Geomicrobiology Journal, 24:559–570, 2007

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490450701670152

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Characterization of Neutrophilic Fe(II)-Oxidizing Bacteria Isolated from the Rhizosphere of Wetland Plants and Description of *Ferritrophicum radicicola* gen. nov. sp. nov., and *Sideroxydans paludicola* sp. nov.

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Iron deposits (Fe plaque) on wetland plant roots contain abundant microbial populations, including Fe(II)-oxidizing bacteria (FeOB) that have not been cultured previously. In this study, 4 strains of Fe plaque-associated FeOB were isolated from 4 species of wetland plants. All 4 isolates grew in tight association with Fe-oxides, but did not form any identifiable Fe-oxide structures. All strains were obligate lithotrophic Fe(II)-oxidizers that were microaerobic, and were unable to use other inorganic or organic energy sources. One strain, BrT, was shown to fix $^{14}CO_2$ at a rate consistent with its requirement for total cell carbon. The doubling times for the strains varied between 9.5 and 15.8 hours. The fatty acid methyl ester (FAME) profiles of 2 strains, BrT and CCJ, revealed that 16:0, 15:1 isoG, and 14:0 were dominant fatty acids. Phylogenetic analysis of the 16S rRNA gene indicated that all the strains were *Betaproteobacteria*. Two of the strains, BrT and Br-1 belong to a new species, *Sideroxydans paludicola*; a third strain, LD-1, is related to *Sideroxydans lithotrophicus,* a recently described species of FeOB. The fourth isolate, *Ferritrophicum radicicola*, represented a new genus in a new order of *Betaproteobacteria*, the *Ferritrophicales*. There are no other cultured isolates in this order. A small subunit rRNA gene-based, cultivation-independent analysis of *Typha latifolia* collected from a wetland revealed terminal restriction fragment profiles (tRFLP) consistent with the presence of these bacteria in the rhizosphere. These novel organisms likely play an important role in Fe(II) oxidation kinetics and Fe cycling within many terrestrial and freshwater environments.

Received 5 April 2007; accepted 26 July 2007
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Keywords iron-oxidizing bacteria, rhizosphere, wetlands, lithotrophs, *Sideroxydans*, *Ferritrophicum*

INTRODUCTION

Bacterial iron oxidation at circumneutral pH is a commonly observed phenomenon when anoxic Fe(II)-enriched waters contact O_2. Such oxic/anoxic boundaries are notable for the presence of rust-colored flocculent material, or iron mats. These have been described at groundwater seeps (Emerson and Revsbech 1994a), wetlands (Roden and Wetzel 1996), and hydrothermal vents (Jannasch and Mottl 1985). The microbial denizens of these habitats include *Leptothrix ochracea* and *Gallionella ferruginea*, organisms that have been commonly described based on the unique morphologies of the Fe-oxides that they produce (Emerson 2000). In addition, the isolation of new unicellular chemolithotrophic Fe(II)-oxidizing bacteria (FeOB) have been reported (Emerson and Moyer 2002; Edwards et al. 2003; Sobolev and Roden 2004). These organisms use O_2 as an electron acceptor and grow at the oxic/anoxic interface where low O_2 concentrations and high Fe(II) concentrations prevail as a result of the mobilization of Fe(II) from soils, sediments, or mineral surfaces. Under these conditions, the microbes can successfully compete for O_2 with abiotic Fe(II) oxidation. Studies demonstrating this have shown that microbes may actively mediate between 18% and 80% of Fe(II) oxidation (Emerson and Revsbech 1994b; Neubauer et al. 2002) with values as high as 90% being reported (Kasama and Murakami 2001; Sobolev and Roden 2001; James and Ferris 2004).

There is widespread evidence demonstrating that Fe(II) oxidation is an important sink for O_2 in the rhizosphere (root-zone) of wetland plants (Van Bodegom et al. 2001). Oxygen released via the root system into the saturated, anaerobic soils that contain Fe(II) results in iron oxidation and the formation of rust-colored precipitates, or Fe plaque, on the root surfaces that may extend several mm into the surrounding soil (Mendelssohn et al. 1995). Fe plaque can account for a large pool of Fe(III) oxides on the roots of wetland and submersed aquatic plant species (Chen et al. 1980; Mendelssohn et al. 1995; Weiss et al. 2003). Though plaque formation has been considered largely an abiotic process, studies have shown that a diverse microbial community is associated with the Fe-coated root surface (Trolldenier 1988; Emerson et al. 1999). We have previously reported that FeOB are common members of this microbial community, observed on the roots of 92% of wetland plants collected in a survey of 13 biogeochemically diverse aquatic environments (Weiss et al. 2003). In the current study, we describe the isolation and characterization of 4 strains of FeOB from the roots of wetland plants. Three of these isolates are related to previously described FeOB from groundwater (Emerson and Moyer 1997), and the fourth is the only cultured representative of a new order within the *Betaproteobacteria*. Their presence is a further line of evidence that FeOB may play a significant role in iron cycling in the rhizosphere.

MATERIALS AND METHODS

Study Site Description

The samples used to enrich FeOB from the rhizosphere were obtained as part of a larger survey on the presence of FeOB in association with wetland plants in the Middle Atlantic region of the United States (Weiss et al. 2003). Isolates described in the current study came from 3 different wetlands (Table 1). Strain CCJ was isolated from *Juncus effusus* growing in the riparian strip of Contrary Creek (near Mineral, VA), at the site of a former pyrite mine with a pH of ca. 4 and soil and root plaque Fe concentrations in excess of 850 and 1230 μmol g dry weight root^{-1} (gdw^{-1}), respectively. Strain LD-1 was isolated from the roots of *Magnolia virginiana* (Fe plaque = 453 μmol gdw^{-1}) collected from a bottomland hardwood ecosystem in Fredericksburg, VA. This system was dominated by high organic matter (ca. 13%), had a pH of 4.5, and much lower levels of soil Fe (72 μmol gdw^{-1}). Strains Br-1 and BrT were both isolated from vegetation growing in a circumneutral constructed herbaceous wetland located on Kent Island, MD, near the Chesapeake Bay. Br-1 and BrT were enriched from the roots of *Schoenoplectus* (*Scirpus*) *americanus* and *Typha latifolia*, respectively. The root Fe plaque concentrations of these plant specimens ranged from 192 μmol gdw^{-1} on the roots of *T. latifolia* to 389 μmol gdw^{-1} on the *S. americanus* specimens. The pH value of the soil at this site was between 5.9 and 6.6.

