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Microbial ferrous iron [Fe(II)] oxidation leads to the formation of iron-rich macroscopic aggregates (“iron snow”) at the redoxcline in a stratified lignite mine lake in east-central Germany. We aimed to identify the abundant Fe-oxidizing and Fe-reducing microorganisms likely to be involved in the formation and transformation of iron snow present in the redoxcline in two basins of the lake that differ in their pH values. Nucleic acid- and lipid-stained microbial cells of various morphologies detected by confocal laser scanning microscopy were homogeneously distributed in all iron snow samples. The dominant iron mineral appeared to be schwertmannite, with shorter needles in the northern than in the central basin samples. Total bacterial 16S rRNA gene copies ranged from $5.0 \times 10^8$ copies g (dry weight)$^{-1}$ in the acidic central lake basin (pH 3.3) to $4.0 \times 10^9$ copies g (dry weight)$^{-1}$ in the less acidic (pH 5.9) northern basin. Total RNA-based quantitative PCR assigned up to 61% of metabolically active microbial communities to Fe-oxidizing- and Fe-reducing-related bacteria, indicating that iron metabolism was an important metabolic strategy. Molecular identification of abundant groups suggested that iron snow surfaces were formed by chemolithotrophic iron oxidizers, such as Acidimicrobium, Ferrovum, Acidithiobacillus, Thiobacillus, and Chlorobium, in the redoxcline and were rapidly colonized by heterotrophic iron reducers, such as Acidiphilium, Albidiferax-like, and Geobacter-like groups. Metaproteomics yielded 283 different proteins from northern basin iron snow samples, and protein identification provided a glimpse into some of their in situ metabolic processes, such as primary production (CO$_2$ fixation), respiration, motility, and survival strategies.

In surface waters the oxidation of ferrous iron [Fe(II)] leads to the formation of ferric iron [Fe(III)], which then precipitates depending on surrounding geochemistry as amorphous or poorly crystalline Fe(III) oxyhydroxides or oxyhydroxysulfates, e.g., ferrihydrite, goethite, or schwertmannite (1, 2). Chemical oxidation of Fe(II) oxidation occurs rapidly under neutral pH conditions. Thus, most neutrophilic Fe-oxidizing microorganisms (FeOM) oxidize iron under microoxic or anoxic conditions (3). However, under extremely acidic pH conditions, Fe(II) is stable in the presence of O$_2$, allowing acidophilic FeOM to oxidize Fe(II) aerobically. The resulting Fe(III) is an attractive terminal electron accep- tor for Fe-reducing microorganisms (FeRM).

Opposing gradients of oxygen and Fe(II) are critical for the formation of iron minerals in streams, lakes, and marine habitats (4–6). These minerals can be the nucleus for pelagic aggregate formation either by adsorption or coprecipitation of organic matter (7) and rapid microbial colonization. Pelagic aggregates become hotspots for microbial processes and are important for the turnover and sinking flux of organic and inorganic matter to the sediment (8, 9). Reactive iron species are important for stabilizing organic matter in ocean and freshwater sediments, pointing to a tight coupling between the biogeochemical cycles of carbon and iron (10). However, we have only a limited understanding of the iron redox reactions occurring in these aggregates before they reach the sediment.

Iron-rich pelagic aggregates have been termed “iron snow” (6) to highlight unique features that are different from the more organically rich snow-like aggregates known from marine and freshwater environments (11). The initial step of iron snow formation, the oxidation of Fe(II), was shown to be a microbial process in an acidic lignite mine lake in which Fe(II) concentrations can reach 10 mM in the anoxic bottom water layer (6). Lignite mine lakes are characterized by low pH and high concentrations of Fe(II) and sulfate (12). The resulting pelagic aggregates link the redoxcline closely with the anoxic sediment, where the reduction of Fe(III) is the terminal electron-accepting process (13, 14). To further understand the microbial formation of these aggregates and their importance for lake biogeochemical processes, we collected aggregates within and below the redoxcline in two basins of the lake that differ in pH. We compared the microbial community structure of the FeOM and FeRM in the iron snow and the corresponding geochemistry and morphology of the aggregates formed under different pH conditions using a combination of quantitative PCR (qPCR), scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), and confocal laser scanning microscopy (CLSM). Furthermore, the application of metaproteomic analyses based on a carefully selected database allowed us to get a glimpse of the proteins mainly derived from active FeOM and FeRM.

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MATERIALS AND METHODS

Lake characteristics and sampling. The acidic Lake 77 located in the Lusatian mining area in east-central Germany has two basins with different stratification patterns caused by a bank that separates the bottom water of the northeastern basin from the rest of the lake (6, 15). The northern basin is meromictic, has a pH of 5.9, and shows higher Fe(II) and sulfate concentrations in the bottom part of the lake due to the inflow of less acidic, contaminated groundwater (16). The central basin undergoes mixing in autumn and spring. Sampling sites were the same as in the study of Reiche et al. (6), and their designations are abbreviated as follows: central basin redoxcline, CR; central basin anoxic bottom water, CB; northern basin redoxcline, NR; and northern basin anoxic bottom water, NB.

