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Bacterial Diversity in a Mine Water Treatment Plant▽†

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We investigated the microbial community in a pilot plant for treatment of acid mine water by biological ferrous iron oxidation using clone library analysis and calculated statistical parameters for further characterization. The microbial community in the plant was conspicuously dominated by a group of *Betaproteobacteria* affiliated with "*Ferribacter polymyxa*".

The operation of opencast pits requires hoisting huge amounts of low-pH groundwater and of iron and sulfate. Due to these contaminants the pumped groundwater must be treated before it is drained into water courses. Pretreatment of mine water by biological oxidation at low pH prior to the conventional chemical treatment involving neutralization with lime can decrease the iron load in the chemical treatment step considerably. By using biological oxidation of ferrous iron at a pH of about 3, iron hydroxysulfates, primarily schwertmannite $\{Fe_{16}[O_{16}(OH)_{9\text{-}12}(SO_4)_{3.5\text{-}2}]\}$, can be precipitated, removed from the treatment system, and used for industrial applications (5). Since the rate of oxidation of ferrous iron only at pH values greater than 5 increases 100-fold per pH unit and the process is very slow at pH values below 4 (1, 15), iron-oxidizing bacteria, which can increase the oxidation rate up to 5 orders of magnitude (1), must be involved in the pretreatment technology. For this biological oxidation step to be performed on a large scale, it is helpful to have some insight into the microbial community responsible for the process. Although iron-oxidizing communities in extremely acidic habitats have been analyzed in various studies, including studies of the Rio Tinto (pH 2) (12), Iron Mountain (pH 0.5 to 1) (2), and a stirred tank for bioleaching (pH 1.3 to 1.6) (14), the treatment system investigated in this study is a different habitat that has not been analyzed previously with respect to flow, higher pH, and forced aeration.

In brief (detailed methods are described in the supplemental material), the microbial communities in the pilot plant and in the groundwater pumped into the plant were investigated by using 16S rRNA gene clone library analysis. Altogether, we analyzed six clone libraries from the pilot plant and one clone library from the groundwater, each comprising 144 to 150 clones. Further characterization of the microbial communities was performed by statistical calculation of diversity and similarity indices based on amplified rRNA gene restriction analysis data, and the habitat was characterized by determining chemical parameters (Table 1).

According to previous studies, amplification with archaeal primers does not yield a PCR product (2, 7, 9). Analysis of the bacterial 16S rRNA gene revealed that representatives of the *Betaproteobacteria* conspicuously dominated the microbial diversity in the solid samples from the oxidation basin, as well as the microbial diversity in both water samples, and were also present in other samples from the plant at significant frequencies (Table 2). The majority of the sequences were affiliated with "*Ferribacter polymyxa*," which was recently isolated from an abandoned copper mine (accession number EF133508) (unpublished data). The species designated "*F. polymyxa*" in sequence databases has not been formally described, and there is considerable evolutionary distance between this species and cultivated relatives, such as the iron-oxidizing organisms *Ferritrophicum radicicola* and *Siderooxidans paludicola*, which were recently isolated from different wetland plants at pH 4 and circumneutral pH (16), and the ammonium-oxidizing organism *Nitrosospira multiformis* (Fig. 1). The average high rate of oxidation of ferrous iron in the plant, which was 35 g m^{-3} h^{-1}, along with the autotrophic growth of an isolate closely related to "*F. polymyxa*" on ferrous iron, corroborated the assumption that iron was oxidized by the *Betaproteobacteria* detected (7). The conspicuous dominance of these species in microbial communities was recently also reported in studies of other mine waters with pHs ranging from 2.4 to 3 (7, 9). Other *Betaproteobacteria* in the plant were related to *Gallionella ferruginea*, a neutrophilic autotrophic iron oxidizer. Relatives of *G. ferruginea* have often been detected in microbial mine water communities, and in some acid mine drainage investigation areas these bacteria seemed to be a dominant group (3, 4, 7). Moreover, sequences related to *Delftia acidovorans* and *Oxalobacter formigenes* were detected at very low relative abundance in all clone libraries. A further significant phylogenetic group was the *Alphaproteobacteria*, which clearly dominated the solid sample from the inflow area (see Fig. S2 in the supplemental material) and was also frequently found in the other clone libraries. The majority of the *Alphaproteobacteria* sequences were related to *Acidocella* species, and several other clones showed 16S rRNA gene similarity to *Acidiphilium*, *Sphingomonas*, *Caulobacter*, and *Caedibacter* species. The function of the heterotrophic *Alphaproteobacteria* in the iron-oxi-