Isolation of Fe(II)-oxidizing Bacteria

For the isolation of rhizosphere bacteria, individual plant specimens were removed from each site in an intact soil block (approximately 15 × 15 × 25 cm) containing the plant and most of its root system in order to minimize potential disturbance and aeration. The intact blocks were immediately transported to the lab, stored at 4°C, and dissected within 24 hours. Roots were washed in sterile deionized water and cut into 1-cm sections. These root sections were inoculated into gel-stabilized Fe gradient tubes containing Modified Wolfe's Minimal Medium (MWMM). The medium and gradient tube technique have been described previously (Emerson and Moyer 1997; Emerson and Merrill Floyd 2005). The tubes were considered positive for FeOB if a discrete band of Fe(III) oxides formed around the root surface see Figure 3 in Emerson et al. (1999). Subsets of samples were examined microscopically to confirm the presence of bacteria within the Fe(III) oxides. A small aliquot (10–20 μL) of the Fe-oxide forming band from each positive sample was transferred to an uninoculated gradient tube. After 2–3 transfers, a dilution series was performed to isolate pure strains of FeOB. Positive tubes at the highest dilution were examined microscopically and streaked out onto R2A (BD, Franklin Lakes, NJ) plates to check for heterotrophic contaminants. If necessary, additional dilution series were performed on each of the enriched strains. All purified strains were characterized by the traditional gram-staining method and a novel gram-staining technique in which two different fluorochromes are applied to the cells

TABLE 1
Characteristics of isolated rhizosphere Fe(II)-oxidizing bacteria

Characteristics	Strain			
	Br-1	BrT	CCJ	LD-1
Habitat				
Location	Kent Island, MD	Kent Island, MD	Mineral, VA	Fredericksburg, VA
Type of habitat	Constructed wetland	Constructed wetland	Riparian strip of mining area	Bottomland hardwood forest
Plant species	*Scirpus americanus*	*Typha latifolia*	*Juncus effusus*	*Magnolia virginiana*
Phylogeny	β-Proteobacteria	β- Proteobacteria	β-Proteobacteria	β-Proteobacteria
Morphology	Curved rod	Curved rod	Straight rod	Curved rod
Cell Diameter (μm)	0.42	0.42	0.89	0.74
Substrate utilization				
Fe(II)[1]	+	+	+	+
Inorganic[2]	−	−	−	−
Organic[3]	−	−	−	−
Doubling time[4] (h)	12.1	15.8	10.7	9.5
pH range[5]	4.5–7.0	4.5–7.0	4.5–7.0	4.5–7.0
G+C mol%		63.4	59.0	
Temperature range[6]	19–37°C	19–37°C	19–37°C	12–26°C

[1]Fe(II) was added a ferrous sulfide or ferrous carbonate in gradient tubes. Ferrous chloride was used in growing larger batch cultures.
[2]Substrates include H_2, ammonium, sulfide, thiosulfate, H_2, Mn(II), and tetrathionate.
[3]Substrates include galactose, pyruvate, acetate, succinate, glycerol, glucose, formate, maltose, ribose, and aspartate.
[4]Calculated at log phase growth on FeS in gradient tubes.
[5]Tested pH range = 4.5–8.0.
[6]Tested temperature range = 4[5] tested temperature range = 4–43°C.

and examined under epifluoresence microscopy (Mason et al. 1998).

For growth of larger cell masses, gradient plates were used in place of gradient tubes. This method is described in Emerson and Weiss (2004), and consists of using a thin agarose-stabilized layer of FeS in a Petri plate overlaid with liquid MWMM medium. The liquid layer is inoculated with a culture of FeOB, and the plates are incubated in an anaerobic jar under microaerophilic conditions using a CampyPak (Becton-Dickinson) with palladium catalyst.

Growth Studies and Phenotypic Analysis

The FeOB were tested for growth on a variety of different substrates and conditions. Cell growth rates on FeS were performed in gradient tubes using direct counts as previously described (Emerson and Moyer 1997). Two sets of controls were also performed: one in which the strains were inoculated in gradient tubes with no FeS in the bottom layer and another in which gradient tubes were left uninoculated.

All of the isolates were tested for growth over a range of different temperatures and pHs. Five tubes of each strain were incubated at each of 7 different temperatures between 4°C to 43°C. The pH range for growth of each strain was tested by varying the buffers used in the top layer of the gradient tubes, followed by subsequent bubbling with CO_2 to adjust the pH (where necessary). Sodium bicarbonate (gassed with CO_2) was used to buffer at pH 4.5 and 5.5, 2-Morpholinoethanesulfonic acid (MES) at pH 6.5 and 7.0, and N-Cyclohexyl-2-aminoethanesulfonic acid (HEPES) at pH 8.0; all buffering agents were used at a final concentration of 5 mM. Gradient tubes were considered positive or negative for growth after incubation for 2 weeks. Positive tubes contained a distinct band of Fe-oxides with associated FeOB at the oxic/anoxic interface.

Strains were also tested for growth in bicarbonate-buffered MWMM on a wide range of substrates including thiosulfate, tetrathionate, galactose, pyruvate, acetate, succinate, glycerol, glucose, formate, maltose, and ribose (final concentration of all = 5 mM). Cells were inoculated into 2 tubes of all substrates and examined both visually and microscopically during a 2-week incubation to determine presence or absence of cell growth. Growth was also tested on a H_2 gas mix (10% H_2:10%O_2:80% N_2) added to the headspace of 5 test-tubes. In addition to R2A, growth was tested on the complex heterotrophic media, tryptic soy, and nutrient agar and broth.

For FAME analysis approximately 400 ml of strains BrT and CCJ were grown in gradient plates until late log phase. The cultures were harvested by centrifugation and then analyzed for FAME as previously described (Emerson et al. in press). For determination of the DNA base composition (mol% G+C), a sensitive fluorometric technique (Gonzales and Saiz-Jimenez 2002, 2004) was used, which employed Sybr Green and a Bio-Rad iCycler real-time PCR instrument. Details of applying this

technique to FeOB have been published previously (Emerson et al. in press).

CO_2 Fixation Experiments

We tested for autotrophy by looking for the incorporation of ^{14}C-labeled inorganic carbon into cellular biomass. Actively growing BrT cells were inoculated into N_2-flushed serum bottles (n = 36) containing MWMM medium buffered with 8.8 mM MES and 10 mM $NaHCO_3$ (final pH $\sim$ 6.9) and supplemented with trace minerals and vitamins. Half of the bottles were poisoned with sodium azide (1 mM final concentration) so that abiotic ^{14}C uptake could be quantified. A subset of all bottles (n = 4 per live/killed treatment) was spiked with $NaH^{14}CO_3$ to a final ^{14}C activity of 0.1 μCi ml^{-1}. At the initiation of the experiment, we added filter-sterilized room air and $FeCl_2 \cdot 4H_2O$ to give final concentrations of 1% O_2 by volume in the bottle headspace and $\sim$400 μM Fe(II) in solution. Daily additions of $FeCl_2$ and air served to replenish these substrates as they were utilized. All bottles were incubated in the dark at 28°C.

At the beginning and end of the 5-day experiment, inorganic C was extracted from the growth medium by lowering the pH to <2 with HCl, bubbling the medium overnight with air, and trapping the evolved CO_2 in 0.5 M NaOH. The ^{14}C activity of aliquots of growth medium (containing incorporated ^{14}C but no $^{14}CO_2$) was measured on a Packard liquid scintillation analyzer. At the end of the experiment, we filtered subsamples of the growth medium through 0.22 μm nitrocellulose filters to quantify ^{14}C incorporation into the particulate and dissolved organic fractions (e.g., cell biomass vs. extracellular polymers). Replicate unlabeled bottles from each treatment were sacrificed daily during the experiment and analyzed for dissolved inorganic carbon concentration (acidification followed by infrared gas analysis) and cell density (microscopy).