Sediment traps were installed within and below the redoxcline where oxygen declines to collect iron snow passively to avoid any mixing. From July 2010 to October 2011, lake water pH, temperature, conductivity, and oxygen content were measured at depth with a U-10 multiparameter water quality checker (Horiba, Japan). Concentrations of Fe(II) and Fe(III) were measured spectrophotometrically using the phenanthroline method (17). Sulfate concentration was measured turbidimetrically by the barium-chloride method (18). Additionally, dissolved CO2 and CH4 were measured at discrete depths in May 2011, as described in the supplemental material.

Geochemical characterization of iron snow. Iron snow was dried (60°C) and ground for elemental analysis. Total organic carbon (TOC), nitrogen, and sulfur were measured by an elemental analyzer (vario EL cube; Elementar, Germany) using thermal oxidation at 1,150°C. Metals, metalloids, cations, and anions were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Spectroflame, Spectro Analytical Instruments) or inductively coupled plasma mass spectrometry (ICP-MS) (XSeries II; Thermo Fisher Scientific) after aqua regia treatment.

SEM and EDX. A droplet from each of four wet iron snow samples was air-dried on an electrically conductive and adhesive Leit-Tab (Plano GmbH, Germany) and coated with carbon (thickness, approximately 15 nm) by vacuum vaporization. Samples were then studied using conventional SEM and material contrast SEM using a LEO-1450 instrument (Carl Zeiss NTS GmbH, Germany) and EDX for Fe, oxygen, and sulfur contents.

CLSM. Iron snow samples were collected from central and northern basins and were examined immediately by CLSM (TCS SP5X; Leica) using a white laser source, an upright microscope, and 63× (numerical aperture [NA], 1.2) water immersion lens. Samples were stained with SYTOX orange; SYBR green, and FM4-64 (Molecular Probes) as appropriate. Images were recorded as follows: SYBR green, excitation at 488 nm and emission at 478 to 498 nm (reflection) and 500 to 575 nm (SYBR green); SYTOX orange, excitation at 547 and 633 nm and emission at 540 to 555 nm (reflection), 560 to 620 nm (SYTOX orange), and 650 to 750 nm (autofluorescence of chlorophylls); FM4-64, excitation at 506 nm and emission at 700 to 775 nm. Optical sections were collected in the Z-direction with a step size of 1 μm. For visualization, image stacks were presented as maximum intensity, volumetric, and isosurface projections.

Total nucleic acid extraction and 16S rRNA clone library construction. Total nucleic acids were extracted from 2 to 3 ml of wet iron snow samples from July 2010 using an RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories, USA), followed by an RNA and DNA separation step using a DNA Elution Accessory Kit (MO BIO Laboratories, USA). Residual DNA was removed from RNA extracts with a Turbo DNA-free kit (Ambion, USA). RNA extract in an amount of 1 to 5 μg was used for reverse transcription to cDNA using an ArrayScript Reverse Transcriptase kit (Ambion, USA). cDNA in an amount of 10 to 50 ng was then used for standard PCR using a HotStarTaq Master Mix Kit (Qiagen, Germany) followed by 16S rRNA clone library construction and analysis (19). Reverse transcription of CR and NB RNA extracts was unsuccessful in several attempts. Additional DNA extracts were obtained for qPCR by extracting 1.5 ml of wet aliquots of iron snow samples with a PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA). All DNA concentrations were determined by a NanoDrop instrument (Thermo Fisher Scientific, USA).

Quantitative PCR. qPCRs for known FeOM- and FeRM-related groups, Acidimicrobium, Ferrovum, Albidiferax ferrureduecs (formerly affinity with Rhodofex ferrureduecs), Geoacter, and Acidiphilium, were performed using 16S rRNA gene-targeted, group-specific primers and the respective thermocycling conditions (see Table S1 in the supplemental material). Because the Acidiphilium cryptum strain JF-5 that was isolated from Lake 77 (13) might be an important FeRM, an additional assay was designed to target its cytochrome c type I gene (ApAc, YP_001325217) using Primer3Plus (20), resulting in primers AphmCytoC-254F (5'-ACA AGT TCC TGG CCA ATC C-3') and AphmCytoC-358R (5'-TCT GCA GAT AGG CGA CCA C-3'). An aliquot of 10 to 50 ng of cDNA and of DNA extracts was used as the template for amplification with a Maxima SYBR green qPCR Mastermix kit (Fermentas, Canada) on an Mx3000P real-time PCR system (Stratagene, USA). Samples were all prepared in duplicates. Standard curves were constructed using a 10-fold dilution series of plasmids containing cloned PCR products obtained from environmental samples (see Table S1 in the supplemental material). Primer specificity was verified using various online probe-match databases (21, 22), followed by PCR verification with plasmids containing cloned PCR products of each target group, various environmental DNA extracts, and DNA from closely related bacterial stains as templates. Acquisition of fluorescence data was performed at 78 to 83°C at the end of each cycle. Dissociation curve analysis was performed to check the specificity of the qPCR products. Standard curves were linear from 5 × 102 to 5 × 105 gene copies per reaction. The amplification efficiencies of all assays were between 85% and 100%, and the R2 values of the correlation curve were between 0.995 and 1.00.

