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Functional Gene Analysis of Freshwater Iron-Rich Flocs at Circumneutral pH and Isolation of a Stalk-Forming Microaerophilic Iron-Oxidizing Bacterium

Shingo Kato,[a] Clara Chan,[b] Takashi Itoh,[a] Moriya Ohkuma[a]

Japan Collection of Microorganisms, RIKEN BioResource Center, Tsukuba, Ibaraki, Japan[a]; Department of Geological Sciences, University of Delaware, Newark, Delaware, USA[b]

Iron-rich flocs often occur where anoxic water containing ferrous iron encounters oxygenated environments. Culture-independent molecular analyses have revealed the presence of 16S rRNA gene sequences related to diverse bacteria, including autotrophic iron oxidizers and methanotrophs in iron-rich flocs; however, the metabolic functions of the microbial communities remain poorly characterized, particularly regarding carbon cycling. In the present study, we cultivated iron-oxidizing bacteria (FeOB) and performed clone library analyses of functional genes related to carbon fixation and methane oxidization (*cbbM* and *pmoA*, respectively), in addition to bacterial and archaeal 16S rRNA genes, in freshwater iron-rich flocs at groundwater discharge points. The analyses of 16S rRNA, *cbbM*, and *pmoA* genes strongly suggested the coexistence of autotrophic iron oxidizers and methanotrophs in the flocs. Furthermore, a novel stalk-forming microaerophilic FeOB, strain OYT1, was isolated and characterized phylogenetically and physiologically. The 16S rRNA and *cbbM* gene sequences of OYT1 are related to those of other microaerophilic FeOB in the family *Gallionellaceae*, of the *Betaproteobacteria*, isolated from freshwater environments at circumneutral pH. The physiological characteristics of OYT1 will help elucidate the ecophysiology of microaerophilic FeOB. Overall, this study demonstrates functional roles of microorganisms in iron flocs, suggesting several possible linkages between Fe and C cycling.

Iron is the fourth most abundant element by weight in the Earth's crust. As a redox-active element, iron is biologically and chemically cycled between reduced and oxidized forms, mainly Fe(II) and Fe(III), respectively. In addition to being an important trace nutrient, iron can be a source of energy for Fe(II)-oxidizing microbes or respired by Fe(III)-reducing microbes. Iron tends to form a variety of minerals; thus, iron redox cycling affects the cycling of other elements by the precipitation of iron sulfides, phosphates, and carbonates and by sorption onto and coprecipitation with iron oxides (1). In the case of biological metabolism, carbon fixation, assimilation, or oxidation can link Fe and C cycles. Although the diversity of Fe-rich microbial communities has previously been characterized, we still know little about how individual microorganisms cycle Fe [particularly Fe(II) oxidizers] and the various roles of associated organisms in iron-rich ecosystems.

Under circumneutral pH and air-saturated conditions, Fe(II) is rapidly oxidized and precipitated as Fe(III) (oxyhydr)oxides. However, under microaerobic conditions, the abiotic oxidation rate of Fe(II) is slow, so iron-oxidizing bacteria (FeOB) can use Fe(II) as an electron donor for growth. To date, several microaerophilic FeOB that can grow chemolithoautotrophically under circumneutral pH have been reported, including isolates of *Gallionella*, *Sideroxydans*, and *Mariprofundus* (2, 3). Our knowledge of FeOB physiology is still limited because of previous difficulties in culturing microaerophilic FeOB, associated with their requirement of low oxygen concentrations (<50 μM) (4). Cultivation methods using opposing Fe(II) and O_2 gradients have largely overcome this problem (5–7), leading to the isolation of FeOB, such as *Gallionella ferruginea* (8), *Mariprofundus ferrooxydans* (9), and strain R-1 (10), which produce twisted iron oxyhydroxide stalks (10–13). The minerals resulting from FeOB activity, bacteriogenic (or biogenic) iron oxides (BIOS), are high-surface-area nanoparticles that accumulate metals and nutrients and, therefore, are significant in elemental cycling (14, 15).

Iron-rich flocs containing BIOS are often found at circumneutral pH groundwater seeps and marine hydrothermal vents, which provide suitable habitats for microaerophilic FeOB. In fact, several microaerophilic FeOB have been isolated from such iron-rich environments (6, 10, 16, 17). PCR-based, culture-independent methods targeting 16S rRNA genes suggest that more diverse FeOB may exist in these environments, as well as iron-reducing bacteria, sulfide-oxidizing bacteria, and methanotrophs (18–23) that potentially cycle iron, sulfur, and carbon in the flocs (24). However, the phylogenies of the 16S rRNA genes are not direct evidence for the physiology of the microorganisms, so the metabolic functions of iron-rich floc microorganisms are poorly understood. Here, we report the characteristics of the microbial community in freshwater iron-rich flocs analyzed by PCR-based, culture-independent methods targeting the *pmoA* and *cbbM* genes, which are related to methane oxidization and carbon fixation, respectively, in addition to bacterial and archaeal 16S rRNA genes. Furthermore, we describe a new isolate from the flocs, a novel stalk-forming, microaerophilic, neutrophilic, chemolithoautotrophic FeOB. The phylogenetic and physiological char-

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Address correspondence to Shingo Kato, skato@jcm.riken.jp.
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acteristics of the isolate, designated strain OYT1, provide insights into the ecophysiology of microaerophilic FeOB.

