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Diversity of Ferrous Iron-Oxidizing, Nitrate-Reducing Bacteria and their Involvement in Oxygen-Independent Iron Cycling

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In previous studies, three different strains (BrG1, BrG2, and BrG3) of ferrous iron-oxidizing, nitrate-reducing bacteria were obtained from freshwater sediments. All three strains were facultative anaerobes and utilized a variety of organic substrates and molecular hydrogen with nitrate as electron acceptor. In this study, analyses of 16S rDNA sequences showed that strain BrG1 was affiliated with the genus *Acidovorax*, strain BrG2 with the genus *Aquabacterium*, and strain BrG3 with the genus *Thermomonas*. Previously, bacteria similar to these three strains were detected with molecular techniques in MPN dilution series for ferrous iron-oxidizing, nitrate-reducing bacteria inoculated with different freshwater sediment samples. In the present study, further molecular analyses of these MPN cultures indicated that the ability to oxidize ferrous iron with nitrate is widespread amongst the *Proteobacteria* and may also be found among the Gram-positive bacteria with high GC content of DNA. Nitrate-reducing bacteria oxidized ferrous iron to poorly crystallized ferrihydrite that was suitable as an electron acceptor for ferric iron-reducing bacteria. Biologically produced ferrihydrite and synthetically produced ferrihydrite were both well suited as electron acceptors in MPN dilution cultures. Repeated anaerobic cycling of iron was shown in a coculture of ferrous iron-oxidizing bacteria and the ferric iron-reducing bacterium *Geobacter bremensis*. The results indicate that iron can be cycled between its oxidation states +II and +III by microbial activities in anoxic sediments.

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INTRODUCTION

Iron is the fourth most abundant element in the Earth's crust, and in most soils and sediments it is the dominant redox-active metal (Cornell and Schwertmann 1996). Acidophilic or neutrophilic bacteria that gain energy from the oxidation of ferrous iron with oxygen as electron acceptor are widespread and have been studied for many decades (reviewed by Blake and Johnson 2000; Emerson 2000). In contrast, only a few years ago it was recognized that ferrous iron can be oxidized also under anoxic conditions either by phototrophic or by nitrate-reducing bacteria (Widdel et al. 1993; Straub, Benz, Schink, and Widdel 1996). Several strains of lithotrophic ferrous iron-oxidizing phototrophs from freshwater and marine sediments were isolated and characterized (Widdel et al. 1993; Ehrenreich and Widdel 1994; Heising and Schink 1998; Heising, Richter, Ludwig, and Schink 1999; Straub, Rainey, and Widdel 1999). Isolation of lithotrophic nitrate-reducing ferrous iron oxidizers turned out to be more difficult: Although this activity was first described with a lithotrophic enrichment culture (Straub et al. 1996), all strains isolated from this culture oxidized ferrous iron only in the presence of an organic cosubstrate (strains BrG1 and BrG3), or the organic cosubstrate accelerated the oxidation of ferrous iron significantly (strain BrG2). Also two other bacterial strains (strain HidR2 and *Dechlorosoma suillum* strain PS) oxidize ferrous iron with nitrate only in the presence of an organic cosubstrate (Benz, Brune, and Schink 1998; Chaudhuri, Lack, and Coates 2001).

Enrichments and most-probable-number (MPN) studies with sediment samples from different locations showed that 1×10^5

to 5×10^8 ferrous iron-oxidizing nitrate reducers per ml were present in these sediments (Straub and Buchholz-Cleven 1998). In accordance with the findings described above, 10^2 to 10^5 times more mixotrophic than lithotrophic ferrous iron-oxidizing, nitrate-reducers were estimated, and bacteria similar to strains BrG1, BrG2, and BrG3 were repeatedly detected. However, these bacteria were not numerically dominant within the ferrous iron-oxidizing, nitrate-reducing community (Straub and Buchholz-Cleven 1998).

The potential for bacterial iron cycling between aerobic oxidation and anaerobic reduction was demonstrated at acidic and at neutral pH (Pronk and Johnson 1992; Johnson, Ghauri, and McGinness 1993; Sobolev and Roden 2002). In both cases a transition between oxic and anoxic incubation conditions was necessary. The discovery of anaerobic ferrous iron oxidation suggests that this process may be tightly coupled to ferric iron reduction in anoxic soils and sediments. It was already demonstrated that the ferrihydrite produced by ferrous iron-oxidizing, nitrate-reducing bacteria is a suitable electron acceptor for ferric iron-reducing bacteria (Straub et al. 1998).

