma* and *Leptospirillum* species (Spearman's $r = -0.642$ and -0.534 , $P < 0.001$). However, a positive correlation between the copper ion concentration and the relative abundance of *Rubrobacter* species was clearly observed (Spearman's $r = 0.538$, $P < 0.001$), implying a critical feature of copper resistance in this dominant genus. Indeed, members of the genus *Rubrobacter* have been found to generate transporters that selectively export copper ions to the extracellular fluid to prevent toxicity (78).

It should be pointed out that the progressive changes in geochemistry in the mine tailings resulted largely from metabolic activities of the wide array of microbes detected, in particular, those capable of iron and sulfur oxidation. Thus, the observed correlations between microbial diversity patterns and geochemical data may mirror the dynamic interactions between the biotic and abiotic components therein. Recent investigations have suggested that different stages of the acidification process are likely associated with different community composition and functions (15). Our dense sampling at the SMC tailing site has captured a wide range of physical and geochemical gradients, enabling a detailed exploration of microbial community succession in the acid generating process (see Fig. S5 in the supplemental material). During the early stage under nearly neutral pH conditions ($\text{pH} > 6$), *Rubrobacter* species were predominant, with a relatively low abundance of *Acidithiobacillus* species. It is likely that the colonization and metabolic activities of *Rubrobacter* species and other pioneer species may lead to a gradual change in biotic and abiotic conditions in the tailings, facilitating the establishment of other populations that play a more important role in the subsequent oxidative pyrite dissolution and acid generation process. In addition, *A. ferrooxidans* has been demonstrated to effectively colonize pyrite under neutral pH conditions, a critical stage in the formation of AMD (79). With acidification progressing and pH declining, *Acidithiobacillus* species increased greatly and dominated the tailing communities under moderately acidic conditions ($\text{pH} 3$ to ~ 6). Meanwhile, *Leptospirillum* species proliferated and some heterotrophic and mixotrophic microorganisms (e.g., *Sulfobacillus* and *Thermogymnomonas* species) increased substantially. Chemoautotrophic bacteria (e.g., *Acidithiobacillus* and *Leptospirillum* species) fix inorganic carbon and release excess organic compounds into environments sustaining heterotrophic and mixotrophic prokaryotes (11, 70, 80). The close interactions between these distinct trophic groups not only facilitate their continued growth but also result in the co-oxidation of minerals (81, 82). During the later stages under extremely acidic conditions ($\text{pH} < 3$), the coop-

eration between autotrophic and heterotrophic species of microbes likely still contributes to the acidification process. The abundances of *Leptospirillum*, *Sulfobacillus*, and *Ferroplasma* species increased dramatically, with *Acidithiobacillus* species continuously decreasing, which might be attributable to the extremely low-pH stress and intense energy competition (4, 83, 84). *Ferroplasma* species apparently have a narrower pH range for growth ($\text{pH} < 3$) but a stronger extreme acid tolerance than other acidophilic microbes (see Fig. S5 in the supplemental material). This result is consistent with previous pure culture studies, which observed no growth of *F. acidiphilum* and “*F. acidarmanus*” at $\text{pH} > 3$ (85, 86). The numerical abundance of *Ferroplasma* species in the extremely acidic tailings may imply a significant role for these cell wall-lacking, iron-oxidizing archaea in the biogeochemical cycling of sulfide minerals at the SMC tailing impoundment, supporting our previous findings at different Pb/Zn tailing sites (14, 20).

The explicit shift of microbial populations over the range of physical and geochemical gradients has enabled the prediction of community composition in the mine tailings. In good agreement with the above analyses, the interaction network showed that microbial taxa were strongly influenced by the direct and indirect effects of pH (10, 30). Besides the pH effect, the abundance of *Euryarchaeota* was also affected by the abundance of *Betaproteobacteria*, indicating competition for ecological niches. The predictive equation for *Nitrospirae* also revealed an influence of the abundance of *Euryarchaeota* and *Gammaproteobacteria* on the abundance of this lineage, and these interactions are readily evidenced by the shifts in the relative abundances of *Leptospirillum* species, *Ferroplasma* species, and *Acidithiobacillus* species during the acidification process of tailings. The relatively lower predictive accuracies of the other lineages likely reflect an effect of other potentially important parameters that were not measured in the present study.

Concluding remarks. The extensive 16S pyrosequencing performed in this study has expanded our knowledge of the biodiversity and distribution of microbes in the extreme mine tailing environment. Although the phylogenetically divergent lineages may respond differently to various environmental conditions, overall microbial diversity patterns in local communities are apparently shaped by pH, and several of the major lineages could readily be predicted by the MAP model. Since pH has recently been found to influence the large-scale ecological distribution of microbes in AMD systems (10), we propose that this variable may serve as a general driving factor of microbial species distribution independent of scale and substrate type in the extreme AMD and associated environments. Now that the importance of pH in structuring microbial communities has been widely documented in diverse physical environments, the focus of the field should be shifted to identifying the processes underlying these consistent pH-dependent patterns. Mine tailings have potential as model systems for such analyses because of their biological and geochemical simplicity and steep gradients of pH and other environmental variables. Exploring the dynamic shifts of microbial diversity and community function associated with the progressive acidification of tailings will reveal how communities shape and are shaped by the explicitly changing environmental conditions. While our recent investigations have started to shed light on this process by using a space-for-time substitution approach (14, 15), such studies should ideally follow the same microbial community through the

key time points along the acidification process in laboratory simulation experiments. In the meantime, as our studies have revealed distinct microbial community compositions and functions associated with different acidification stages, the prevention and mitigation of AMD production at different tailing sites may require diversified strategies developed on the basis of a thorough knowledge of the ecology of the microbial communities populating this extreme environment.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (40930212 and U1201233).

We thank Alan Baker for his valuable review.

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