MATERIALS AND METHODS

Site description and sampling. Orange/ochre-colored flocs (see Fig. S1 in the supplemental material) up to 20 cm thick were observed in a groundwater seep in Ohyato Park, Tokyo, Japan (35°38′22.76″N, 139°27′29.24″E). The surface portion (top ~2 cm) of the flocs was collected into sterile plastic tubes using a sterile spatula in July 2010. Samples for cultivation and microscopy were immediately transferred to and stored in a cooler box with refrigerants, and samples for DNA analysis were frozen and stored with dry ice. These samples were transported to our laboratory within a day and stored at 4°C for microscopy and cultivation and at −80°C for DNA analysis.

Chemical analysis. The temperature and pH of the discharged groundwater were determined by a pH meter with a thermometer (9621-10D; Horiba, Kyoto, Japan). Dissolved oxygen (DO) concentrations were measured with a DO meter (9520-10D; Horiba, Kyoto, Japan) calibrated with air as a standard. The groundwater sample was filtered with a 0.2-μm-pore-size cellulose-acetate membrane filter in the field and stored at 4°C in the field until chemical analysis. The ferrous iron concentration was determined by ferrozine assay (25). Chloride, sulfate, and nitrate concentrations were determined by ion chromatography (ICS-2100; Dionex, Osaka, Japan).

Isolation and characterization. To isolate FeOB, we used a gradient culture method (5) with minor modifications. A portion of the collected flocs was inoculated into agarose-stabilized gradient tubes containing modified Wolfe's mineral medium (MWMM) with an FeS plug. This medium was buffered with MES (morpholineethanesulfonic acid; 10 mM) to pH 6.2. A gas mixture of N_2-CO_2-O_2 (79:20:1, 0.1 MPa) was used in the headspace. The tubes were incubated at 25°C in the dark for 1 week. Enrichments were initially transferred 3 times, with cultivation as described above. Cells were purified using the serial dilution-to-extinction method, with 5 transfers. We used 96-well plates for the serial dilution-to-extinction method (see Fig. S2 in the supplemental material). Each well contained 20 μl of FeS plug on the bottom and 180 μl of agarose-stabilized MWMM on the plug. After inoculation (5 μl), the plates were incubated in polycarbonate boxes (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) with the gas mixture of N_2-CO_2-O_2 (79:20:1). In the end, the culture showing growth at the highest dilution was designated strain OYT1 (deposited in JCM as JCM 18545 and in DSMZ as DSM 26810). Purity was confirmed by microscopic observation, streaking cell suspensions on R2A agar plates to check for the presence of contaminating heterotrophs, and repeated partial sequencing of the 16S rRNA genes amplified by PCR using the bacterial and archaeal universal primer sets Bac27F (26) or Arc9F (27) and Uni1406R (18), as described below. Cells were stained with SYBR green I for visualization by fluorescence microscopy. Growth experiments were conducted in quintuplicate; growth was determined by direct cell counts as described previously (6).

Strain OYT1 was tested for growth on organic and inorganic substrates, including ferrous chloride (5 mM), sodium thiosulfate (5 mM), sodium sulfide (5 mM), sodium nitrite (10 mM), manganese chloride (10 mM), sodium pyruvate (10 mM), glucose (10 mM), and sodium acetate (10 mM). Test substrates were added to the agarose plug in gradient tubes. Zero-valent iron (Fe^0) was also tested as an iron source, as an Fe^0-agarose plug prepared as previously described (20). Growth was checked by fluorescence microscopy, as described above.

To determine the effect of pH on growth, the pHs of the gradient culture medium were adjusted to various levels with acetate-acetic acid buffer (pH 5.0 and 5.4, 10 mM), MES (pH 5.6, 6.1, and 6.5, 10 mM), and HEPES (pH 7.1, 7.3, and 7.5, 10 mM). To determine the range of growth temperatures and the optimum growth temperature, cells were grown in the agarose-stabilized gradient tubes at 4, 8, 10, 15, 20, 25, 30, and 35°C under microaerobic conditions (N_2-CO_2-O_2, 79:20:1) at pH 6.2.

The total iron concentrations in cultures were determined by ferrozine assay as described previously (6); absorbance was measured at 562 nm with a spectrophotometer (DU800; Beckman Coulter, Brea, CA, USA).

DNA extraction, PCR, cloning, sequencing, and phylogenetic analysis. A PCR-based phylogenetic analysis of diagnostic genes was performed as described previously (18), with minor modifications. DNA was extracted from the samples (approximately 0.5 g) using the FastDNA spin kit for soil and the FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA). PCR was performed with the following oligonucleotide primers: Bac27F (26) and Uni1406R (18) for the bacterial 16S rRNA gene, Arc9F (27) and Uni1406R for the archaeal 16S rRNA gene, A189f and A682r (28) for *pmoA*, and cbbM343F and cbbM1226R (29) for *cbbM*. In addition, PCRs for *pmoA* and *cbbM* were also performed for the DNA extracts from the iron-rich microbial mat collected previously in Budo Pond, Hiroshima University, Japan (20). We failed to amplify *cbbL* (forms IA and IC), *mcrA*, and *amoA* from the DNA extracts from the flocs of Ohyato Park and Budo Pond using the previously reported methods (30–32). PCR products were cloned, and the nucleotide sequences of randomly selected clones were determined. The 16S rRNA gene sequence of OYT1 was also determined as described above. The *cbbM* gene sequence of OYT1 was determined by direct sequencing of the PCR amplicon of *cbbM* because the cloning of this gene was unsuccessful. Chimera sequences were identified using Mallard (33) and removed. The nonchimeric sequences of clones were assigned to operational taxonomic units (OTUs) at a definition level of 0.03 using mothur (34). The sequences of representative OTUs were aligned using MUSCLE (35), and the gap positions were removed from the alignment data set. Maximum-likelihood trees were constructed using PhyML (36). Bootstrap values were estimated from 100 replicates. Calculations of coverage, Shannon diversity index, and Chao1 species richness estimates were performed using mothur (34).