In this study, we describe the physiological features and phylogenetic affiliations of strains BrG1, BrG2, and BrG3 in more detail. Molecular analyses of higher-dilution MPN cultures give first indications on some numerically more dominant ferrous iron-oxidizing bacteria. Furthermore, biologically produced ferrihydrite proved to be well suited as electron acceptor for dissimilatory ferric iron-reducing bacteria in MPN dilutions. With this material, anaerobic cycling of iron was demonstrated in a coculture experiment with ferrous iron-oxidizing, nitrate-reducing bacteria and the ferric iron-reducing bacterium *Geobacter bremensis*.

MATERIALS AND METHODS

Microorganisms

Strains BrG1, BrG2, BrG3, and *Geobacter bremensis* (DSM 12179) were from subcultures that had been kept in our laboratory since their isolation (Straub et al. 1996; Buchholz-Cleven, Rattunde, and Straub 1997; Straub, Hanzlik, and Buchholz-Cleven 1998; Straub and Buchholz-Cleven 2001). MPN cultures of ferrous iron-oxidizing, nitrate-reducing bacteria were obtained from MPN studies described before (Straub and Buchholz-Cleven 1998). A ferrous iron-oxidizing, nitrate-reducing enrichment culture had been kept in our laboratory since it was established (Straub et al. 1996).

Medium Composition and Growth Conditions

Techniques for preparation of media and cultivation of bacteria under anoxic conditions have been described (Widdel and Bak 1992). In the present study, a defined, bicarbonate-buffered freshwater medium was used; the medium contained per liter of distilled water 0.3 g of NH_4Cl, 0.05 g of $MgSO_4 \times 7H_2O$, 0.4 g of $MgCl_2 \times 6H_2O$, 0.6 g of KH_2PO_4, and 0.1 g of $CaCl_2 \times 2H_2O$. After autoclaving and cooling under an atmosphere of N_2—CO_2 (90:10), 30 ml $NaHCO_3$ solution (1M, autoclaved under CO_2), vitamins, an EDTA-chelated mixture of trace elements, and a selenite and tungstate solution were added (Widdel and Bak 1992). The pH was adjusted to 7.0. Cultures were incubated at 28°C in the dark. When cultures were grown with iron, tubes or bottles were incubated horizontally and shaken every other day to allow even distribution of bacteria and iron minerals (Straub et al. 1996).

Ferrous sulfate was added as electron donor from an anoxic stock solution. Fermented peptone was prepared as described (Widdel and Bak 1992). Sediment extract was obtained by shaking sediment on a rotary shaker at 4°C for five days. After centrifugation ($6,000 \times g$; 15 min), the supernatant was sterilized by filtration (0.2 μm pore diameter).

Enumeration of Ferrihydrite-Reducing Bacteria

Numbers of ferrihydrite-reducing bacteria were estimated in MPN dilution series with two different media containing 10 mM of either synthetically or biologically produced ferrihydrite as electron acceptor. In both media, acetate (2.5 mM) was supplied as electron donor and carbon source, and ferrous chloride (2 mM) as reducing agent.

Sieved and homogenized sediment samples obtained from Bremen (Germany, town ditch), Tübingen (Germany, pond), Carpi (Italy, ditch), and Perpignan (France, stream) were used as inocula for the MPN dilution series.

Three replicate ten-fold dilutions of sediment samples were prepared in each medium and incubated for 12 weeks at 28°C. Tubes were scored positive on the basis of production of ferrous iron; the numbers of ferrihydrite-reducing bacteria were calculated by standard procedures (de Man 1975).

Microbial and Synthetic Production of Ferrihydrite

A lithotrophic, nitrate-reducing enrichment culture oxidized ferrous iron to 2-line ferrihydrite (Straub et al. 1996, 1998). The produced ferrihydrite was washed 5 times with a 10-fold volume of distilled water to remove medium components. The resulting ferrihydrite suspension was deoxygenated by stirring under N_2 and repeated flushing of the atmospheric headspace in a tightly sealed flask. The suspension was autoclaved for 15 minutes and stored in the same tightly sealed flask under N_2. According to electron diffraction analysis, the pattern of the 2-line ferrihydrite was identical before and after autoclaving (M. Hanzlik, personal communication). Furthermore, *G. bremensis* reduced autoclaved ferrihydrite at the same rate as ferrihydrite that was not autoclaved (K. L. Straub, unpublished data).