DNA Extraction

Late log phase cultures of each of the strains were grown on gradient plates and used for DNA extraction. The cells and iron oxides were harvested by centrifugation, washed with phosphate buffer (50 mM, pH 8.0), and DNA was extracted using the PowerMax soil DNA isolation kit (MoBio). This minimal procedure produced DNA of good quantity and quality, which was assessed by measuring the absorbances at 260 and 280 nm, respectively, on a NanoDrop1000 spectrophotometer, and by running the samples on agarose gel electrophoresis.

Phylogenetic Analysis

The 16S rRNA gene was PCR amplified using the universal bacterial primers 27F and 1525R with the Roche PCR Core kit. PCR reactions were initiated with a 10-min incubation at 20°C, and then continued with an initial heating to 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 45°C for 30 sec, extension at 72°C for 30 sec, and concluded with an extension cycle at 72°C for 1 min. PCR products were cleaned using Wizard columns and used in subsequent sequencing reactions (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit) with primers including 27F, 519R, 907F, 922R, and 1525R. Sequencing products were purified through Centri-Sep spin columns and then read on an ABI Prism 377 DNA sequencer.

Nearest neighbors for isolates Br-1, BrT, and LD-1 were found using a BLAST search (www.ncbi.nlm.nih.gov/blast), whereas the nearest neighbor for CCJ was found using the NAST approach (greengenes.lbl.gov). FeOB and nearest neighbor sequences were manually aligned with members of the *Betaproteobacteria* using the Ribosomal Database Project–II Release 9 alignment as a template (Cole et al. 2005). Neighbor-joining dendrograms (50% consensus) with bootstrapping (n = 1000) were created using MEGA3 (Cole et al. 2005).

tRFLP Analysis

A *Typha latifolia* plant including its root mass was collected from a tidal freshwater marsh at Jug Bay on the Patuxent River in Maryland. Within 2 hours of collection, Fe-plaque encrusted roots and associated bulk soil were subsampled as described previously (Weiss et al. 2004). A sample containing multiple fine roots (wet wt = 0.31 mg) was rinsed to remove loosely adhering soil and placed in a sterile 10 cm petri dish and minced with a sterile razor blade. Bulk soil (0.26 mg) from which visible roots had been removed was extracted directly. DNA was extracted from each sample and quantified as described before.

A fragment of the bacterial 16S rRNA gene was amplified using two primers (27F-5′ AGAGTTTGATCMTGGCTCAG labeled with D4 Well Red dye on the 5′ terminus, and 519R-5′ GTATTACCGCGGCTGCTG). PCR conditions were similar to those described above. Approximately 250 nanograms of each amplicon were digested with each of the following restriction endonucleases: *Alu I*, *BstU I*, *Hae III* and *Hha I* (New England BioLabs, Inc.). Digests were performed according the manufacturer's protocol in 50 μL reaction volumes in 0.2 mL thin-walled tubes at the appropriate temperature(s) (37°C or 60°C), and inactivated by heating at 80°C for 20 minutes in the thermal cycler. Desalted preparations were resuspended in 50 μL of sterile water and stored at -20°C to -80°C until fragment analysis with the sequencer.

tRFLP fragments were prepared in a 96-well microcentrifuge plate and analyzed using the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc.). Ten-fold dilutions of each purified sample digest were prepared in a 40 μL injection volume containing 0.5 μL of GenomeLab DNA Size Standard 600 (Cat# 608095, Beckman Coulter) and SLS (Sample Loading Solution). Dilutions of the sample digests were necessary in order to determine the proper sample load and sample:size standard signal ratios. The sequencer was run using the Frag-4 method with default settings. Resulting data files were assessed for quality using CEQ 8000 Genetic Analysis System software, and a data file including sample ID, peak area and fragment size was exported as Fragments/Genotypes data (*.csv file) to Microsoft Excel.

Comparative Analysis of Community and Isolate Profiles

NEBcutter v2.0 (New England BioLabs, Inc; http://tools.neb.com/NEBcutter2/index.php) software (Vincze et al. 2003) was

used to generate *in silico* digest profiles for each of the 4 isolates, 1 for each of 4 four restriction enzymes used. DNA from pure cultures of the isolates was also analyzed using the four enzymes to confirm that *in silico* digests matched the actual organisms. T-Align software (http://inismor.ucd.ie/~talign/) (Smith et al. 2005) was used to compare replicate tRFLP profiles for each sample digest and generate a consensus profile containing only terminal restriction fragments (TRFs) present in both original tRFLP profiles. The confidence interval was set to the default 0.5. The resulting profiles representing different communities were compared to produce a list showing whether a TRF was present in a particular sample profile, bulk soil and root, and displayed its relative fluorescence intensity. Predicted microbial isolate TRFs were compared to the community peak data.

RESULTS

Enrichment and Isolation

In the strains that were successfully isolated, cultures generally became pure within two to three dilution series; transferring the cultures early in their growth phase appeared to facilitate isolation. Other enrichments ended up with binary cultures of an Fe-oxidizer and a heterotroph that could not be eliminated even after repeated dilutions. These enrichments were abandoned.

Among the rhizosphere isolates, 3 of the strains were vibroid or curved rods (Br-1, BrT, and LD-1), and one, CCJ, was a straight rod (Figure 1). The cell diameters ranged from 0.42 μm (Br-1 and BrT) to 0.74 μm and 0.89 μm for LD-1 and CCJ, respectively (Table 1). All the strains grew in close association with Fe-oxides (Figure 1); however, none of them formed morphogically unique Fe-oxide structures such as sheaths or stalks. All four strains were motile and all appeared to be microaerobes and aerotactic based on observations in FeS gradient tubes. Although phylogenetically all strains belong to the gram-negative bacteria (see later), they consistently stained gram-positive both with the standard gram-staining procedure and using a fluorescent dye technique (results not shown). The same result was found for two other recently described freshwater FeOB (Emerson et al. in press).

GROWTH STUDIES

The growth curves of the 4 rhizosphere FeOB are shown in Figure 2. The doubling times for the strains varied between 9.5 hours for LD-1 to 15.8 hours for BrT (Table 1). Following the exponential growth phase, cell densities leveled off, and the cells remained viable for at least 6 weeks when left at room temperature. There was no growth in gradient tubes that lacked FeS in the bottom layer (data not shown). The maximum growth yield for the rhizosphere strains in gradient tubes was 4×10^7 cell ml^{-1}; whereas in gradient plates maximum yields of up to 3×10^8 cells ml^{-1}have been recorded (Emerson and Weiss 2004).