Quantitative PCR. Bacterial and archaeal rRNA gene copy numbers in the extracted genomic DNA were determined by quantitative PCR (Q-PCR) as previously described (20), with minor modifications. A dilution series of PCR products of the *Escherichia coli* 16S rRNA gene was used as a standard for bacterial Q-PCR analysis (the r^2 value of the standard curve was 0.999). A dilution series of the PCR products of an archaeal 16S rRNA gene clone (oytA093) obtained from the flocs in this study was used as a standard for archaeal Q-PCR analysis ($r^2 = 0.995$). All assays were performed in triplicate. Bacterial and archaeal cell numbers were estimated from Q-PCR data, assuming an average 16S rRNA gene copy number of 4.2 or 1.7 per cell, using the database rrnDB, version 3.1.221 (37).

Light and electron microscopy. A portion of the collected flocs was fixed with formaldehyde (final concentration, 3.7%) at 4°C overnight and then stored in phosphate-buffered saline (PBS)-ethanol (1:1) at −20°C until microscopic inspection. Portions of the sample were stained with 4′,6′-diamidino-2-phenylindole (DAPI) or SYBR green I in the dark for 5 min. The stained samples were placed on a glass slide and observed with a fluorescence microscope (BX60; Olympus, Tokyo, Japan). Detailed morphology was observed by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For the SEM and TEM sample preparation, OYT1 was cultured in liquid MWMM medium as previously described (10). Samples for SEM were mounted on a 0.2-μm-pore-size polycarbonate filter, rinsed with ultrapure water, air dried, and coated with Au/Pd. Samples for TEM were washed with ultrapure water, air dried on a Formvar-coated copper grid, and coated with carbon. Electron micrographs of the stalks produced by OYT1 were taken with a field emission SEM (S-4700; Hitachi) and a TEM (LIBRA 120; Zeiss) at the Delaware Biotechnology Institute (DBI) Bioimaging Center.

Nucleotide sequence accession numbers. The sequence data reported in this paper have been submitted to GenBank under accession numbers AB722175 to AB722188 for archaeal 16S rRNA genes, AB722189 to AB722261 for bacterial 16S rRNA genes, AB722305 to AB722363 for *cbbM* genes, and AB722404 to AB722449 for *pmoA* genes from Ohyato Park; AB722262 to AB722304 for *cbbM* genes and AB722364 to AB722403 for *pmoA* genes from Budo Pond; and AB720115 and AB720116 for 16S rRNA and *cbbM* genes of OYT1.

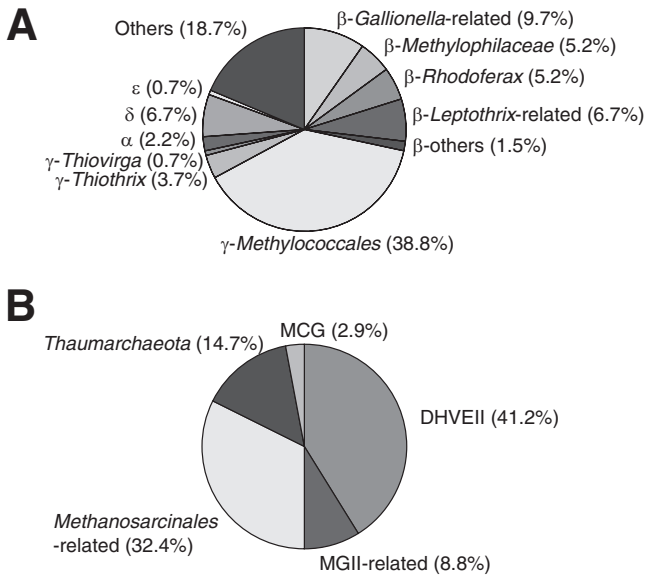


FIG 1 Bacterial (A) and archaeal (B) community structures based on the 16S rRNA gene clone libraries. Greek letters from α to ε represent *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and *Epsilonproteobacteria*. Numbers in parentheses indicate the detection frequency for each taxonomic group in the clone libraries.

RESULTS

Groundwater chemistry, biomineral morphology, and microbial abundance. The groundwater discharging into the iron flocs had a pH of 6.7 and temperature of 15.8°C. The concentrations of DO, ferrous iron, chloride, sulfate, and nitrate were 20.3, 37.6, 395, 56.2, and 6.77 μM, respectively. The flocs contained sheaths resembling structures produced by *Leptothrix* spp. (data not shown), which are often found in freshwater iron-rich flocs or mats (38–41). The sheaths may contribute to the accretion of flocs, as mentioned previously (40, 41). Although some *Leptothrix* have been characterized as iron oxidizers, no obligate chemolithoautotrophic species belonging to *Leptothrix* have been isolated. The presence of sheaths is consistent with the detection of 16S rRNA genes related to *Leptothrix*, described below. No *Gallionella*-like twisted stalk structures were observed in the flocs.