Synthetic ferrihydrite was produced as described by Lovley and Phillips (1986) and characterized by electron diffraction analysis. The suspension of synthetically produced ferrihydrite was deoxygenated, sterilized, and stored as described above.

Analytical Methods

Ferrous iron was quantified photometrically at 510 nm wavelength after chelation with 2 mM *o*-phenanthroline in 0.7 M sodium acetate buffer, pH 5, in a test volume of 1 ml as described before (Straub et al. 1996).

Nitrate, nitrite, and benzoate were measured by high performance liquid chromatography as described by Rabus and Widdel (1995a, 1995b).

Ammonium ions were quantified using the indophenol formation reaction (Greenberg, Clesceri, and Eaton 1992).

N_2 was detected by gas chromatography (detection by thermal conductivity) of cultures grown under a headspace of He—CO_2 (90:10).

16S rDNA Sequence Determination, Phylogenetic Analyses, and DNA Base Composition

Genomic DNA was extracted from freshly grown MPN cultures, 16S rDNA was amplified, and PCR products were sequenced using the same primers and methods as described previously (Buchholz-Cleven et al. 1997; Straub and Buchholz-Cleven 1998). Phylogenetic analyses were performed using the ARB software package (Ludwig et al. 2004). For tree reconstruction, distance matrix, maximum-parsimony, and maximum-likelihood methods with different filters were applied as implemented in ARB and the resulting trees were combined for a consensus tree as suggested by Ludwig et al. (1998).

The G+C content of DNA was determined by HPLC (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences obtained from MPN cultures are deposited in the GenBank database under the accession numbers AY521560 for DGGE-type D, AY521561 for DGGE-type E, AY521563 for DGGE-type F, and AY521562 for DGGE-type G, respectively.

RESULTS AND DISCUSSION

Bacterial Oxidation of Ferrous Iron with Nitrate

Oxidation of ferrous iron with nitrate as electron acceptor was first observed in lithoautotrophic enrichment cultures inoculated either with brackish water sediment from the Baltic Sea, or with freshwater sediments from town ditches in Bremen (Straub et al. 1996). From the latter lithotrophic enrichment culture, strains BrG1, BrG2, and BrG3 were isolated in agar dilution series (Straub et al. 1996; Buchholz-Cleven et al. 1997). However, ferrous iron oxidation by these strains was not as rapid as in the enrichment culture, and was stimulated by addition of organic substrates at low concentrations. All attempts to isolate strains that oxidize ferrous iron lithoautotrophically as fast as the enrichment culture failed although we tried to stimulate growth by addition of further vitamins (lipoic acid, folic acid, riboflavin), 2-mercaptoethane sulfonate, reducing agent (0.5 or 2 mM cysteine), yeast extract (0.005%), fermented peptone (0.005%), or sediment extract (5%). It remains unclear how ferrous iron oxidation is stimulated in the lithotrophic enrichment culture since, e.g., homoacetogenic acetate synthesis would require an electron source of a redox potential considerably lower than that of the $Fe^{2+}/Fe(OH)_3$ couple (Schink 1999).

Rapid and complete ferrous iron oxidation was observed only within a narrow pH range (pH 6.4 to 6.7). Furthermore, the phosphate concentration in the medium appeared to be crucial. Upon addition of ferrous sulfate (10 mM) to the medium, white ferrous precipitates formed which presumably consisted of ferrous carbonates and ferrous phosphates. With 30 mM bicarbonate and 4.4 mM phosphate in the medium, 4 mM ferrous iron was found in the filtrate after filtration through 0.2 μm filters. If the phosphate concentration in the medium was low (0 or 2 mM), only little ferrous iron phosphate precipitated and the filtered medium contained 6 to 6.5 mM ferrous iron. If the phosphate concentration in the medium was high (6 or 8 mM), more ferrous phosphate precipitates formed and the filtrate contained only 0.7 to 1.7 mM residual ferrous iron. Under both conditions, with high or low phosphate concentration, ferrous iron oxidation was strongly impaired. Low rates of ferrous iron oxidation were also observed in media with low (10 mM) bicarbonate concentrations that contained 5.5 mM filterable ferrous iron. Modifications of the ammonium, calcium, or sulfate concentrations had no effect on ferrous iron oxidation. These findings may indicate that iron oxidation in these cultures depended on a delicate balance between availability and toxicity of ferrous iron. On the other hand, limitation of phosphate and/or bicarbonate could be another plausible explanation for the observations.

