Spatial and Temporal Analysis of the Microbial Community in the Tailings of a Pb-Zn Mine Generating Acidic Drainage\textsuperscript{V,†}

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Received 17 October 2010/Accepted 9 June 2011

Analysis of spatial and temporal variations in the microbial community in the abandoned tailings impoundment of a Pb-Zn mine revealed distinct microbial populations associated with the different oxidation stages of the tailings. Although Acidithiobacillus ferrooxidans and Leptospirillum spp. were consistently present in the acidic tailings, acidophilic archaea, mostly Ferroplasma acidiphilum, were predominant in the oxidized zones and the oxidation front, indicating their importance to generation of acid mine drainage.

Mine tailings represent an important source of microbiologically mediated generation of acid mine drainage (AMD), a major environmental challenge worldwide. During mining and after separation of economically important minerals, typically up to 80 to 99% of the crushed ore is dumped as tailings waste (2), and rates of metal sulfide oxidation are significantly accelerated as a result of far greater mineral surface areas being exposed to oxygenated water. The microorganisms involved in the acid generation process of sulfidic mine waste have been explored mainly by cultivation-based methods (4, 10, 12, 16). While recent culture-independent microbial community analysis of water samples from AMD sites has expanded our knowledge of the biodiversity and ecology of extremely acidic environments (1, 7), surprisingly little information is currently available concerning the diversity and distribution of microorganisms in tailings dumps. To date, the molecular characterization of microbial communities at the oxidation front in a porphyry copper tailings impoundment has identified bacteria related to Leptospirillum (groups III and II) and Sulfolobus as the dominant iron/sulfur oxidizers (2), while in another study of an abandoned Pb-Zn tailings site, phyotypes affiliated with Acidithiobacillus ferrooxidans, Sulfolobus, and Acidimicrobiurn were frequently identified in the acidic tailings bacterial 16S rRNA clone library (9). In contrast, we have recently shown that an extremely acidic (pH 1.9) Pb-Zn mine tailings site harbored a remarkably simple, archaeon-dominated community, with the vast majority of the detected 16S rRNA sequences phylogenetically affiliated with the iron oxidizer Ferroplasma acidiphilum and tentative groups within the Thermoplasmata lineage so far represented by only a few environmental sequences (14). In addition to their differences in ore mineralogy, the three studied tailings sites are located in dramatically different climate zones. Consequently, geochemical and physical conditions, although not equally sufficiently characterized, vary considerably among different tailings, and these may have significant influence on the microbial community composition. The observed differences between these tailings communities also suggest a limited knowledge of the indigenous microbial populations and their relative contributions in sulfide mineral oxidation and AMD generation in situ. Furthermore, how and why the dominant species fluctuate temporally and spatially within the tailings is poorly understood.

We hypothesized that microbial community structure and function evolve with progressing oxidation of sulfidic mine tailings interacting with the changing geochemical and physical conditions. To test this, we explored the diversity and spatial distribution (depth profile and horizontal variability) along with seasonal variations of microorganisms in a massive acid-generating mine tailings site by culture-independent approaches. Samples were collected from the tailings impoundment of the Fankou Pb-Zn mine, Renhua, Guangdong Province, People’s Republic of China (Fig. 1). The region has a warm and humid subtropical climate, with an annual precipitation around 1,670 mm and an annual mean temperature of 18°C. The average daily temperature is highest in July (ca. 29°C). The tailings dump is typically stratified in July (ca. 29°C). The tailings dump is typically stratified into an oxidized zone (0 to 10 cm of depth, pH < 2.5) with a thin cemented layer on the surface, an active oxidation zone (i.e., oxidation front, 10 to 15 cm, pH ~5), and a primary zone with unaltered tailings material (below 15 cm, pH ~7) (Fig. 1). Replicate tailings cores were obtained in summer (July) 2007 from four separate locations of the tailings. This was done at each location by driving five metallic tubes side by side (in a circular pattern) into the impoundment. Each of these cores was sectioned into distinct layers, namely, the orange-colored oxidized zone (OZ), active oxidation zone (AOZ), and primary zone (PZ). The five subfractions at each location were pooled, yielding four composite tailings samples of each zone. A horizontal oxi-

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\textsuperscript{†} Supplemental material for this article may be found at http://aem.asm.org/.