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TABLE 1. Chemical parameters for the inflowing groundwater and the oxidation basin of the plant[a]

Location	pH	E_h (mV)	Temp (°C)	Fe^{2+} concn (mg/liter)	Fe^{3+} concn (mg/liter)	SO_4^{2-} concn (mg/liter)	Total inorganic carbon concn (mg/liter)	Total organic carbon concn (mg/liter)	NH_4 concn (mg/liter)	Cl^- concn (mg/liter)
Inflow	4.9	270	17	630	3	2,700	98	50	0.6	21
Oxidation basin	3	480	17	100	300	2,400	ND[b]	ND	ND	ND

[a] The oxygen concentration 20 cm below the water surface decreased from approximately 8 mg/liter around the inflow area to about 5 mg/liter in the middle of the oxidation basin.
[b] ND, not determined.

dizing community has not been completely clarified, but the presence of *Acidocella* and *Acidiphilium* species in acidic mine waters is quite common and has been reported in various studies (8, 11, 13). *Gammaproteobacteria*, including the well-known iron oxidizer *Acidithiobacillus ferrooxidans*, as well as other representatives, such as *Acinetobacter*, *Stenotrophomonas*, and *Legionella*, constituted another major sequence group. Sequences related to *A. ferrooxidans* were detected in all of the clone libraries obtained from the plant except the library from the water sample from the inflow area. For other clones that could be assigned to this phylogenetic group there were no close cultivated relatives. Representatives of the *Deltaproteobacteria* formed a considerable group only in clone libraries from water samples. Except for one clone, which was related to the sulfur-reducing *Desulfuromonas* species, all these clones were distantly related to the sulfate-reducing bacterium *Desulfobacca acetooxidans*. The *Actinobacteria* were represented in the microbial community by species related to "*Ferrimicrobium acidiphilum*," a heterotrophic iron oxidizer which has been observed in several mine waters (4, 6), by relatives of *Rhodococcus*, and by other uncultured species. Sequences affiliated with the *Firmicutes* were detected mainly in water samples (see Fig. S3 in the supplemental material). Besides a few sequences related to *Bacillus subtilis*, the majority of the clones were related to isolate SLC66, which has been described as a gram-positive iron-oxidizing acidophile (10). Within the *Nitrospira* class, relatives of *Leptospirillum ferrooxidans*, a well-studied iron oxidizer which has often been detected in extremely acidic environments (2, 6), were detected only in two clone libraries from the inflow area. It is remarkable that the *Leptospirillum*-related sequences were detected close to the point where there was continuous inflow of higher-pH groundwater and not in other areas of the plant. Representatives of *Acidobacteria* detected in clone libraries from the water samples were distantly related to the heterotrophic organism *Acidobacterium capsulatum*.