After numerous transfers, the enrichment culture showed growth only with nitrate, nitrite, or dinitrogen oxide as electron acceptors; no growth was observed with ferrihydrite, sulfate, sulfite, thiosulfate, elemental sulfur, fumarate, or malate in the presence of acetate as electron donor.

Physiological Characterization of Strains BrG1, BrG2, and BrG3

Although strains BrG1, BrG2, and BrG3 were obtained from a strictly lithoautotrophic ferrous iron-oxidizing, nitrate-reducing enrichment culture, they oxidized ferrous iron only in the presence of an organic cosubstrate (strains BrG1 and BrG3), or the presence of an organic cosubstrate accelerated the oxidation of ferrous iron significantly (strain BrG2; Straub et al. 1996; Buchholz-Cleven et al. 1997). However, ferrous iron oxidation in growth experiments ceased if electrons from organic cosubstrates exceeded those from ferrous iron by a factor of 10. Continuous cultivation of all three strains in the presence of ferrous iron was possible and turned out to be necessary: after strains were subcultivated for some transfers on organic substrates only, they lost the capacity to oxidize ferrous iron. This observation may indicate that the ferrous iron-oxidizing enzyme

Table 1
Comparison of physiological and biochemical features

Feature[a]	Strain BrG1	Strain BrG2	Strain BrG3
Reduction of:			
O_2, NO_3^-, NO_2^-,	+[b]	+[b]	+
N_2O	+	+	−
Ferrihydrite (synthetic or biological)	−	−	−
Malate, fumarate	−	−	−
Sulfate, S°	−	−	−
Cell shape and size (μm)	Rod; 2 × 0.6[b]	Slightly curved rod; 1.5 × 0.6[b]	Rod; 2.5 × 0.6
Temperature range and optimum (°C)	6–40; 28–35	6–40; 28	6–40; 32–35
pH range with ferrous iron[c]	6.7	6.4–6.7	6.7
pH range and optimum with acetate[d]	6–8.5; 6.7–7.5	6–8.5, 7–7.5	6–9; 7-8
NaCl concentration (% w/v)	≤1.5	≤1	≤1.5
Vitamin requirement	B_{12}	None	B_{12}
G+C content (mol%)	66	64	69

[a] Symbols: +, utilized; −, not utilized.
[b] Data from Straub et al. 1996.
[c] In the presence of acetate (0.5 mM) as an organic cosubstrate.
[d] Acetate as the only electron donor and carbon source.

system of these strains may be plasmid-coded in parts or even in its entirety. However, neither the biochemistry nor the genetics of ferrous iron oxidation in these strains have been studied. With regard to the pH range and optimum, the nitrate-reducing strains behaved similar to ferrous iron-oxidizing phototrophs (Straub et al. 1999): with acetate as electron donor, growth was possible within a much broader pH range (approximately pH 6 to 9, Table 1) than with ferrous iron as electron donor. Ferrous iron oxidation occurred only at pH values of approximately 6.7, similar as observed with the enrichment culture (Table 1).

All three strains were also able to oxidize molecular hydrogen and a variety of organic substrates (Table 2). They also grew aerobically under air. Nitrate, nitrite, and dinitrogen oxide (strains BrG1 and BrG2 only) were reduced to molecular nitrogen; ammonium was not formed. No strain was able to use in turn the produced ferrihydrite as electron acceptor. Further physiological and biochemical features of the isolated strains are summarized in Table 1.

Phylogenetic Affiliation of Strains BrG1, BrG2, and BrG3

At the time of isolation, the phylogenetic affiliations of strains BrG1, BrG2, and BrG3 were either unclear or the strains represented new lines of descent within the beta- and gamma-subgroup of *Proteobacteria* (Buchholz-Cleven et al. 1997). In the meantime some new species, genera, and families have been described that allowed us to define the phylogenetic positions of the three strains.