\textsuperscript{†} Published ahead of print on 24 June 2011.
A series of samples was collected in the same manner from areas that roughly mirror the time of the deposition of the tailings and represent oxidized and extremely acidic regions (OZ-SUM) (pH 2.5), slightly acidic regions (SA) (pH 5.1), and neutral regions (NEU) (pH 7.2). To study the pattern of microbial populations in relation to the seasonal variations in environmental conditions, additional replicate OZ samples were collected from near the OZ-SUM sampling site in winter (OZ-WIN) and spring (OZ-SPR). For comparison of their respective microbial communities, water samples (AMD) were collected in summer 2007 from the acidic runoff ponds associated with the Fankou tailings, and tailings samples (LP) were collected from the oxidized zone of the tailings dump of the Lianping mill site, another large-scale Pb-Zn mine in Guangdong 100 km from Fankou.

Geochemical and physical parameters of tailings were measured as described previously (14). For total cell counts, tailings samples were fixed on site in a freshly prepared paraformaldehyde solution (3% in 10× phosphate-buffered saline) and subsequently processed and stained with SYBR green II following the procedures of Diaby et al. (2). Cells were counted using a Carl Zeiss Axio Observer Z1 epifluorescence microscope at magnification ×630. Total DNA was extracted from the composite tailings, and cells were harvested by centrifugation from the AMD samples using the FastDNA Spin kit for soil (Qbiogene), following the manufacturer’s instructions. For clone library construction, 16s rRNA gene fragments were obtained by PCR using the universal primers 533F and 1492r as described previously (13). Amplification was performed using the same conditions as for clone library construction, except that primer 533F was labeled with 6-carboxyfluorescein (FAM) at its 5′ end. PCR products from each field replicate were pooled and purified, digested separately usingMspI and RsaI, and resolved by multi-capillary electrophoresis with an ABI Prism 310 genetic analyzer using the GeneScan software program. The internal size standard GS1000-ROX was loaded in each lane. Abundance data were obtained from the relative peak area following sample standardization. Principal-component analysis (PCA) was performed in the SPSS 11.0 software program to determine relationships between microbial communities on the basis of the abundance and size of terminal restriction fragments (TRF).

Compared to the oxidizing and the unaltered tailings, the oxidized tailings were characterized by a brownish color, low pH and total organic carbon, depleted sulfide contents, and high electrical conductivity (EC) and redox potential values (Table 1). Moisture contents decreased with depth along the vertical profiles, and the active oxidation zone contained relatively high levels of Zn, Cd, Mn, Fe, and S (Table 1; see also Table S1 in the supplemental material). Despite the seasonal changes at Fankou (e.g., as reflected by the recorded temperature and moisture content values), pH, EC, and heavy metal...
contents did not vary significantly between tailings samples collected in summer, winter, or spring. Microbial numbers in the tailings (Table 2) ranged between \(10^9\) and \(10^{10}\) cells g\(^{-1}\) dry weight and also exhibited little seasonal variation in the oxidized zone but decreased with depth in the tailings profile (\(P < 0.05\)).

Analysis of 674 randomly selected clones from the nine gene libraries revealed 80 unique OTUs grouped within the Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Nitrospirae, and several other divisions in the domain Bacteria, as well as the Thermoplasmatales within the Archaea (Table 2; see also Fig. S1 in the supplemental material). The predicted minimum numbers of microbial species present ranged from 11 to 47 (Chao1 estimator) and 13 to 64 (ACE), and the lowest richness was observed in the oxidation front and AMD samples (Table 2). In general, the unaltered tailings materials (PZ and NEU) harbored relatively more diverse microbial communities. Archaeal phylotypes, particularly those affiliated with the iron-oxidizing genus Ferroplasma (6), dominated (51 to 64%) the gene libraries constructed with the seasonal tailings samples from the oxidized zone and were most frequently recovered (42%) from the oxidation front library (Fig. 2; see also Fig. S1). In marked contrast, no archaeal OTUs were detected in the unaltered, nonacidic tailings samples, which included diverse proteobacterial groups. Bacterial 16S rRNA sequences, mostly affiliated with the well-studied A. ferrooxidans, made up 76% of the AMD clone library, while the clone library constructed from the LP oxidized tailings was dominated by sequences affiliated with the iron oxidizer “Leptospirillum ferrodiazotrophum” (15). Importantly, sequences affiliated with the Archaea, A. ferrooxidans, and Leptospirillum spp. collectively accounted for >70% of the clone libraries of the acidic tailings and AMD samples, while these populations represented a minor fraction (<3%) of the unaltered tailings clone libraries (Fig. 2).