Based on the Q-PCR analysis, bacterial populations dominated the microbial community of the iron-rich floc sample ($1.09 \times 10^9 \pm 0.22 \times 10^9$ cells/g wet weight). The archaeal population represented 0.1% of the floc community ($1.67 \times 10^6 \pm 0.63 \times 10^6$ cells/g wet weight).

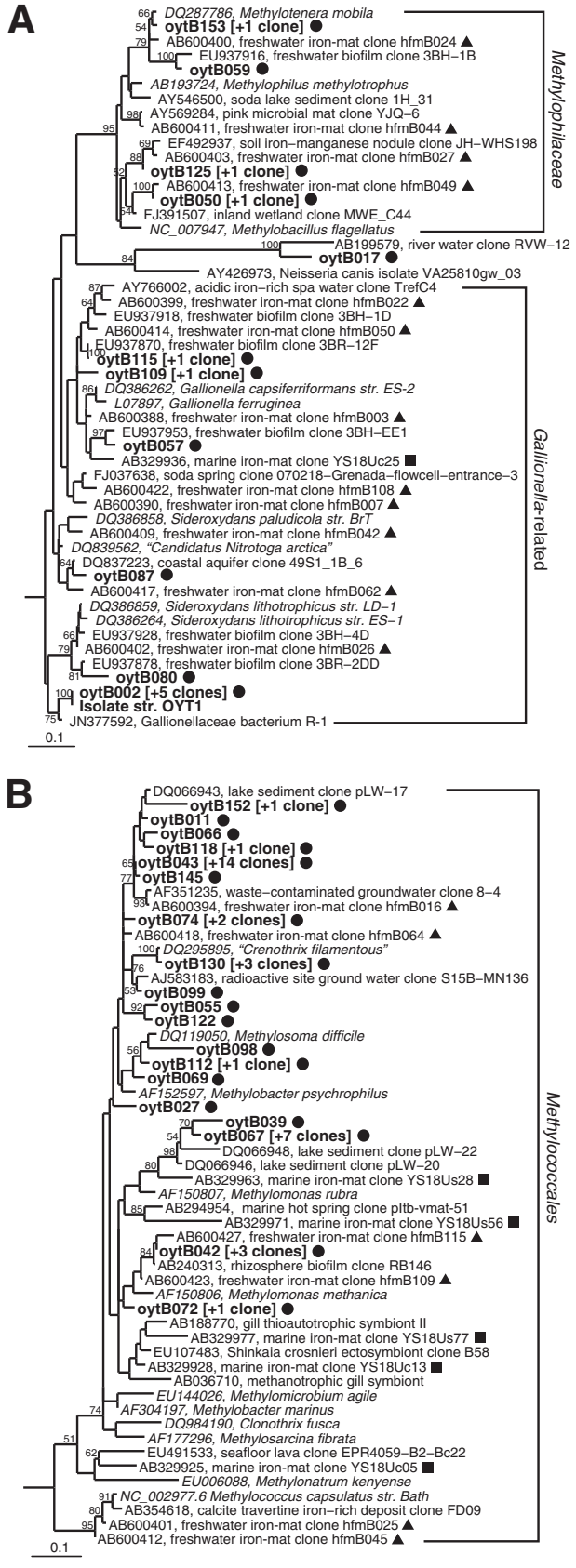
Detection of bacterial and archaeal 16S rRNA, *cbbM*, and *pmoA* genes. Phylogenetically diverse OTUs of bacterial and archaeal 16S rRNA, *cbbM*, and *pmoA* genes were detected in the flocs. The frequencies of detection of each taxon in the bacterial and archaeal 16S rRNA gene clone libraries are shown in Fig. 1. The coverage, Shannon diversity index, and Chao1 species richness estimates for the clone libraries are summarized in Table 1. Phylogenetic trees for all OTUs are shown in Fig. S3 to S6 in the supplemental material. The parts of the trees for the *Gallionella*-related group, *Methylophilaceae*, and *Methylococcales* are shown in Fig. 2. Some OTUs were shared between the clone libraries from Budo Pond (20) and Ohyato Park for bacterial and archaeal 16S rRNA, *cbbM*, and *pmoA* genes (Fig. 3).

Clones belonging to *Proteobacteria* were the most abundant in the library, accounting for 81.3% of the total number of clones (Fig. 1A). In particular, clones belonging to *Betaproteobacteria* and *Gammaproteobacteria* were abundant in the library, accounting for 28.3% and 43.2% of the total number of clones, respectively (Fig. 1A). The OTUs related to FeOB such as *Gallionella*, *Sideroxydans*, and R-1 (a member of the *Gallionellaceae*), as well as *Leptothrix* (Fig. 2A; see S3A in the supplemental material). OTUs related to methano/methylotrophs in *Methylophilaceae* and *Methylococcales*, iron reducers in *Rhodoferax*, and sulfide-oxidizing bacteria (i.e., *Thiothrix*, *Thiovirga*, and *Sulfuricurvum*) were also detected (Fig. 2A and B; see also Fig. S3A to C). Clones belonging to *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, and the clone groups (i.e., OD1, OP11, and OP3) accounted for small portions of the library (0.7 to 3.7% of the total number of clones) (Fig. 1; see also Fig. S3D and E). Twenty bacterial OTUs, accounting for 17.9% of the total number of OTUs in the library from Ohyato Park, were shared with the library from Budo Pond (Fig. 3A). The shared OTUs were classi-

TABLE 1 Summary of the results of clone library analyses of 16S rRNA and functional genes from three iron-rich flocs at circumneutral pH