According to 16S rDNA sequence analyses, strain BrG1 is affiliated with the genus *Acidovorax* which belongs to the family *Comamonadaceae* within the beta-subgroup of *Proteobacteria* (Figure 1; Wen, Fegan, Hayward, Chakraborty, and Sly 1999). Strain BrG1 exhibits the highest 16S rDNA sequence similarity (98.8%) to *A. temperans*. Described strains of *A. temperans* have several features in common with strain BrG1 including morphology, utilization of organic electron donors, reduction of

Table 2
Comparison of organic compounds tested as electron donors for growth with nitrate as the electron acceptor[a]

Compounds (mM)[b]	Strain BrG1	Strain BrG2	Strain BrG3
Acetate (5, 10)	+[c]	+[c]	+
Propionate (2, 5)	+[c]	+[c]	−
Butyrate (2, 5)	+	+	−
Valerate (2, 3)	+	+	+
Pyruvate (5, 10)	+	+	+
Lactate (5, 10)	+[c]	−[c]	−
Malate, succinate, fumarate (5, 10)	+[c]	+[c]	−
Citrate (5, 10)	+	−	−
Aspartate, glutamate (4)	+	−	+
Alanine (4)	+	−	−
Serin (4)	−	−	+
Glucose, maltose (2, 5)	−	−	+
Ethanol, 1-propanol (5, 10)	+	+	−

[a] Symbols: +, utilized; −, not utilized.
[b] Further organic compounds tested but not utilized as electron donors by any strain: formate (5, 10), benzoate (1, 3), ribose (2, 5), xylose (2, 5), fructose (2, 5), saccharose (1, 3), cellobiose (1, 3), methanol (5, 10), 2-propanol (5, 10), acetone (5, 10).
[c] Data from Straub et al. 1996.