Metaproteomics analysis. Total proteins were extracted from 11 to 22 g of thawed iron snow (~0.15 to 0.4 g of dry weight) using a direct soil protein extraction method (SDS-trichloroacetic acid [TCA]), as described in Chourey et al. (23) and in the supplemental material. Proteins were digested, and peptides were purified and quantified as described earlier (23, 24). All nanofluid liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) measurements were made using a hybrid linear trap quadrupole (LTQ)/Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Germany) interfaced with an Ultimate 3000 high-performance liquid chromatography (HPLC) system (Dionex, USA) and operated in data-dependent mode with Xcalibur software, version 2.1.0. Instrument setup and parameters were similar to those described earlier (24, 25) and in the supplemental material.

We created a database from sequenced microbial genomes from the Integrated Microbial Genomes server (26) (December 2010 version). The iron snow reference database included 114 full bacterial genomes, 9 bacterial plasmids, 38 full archaeal genomes, and 14 archaeal plasmids (see Table S2 in the supplemental material). Genomes and plasmids were selected based on the presence of genera detected in several Lake 77 clone libraries (6, 19). The selection also reflected the genera of more than 100 isolates from Lake 77, of which 22 were able to both oxidize and reduce iron (19). In addition, genome sequences of known acidophiles or microbes with either Fe-oxidizing or Fe-reducing capacities were included (26, 27). All data sets and the database can be accessed at http://compbio.ornl.gov/Iron_Snow/microbial_communities. Bioinformatic and statistical analyses were performed as previously described (23, 24, 28). In brief, peptide fragmentation spectra obtained for each sample were searched for database matches via SEQUEST, version 27. Output files were filtered using DTASelect for the SEQUEST searches. Detection of at least two peptides per protein sequence was set as a prerequisite for protein identification. The data sets were further evaluated using a Poisson regression model. More details can be found in the supplemental material.

Nucleotide sequence accession numbers. The 16S rRNA sequences determined in this study have been deposited in the EMBL database under accession numbers HE603991 to HE604098.
Dissolved CO$_2$ and CH$_4$ reached up to 5.7 mM and 5.2 mM, respectively, in the hypolimnion that had a pH of 5.9. Concentrations of Fe(II) and sulfate increased up to 12 mM and 20 mM, respectively, below the redoxcline. In the northern basin, oxygen was already depleted at 4.2-m depth, and concentrations of Fe(II) and sulfate increased from 1 mM to a maximum of 2.5 mM and 3.2 mM, respectively, below the redoxcline.

### RESULTS

#### Lake biogeochemistry.
When iron snow was sampled in summer within and below the redoxcline in both basins, sedimentation rates were ~1.8 times higher in the northern basin than in the central basin (Table 1). In the acidic central basin (pH 3.3), oxygen decreased to zero from 3.5- to 4.4-m depth, whereas Fe(II) increased from 1 mM to ~3 mM in the anoxic hypolimnion (see Fig. 1G, white arrow).

Nucleic acid- and lipid-stained microbial cells were detected in high abundances using CLSM and were homogeneously distributed in iron snow samples from both basins (Fig. 2). Cells with various morphologies were observed by CLSM and by SEM with or without oxalate extraction (data not shown). The bacterial community was dominated by cocci, rods, and filamentous cells. One single filamentous cell (>120 μm in length) was found interconnecting different mineral particles separated from each other (Fig. 2C). Microbial cells were often found wrapped within the Fe mineral spheres in central basin samples but not often in northern basin samples (Fig. 2D). Autofluorescence from phototrophic organisms was rare in iron snow from the central basin (~18 μm in length) (Fig. 2B) but was not found in the northern basin. SEM examinations showed unicellular diatoms (~22 μm in length), morphologically resembling *Eunotia* sp., in close association with schwertmannite spheres and decorated by coral-like iron minerals on one terminal of the cell (see Fig. S3 in the supplemental material).

#### Characterization and morphology of iron snow.
The chemical composition of iron snow showed greater differences between basins than between depths in a given basin. TOC, P, and N contents were significantly elevated in the northern basin. Total Fe was similar in the two redoxcline samples, CR and CB, but Fe was lowest in northern bottom water. In contrast, S contents were highest in NB samples. Cd, Co, Cu, Ni, Pb, and U concentrations were all below 0.12 μM g (dry weight)$^{-1}$ (Table 1). The unique hedgehog-like morphology indicative for schwertmannite was observed in all four iron snow samples by SEM (Fig. 1B, D, F, and H). The presence of schwertmannite as a dominant mineral was also indicated by the mass ratios of Fe, S, and O detected using EDX (see Table S3 in the supplemental material). However, hedgehog-like spheres were smaller with shorter needles in the northern basin than in the central basin samples, and planar structures were also observed in northern samples (Fig. 1H). Back-scattered electron micrographs (Fig. 1A, C, E, and G) confirmed high iron contents in schwertmannite and revealed the sharp and narrow pin structures measuring about 30.3 nm in diameter (Fig. 1G, white arrow).