Target gene and clone library	Total no. of clones	No. of $OTU_{0.03}$[a]	Chao1 richness (95% CI[b])	Shannon diversity index	% coverage	Reference or source
Bacterial 16S rRNA gene						
OytB	134	67	184 (124–310)	3.92	61.2	This study
HfmB	107	48	103 (70–190)	3.52	72.0	20
Archaeal 16S rRNA gene						
OytA	34	14	42 (21–119)	2.34	76.5	This study
HfmA	48	28	56 (38–110)	3.10	56.3	20
pmoA						
OytpmoA	60	16	34 (20–91)	1.87	81.7	This study
HfmpmoA	43	14	21 (15–48)	2.12	81.4	This study
cbbM						
OytcbbM	63	13	21 (15–51)	2.07	88.9	This study
HfmcbbM	55	18	36 (23–83)	2.28	78.2	This study

[a] $OTU_{0.03}$, OTUs at a definition level of 0.03.
[b] CI, confidence interval.



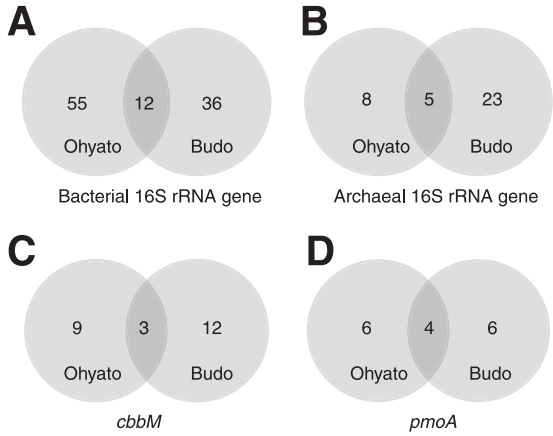


FIG 3 Venn diagrams comparing the community structures of flocs in Ohyato Park (Ohyato) and Budo Pond (Budo). Communities of bacterial (A) and archaeal (B) 16S rRNA genes, *cbbM* (C), and *pmoA* (D) are shown. Numbers in circles indicate the number of OTUs. Numbers in lenses indicate the number of shared OTUs.

fied as *Gallionella*-related, *Methylophilaceae*, *Methylococcales*, and *Thiothrix* (Fig. 2; see also Fig. S3A and B).

In the archaeal clone library, clones in deep-sea hydrothermal vent euryarchaeotic group II (DHVEII) (42) were abundant, accounting for 41.2% of the total number of clones (Fig. 1B). Our OTUs in DHVEII were related to environmental clones detected in freshwater iron-rich mats, marine mud volcanoes, and cold seep sediments (94 to 99% similarity) (see Fig. S4 in the supplemental material). The other OTUs were affiliated with marine group II (MGII) (43) and *Methanosarcinales* in *Euryarchaeota*, with *Thaumarchaeota*, and with the miscellaneous crenarchaeotic group (MCG) (44) (see Fig. S4). Five archaeal OTUs, accounting for 35.7% of the total number of OTUs in the Ohyato Park library, were shared with the library from the iron-rich flocs of Budo Pond (Fig. 3B). The shared OTUs were in DHVEII, MCG, and the *Methanosarcinales*-related group (see Fig. S4).

Clones of *cbbM* genes were detected in the flocs of both Ohyato Park and Budo Pond (see Fig. S5 in the supplemental material). Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a key enzyme in the Calvin-Benson-Basham (CBB) cycle, one of the carbon fixation pathways. RubisCO large-subunit genes (i.e., *cbbL* and *cbbM*) serve as phylogenetic markers for autotrophs (45). Microaerophilic or facultative anaerobic autotrophs often possess *cbbM* (46). The *cbbM* clones detected were related to the genes of cultured species, such as *Leptothrix cholodnii*, *Dechloromonas aromatica*, *Thiobacillus denitrificans*, "*Sideroxydans lithotrophicus*"

FIG 2 Parts of the phylogenetic trees of bacterial 16S rRNA genes for the *Gallionella*-related group and *Methylophilaceae* in *Betaproteobacteria* (A) and *Methylococcales* in *Gammaproteobacteria* (B). The whole topologies of the trees of *Betaproteobacteria* and *Gammaproteobacteria* are shown in Fig. S3A and B, respectively, in the supplemental material. Symbols following clone names indicate the sampling points: circle, Ohyato Park; triangle, Budo Pond; square, Southern Mariana Trough. Maximum-likelihood trees were constructed using 795 (A) or 761 (B) homologous positions in each alignment data set with the nucleotide substitution model GTR+I+G. Bootstrap values (>50%) are shown at the branch points. The scale bar represents 0.1 nucleotide substitution per sequence position. Numbers following the detected clones indicate the number of clones.

strain ES-1, and *Halothiobacillus neapolitanus*, as well as environmental clones detected in groundwater and an oil-contaminated aquifer (see Fig. S5). Three of the *cbbM* OTUs detected in Budo Pond and Ohyato Park were shared (Fig. 3C).