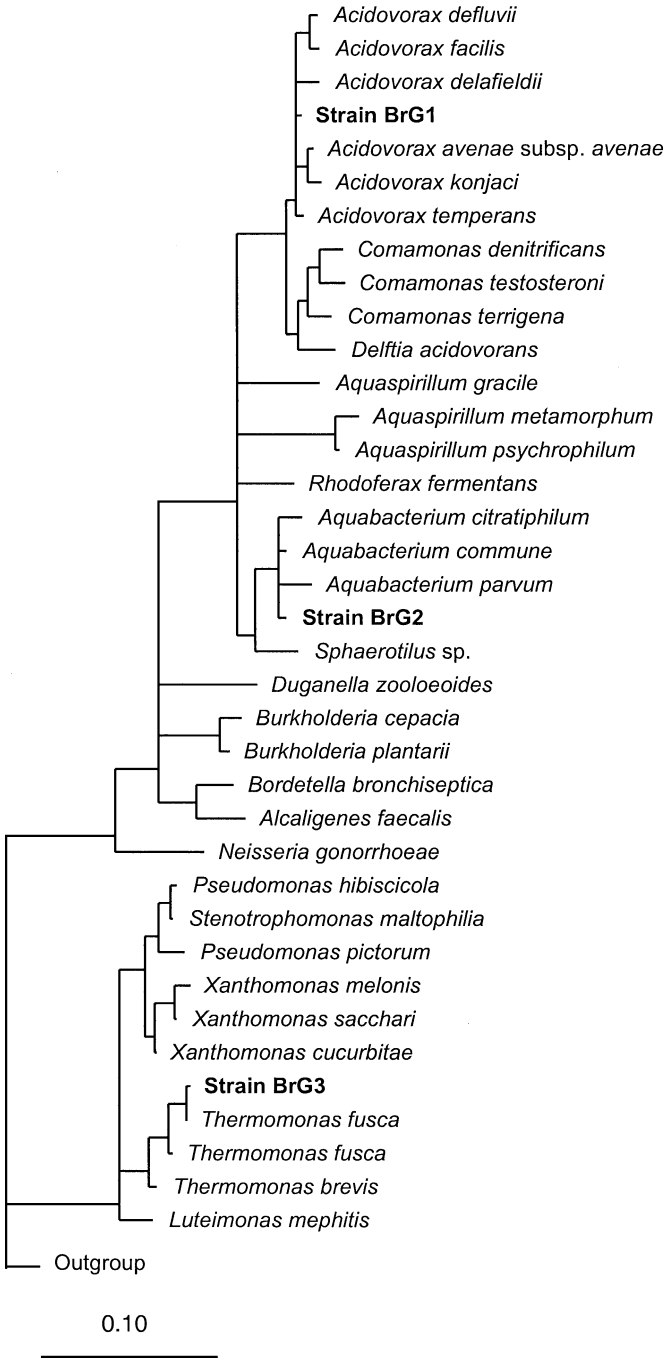

Figure 1. Phylogenetic consensus tree based on 16S rDNA sequence comparisons showing the affiliation of ferrous iron-oxidizing, nitrate-reducing strains BrG1, BrG2, and BrG3 within the beta- and gamma-subgroup of *Proteobacteria*. The outgroup comprises 117 bacterial 16S rDNA sequences. Bar represents 0.10 estimated changes per nucleotide.

nitrate and nitrite to dinitrogen, temperature range, NaCl tolerance, and G+C content of DNA (Willems et al. 1990). In the last few years bacteria belonging to the genus *Acidovorax* were isolated or detected by molecular techniques in various environments, including a municipal wastewater treatment plant, a bioreactor for denitrification of drinking water, soils, and sediments (Willemsen et al. 1990; Schulze et al. 1999; Schloe et al. 2000; Song, Palleroni, and Häggblom 2000). It is not known whether cultivated strains of the genus *Acidovorax* or strains of *A. temperans* are capable of ferrous iron oxidation.

Also strain BrG2 is affiliated with the beta-subgroup of *Proteobacteria* (Figure 1). Closest relatives were species of the recently described genus *Aquabacterium*, with approximately 98% sequence similarity of the 16S rDNA genes (Kalmbach, Manz, Wecke, and Szewzyk 1999). Like strain BrG2, *A. citratiphilum*, *A. commune*, and *A. parvum* were able to grow by denitrification with a similar variety of organic electron donors. All *Aquabacterium* species grow at moderate temperatures, tolerate 0.4 to 1.8% NaCl and have a G+C content of 65–66 mol% (Kalmbach et al. 1999). *A. citratiphilum*, *A. commune*, and *A. parvum* were all isolated from a drinking water house installation system. *Aquabacterium*-like bacteria were detected with molecular techniques also in hyporheic microbial communities in rivers of the Western United States (Feris et al. 2003). It is not known whether any other strain of the genus *Aquabacterium* can oxidize ferrous iron.

Strain BrG3 is related to the genus *Thermomonas* within the gamma-subgroup of *Proteobacteria* which was described only recently (Figure 1; Busse et al. 2002). The type species of this genus, *T. haemolytica*, grows optimally at temperatures between 37 and 50°C, and does not reduce nitrate. However, two further members of this genus, *T. brevis* and *T. fusca*, were isolated from a denitrification reactor in the meantime. Like strain BrG3, both species grow at moderate temperatures and reduce nitrate or nitrite (Mergaert, Cnockaert, and Swings 2003). 16S rDNA sequences of *T. fusca* and strain BrG3 share 99.6% sequence similarity. Their morphology and G+C content of DNA are similar. The ability of *Thermomonas* species to oxidize ferrous iron was not yet tested.

Phylogenetic Analyses of MPN Cultures

A first study on the abundance of ferrous iron-oxidizing, nitrate-reducing bacteria showed that type BrG1, BrG2, and BrG3 bacteria could be enriched from several sediment samples. However, analysis of MPN dilution series by denaturing gradient gel electrophoresis (DGGE) followed by hybridization with specific probes showed that the three strains did not constitute the numerically dominant group amongst the ferrous iron-oxidizing, nitrate-reducing microorganisms (Straub and Buchholz-Cleven 1998). In particular in higher-dilution MPN tubes (10^4 to 10^8), the 16S rDNA segments differed in electrophoretic mobility from the segments obtained from strains BrG1, BrG2, and BrG3 which were previously described as DGGE-type A, B, and C, respectively (Buchholz-Cleven et al. 1997). Some higher-dilution MPN tubes were further analyzed for the present study. According to the differences in electrophoretic mobility, four further types (DGGE-type D, E, F, and G) were assigned. DNA was extracted from four MPN cultures (inoculated with sediment