### TABLE 1 Characterization of lake water and iron snow of Lake 77 obtained in July 2010 from sediment traps exposed at the redoxcline and in the anoxic bottom water of the central basin and northern basin

<table>
<thead>
<tr>
<th>Water and iron snow characteristic</th>
<th>Value for the parameter at the indicated site$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling depth (m)</td>
<td>CR</td>
</tr>
<tr>
<td>pH</td>
<td>3.3</td>
</tr>
<tr>
<td>Sedimentation rate (g m$^{-2}$ day$^{-1}$)</td>
<td>2.6</td>
</tr>
<tr>
<td>Fe (mM g$^{-1}$ wet)</td>
<td>6.38</td>
</tr>
<tr>
<td>C (mM g$^{-1}$)</td>
<td>1.65</td>
</tr>
<tr>
<td>S (mM g$^{-1}$)</td>
<td>1.87</td>
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<tr>
<td>Al (mM g$^{-1}$)</td>
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<tr>
<td>Ca (mM g$^{-1}$)</td>
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</tr>
<tr>
<td>Mg (mM g$^{-1}$)</td>
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</tr>
<tr>
<td>Mn (μM g$^{-1}$)</td>
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</tr>
<tr>
<td>P (μM g$^{-1}$)</td>
<td>5.97</td>
</tr>
<tr>
<td>Fe (μM g$^{-1}$)</td>
<td>2.75</td>
</tr>
<tr>
<td>Ni (μM g$^{-1}$)</td>
<td>0.76</td>
</tr>
<tr>
<td>As (μM g$^{-1}$)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

$^a$Values of element analyses were the average of triplicate measurements. The analytical error for carbon measurement was less than 0.5% of the average value, and for sulfur and nitrogen it was less than 5%. The analytical errors for other elements were less than 2%.

$^b$Concentration below the detection limit of phosphorus (3.87 μM g of dry weight$^{-1}$).

$^c$Measured by ICP-OES.

$^d$Measured by ICP-MS.

$^e$All weights are dry weights.

$^f$CR, central redoxcline; CB, central bottom; NR, northern redoxcline; NB, northern bottom.
library, *Betaproteobacteria* was the dominant group (36.2%), in which most of the clones were related to FeRM *Albidiferax* (32.6%). *Chlorobium ferrooxidans*-related clones were observed only in the NR cDNA library, while those related to *Acidiphilium* spp. and neutrophilic FeRM *Geobacter* spp. were retrieved from both sites (Table 2).

The relative proportion of five groups known as FeOM or FeRM, *Acidimicrobium*, *Ferrovum*, *Albidiferax*, *Geobacter*, and *Acidiphilium*, were estimated using qPCR and genomic DNA as templates. Total bacterial 16S rRNA gene copies reached up to $3.97 \times 10^{10}$ (dry weight)$^{-1}$ in NB iron snow; however, copy numbers were ~80 times lower in CR iron snow (Fig. 3). Differences between CR and CB or between NR and NB were not significant. The 16S rRNA gene copy numbers were 1.37 $\times 10^{12}$ (dry weight)$^{-1}$ in CB cDNA and 9.31 $\times 10^{11}$ (dry weight)$^{-1}$ in NR cDNA samples. The *Acidimicrobium*-related group showed high relative abundance in the central basin samples, comprising 24.8% of the total 16S rRNA gene copies in CB DNA, 21.6% in CR DNA, and 19.7% in CB cDNA. In all northern basin samples, the *Acidimicrobium*-related group comprised less than 7.5% of the total 16S rRNA gene copies. The *Ferrovum*-related group was found to be abundant in the CB community, as revealed by the high ratio of the 16S rRNA gene copies in both DNA-based (34.7%) and RNA-based (19.2%) samples. In contrast, the *Albidiferax*-like group was detected at extremely low abundance in central basin samples but comprised 15.0% of total 16S rRNA gene copy numbers in the north basin samples and 33.5% of active bacteria in the NR cDNA. Similarly, *Geobacter*-like sequences were found at low relative abundance (<0.6%) in the central basin samples (Table 2) but were >15.8% of active bacteria in the NR cDNA. The *Acidiphilium*-related 16S rRNA gene copy was abundant in CB DNA (20.4%) and CB cDNA (10.1%) but not in CR DNA (0.4%) or in the northern basin (8.7% in NR DNA, 6.2% in NB DNA, and 3.0% in NR cDNA). Wide-ranging detection of the ApcA gene of *A. cryptum* JF-5 showed that this organism was ubiquitous in all samples, reaching 83.4% of total *Acidiphilium* in the CR DNA sample (Fig. 3), which is significant given that there are two 16S rRNA gene copies in the *A. cryptum* JF-5 genome but only one copy of the ApcA gene. The difference in the ApcA gene and the 16S rRNA gene abundances in RNA samples can be explained by the different RNA targets of the primer sets. The ApcA gene-specific primer set targets mRNA-derived templates, whereas the *Acidiphilium*-specific primer set targets the much more abundant rRNA-derived templates.