None of the strains grew on any other substrate than Fe(II), either when presented with different inorganic or organic substrates as sole energy and/or carbon sources (Table 1), or on different complex heterotrophic media including R2A, tryptic soy agar, or nutrient broth or plates. These latter substrates were tested both aerobically and microaerobically. The inorganic energy sources that were tested included: thiosulfate, elemental sulfur, hydrogen, formate, and ammonium. In terms of temperature tolerance, all four strains grew between 19 and 26°C; LD-1 also grew at 12°C while Br-1, BrT, and CCJ all grew at 37°C. All the strains grew between pH 4.5 and 7.0 with no growth observed at pH 8.0. The primary fatty acids identified by FAME for strains BrT and CCJ are shown in Table 2. The identified fatty acids were qualitatively similar for both strains but quantitatively 16:0 was the major identified fatty acid in strain BrT, while in CCJ 16:0, 14:0, and 15:1 isoG, and 18:1 isoH were relatively close in abundance. Over 50% of the fatty acids in both BrT and CCJ either could not be distinguished by MIDI system, the summed features in Table 2, or were not identified at all.

TABLE 2
FAME analysis of BrT and CCJ

Fatty Acid	BrT (%)	CCJ (%)
10:0 3OH	2	2.7
12:0	1	4.4
14:0	5.1	14.1
13:0 2OH	2.4	5.6
15:1 isoG	4.2	9.9
16:0	36.3	15.7
18:1 isoH	2.1	7.5
16:1 w7c/16:1 w6c	32.7	26
Summed in Feature 8	6.5	7.2

CO_2 Fixation

For strain BrT, there was a 6.7-fold increase in the amount of $H^{14}CO_3^-$ assimilated into organic matter in bottles inoculated with live cells relative to killed control bottles (Figure 3). Of this amount, about 87% of the ^{14}C activity was associated with the particulate fraction (trapped on a 0.22 μm filter after acidification) with only 13% in the dissolved phase. There was a slight increase in the ^{14}C activity of killed control bottles relative to initial (T_0) samples that may reflect sorption onto cells or Fe oxides, or the abiotic formation of iron-carbonate minerals such as siderite. In parallel (unlabelled) bottles, cell density increased from 4.1×10^5 cells ml^{-1} at the beginning of the experiment to 2.7×10^6 cells ml^{-1} after 5 days. Based on estimates of cellular C uptake calculated using the ^{14}C data, the concentration of non-labeled HCO_3^-, and a ^{14}C discrimination factor of 1.05, the uptake of HCO_3^- accounted for the C needed to support the observed cell growth [assuming 1.2×10^{-14} g C $cell^{-1}$; ref (Neubauer et al. 2002)].

Phylogeny

All 4 strains were *Betaproteobacteria*, and 2 of them represent new species. Strains Br-T, Br-1, and LD-1 clustered

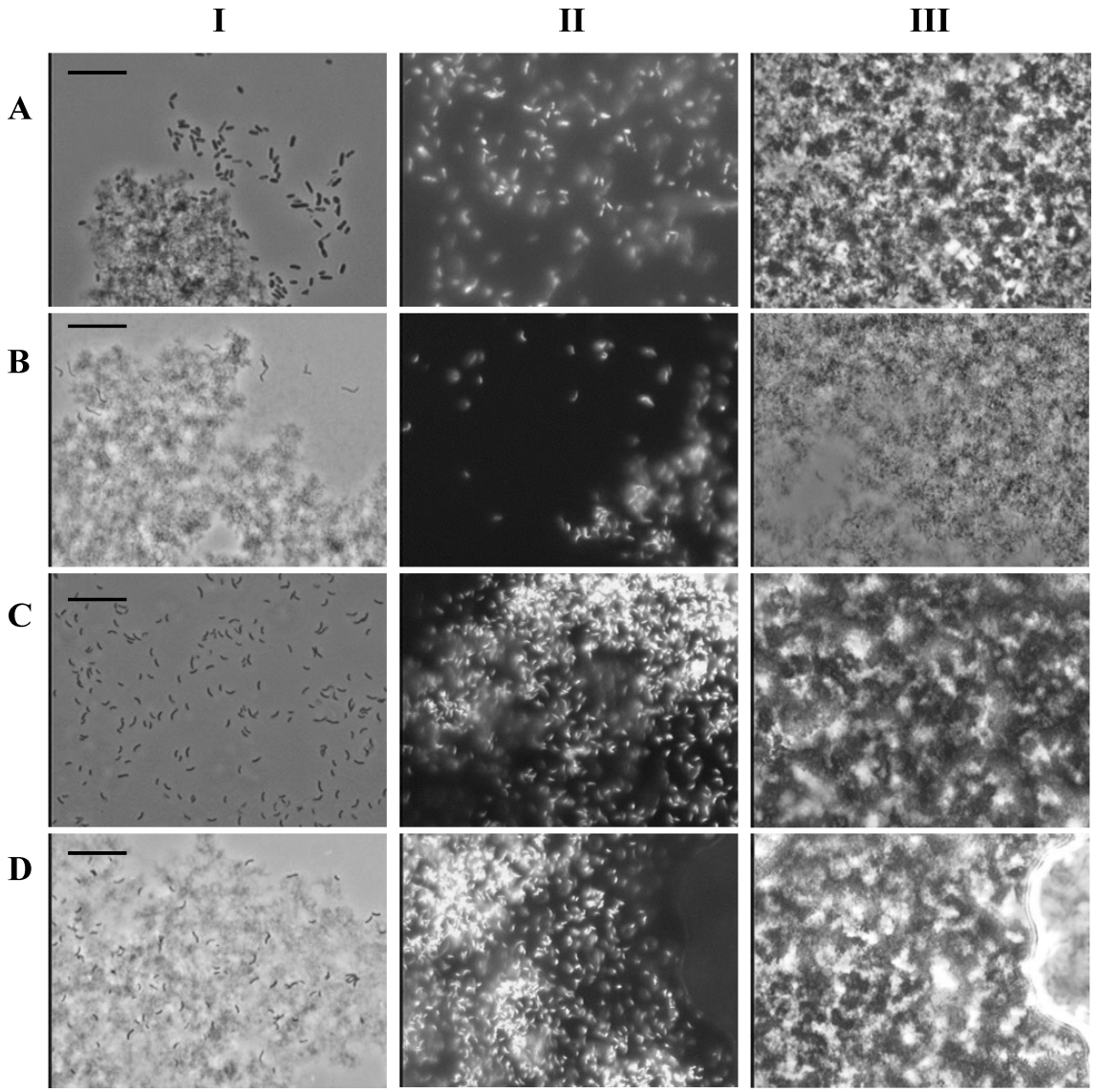

FIG. 1. Photomicrographs of rhizosphere Fe(II)-oxidizing bacteria. The 4 strains, CCJ, LD-1, Br-1, and BrT are represented by A, B, C, and D, respectively. The first panel shows free cells not trapped by the Fe(III) oxides, visualized by phase-contrast. Cells in the second panel, embedded in an agarose-Fe(III) matrix, are stained with SYTO and captured under epifluoresence. The third panel shows the images in Panel 2 under light microscopy. All samples were taken from 1-month-old gradient tubes and examined under 100x. Each bar in the first set of panels represents approximately 10 microns.

with previously described isolates of lithotrophic FeOB (Figure 4), including *Gallionella* spp. and the newly described genus *Sideroxydans* (Emerson et al. in press). Strains Br-T and Br-1 are essentially identical, both phenotypically, and by comparison of their SSU rRNA genes, and are proposed to comprise a new species, *Sideroxydans paludicola*. Strain LD-1 shares 98.1% sequence similarity with the SSU rRNA gene from the recently described *Sideroxydans lithotrophicus* and 94.2% sequence similarity with the *S. paludicola* SSU rRNA gene. Given that it appears physiologically and morphologically similar to *S. lithotrophicus,* which was isolated from groundwater (Emerson and Moyer 1997), we are designating it a new strain of this species. The other closely related phylogenetic relatives of these three strains are all environmental clones (Figure 5). The phylogenetic coherence of these organisms has led us to propose that they constitute a new order of *Betaproteobacteria*, the *Gallionellales*. We further propose that the family *Gallionellaceae* be transferred to the new order from the order *Nitrosomonadales*.