Table 3
Similarities of 16S rDNA sequences derived from MPN cultures of ferrous iron-oxidizing, nitrate-reducing bacteria

DGGE-type[a]	Sequence length (bp)	Similarity of 16S rDNA sequences (accession number)
D	1490	93% to *Pelobacter propionicus* (X70954)
E	1487	94% to *Geobacter* sp. strain CdA-2[c] (Y19190)
F	541[b]	99% to *Nocardioides* sp. strain JS614[d] (AF498652)
G	1421	98% to *Bradyrhizobium japonicum* (AF530468)

[a] According to the electrophoretic mobility of 16S rDNA segments in DGGE gels. Strains BrG1, BrG2, and BrG3 represent DGGE-types A, B, and C, respectively (Buchholz-Cleven et al. 1997).
[b] Corresponding to *Escherichia coli* positions 358-906 (Brosius, Dull, Sleeter, and Noller 1981).
[c] Cummings et al. 2000.
[d] Coleman, Mattes, Gossett, and Spain 2002.

samples taken in Bremen) that showed only a single new type of band in the DGGE analysis, and the 16S rDNA genes were amplified and sequenced. Comparison of the 16S rDNA sequences showed an affiliation of DGGE-type D and E with members of the family *Geobacteraceae* of the delta-subgroup of *Proteobacteria* (Table 3). Many members of this family are known to reduce ferrihydrite, but none is known to oxidize ferrous iron with nitrate. However, several species of acidophilic bacteria have been described that catalyze both the (aerobic) oxidation and the anaerobic reduction of iron (reviewed by Blake and Johnson 2000). In addition, *Desulfitobacterium frappieri* strain G2 was recently reported to reduce ferrihydrite and oxidize ferrous iron with nitrate at circumneutral pH values (Shelobolina, Vanpraagh, and Lovley 2003). Therefore, it may as well be possible to find members amongst the *Geobacteraceae* that catalyze both, iron oxidation and iron reduction, in particular as nitrate is used as electron acceptor by some of these strains.

A partial sequence (541 bp) of DGGE-type F was almost identical to a 16S rDNA sequence of a strain belonging to the family *Nocardioides* of Gram-positive bacteria with high GC content of DNA (Table 3). The 16S rDNA sequence of DGGE-type G showed a high similarity to *Bradyrhizobium* species of the alpha-subgroup of *Proteobacteria* (Table 3). As *Bradyrhizobium* species are members of the microbial community associated with plant roots, this finding may be of interest with respect to microbial oxidation of ferrous iron observed in the rhizosphere.

Assuming that the 16S rDNA sequences analyzed were actually derived from the metabolically active, ferrous iron-oxidizing bacteria of the MPN cultures, our results indicate that the ability to oxidize ferrous iron with nitrate as the electron acceptor is widespread among bacteria.

Anaerobic Microbial Cycling of Iron

In a previous study it was shown that ferrous iron-oxidizing, nitrate-reducing bacteria oxidized ferrous iron to poorly crystallized 2-line ferrihydrite. Furthermore, biologically produced ferrihydrite was found to be a suitable electron acceptor for species of the genus *Geobacter* which completely reduced it to the ferrous state (Straub et al. 1998). To further investigate the suitability of the biologically produced ferrihydrite as electron acceptor, medium for MPN dilutions was supplied with either biologically or synthetically produced ferrihydrite. These two media were inoculated in parallel with sediment samples from four different European locations. In all these sediments, the acetate-oxidizing, ferrihydrite-reducing community constituted approximately 10% of the total number of bacteria; this ratio was determined by comparing MPN numbers with DAPI counts of total cell numbers (Straub and Buchholz-Cleven 1998). The estimates were higher in MPN dilutions supplied with biologically produced ferrihydrite than in MPN dilutions supplied with synthetically prepared ferrihydrite. The differences were statistically significant for two (Bremen and Perpignan) of the four samples (Table 4). Hence, both ferrihydrite preparations are well suited to serve as electron acceptor for dissimilatory ferric iron-reducing bacteria; the biologically produced ferrihydrite may even be the better.