The relative abundances of major phylogenetic lineages identified in the environmental samples were explored using community profiles generated with MspI, which produced the largest numbers of TRFs (Fig. 3). A total of 24 distinct TRFs were identified in all T-RFLP profiles, with the average number of TRFs ranging between 5 and 13. Four of these (41, 71, 98, and 205 bp in size) were the most dominant, though their relative magnitudes varied significantly among the samples. To identify taxonomic affiliations of these TRFs, we performed in silico T-RFLP analysis of all retrieved 16S rRNA sequences and experimentally determined TRFs of representative clones of the abundant OTUs (>5%) from each clone library. While TRF-41 did not correspond to any specific phylogenetic group but was most frequently associated with the Firmicutes, Actinobacteria, and Bacteroidetes, TRF-71 was related exclusively to the archaeal lineages. This TRF dominated (on average, 42%) the tailings populations in the oxidized zone and the LP oxidized tailings samples and accounted for 28% of the T-RFLP fingerprints at the oxidation front. TRF-98 was most frequently associated with A. ferrooxidans, although this signature was shared by many other proteobacterial phylotypes re-

### Table 2. Total cell counts and summary of 16S rRNA gene libraries and diversity indices of mine tailings and associated AMD microbial communitiesa,b

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell no. (log (n) g(^{-1}) dry wt)b</th>
<th>No. of clones analyzed</th>
<th>No. of OTUs</th>
<th>Good’s coverage (%)</th>
<th>Chao1 value</th>
<th>ACE value</th>
<th>Shannon index ((H'))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OZ-SUM</td>
<td>10.6</td>
<td>83</td>
<td>20</td>
<td>96</td>
<td>25 (21, 43)</td>
<td>30 (23, 55)</td>
<td>2.40</td>
</tr>
<tr>
<td>OZ-WIN</td>
<td>10.6</td>
<td>75</td>
<td>13</td>
<td>96</td>
<td>14 (11, 34)</td>
<td>14 (12, 30)</td>
<td>1.75</td>
</tr>
<tr>
<td>OZ-SPR</td>
<td>10.8</td>
<td>72</td>
<td>19</td>
<td>97</td>
<td>25 (19, 52)</td>
<td>29 (21, 59)</td>
<td>2.37</td>
</tr>
<tr>
<td>AOZ</td>
<td>10.2</td>
<td>74</td>
<td>11</td>
<td>97</td>
<td>11 (11, 19)</td>
<td>13 (11, 22)</td>
<td>1.84</td>
</tr>
<tr>
<td>SA</td>
<td>10.8</td>
<td>69</td>
<td>16</td>
<td>91</td>
<td>21 (17, 49)</td>
<td>32 (20, 76)</td>
<td>2.23</td>
</tr>
<tr>
<td>PZ</td>
<td>9.6</td>
<td>74</td>
<td>30</td>
<td>82</td>
<td>47 (37, 76)</td>
<td>64 (45, 112)</td>
<td>3.12</td>
</tr>
<tr>
<td>NEU</td>
<td>10.7</td>
<td>74</td>
<td>21</td>
<td>91</td>
<td>24 (21, 36)</td>
<td>26 (22, 40)</td>
<td>2.81</td>
</tr>
<tr>
<td>LP</td>
<td>10.0</td>
<td>73</td>
<td>16</td>
<td>90</td>
<td>22 (18, 40)</td>
<td>31 (21, 67)</td>
<td>1.95</td>
</tr>
<tr>
<td>AMD</td>
<td>7.6</td>
<td>80</td>
<td>6</td>
<td>99</td>
<td>12 (7, 43)</td>
<td>13 (7, 48)</td>
<td>0.73</td>
</tr>
</tbody>
</table>

a OTUs were defined by a 3% difference in the nucleic acid sequence alignment. Numbers in parentheses are lower and upper 95% confidence intervals for the Chao1 and ACE estimators.