Strain CCJ was the most novel of the isolates. It was closely related to only one recently described 16S rRNA gene clone in Genbank, DQ337058, which came from subsurface water associated with the Kalahari Shield in South Africa. Different treeing algorithms (parsimony and maximum likelihood) created slightly different branching orders, but in every case CCJ clustered by itself (results not shown). Due to its phylogenetic

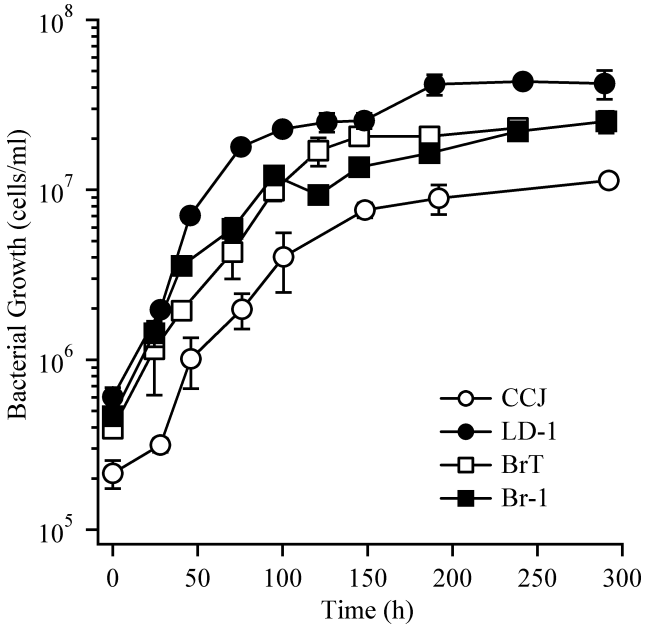

FIG. 2. Growth curves of rhizosphere Fe(III)-oxidizing bacteria. Each data point represents two harvested gradient tubes ± 1 standard deviation. Controls (inoculated tubes with no FeS), were within 5% of the cells numbers enumerated at Day 0 (data not shown).

novelty, as well as the fact that it is morphologically unique from the other strains of obligate, lithotrophic FeOB, we place it in a new order, the *Ferritrophicales*, which circumscribes the family *Ferritrophicaceae* and the genus as *Ferritrophicum*. Strain CCJ, which we have named *Ferritrophicum radicicola*, is the type strain of this genus.

tRFLP Results

Fragment sizes corresponding to all 4 of the Fe-oxidizing strains were present in both bulk soil and directly associated with roots of *Typha* based on the tRFLP analysis. Examples of restriction digests comparing roots and soil with *BstU I* and *HaeIII* are shown in Figure 6 and 6A, respectively. Both *in silico* analysis and comparison with pure cultures indicated that strains BrT, BR-1, and LD-1 had matching *BstU I* TRFs of 390 nt. Similarly, strain CCJ was predicted to have a TRF of 387 nt when cut with BstU. The *Hae III* digest could distinguish between Br-T/Br-1, TRF = 201 nt, and LD-1, TRF= 220 nt based on *in silico* analysis and digests of pure cultures. Again these correspond well with peaks in the environmental samples, Figure 6B. Likewise a peak in the *Hae III* digest that corresponded to strain CCJ, TRF = 217 nt, was also present. Any individual TRF may represent multiple genera, therefore the correspondence of an individual TRF with that of a known culture is at best presumptive evidence for its presence in an environmental sample (Marsh 2005).

Cutting with multiple restriction enzymes increases the confidence that an individual phylotype may be present in a sample. Overall, a minimum of three of the four restriction enzyme profiles displayed TRFs comparable to the FeOB described here. Digests with *Alu I* and *Hha I* also demonstrated similar correlations between expected and observed TRFs for the isolates and bulk soil and root microbial community analysis (data not shown). Other notable species matches included the FeOB *Sideroxydans lithtrophicus,* ES-1 and*Gallionella capsiferriformans,* ES-2 (Emerson et al. in press), as well as the *Betaproteobacterium,* TW-2 (Sobolev and Roden 2004).

FIG. 3. Fixation of $H^{14}CO_3^-$ in bottle cultures containing live or killed BrT cells. Particulate and dissolved C was separated by filtration through a 0.22 μm filter after the samples were acidified to remove inorganic C. For the initial samples, the live and killed treatments were similar and were therefore pooled for presentation. Error bars are ±1 standard deviation (n = 4 for initial samples, n = 2 for 5-day samples) and provide an estimate of variability for total ^{14}C uptake (particulate + dissolved).

DISCUSSION

Novel Strains of FeOB

The wetland rhizosphere appears to be an excellent habitat for circumneutral FeOB due to the interacting gradients of O_2 (from root radial O_2 loss) and Fe(II) (from anoxic soils), combined with high inorganic C concentrations produced by high rates of soil metabolism that are driven, in part, by the activity of the plants themselves (Neubauer et al. 2005). This group of Fe-plaque-associated bacteria was isolated from the roots of 4 different plant species from three separate wetlands. All strains are all obligate, lithotrophic Fe-oxidizers. The observation that they are unable to utilize other potential substrates that might be common in the rhizosphere (i.e., organic acids or reduced S-compounds) suggests they are uniquely adapted for Fe-oxidation in the rhizosphere.

Furthermore, the fact that *S. paludicola* strain BrT can take up sufficient $H^{14}CO_3^-$ to meet its cell carbon requirements suggests these FeOB are lithoautotrophs. This capacity for lithotrophic growth appears to be common across known circumneutral FeOB, including *Gallionella ferruginea* (Hallbeck and Pederson 1991), and organisms enriched from the wetland rhizosphere [this study, (Sobolev and Roden 2004)], groundwater springs (Emerson and Moyer 1997), and marine hydrothermal vents (Emerson and Moyer 2002; Edwards et al. 2003). Some of these organisms are also capable of heterotrophic or mixotrophic