Coculture experiments were performed to further investigate the potential for microbial anaerobic cycling of iron. For these experiments, the lithoautotrophic ferrous iron-oxidizing, nitrate-reducing enrichment culture and *Geobacter bremensis* were

Table 4
Numbers of ferric iron-reducing bacteria per g dry weight of sediment[a]

	Estimation with	
Source of sediment	biologically produced ferrihydrite	synthetically produced ferrihydrite
Bremen	1.2×10^9 (6.0×10^8–8.4×10^9)	9.0×10^7 (3.0×10^7–3.1×10^8)
Tübingen	1.6×10^9 (6.4×10^8–7.7×10^9)	6.4×10^8 (3.2×10^8–4.5×10^9)
Carpi	7.5×10^7 (3.7×10^7–3.9×10^8)	3.9×10^7 (1.5×10^7–1.2×10^8)
Perpignan	3.3×10^8 (1.7×10^8–2.3×10^9)	3.3×10^6 (1.7×10^6–2.3×10^7)

[a] The values in parentheses are the 95% confidence limit values.

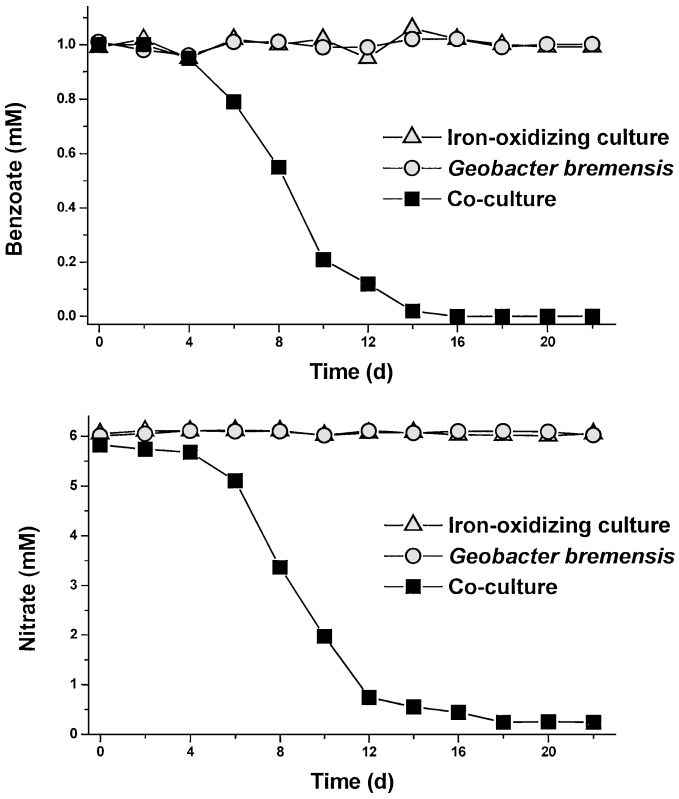

Figure 2. Oxidation of benzoate with nitrate as the electron acceptor occurs only in the coculture of ferrous iron-oxidizing and ferric iron-reducing bacteria, additionally supplied with 5 mM biologically produced ferrihydrite. Data are from representative cultures.

chosen. The ferrous iron-oxidizing, nitrate-reducing culture was unable to oxidize benzoate with nitrate (Figure 2); in a control experiment, 1 mM benzoate was shown not to be toxic. Furthermore, it is known from a previous study that nitrate is reduced to dinitrogen (Straub et al. 1996). In contrast, *G. bremensis* known to oxidize benzoate with ferrihydrite was not able to oxidize benzoate with nitrate as electron acceptor nor to ferment benzoate (Figure 2; Straub et al. 1998). In a further control experiment, 6 mM nitrate was found not to be toxic for *G. bremensis*. The medium for the coculture experiment was supplied with 1 mM benzoate, 6 mM nitrate and 5 mM biologically produced ferrihydrite. After inoculation with both cultures (2% inoculum each), benzoate was completely oxidized with nitrate in 16 days (Figure 2). In controls with pasteurized inoculum neither benzoate oxidation nor nitrate reduction was observed (data not shown). Complete oxidation of 1 mM benzoate yields 30 mM electrons while for reduction of 6 mM nitrate to dinitrogen 30 mM electrons are needed. Thus, iron changed in the coculture experiment approximately 6 times between the oxidized and the reduced state. Although such laboratory studies are always somehow artificial and cannot replace in situ investigations they demonstrate that anaerobic microbial iron cycling is possible and may be of major importance in the complex electron flow network in anoxic environments.

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