Clones of *pmoA* genes were detected in the flocs of both Ohyato Park and Budo Pond (see Fig. S6 in the supplemental material). The *pmoA* gene encodes the alpha subunit of the particulate methane monooxygenase (47), which is a functional gene for methanotrophs (48). Methanotrophs have been divided into two distinct groups, type I (*Gammaproteobacteria*) and type II (*Alphaproteobacteria*), on the basis of phylogeny, physiology, and morphology (49). Furthermore, type I methanotrophs have been divided into two distinct subgroups, types Ia and Ib (50). The *pmoA* genes of types Ia and Ib were detected in the present study (see Fig. S6). The ratios of types Ia and Ib in the total number of clones for each clone library differed (96.7% and 3.3% for the *pmoA* library of Ohyato Park and 39.5% and 60.5% for the *pmoA* library of Budo Pond). The detected *pmoA* OTUs of type Ia were related to *Methylomonas methanica* and environmental clones detected in soils and lake sediments (see Fig. S6). The *pmoA* OTUs of type Ib were related to the gene of *Methylococcus capsulatus* and environmental clones detected in soils, lake sediments, and subsurface geothermal water (see Fig. S6). No type II *pmoA* genes were detected, consistent with the absence of 16S rRNA genes related to alphaproteobacterial methanotrophs.

Physiology and phylogeny of isolate strain OYT1. Floc from Ohyato Park was used as an inoculum in an FeS-containing gradient tube; a band of iron oxides appeared 4 days after inoculation. Dense cells associated with the iron oxides were observed by fluorescence microscopy. Strain OYT1 was isolated from the enrichment culture by serial 10-fold dilutions in gradient tubes. A band of iron oxides appeared in the gradient tube 2 days after inoculation with OYT1, which was clearly distinguishable from the iron oxides in the gradient tube without inoculation (see Fig. S7 in the supplemental material). Purification checks did not detect any contaminants. Cells of OYT1 stored in 10% glycerol solution at −80°C remained viable for at least 6 months and retained their stalk-forming ability.

OYT1 was able to grow between 8°C and 30°C and pH 5.6 and 7.3. Optimum growth was observed from 25 to 30°C and pH 6.1 to 6.5. An amorphous cloud of iron oxides, instead of the band, was observed at 10°C or lower. No growth was observed at 4°C or 35°C. OYT1 grew on iron-containing medium with FeS, $FeCl_2$, or Fe^0 but not on iron-free medium with the other inorganic and organic substrates. OYT1 grew on FeS at 25°C and pH 6.2, with a doubling time of 10.9 h during exponential growth (Fig. 4A). The rate of iron oxidation, as measured by total iron accumulation, in the culture medium with OYT1 was clearly higher than that in the abiotic control (Fig. 4B).

OYT1 cells are gently curved short rods (0.8 to 1.9 μm long and 0.7 to 0.9 μm wide) (Fig. 5A and B). Stalk structures, including serpentine-like or Y-shaped filaments, were observed in the culture medium (1.0 to 1.5 μm wide) (Fig. 5A), often entwined with one another (see Fig. S8A and B in the supplemental material). The stalk structures were composed of thin fibrils, 25 to 200 nm wide (Fig. 5C and D; see also Fig. S8C and D). The stalk morphology of OYT1 resembles that of *G. ferruginea* (8), *M. ferrooxydans* (9), and R-1 (10). The stalks were common at pH 7.1 or higher but rare at pH 6.5 or lower.

OYT1 is affiliated with the *Betaproteobacteria* (Fig. 2A). The sequence closest to the 16S rRNA gene of OYT1 was that of R-1 (97.8% similarity). OYT1 and R-1 are distant from *Gallionella* species (92.9 to 94.0% similarity) and formed a distinct lineage (Fig. 2A; see also Fig. S9 in the supplemental material). The OTU (i.e., the representative clone, oytB002) almost identical to the 16S rRNA gene of OYT1 (99.9% similarity) included the most abundant clones in the *Gallionella*-related group (6 out of 13 clones). The deduced amino acid sequence of the *cbbM* gene of OYT1 was closest to that of "*Gallionella capsiferriformans*" strain ES-2 (97.8% similarity) (see Fig. S5).

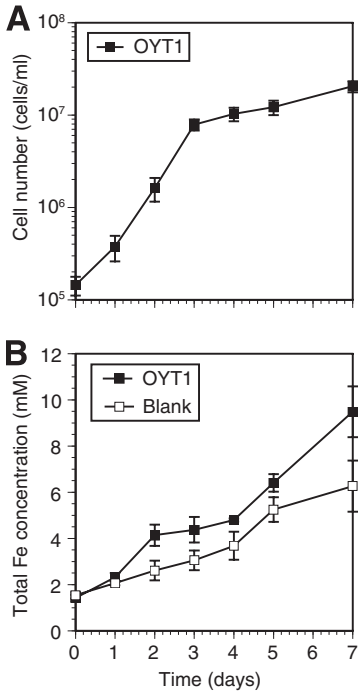


FIG 4 Growth curve of OYT1 (A) and temporal change of total iron concentration in culture medium (B). Solid and open squares indicate the culture medium with and without OYT1, respectively. Error bars indicate standard deviations of the means.

DISCUSSION

In the present study, we report the characteristics of microbial communities in freshwater iron-rich flocs at circumneutral pH determined by culture-dependent and culture-independent analyses, including the first analysis of functional genes in such flocs. In addition to 16S rRNA gene analysis, *cbbM* and *pmoA* gene analyses indicate that a variety of putative autotrophs and methanotrophs are present in the iron-rich flocs. Furthermore, the isolation and characterization of the stalk-forming bacterium, strain OYT1, demonstrate the presence of chemolithoautotrophic microaerophilic FeOB in the floc.