The clones obtained from the groundwater sample were partially sequenced, which yielded 600 to 900 bases. The majority of the clones did not exhibit high levels of similarity to cultivated species, and *Alphaproteobacteria* and *Betaproteobacteria*, which dominated the microbial community in the pilot plant, were detected at only very low frequencies. However, individual phylotypes discovered in the groundwater that were relatives of "*F. acidiphilum*" and *Legionella*, as well as of uncultured *Actinobacteria*, were similar to phylotypes in the clone libraries from the pilot plant. Characterization of the microbial community by statistical evaluation revealed that the Shannon indices of the clone libraries decreased from the inflowing groundwater to the oxidation basin, whereas the Shannon indices of the clone libraries from the water samples were higher

TABLE 2. Distribution of phylogenetic groups in seven clone libraries

Putative phylum or class	% of clones in clone libraries[a]						
	Solid samples				Water samples		Groundwater sample (n = 145)
	Inflow		Oxidation basin		Inflow (n = 150)	Oxidation basin (n = 150)	
	Preparation step 1 (n = 160)[b]	Preparation step 2 (n = 144)	Preparation step 1 (n = 147)	Preparation step 2 (n = 150)			
Betaproteobacteria	34	29	60	76	31	37	1
Alphaproteobacteria	51	54	32	5	17	12	1
Gammaproteobacteria	14	14	6	5	4	8	16
Deltaproteobacteria	0	1	0	0	13	13	11
Actinobacteria	0.5	1	1	10	7	7	23
Nitrospira	0.5	0	0	0	3	0	0
Verrucomicrobia	0	0	1	2	3	2	6
Chloroflexi	0	0	0	1	1	0	6
Firmicutes	0	0	0	1	14	13	3
Acidobacteria	0	0	0	0	7	8	6
Chlorobi	0	0	0	0	0	0	13
Candidate division OP11	0	0	0	0	0	0	3
Gemmatimonadetes	0	0	0	0	0	0	1
Planctomycetes	0	0	0	0	0	0	1
Unclassified	0	1	0	0	0	0	9

[a] Assignment of clones resulted from digestion with RsaI (and in some cases also with TaqI) and sequencing of at least one representative for each digestion pattern.
[b] n is the number of clones analyzed.