**Metaproteomics.** A total of 283 different proteins were retrieved from iron snow collected in the northern basin, including 140 in NR and 66 in NB (see Table S7 in the supplemental material). The molecular masses ranged from 7.2 to 156.3 kDa. The proteins could be assigned to 43 genera. The most represented groups were *Chlorobium* (19.1%, 54 proteins), *Acidiphilium* and *Burkholderia* (8.1%, 23 proteins each), *Acidovorax* (7.8%, 22 proteins), and *Albidiferax* (6%, 17 proteins). Other Fe cycling-related groups, *Geobacter*, *Acidithiobacillus*, *Pelobacter*, *Leptothrix*, and *Acidimicrobium*, were also detected. Various functional categories were identified, such as the heat shock hsp60 and chaperonin GroEL proteins, ATP synthases, translation elongation factor proteins, Gram-negative-type outer membrane porins, ribosomal proteins, RNA polymerases, and gas vesicle-related proteins. In addition, flagellin and OmpA/MotB domain proteins were detected, mainly from *Acidovorax*, *Acidiphilium*, *Albidiferax*, and *Diaphorobacter*-related groups. Proteins representing carboxysome shell/microcompartment were found in NR, possibly de-
rived from Acidithiobacillus species (protein accession numbers YP_002426105, YP_002426106, ZP_05293097 to ZP_05293099, YP_002219821, and YP_002219822), Thiobacillus (YP_316402 to YP_316404), and Acidimicrobium (YP_003108766 to YP_003108768) (see Table S7 in the supplemental material). The periplasmic protein cytochrome c class I was also retrieved in NR samples. Another cytochrome c oxidase (YP_002361568) from Methylocella was detected in CB samples. Bacteriochlorophyll (BChl) c-binding proteins from Chlorobium were found in high abundance (ZP_01386130 and YP_002019351). In total only 70 proteins were detected from central basin iron snow (25 proteins only from CR and 27 only from CB samples), which might be due to the low biomass or extraction limitations from iron minerals at low pH (see Table S8 in the supplemental material). The periplasmic protein cytochrome c class I was also retrieved in NR samples. Another cytochrome c oxidase (YP_002361568) from Methyllocella was detected in CB samples. Bacteriochlorophyll (BChl) c-binding proteins from Chlorobium were found in high abundance (ZP_01386130 and YP_002019351).

In total only 70 proteins were detected from central basin iron snow (25 proteins only from CR and 27 only from CB samples), which might be due to the low biomass or extraction limitations from iron minerals at low pH (see Table S8 in the supplemental material). Detection of proteins from Acidiphilium, Chlorobium, and Geobacter was in accordance with results acquired from 16S cDNA analysis of the same iron snow samples. Several gas vesicle-related proteins were attributed to those from Chlorobium and the outer membrane protein OmpA/MotB and the periplasmic protein cytochrome c class I from Acidiphilium spp. Other proteins were annotated as ATP synthase, stress response proteins, and chaperonin GroEL proteins (see Table S8 in the supplemental material).

**DISCUSSION**

**Biogeochemistry of iron snow.** Redoxclines present in the central and northern basins of Lake 77 favored the activity of aerobic FeOM by providing strong vertical gradients of oxygen and Fe(II) within a centimeter range. The 2-fold-higher sedimentation rates observed in the northern basin were likely caused by Fe(II) concentration rates that were 4-fold higher in the northern than in the central hypolimnion because of the Fe(II)-rich groundwater inflow (15). Previous experiments showed that the oxidation of Fe(II) is microbially mediated in water samples obtained from the epilimnion and the redoxcline, with rates varying from 0.2 to 0.7 mmol Fe(II) liter$^{-1}$ day$^{-1}$ under acidic conditions (6). Nonetheless, chemical oxidation could contribute to Fe(II) oxidation in the deeper, microoxic part of the redoxcline of the northern basin where the pH increases to 5.9. Despite pH differences, all iron-rich precipitates were dominated by schwertmannite, with shorter
needles of hedgehog-like spheres in the samples collected in the northern basin (Fig. 1). The higher TOC content of iron snow collected in the northern basin than that of the central basin might reflect the higher numbers of microorganisms colonizing these aggregates (Fig. 2) or the larger amount of organic carbon adsorbed to or coprecipitated with these reactive iron species (29–31) where there is more dissolved organic carbon (DOC) during mineral formation. DOC concentration was 6-fold higher in the northern basin than in the central basin (6). The tight association between iron minerals and organic matter in iron snow could play a role in the long-term sequestration of carbon in the lake, similar to that in marine sediments (32). The high iron snow sedimentation rates in this lake provided a rapid removal mechanism of not only iron and organic carbon but also living microorganisms from the water column to the sediment.