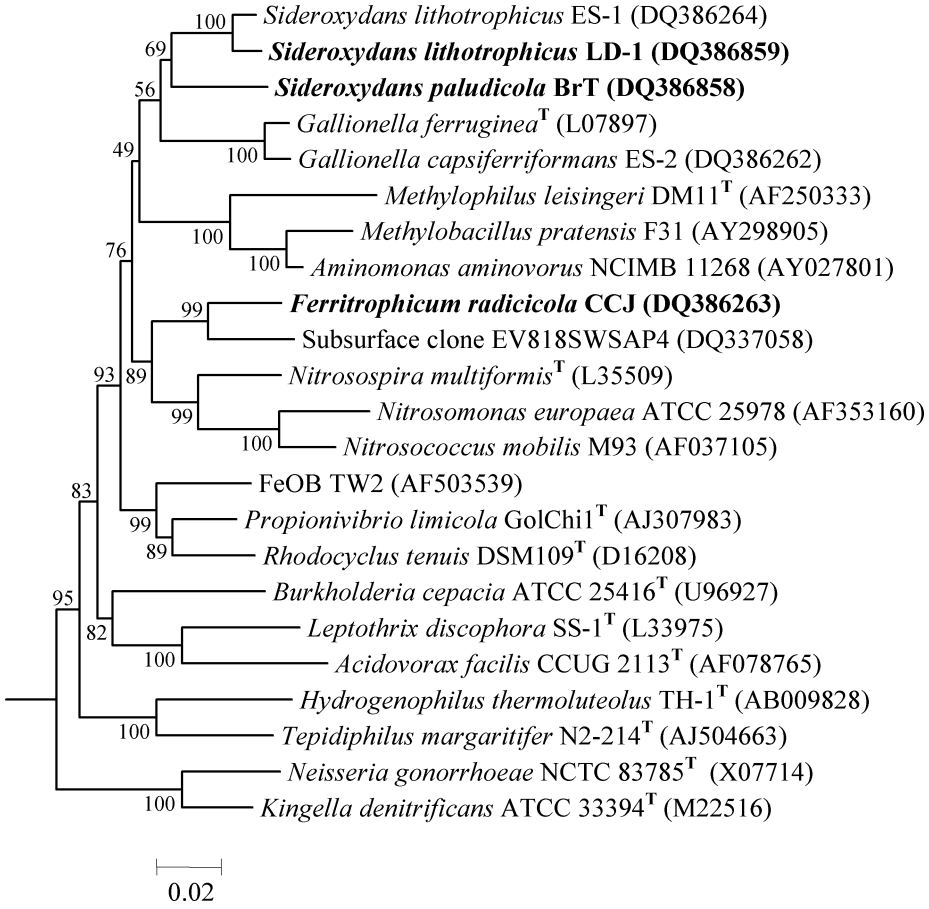

FIG. 4. Neighbor-joining dendrogram based on 16S rRNA gene sequences (~1388 bases) showing the position of *Ferritrophicum radicicola* CCJ (DQ386263), *Sideroxydans paludicola* BrT (DQ386858), and *Sideroxydans lithotrophicus* LD-1 (DQ386859) within the *Betaproteobacteria*. The tree was rooted with members of the *Gammaproteobacteria*, *Xanthomonas oryzae* LMG 5047-T^T (X95921) and *Pseudomonas aeruginosa* LMG 1242T^T (Z76651). Bootstrap support values (n = 1000 replicates) are shown at branch nodes and the bar represents 2 changes per 100 bases.

growth (Hallbeck and Pedersen 1991; Sobolev and Roden 2004), a trait that was not observed in our rhizosphere FeOB strains.

The fact that these four strains represent one new genus and two new species suggests that the obligate FeOB are phylogenetically diverse. A more comprehensive phylogenetic analysis based on the SSU rRNA gene suggests that freshwater, lithotrophic FeOB are primarily associated with the *Betaproteobacteria*. This includes all of our isolates, the newly described *Sideroxydans lithotrophicus* and *Gallionella capsiferriformans* (Emerson et al. in press), strain TW-2 (Sobolev and Roden 2004), and another recently described anaerobic, lithoautotrophic FeOB that couples Fe(II) oxidation to nitrate reduction (Weber et al. 2006).

Our phylogenetic analysis (Figure 4) indicates that *Sideroxydans* spp. and *Gallionella* spp. form a clade. This phylogenetic grouping is consistent with their physiological attributes, that is, they are all microaerophilic, lithotrophic Fe-oxidizers, and confirms that the order *Gallionellales* is comprised of neutrophilic FeOB. It is important to note, however, that in one respect these newly described species differ markedly from the prototypical iron-oxidizer, *G. ferruginea*; they do not produce helical stalk-like Fe-oxide structures. For these rhizosphere isolates the lack of a stalk may be advantageous, since they are growing in soil where a stalk is not necessary to maintain position in moving water, as it is for *G. ferruginea*. Furthermore, a stalk would only impede their ability to move through the dense soil matrix.

The environmental clones that fall within this clade are primarily associated with groundwater, aquifer sediments, or the rhizosphere of *Phragmites*, a common wetland plant (Alfreider et al. 2002; Bakermans and Madsen 2002; Brummer et al. 2003; Blute et al. 2004; Hallberg et al. 2006). While none of these clones are explicitly described as coming from Fe-oxidizing environments, the habitats in which they have been found are consistent with kinds of environments where microbial Fe-oxidation may be occurring. For example, we have observed Fe-plaque associated with *Phragmites* and shown that FeOB are present (Weiss et al. 2003). The phylogenetic similarity between the environmental clones and the FeOB described herein and elsewhere (Emerson et al. in press) suggests that these clones represent putative, uncultured FeOB.

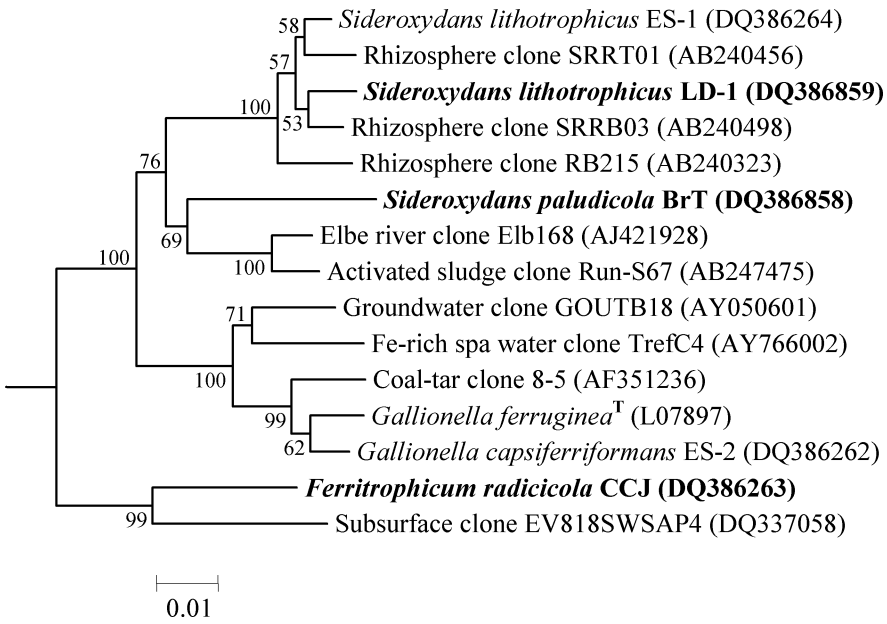

FIG. 5. Neighbor-joining dendrogram based on 16S rDNA sequences (~1388 bases) showing the relationship of environmental sequences to the FeOB described here (bold type). The tree was rooted with members of the order *Nitrosomonadales*, *Nitrosomonas europaea* ATCC 25978 (AF353160), *Nitrosococcus mobilis* M93 (AF037105), and *Nitrosospira multiformis*^{T} (L35509). Bootstrap support values (n = 1000 replicates) are shown at branch nodes and the bar represents 1 change per 100 bases.