Putative autotrophs. The *cbbM* analysis suggests that a variety of putative microaerophilic autotrophs possessing *cbbM* exist in the freshwater iron-rich flocs. Previously, *cbbM* genes similar to our clones were detected in low-oxygen groundwater environments (32). It should be noted that some heterotrophs, such as *Dechloromonas aromatica* (GenBank accession no. CP000089) and *Rhodoferax ferrireducens* (GenBank accession no. CP000267), possess *cbbM* in their genome. Hence, it is possible that not all species possessing the detected *cbbM* genes are necessarily au-

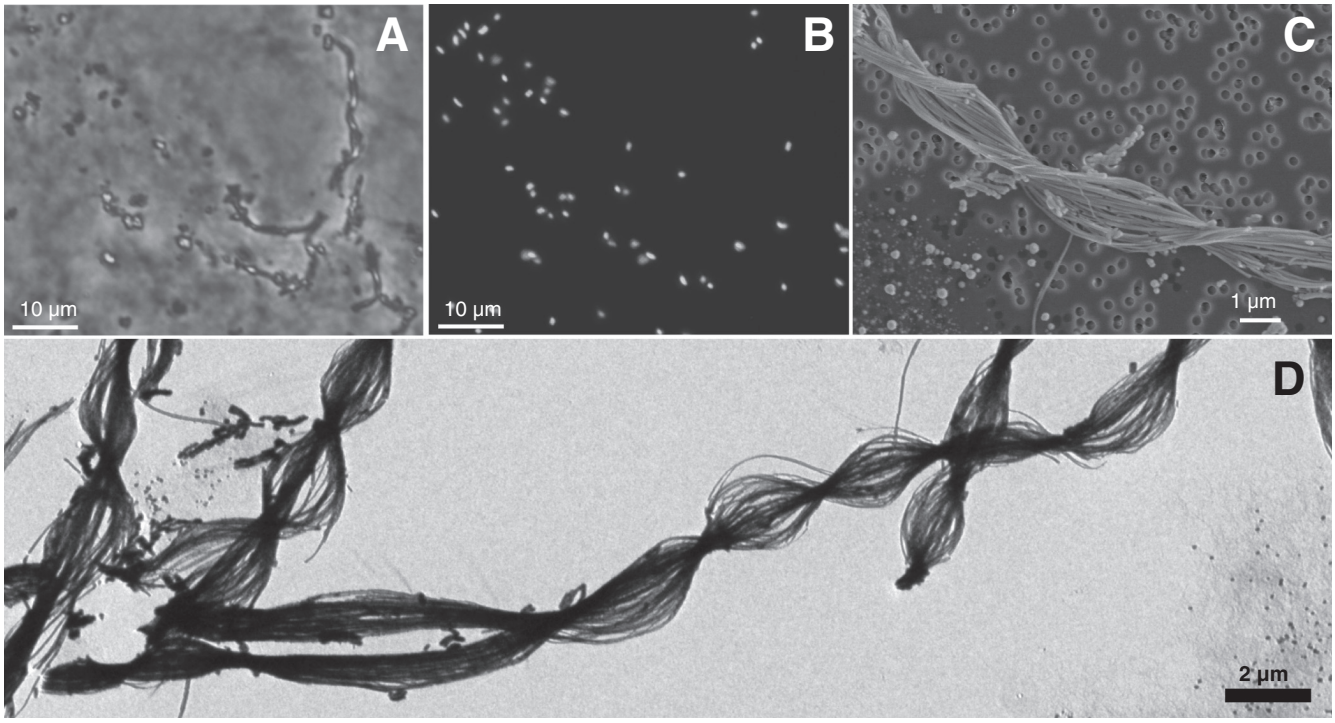


FIG 5 Microscopic observation of OYT1. Images were obtained by differential interference contrast microscopy (A), fluorescence microscopy (B), SEM (C), and TEM (D). (B) Cells were stained with SYBR green I. The images in panels A and B are in the same view.

totrophs. A significant number of the *cbbM* genes (22 clones) were similar to those of *Sideroxydans lithotrophicus* ES-1, suggesting that chemolithotrophic Fe oxidation provides energy for carbon fixation in iron-rich flocs. Although no *cbbM* genes related to OYT1 were detected in the flocs, 16S rRNA genes almost identical to OYT1 were detected (oytB002, 99.9% similarity) (Fig. 2A). This is probably due to a methodological bias, as supported by the fact that the cloning of the *cbbM* gene of OYT1 was unsuccessful in the present study.

Methanotrophs. The detection of the 16S rRNA genes in *Methylococcales*, together with those in the *Gallionella*-related group, has been reported in freshwater iron-rich environments, including Budo Pond (20, 23, 51, 52), although *pmoA* gene analysis had not been performed for these environments. The present results of bacterial 16S rRNA and *pmoA* gene analyses indicate that methanotrophs coincide with iron-oxidizing bacteria in the iron-rich flocs of Ohyato Park and Budo Pond. A nucleotide sequence similarity lower than 97% for the 16S rRNA gene (53) or a deduced amino acid sequence similarity lower than 93% for the *pmoA* gene (54) suggests a novel species. The bacterial 16S rRNA and *pmoA* genes detected in the present study represented low similarities to cultured methanotrophic species. The phylogeny of *pmoA* is congruent with that of the 16S rRNA gene (55). Hence, although the *pmoA* and 16S rRNA genes detected in parallel PCR analyses cannot be directly linked, some of the *pmoA* genes detected may be derived from the methanotrophs possessing the detected 16S rRNA genes clustered in *Methylococcales*.