For visualizing the microbial cells in the iron snow, we tested several methods. While SEM was ideal to visualize mineral morphologies (Fig. 1) and to detect larger unicellular eukaryotic organisms, like diatoms, it was not suited for detecting single microbical cells. These cells were either too small or masked by the large amount of iron minerals present. CLSM was ideal to visualize microorganisms in such an iron mineral-rich environment when we used dyes for total nucleic acid or lipid staining (Fig. 2). Then CLSM allowed the detection of cells with various cell morphologies and even of filamentous cells bridging different parts of the aggregates. Three-dimensional (3D) reconstruction of the optical sections showed a clear association of microbial cells with minerals.

Fe-related bacteria dominate iron snow. Quantitative PCR results suggested that at least 52.9% and 60.6% of total active bacterial 16S rRNA gene copies of the iron snow could be assigned to FeOM and FeRM in the central and northern basins, respectively. Similarly, 25.3% and 38.2%, respectively, of sequences detected in the 16S rRNA clone libraries of the central and northern basins were 97% related to cultured FeOM and FeRM representatives (Table 2), suggesting the dominance of activities related to the cycling of Fe. The two methods yielded comparable results showing, for instance, that Acidimicrobium and Ferrovum were the main FeOM in the CB but not in the NR, while Albidiferax was the main FeRM in the NR. The general consistency of these results confirmed the reliability of 16S rRNA gene library techniques for

<table>
<thead>
<tr>
<th>Fe-related function and organism</th>
<th>Relative abundance by detection method and location (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>16S rRNA clone libraries</td>
</tr>
<tr>
<td></td>
<td>CB</td>
</tr>
<tr>
<td>Iron oxidizers</td>
<td></td>
</tr>
<tr>
<td>Acidimicrobium</td>
<td>13.0</td>
</tr>
<tr>
<td>Chlorobium</td>
<td>nd</td>
</tr>
<tr>
<td>Ferrovum</td>
<td>3.1</td>
</tr>
<tr>
<td>Iron reducers</td>
<td></td>
</tr>
<tr>
<td>Acidiphilum</td>
<td>5.3</td>
</tr>
<tr>
<td>Albidiferax</td>
<td>nd</td>
</tr>
<tr>
<td>Geobacter</td>
<td>0.8</td>
</tr>
<tr>
<td>Acidobacteria spp.</td>
<td>3.1</td>
</tr>
<tr>
<td>Total</td>
<td>25.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>The percentage of sequences in cDNA libraries which were >97% identical to known Fe cycling-related bacteria was used for calculations. CB, central basin bottom water column; NR, northern basin redoxcline; nd, not detected; ND, not determined.

![FIG 3](http://aem.asm.org/) Quantitative PCR analyses of cDNA and DNA extracts from iron snow of the central basin (central redoxcline, CR; central bottom CB) and northern basin (northern redoxcline, NR; northern bottom, NB). Total bacterial 16S rRNA genes and Fe-related bacterial phylogenetic groups were detected. Fe(III) reduction-related cytochrome c class I (ApcA) gene copies of Acidiphilium cryptum JF-5 were also determined. The results of each assay were the mean values of two analytical duplicate reactions.
determining dominant phylogenetic groups. Since nearly full-length sequences of the genes were retrieved, reliable phylogenetic analyses were possible. The microbial community structure of iron snow collected from anoxic bottom water shared great similarities with the corresponding sediment surface (6) from which a broad range of microorganisms has been isolated or detected (species related to Acidithiobacillus, Ferrovum, Ferrimicrobium, Acidimicrobium, Alicyclobacillus, Acidocella, Acidithiobacillus, Thiobacillus, Leptospirillum, Dyella, Geobacter, and Sulfolobus) (13, 19, 33, 34). Many of the FeOM are capable of reducing schwertmannite even under microoxic conditions (19, 35), suggesting that microbial iron cycling might occur in the redoxcline.

While the 16S rRNA gene-based analyses provided reasonable information on microbial community abundance, they were not able to elucidate the in situ metabolic functions of the dominating groups. For both FeOM and FeRM, there is no common functional gene marker related to either Fe oxidation or reduction. Fe respiration pathways vary between neutrophilic and acidophiles and between species. The best known acidophilic model FeOM, Acidithiobacillus thioxidans, comprises at least four distinct species that use at least two different pathways to oxidize ferrous iron (3). And until now there has been no comprehensive study on the Fe-reducing pathway of acidophiles (36). Thus, metaproteomic analysis provided us with a chance to unveil some metabolic functions of FeOM and FeRM.