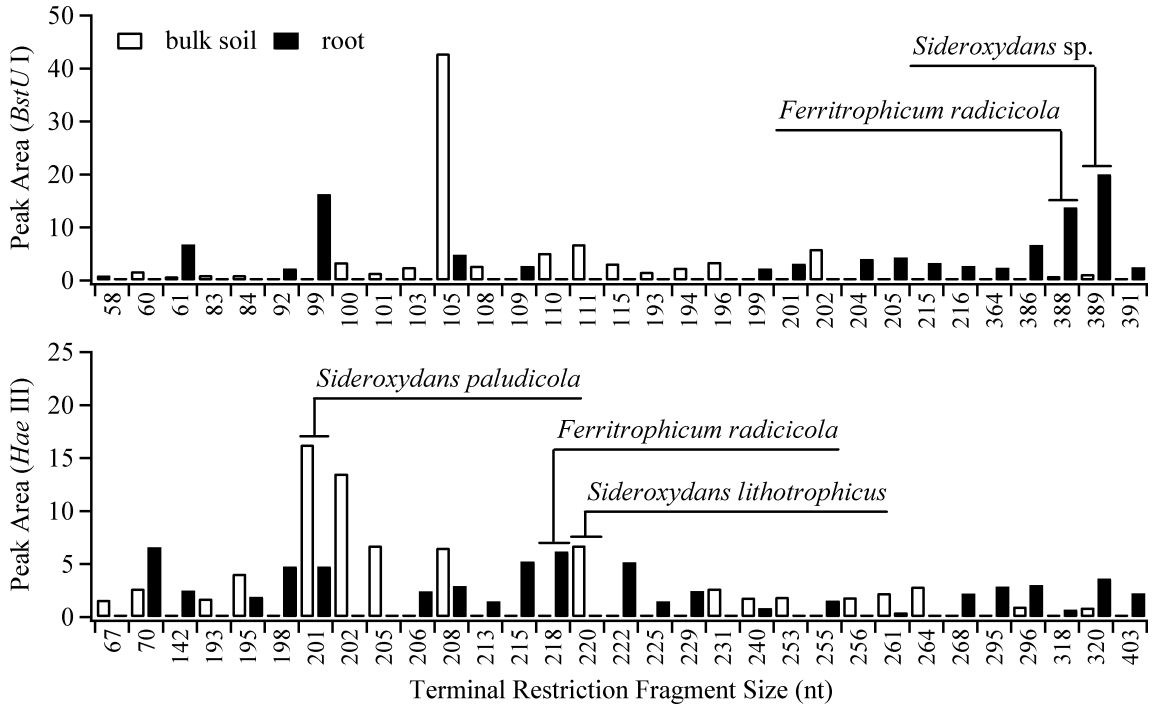

FIG. 6. *Typha latifolia* bulk soil and root tRFLP fragment data. Consensus terminal restriction fragments (TRFs) generated from T-Align analysis of bulk soil and root *BstU I* 16S tRFLP data (A) and *HaeIII* 16S tRFLP data (B) display the fragment size and peak area (relative fluorescence) for each TRF present in either bulk soil, root or both samples following phylogenetic assignments using the PAT algorithm and bin configurations. TRFs are rounded to the nearest whole nucleotide (nt).

Ferritrophicum radicicola, strain CCJ, is the most unusual of the new isolates by phylogeny, phenotype, and habitat. As mentioned above, because of its uniqueness, we propose that it be the type genus of a new order of *Betaproteobacteria*. Its closest relative, an uncultured SSU rRNA clone from groundwater, like the clones in the *Gallionellaceae*, comes from an environment where FeOB might well be present. Until more isolates or clones from specific Fe-rich habitats become available, it would be premature to state that this order consists solely of lithotrophic FeOB. *F. radicicola* is physiologically very similar to the other obligate, microaerophilic FeOB; however, its straight rod shape represents a morphological distinction. While *F. radicicola* is not an acidophile, it was isolated from a plant growing in an acid mine drainage system where the soil pH was ∼4 compared to the other isolates that were from more neutral habitats.

Microbial Role in Fe(II) Oxidation

Though iron plaque has long been considered the result of abiological Fe(II) oxidation, we have found a microbial community on the order of 10^7 cells g^{-1} dry weight root associated with the roots of wetland plants (Emerson et al. 1999; Weiss et al. 2003); based on cultivation studies, FeOB accounted for ∼1% of the total cell number (Weiss et al. 2003). Our analysis using tRFLP as a cultivation-independent method provides further evidence for the potential existence in the rhizosphere of FeOB closely related to the strains described here. The tRFLP results indicate that FeOB may be present both on the root surfaces and in the soil surrounding the roots. The finding that lithotrophic FeOB are present and potentially abundant in the rhizosphere and other environments leads to questions of how these organisms affect overall Fe(II) oxidation.

Although we presently do not have any methods to directly measure the *in situ* activity of rhizosphere FeOB, studies examining the role of the indigenous community and individual isolates on Fe(II) oxidation have also shown that FeOB may play a significant role, mediating up to 90% of the Fe(II) oxidation (Emerson and Revsbech 1994; Sobolev and Roden 2001). A study with strain BrT done in a bioreactor to specifically address the kinetics of neutrophilic, microaerophilic iron oxidation showed that its activity accounted for 18–53% of total Fe(II) oxidation, and that the cell yields could be accounted for by the amount of Fe(II) oxidized (Neubauer et al. 2002). Another study that used hydroponic plant microscosms to specifically examine the influence of FeOB on formation iron plaque found there was a positive correlation between the presence of the FeOB and the accumulation of Fe plaque (Neubauer, et al 2007). These studies, combined with environmental studies cited here, collectively illustrate the potential of FeOB to influence Fe(II) oxidation kinetics at circumneutral pH, especially when oxygen concentrations are very low, as is the case in the wetland plant rhizosphere.

Environmental Significance

Another important environmental role that unicellular FeOB may play in natural environments is to dramatically alter the dynamics of Fe(II) and O_2 consumption at the oxic-anoxic interface. When grown in gradient systems, as described here, the presence of FeOB commonly leads to a discrete zone of Fe(III) deposition and steeper oxygen gradients than in systems without FeOB (Sobolev and Roden 2001). A recent study also found the presence of FeOB led to a suboxic deposition of Fe(III) oxides, possibly due to the production of chelation compounds by the FeOB that prevented its deposition in the microaerophilic layer (Sobolev and Roden 2001). FeOB will deposit Fe(III) oxides in very close vicinity to or potentially within the anoxic zone where Fe(III) reduction then could occur (Weiss et al. 2004). The mineralogy of these Fe oxides is predominantly amorphous ferrihydrite which is the preferred substrate of Fe(III)-reducing bacteria (FeRB) (Lovley 2000). Thus, another important role that FeOB may play *in situ* is through participation in a microbially mediated Fe cycle. This will be especially important in the mosaic of aerobic and anaerobic microsites existing in the rhizosphere, resulting from both temporal and spatial variations of O_2 in this environment.