b Units are \(n\) ml\(^{-1}\) for the AMD samples.
covered from the unaltered and moderately acidic tailings samples. This TRF had the highest relative abundance in the AMD profiles but represented a minor component of the extremely acidic tailings fingerprints (except for SPR). TRF-205 represented a characteristic signature of *Leptospirillum* spp. and several deltaproteobacterial phylotypes detected in the unaltered or moderately acidic tailings. This TRF constituted a substantial fraction of the T-RFLP fingerprints of most acidic tailings and the AMD samples. PCA revealed that the microbial community profiles formed groups that generally reflect the oxidation stages of the corresponding mine tailings samples, and the tailings communities were clearly distinct from the AMD communities (Fig. 4).

*Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, the two iron-oxidizing species most commonly isolated from acidic drainage waters, are widely considered to be significant contributors to AMD generation. In the Fankou tailings, however, archaea mostly affiliated with *F. acidiphilum* were significant prokaryotic community members in the oxidized zone and oxidation front, and seasonal variation did not cause a significant shift within the indigenous microbial community. *Ferroplasma* spp. also represented the predominant populations in the extremely acidic Lianping (this study) and Lechang (14) tailings, indicating their potentially significant role in the biogeochemical processes at these adjacent Pb-Zn mine tailings sites. In supporting this, recent culture-independent studies at a number of geochemically distinct, acid-generating sites have suggested that acidophilic archaea related to the ferrous-iron-oxidizing *F. acidiphilum* are numerically significant and thus ecologically important in acidic environments in diverse geographical locations (5). These cosmopolitan archaea are well suited to influencing the biogeochemical cycling of sulfur and sulfide minerals in highly acidic mine environments since they are well adapted to conditions of very low pH and high concentrations of iron and other metals (3, 5). In spite of this, it remains unclear how readily our findings may be extended to other tailings sites with different mineralogy and that are located in other geographical regions. Indeed, amplification of 16S rRNA genes using archaeon-specific primers was not successful for DNA samples isolated from a copper tailings impoundment (2), and quantitative microbial community analysis has only occasionally, or not at all, detected archaea in three different sulfidic mine tailing dumps (8). Future large-scale molecular surveys of geographically separated and geochemically diverse tailings sites are needed to elucidate the distribution pattern and the influencing factors of microbial diversity in these important AMD generating ecosystems.

Another important observation of this work is that the microbial populations in the unaltered tailings contrast greatly with those associated with the moderately or extremely acidic
tailings, demonstrating limited occurrence of archaea, *A. ferrooxidans*, and *Leptospirillum* spp. in the stage prior to pyrite oxidation. Future investigations involving intensive sampling and analysis of tailings at different fine-scale oxidation stages are needed to elucidate how the original microbial community with diverse proteobacterial groups evolves gradually via the oxidation front to the significantly different, archaeae-dominated acidophilic community and how community structure and function correlate with biogeochemical processes of mine tailings. It is likely that the colonization of certain pioneer species and their activities in the unaltered tailings zone lead to a gradual change in geochemical conditions, facilitating the establishment of other populations that play a more important role in the subsequent oxidative pyrite dissolution and acid generation process of mine tailings. Thus, cultivation, characterization, and quantitative microbial analysis of the dominant, as yet uncultured pioneer colonizers may provide new clues for the prevention and mitigation of AMD production at this site. Nucleotide sequence accession numbers. The 16S rRNA gene sequences from this study have been deposited in the EMBL/GenBank/DDBJ databases under accession numbers FR682998 to FR683084.

Financial support was provided by the National Natural Science Foundation of China (40930212).

REFERENCES