Methanotrophs probably play a role as primary producers in the iron-rich flocs at the groundwater-discharging points of Budo Pond and Ohyato Park. In groundwater systems, methane can be a carbon and energy source that sustains microbial ecosystems. In fact, methanotrophs have been detected in groundwater by culture-independent and culture-dependent analyses (56–58). Although the methane concentration of the discharging groundwater or the pore water within the flocs is unknown, methanogens can produce methane in a surrounding aquifer, soil, and/or iron-rich floc.

Uncultured archaea. The archaeal diversity and distribution in iron-rich flocs at circumneutral pH are not well known (18, 20). The archaeal 16S rRNA gene analysis indicates that yet-uncultured archaea, i.e., DHVEII, MCG, and MGII-related, are present in the iron-rich floc of Ohyato Park. Further cultivation efforts and metagenomic analysis are required to elucidate the physiology of these yet-uncultured archaea. Five OTUs in the DHVEII, MCG, and *Methanosarcinales*-related groups were shared with the archaeal community of the iron-rich floc of Budo Pond (Fig. 3B; see also Fig. S4 in the supplemental material), suggesting that these archaea are common members in freshwater iron-rich flocs. Although the archaeal population was relatively small (less than 1% of the total numbers of cells) in the iron-rich floc communities at both Budo Pond (20) and Ohyato Park as shown by Q-PCR, they potentially play a role in carbon cycling in the microbial ecosystem.

Stalk-forming microaerophilic iron-oxidizing bacteria. Chemolithoautotrophic microaerophilic FeOB are likely to play roles as primary producers in freshwater iron-rich flocs. The isolation and characterization of OYT1 provide direct evidence for the presence of such FeOB in the floc of Ohyato Park. Several OTUs related to OYT1 and other isolates in the *Gallionella*-related group (>95% nucleotide sequence similarity) were detected in the floc sample (Fig. 2A), suggesting that OYT1 is representative of community members. Moreover, *cbbM* genes related to *S. litho-*

trophicus strain ES-1 were also detected (89 to 94% deduced amino acid sequence similarity) (see Fig. S5 in the supplemental material), providing further evidence of autotrophy by FeOB. Although one member of the *Gallionella*-related group, "*Candidatus* Nitrotoga arctica," is a nitrite-oxidizing bacterium (60), to date, most isolates related to *Gallionella* are chemolithoautotrophic microaerophilic FeOB (6, 10, 17, 59), strongly suggesting that the 16S and *cbbM* sequences also represent this metabolism.

The physiological characteristics of OYT1 are similar to those of other freshwater microaerophilic FeOB isolates growing at circumneutral pH, such as R-1, *S. lithotrophicus* strains ES-1 and LD-1, *G. capsiferriformans* strain ES-2, and *Sideroxydans paludicola* strain BrT (6, 10, 17). All of the FeOB isolates, including OYT1, can grow at about pH 6 and 25°C and in low O_2 concentrations in gradient culture media. They use Fe(II) as their sole energy source and are likely to fix CO_2 via the CBB cycle. The genomes of *S. lithotrophicus* ES-1 and *G. capsiferriformans* ES-2 contain *cbbM* genes (GenBank accession numbers CP001965 and CP002159, respectively); similarly, OYT1 also possesses a *cbbM* gene (see Fig. S5 in the supplemental material).

Despite the presence of the stalk-forming FeOB in the iron-rich flocs, no stalks were observed in the floc from Ohyato Park. The same situation has been reported in Budo Pond (20). Some microbes related to *Gallionella* do not normally produce stalks (6, 17, 61), while others do not produce stalks under conditions of very low O_2 or pH below 6 (62). In OYT1 cultures, stalk formation was rarely observed at pH 6.5 or lower. The lower pH of the discharging groundwater of Ohyato Park and Budo Pond (6.2 to 6.7) is consistent with the lack of stalks. These results indicate that the stalk formation may be unnecessary for some FeOB to thrive under weak acidic conditions. Chan and her coworkers suggest that at neutral and alkaline pH, extracellular polymers produced by FeOB localize Fe mineral precipitation, which generates protons and increases the pH gradient between the outside and inside of the cells, thus increasing the energy-generating potential (63). This may explain the present result that OYT1 produced stalks well at higher pH (>7). In addition, because the rate of abiotic iron oxidation increases dramatically with an increase in pH, OYT1 and other stalk-forming FeOB may produce stalks at higher pH in order to nucleate abiotic Fe precipitation and prevent encrustation with iron oxides.

Concluding remarks. We have documented the diversity of an iron floc community and shown that members have the genetic potential to fix carbon and transform methane. Fe oxidation can fuel carbon fixation, while methane oxidation can result in carbon assimilation, as well as fixation. Although Fe oxidation and methanotrophy are not necessarily linked, the co-occurrence here and in other systems causes us to surmise some possible ecological relationships. Both metabolisms may be the consequence of organic degradation in the adjacent soil/aquifer from which the groundwater emerges; Fe(III) reduction would provide a source of Fe(II), and methanogenesis would result in methane. There may also be more localized cycling, either in anoxic microenvironments within the floc or in the Fe- and organic-rich sediment that accumulates beneath the Fe floc; this is suggested by the presence of a few sequences similar to methanogens and Fe(III)-reducers in the 16S rRNA clone library. If localized, there could be a tight cycling of primary production (by FeOB and methanotrophs), organic degradation (by Fe reducers and other organisms), and methanogenesis. Further environmental work coupled to laboratory experiments with the new autotrophic FeOB OYT1 and other isolates will help elucidate links between Fe floc ecology and elemental cycling.

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