Because of the potential abundance of Fe plaque associated with the rhizosphere in a variety of wetlands (Chen et al. 1980; Gonzalez and Saiz-Jimenez 2002; Weiss et al. 2003), rhizosphere Fe(III) deposition could have important links with the wetland biogeochemical cycles of carbon, phosphorus, sulfur, manganese, and other trace metals. Specifically, Fe-oxides are well known for their reactivity. They can bind other elements, most notably P (Christensen and Wigand 1998; Blute et al. 2004; Weis and Weis 2004) and trace metals such as Cu, As, Zn, and Ni (Greipsson and Crowder 1992; Hansel et al. 2001; Neubauer et al. in press), thus affecting both plant nutrition and the mobility of potentially toxic elements.

Furthermore, the dominance of Fe(III) in the rhizosphere can affect other carbon-consuming processes such as methanogenesis. Competition between methane-oxidizing bacteria and FeOB for oxygen in the rhizosphere may lead to an increase in methane emissions. On the other hand, a rhizosphere Fe cycle where Fe(III) is continually being generated for potential Fe(III) reduction may lead to decreased methanogenesis because Fe(III)-reducing bacteria can outcompete methanogens for carbon in the rhizosphere (Roden and Wetzel 1996; Roden and Wetzel 2002; Neubauer et al. 2005). Expanding our knowledge of the microbes that influence plaque formation, especially FeOB, is therefore necessary to facilitate our understanding of other biogeochemical processes occurring in the wetland plant rhizosphere.

Description of Gallionellales ord. nov. *Gallionallales* (Gal.lion.el.lales). N.L. masc. n. *Gallionella* the type genus of the order; N.L. *-ales* ending denoting an order; N.L. masc. n. *Gallionellales* (the order of *Gallionellales*). Members of the order *Gallionellales* are Gram negative microaerophiles that can use ferrous iron as an energy source for chemolithotrophic growth, with CO_2 as the sole carbon source. The order includes 2 genera, *Gallionella* and *Sideroxydans*, and the following species: *Gallionella ferruginea, Gallionella capsiferriformans,*

Sideroxydans lithotrophicus, and *Sideroxydans palludicola*. The type genus is *Gallionella* (Ehrenburg, 1838). Phylogenetic analysis of SSU rRNA sequences indicates that these strains form a robust group, distinct from the *Nitrosomonadales* and other orders of *Betaproteobacteria*.

Description of Sideroxydans paludicola sp. nov. *Sideroxydans paludicola* (pa.lu.di.co'la. L. n. palus -udis, a swamp, marsh; L. suff. -cola (from L. n. incola), a dweller, an inhabitant; N.L. n. (nominative in apposition) paludicola, an inhabitant of marsh). Cells are short to medium length curved rods, approximately 0.42 μm in diameter that stain gram positive. The cells are motile. They grow exclusively on Fe(II) as an energy source and fix CO_2. The cells are microaerophilic and the best growth is with opposing gradients of Fe(II) and O_2. The doubling time is approximately 14 h.

The following substrates did not support growth: sulfide, elemental sulfur, thiosulfate, tetrathionate, H_2, formate, ammonia, galactose, pyruvate, acetate, succinate, glycerol, glucose, maltose, and ribose. The pH range for growth is between 4.5 and 7.0, and the temperature range for growth is 19°C–37°C. The predominant fatty acids are 16:0, 14:0, and 15:1 isoG. The mole % G+C is 63.4%. Source, Fe-plaque associated with the rhizosphere of wetland plants in a constructed wetland on Kent Island, Maryland. Two strains exist: strain BrT (ATCC BAA-1019; JCM 14765) and strain Br-1 (ATCC BAA-1017). The type strain is BrT^T.

Description of Ferritrophicales ord. nov. *Ferritrophicales* (Fer.ri.trophi.cales). N.L. masc. n. *Ferritrophicum* the type genus of the order; N.L. *–ales,* ending denoting an order; N.L. masc. n. *Ferritrophicales* (the order of *Ferritrophicales*).The order *Ferritrophicales* is circumscribed on the basis of phylogenetic analysis of the SSU rRNA gene of the type genus. The order contains the family *Ferritrophicaceae*. Description is the same as for the family *Ferritrophicaceae*. The type genus is *Ferritrophicum*.

Description of Ferritrophicaceae fam. nov. *Ferritrophicaceae* (Fer.ri.trophi.ca.ceae). N. L. masc. n *Ferritrophicum*, type genus of the family; suffix –aceae, ending to denote a family; N.L. fem. Pl. n. *Ferritrophicaceae*, the *Ferritrophicum* family. The description is the same as for the genus *Ferritrophicum*. The type genus is *Ferritrophicum*.

Description of Ferritrophicum gen. nov. *Ferritrophicum* (Fer.ri.tro.phi'cum. L. n. ferrum, iron; Gr. adj. trophikos, nursing, tending or feeding; N.L. neut. n. Ferritrophicum, one that feeds on iron, a bacterium that gains its energy from oxidizing ferrous iron). The cells are rod shaped and motile. Gram negative, belonging to the class *Betaproteobacteria*. They utilize ferrous iron as an energy source for lithotrophic growth. The cells are microaerophilic and the best growth is with opposing gradients of Fe(II) and O_2. The type species is *Ferritrophicum radicicola*.

Description of Ferritrophicum radicicola sp. nov. Ferritrophicum radicicola (ra.di.ci.co'la. radix -icis, a root of a plant; L. suff. -cola (from L. n. incola), a dweller, an inhabitant; N.L. n. (nominative in apposition) radicicola, an inhabitant of roots). Cells are short to medium length straight rods, approximately 0.9 μm in diameter that stain gram positive. The doubling time is approximately 11 h. The following substrates did not support growth: sulfide, elemental sulfur, thiosulfate, tetrathionate, H_2, formate, ammonia, galactose, pyruvate, acetate, succinate, glycerol, glucose, maltose, and ribose. The pH range for growth was between 4.5 and 7.0, and the temperature range for growth is 19°C–37°C. The predominant fatty acids are 16:0, 14:0, 15:1 isoG, and 18:1 isoH. The mole % G+C is 59%. Source Fe-plaque associated with the rhizosphere *Juncus effusus* growing along the creek bank of an acid mine site near Mineral, Virginia. The type strain is CCJ^T (ATCC BAA-1016; JCM 14764).

ACKNOWLEDGMENTS

We thank Paul Krader (ATCC) for the FAME analysis, and Jeff Benson and Jason Cooper of the molecular authentication resource center at the ATCC for assistance with tRFLP analysis. This work was funded by grants from the NASA Astrobiology Institute and the National Science Foundation (DEB-9985922 and DEB-0516197), the Jeffress Memorial Trust, an NSF Dissertation Improvement Grant to JW (DEB-0073080), and an American Association of University Women American Fellowship to JW.

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