We used a reference database focusing on FeOM/FeRM and acidophiles. All available databases can be searched for the MS data sets; however, this is likely to boost the chances of peptides being matched to multiple proteins from different organisms (including proteins with low sequence identity), leading to false protein identifications and erroneous conclusions. Use of a reference database has been reported in many studies in which the actual genome or metagenome for the sample was unavailable (37, 38). The reference database was chosen on the basis of some functional similarities between organisms whose genomes were chosen for protein matching. On the other hand, the lack of some genome-sequenced relatives of certain dominating species, such as Ferrovum, had prevented a sophisticated interpretation of its ecological role in iron snow.

Nonetheless, part of the mystery of in situ activity and metabolism of both FeOM and FeRM inhabiting these aggregates was unveiled. Many flagellin domain proteins from FeOM and FeRM, such as Acidovorax, Acidithiobacillus, and Albidiferax, were detected in different iron snow samples, suggesting that flagellar motility might help organisms reach surfaces and switch from a pelagic to an attached lifestyle. These complex organelles play an important role in adhesion to substrates and biofilm formation (39). Large amounts of shock- or protein refolding-related chaperones, such as DnaK or GroEL, were also revealed in iron snow samples. Such proteins were found in other acidic environments (40–42) and in Ferroplasma acidarmanus cultured aerobically (43), suggesting that low-pH conditions might be a key challenge for cell survival due to its potential damage to DNA and proteins.

Primary production as an important metabolic strategy for Fe(II) oxidizers. A number of autotrophic acidophiles use the Calvin-Benson-Bassham cycle to obtain their cellular carbon (44). Its key enzyme is ribulose biphosphate carboxylase/oxygenase (RuBisCO) that is detected in Acidithiobacillus thiooxidans (45), Acidithiobacillus ferrooxidans (46, 47), and Acidimicrobium ferrooxidans (44). While RuBisCO may be present in the cytoplasm, it is often found packaged in microcompartments called carboxysomes (48, 49). Clusters of carboxysome genes in Acidithiobacillus and Thiobacillus denitrificans are closely associated to RuBisCO-related genes, mostly in identical operons (48, 50, 51). Because RuBisCO- or carboxysome-related genes are putatively identified in the FeOM Acidithiobacillus, Acidimicrobium, and Thiobacillus species based on bioinformatic analysis (44, 50–52), the detection of carboxysome shell proteins related to these three species in northern basin iron snow indicated their function in CO2 fixation. Thus, these FeOM are probably acting as primary producers in this lake. Additionally, we observed, using MicrobesOnline (53), that genes coding for Acidimicrobium ferrooxidans microcompartment proteins (Afer_0124 to Afer_0126) are located downstream of the two RuBisCO subunit genes (Afer_0119 and Afer_0120), probably in a same operon. This suggests that they may be concurrently expressed and packed as intact carboxysomes for CO2 fixation.

Another primary producer, the green sulfur bacteria Chlorobium-like group, was recognized by 16S rRNA cloning, and a number of different proteins were assigned to this group, including bacteriochlorophyll (BChl) c-binding proteins of the FeOM C. ferrooxidans (54). BChl serves as a light-harvesting and energy-transforming chromophore in photosynthetic microorganisms (55). Gas vesicle proteins from Chlorobium-like groups were found in both central and northern basin samples. Gas vesicles of aquatic bacteria are small, hollow protein structures that provide buoyancy, allowing positioning at a favorable depth for growth (56). Therefore, the CO2-fixing Chlorobium-like species attached to or captured within the iron snow may utilize this system in the redoxcline, where there is enough light for photosynthesis and sufficient Fe(II) for oxidation.

Acidithiobacillus as an active acidophilic Fe(III) reducer. The acidophilic FeRM Acidithiobacillus-related group was detected in all iron snow samples, suggesting their ubiquitous distribution. Acidithiobacillus-related proteins were one of the most abundant groups. Although the Fe(III) reduction mechanism in acidophiles has not yet been revealed completely, the membrane-associated proteins related to the electron transport chain are of high interest. Five proteins detected in iron snow may be related to the electron transfer chain of Acidithiobacillus: OmpA/MotB domain proteins (YP_001233320, YP_001233321, and YP_001233507), TonB-dependent receptor (YP_001220344), and ApcA (YP_001235217). Genes encoding two OmpA/MotB domain proteins (Acry_0173 for YP_001233320 and Acry_0174 for YP_001233321) were located downstream of a gene coding an NADH-ubiquinone oxidoreductase subunit (Acry_0170), a component participating in the electron transport chain during Fe oxidation and reduction in Ferroplasma (43). Recently, one TonB-dependent receptor homologue from Shewanella oneidensis (SO2907) was shown to be involved in the dissimilatory reduction of chelated Fe(III) species but not of solid iron oxides (57). However, more experimental evidence is required to explain the roles of OmpA/MotB domain and TonB-dependent receptor proteins in acidophiles, which have the advantage of also accessing soluble Fe(III) at a pH below 3. The ApcA gene of A. cryptum JF-5 was quantified in all iron snow DNA and cDNA samples, and protein ApcA was observed in both basins, indicating its active role. ApcA is an essential electron transfer protein in respiratory chains involved in the reduction of electron acceptors like Fe(III) or hexavalent chromium in the neutrophile S. oneidensis and Geobacter sulfurreducens (58–60). How-
ever, very little is known about such enzymology in acidophilic FeRM although purified and reduced ApoA from *A. cryptum* IF-5 can reduce chromate at low pH (61). The results of our polyphasic approach suggest that *Acidiphilum* was active in the iron snow formed in both basins and that it may be involved in the reductive dissolution of schwertmannite under oxic to suboxic conditions in the redoxcline and under anoxic conditions during sedimentation of iron snow.