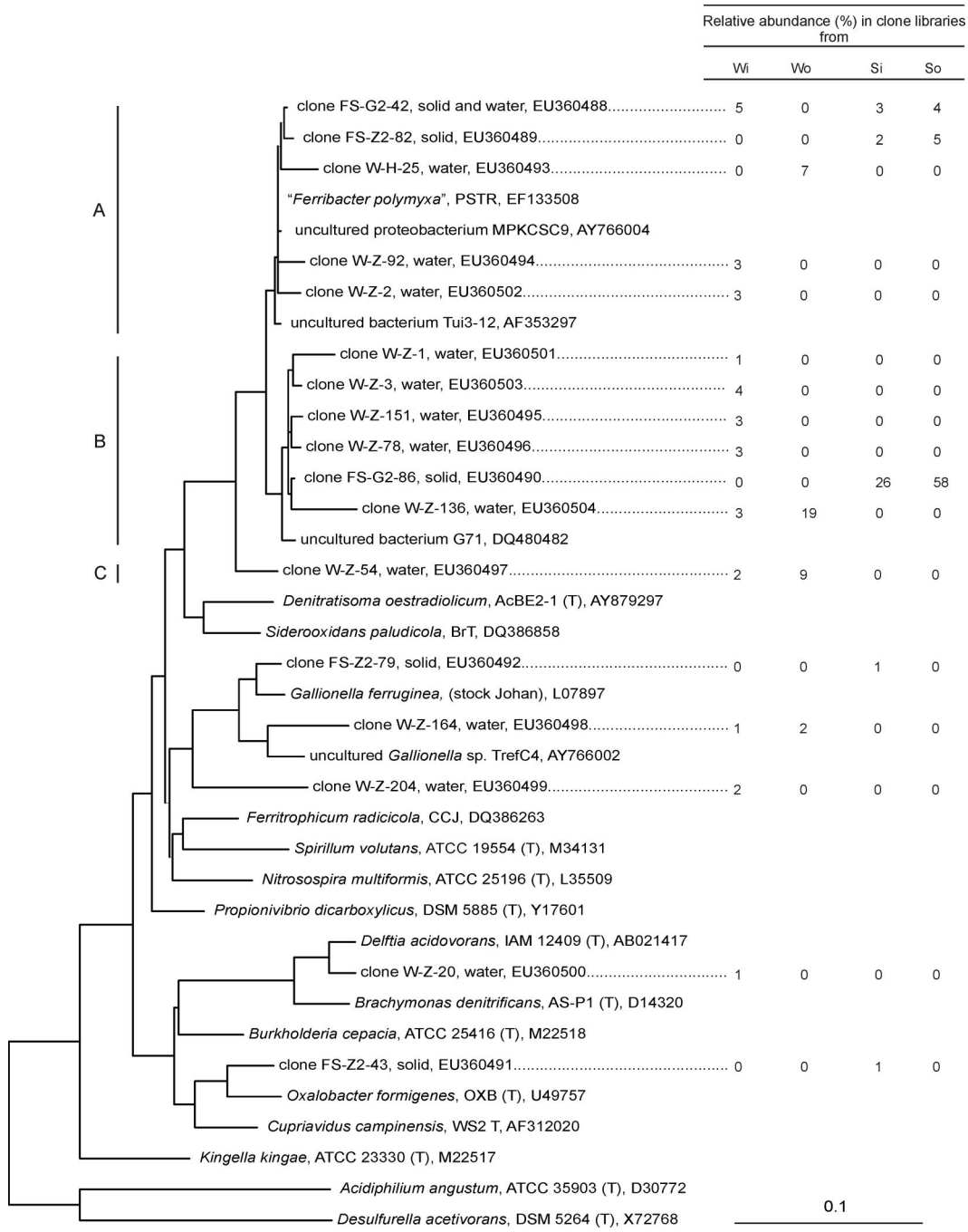

FIG. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Betaproteobacteria* from the pilot plant. The relative abundances of the operational taxonomic units were based on the frequencies of the characteristic restriction patterns of the sequences belonging to one operational taxonomic unit divided by the total number of clones in the samples as shown in Table 2. 16S rRNA gene sequences of *Acidiphilium angustum* and *Desulfurella acetivorans* were used as outgroups. Lines A, B, and C indicate single clusters in the class *Betaproteobacteria*. Wi, water sample from the inflow area; Wo, water sample from the oxidation basin; Si, solid sample from the inflow area; So, solid sample from the oxidation basin. The relative abundances from the first and second DNA preparation steps (see the supplemental material) were not divided.

than the corresponding indices of the clone libraries from the solid samples (see Table S1 in the supplemental material). Calculation of similarity indices revealed that despite the different characteristics of the groundwater and the water in the inflow area, the clone libraries from these sampling points exhibited significantly higher levels of similarity than the clone libraries from solid and water samples from the same sampling point in the pilot plant, which were very similar habitats based on the pH (see Table S2 in the supplemental material). The high level of similarity between the groundwater sample and the water sample from the inflow showed the impact of the groundwater bacteria on the composition of the unattached

population in the pilot plant. This impact was also shown by the similar frequencies of the *Deltaproteobacteria* in the clone libraries from the groundwater and from the water samples from the pilot plant (11 to 13%), whereas several sequences were affiliated with sulfate-reducing species. The aerobic, acidic conditions in the plant presumably did not provide a favorable environment for these bacteria, which were expected to just pass through the oxidation basin. However, since the frequencies of the phylogenetic groups that dominated the pilot plant were very low in the groundwater sample, the low similarity indices for solid and water samples from one sampling point did not result only from the impact of inflowing groundwater bacteria on the unattached population. In fact, the low level of similarity resulted from the conspicuously higher relative number of *Alphaproteobacteria* in the clone libraries from the solid samples (Table 2). We concluded that the microbial community in the pilot plant, which was conspicuously dominated by relatives of "*F. polymyxa*," could be divided into an attached population and an unattached population.

Further investigations of the pilot plant may show how different operating conditions, particularly the relative flow rate and retention time, as well as the pH, affect the microbial diversity in the plant and may provide evidence of the stability of this microbial community.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers EU360471 to EU360508.

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