The dominance of neutrophile-like Fe(III) reducers in the northern basin. Although *Albidiferax ferrireduces* and *Geobacter* are neutrophilic FeRM, their relatives were detected as the two most abundant groups in the northern basin, suggesting their tolerance of slightly acidic conditions. *A. ferrireduces* is a metabolically versatile microorganism that likely plays an important role in subsurface carbon and metal cycles (62, 63). It has a pH optimum of 7.0 (62); therefore, it was not surprising to find much higher abundances of the *Albidiferax* genus in the northern basin than in the central basin using cloning and qPCR approaches. Although some of these members are capable of autotrophic growth, *A. ferrireduces* has only an incomplete pathway associated with CO2 fixation in its genome (63). We detected mostly chaperonin GroEL and nucleic acid/protein synthesis-related proteins in iron snow samples. Interestingly, retrieved flagellin-like proteins (Rfer_0630 and Rfer_0631) indicated motility, which agrees with previous findings (62, 63). Malate dehydrogenase (Rfer_1803), a carbon metabolism protein involved in the citric acid cycle, was also detected.

The *Geobacter*-like group was the second most abundant bacterial group in the northern basin. Proteins identified from the *Geobacter*-like group were referred mostly to ATP synthase, shock-related proteins, and various chaperones. However, since no protein potentially involved in an electron transport chain was found, it remains unclear if these organisms actively participate in the reduction of Fe(III) in this lake.

Formation and transformation of iron snow. The dominance of Fe-related microorganisms of the microbial community inhabiting iron snow appeared to be another unique feature of this freshwater snow-like aggregate. Our data suggested that mainly acidophilic autotrophic FeOM, such as *Ferrovum*, *Acidimicrobium Acidithiobacillus*, and *Thiobacillus*, and a small population of neutrophilic *Sideroxydans* sp. were likely responsible for the initial step of Fe(II) oxidation in the redoxcline, where Fe(II) meets oxygen. "Ferrovum myxofaciens," known for its copious quantities of excreted exopolysaccharides (64, 65), might serve as a nucleation site for the precipitation of schwertmannite as a dominant Fe mineral. The lack of encrustation of most acidophilic FeOM is advantageous since the cells are still able to contact ferrous iron and to proceed with oxidation (65). Nonetheless, some microorganisms may become enclosed by schwertmannite minerals. Phototrophic *Chlorobium* organisms may also serve as primary producers for the next trophic level, but chemolithoautotrophy was likely advantageous in this lake, where light penetration is limited by large amounts of iron aggregates in the water column.

Autotrophically fixed carbon may feed heterotrophs, including FeRM like *Acidovorax*, *Acidiphilum*, and *Albidiferax*, that are favored by their flagellar motility to reach solid-phase Fe(III). A two-way transfer of carbon in a mixed culture of *Acidithiobacillus ferrooxidans* and *Acidiphilum cryptum* was identified (66). The organic substances, mostly peptides, secreted by the chemosynthetic bacteria are utilized by the heterotroph leading to CO2 formation. The pincushion-like structure of schwertmannite provides a large specific surface area highly suited for adsorption of DOC or organic matter excreted by phototrophs or other iron snow inhabitants, making the iron snow an attractive ecological niche on its descent through the redoxcline to the sediment. However, the rapid sinking velocity of iron snow compared to that of the more organically rich lake snow (6) may not allow for all the complex aspects of aggregate formation and transformation. The short residence time of the iron snow in the lake will limit metabolic interaction, organic carbon decomposition processes, and the reductive dissolution of schwertmannite by the abundant heterotrophic FeRM, e.g., *Albidiferax*, *Geobacter*, and *Acidiphilum*. The majority of solid-phase Fe(III) will reach the sediment within 1 day in this shallow lake. Precipitated iron snow will fuel Fe(III) reduction as the dominating electron-accepting process for the oxidation of organic matter (14). The reduced Fe(II) will accumulate in the hypolimnion until it becomes mixed with oxygenated surface water in autumn, closing the iron cycle in the central basin. In the separated northern basin, the sedimentation velocity of iron snow is reduced due to the higher salinity in the permanently anoxic bottom water, where reoxidation of Fe(II) is limited to diffusion through the shallow redoxcline.

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