The ISME Journal (2010) 4, 267–278

www.nature.com/ismej

ORIGINAL ARTICLE

Identification of iron-reducing microorganisms in anoxic rice paddy soil by ^{13}C-acetate probing

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In anoxic rice field soil, ferric iron reduction is one of the most important terminal electron accepting processes, yet little is known about the identity of iron-reducing microorganisms. Here, we identified acetate-metabolizing bacteria by RNA-based stable isotope probing in the presence of iron(III) oxides as electron acceptors. After reduction of endogenous iron(III) for 21 days, isotope probing with ^{13}C-labeled acetate (2 mM) and added ferric iron oxides (ferrihydrite or goethite) was performed in rice field soil slurries for 48 and 72 h. Ferrihydrite reduction coincided with a strong suppression of methanogenesis (77%). Extracted RNA from each treatment was density resolved by isopycnic centrifugation, and analyzed by terminal restriction fragment length polymorphism, followed by cloning and sequencing of 16S rRNA of bacterial and archaeal populations. In heavy, isotopically labeled RNAs of the ferrihydrite treatment, predominant ^{13}C-assimilating populations were identified as *Geobacter* spp. (~85% of all clones). In the goethite treatment, iron(II) formation was not detectable. However, *Geobacter* spp. (~30%), the δ-proteobacterial *Anaeromyxobacter* spp. (~30%), and novel *β-Proteobacteria* were predominant in heavy rRNA fractions indicating that ^{13}C-acetate had been assimilated in the presence of goethite, whereas none were detected in the control heavy RNA. For the first time, active acetate-oxidizing iron(III)-reducing bacteria, including novel hitherto unrecognized populations, were identified as a functional guild in anoxic paddy soil.

The ISME Journal (2010) **4,** 267–278; doi:10.1038/ismej.2009.100; published online 24 September 2009
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: RNA-based stable isotope probing; iron(III)-reducing microorganisms; rice paddy soil; *Geobacter*, *Anaeromyxobater*

Introduction

The degradation of organic matter in flooded rice field soils is accomplished by several guilds of microorganisms that operate the anaerobic microbial food chain (Conrad, 1996; Schink, 1997). Most of the electrons from substrate oxidation flow into methanogenesis as predominant terminal reduction processes in rice paddies; however, iron oxide reduction is the second most important electron sink (Yao *et al.*, 1999). Conceptually, iron oxide reduction occurs directly after flooding of rice field soil, and at interfaces such as water–soil and the rhizosphere, in which oxygen diffusion fuels the re-oxidation of iron(II) in steep chemical gradients (Conrad and Frenzel, 2002; Conrad, 2007). After flooding, oxidants (oxygen > nitrate > sulfate and iron(III) oxides) are reduced sequentially according to the thermodynamic theory (Ponnamperuma, 1972; Patrick and Reddy, 1978). In many rice field soils, the duration of the iron reduction phase determines the onset of methanogenesis, whereas in iron-rich acidic sulfur soils methanogenesis might not commence at all because of the presence of oxidants (that is nitrate, sulfate, iron(III) oxides) (Yao *et al.*, 1999). The suppression of methanogenesis most likely originates from outcompetition of methanogens by iron-reducing microorganisms for common electron donors (Achtnich *et al.*, 1995a; Chidthaisong and Conrad, 2000), but direct inhibition of methanogens by ferric iron is also discussed (van Bodegom *et al.*, 2004). Accordingly, addition of weakly crystalline ferric iron oxides (that is ferrihydrite) effectively suppresses methanogenesis (Achtnich *et al.*, 1995a; Jäckel and Schnell, 2000;

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Received 23 March 2009; revised 6 July 2009; accepted 30 July 2009; published online 24 September 2009

Lueders and Friedrich, 2002; Qu *et al.*, 2004), whereas more crystalline iron oxides such as lepidocrocite, goethite, and hematite are less effective (Qu *et al.*, 2004). Iron fertilization has been suggested as a strategy for mitigation of methane emission from rice paddies (Furukawa and Inubushi, 2002; Jäckel *et al.*, 2005). However, the biogeochemical details of microbial iron cycling and methane suppression in rice field soils are neither well known nor are the microorganisms involved (Conrad, 2007). Compared with the numerically abundant polymer hydrolyzing and fermenting microorganisms in rice field soil (up to 10^{10} cells g^{-1} dry weight soil), dissimilatory iron-reducing populations are three orders of magnitude smaller, but still significantly more abundant (one order) than methanogens (Frenzel *et al.*, 1999). Potentially involved in iron reduction are *Geobacter* spp. and *Anaeromyxobacter* spp., which were detected by molecular surveys in bulk soil, on rice roots, or were retrieved by cultivation (Hengstmann *et al.*, 1999; Treude *et al.*, 2003; Scheid *et al.*, 2004). However, dissimilatory iron reduction has so far not been directly linked to specific populations in rice field soil.

A direct way of linking identity of microorganisms to a specific function is stable isotope probing (SIP) of nucleic acids (Radajewski *et al.*, 2000), and in particular of RNA (Manefield *et al.*, 2002). Nucleic acid SIP capitalizes on the incorporation of heavy stable isotopes (^{13}C, ^{15}N, ^{18}O) into RNA (or DNA), the physical separation of labeled, isotopically 'heavy' RNA from unlabeled, 'light' RNA, and subsequent identification of actively label incorporating populations by cloning and sequencing of 'heavy' RNA. In rice field soil, the known iron-reducing bacteria *Geobacter* spp. and *Anaeromyxobacter* spp. were recently found by SIP to assimilate ^{13}C-labeled acetate in the methanogenic phase that is when the iron reduction phase had already ceased and presumably easily bio-reducible, amorphous iron(III)oxides were not available (Hori *et al.*, 2007).

Crystalline iron (III) oxides, such as goethite, are the most abundant ferric iron minerals in soils (Cornell and Schwertmann, 2003); however, these phases were found unavailable to a large extent for microbial iron reduction (Lovley and Phillips, 1986, 1987, 1988; Komlos *et al.*, 2007). Microorganisms reduced iron mineral phases with low crystallinity such as hydrous ferric oxide and ferrihydrite at higher rate than phases with a higher crystallinity such as goethite in pure culture experiments (Roden, 2003). In soils, not much is known about the identity of iron-reducing bacteria capable of reducing iron mineral phases.

The objective of our study was to identify the active acetate-assimilating microbes in anoxic paddy soil that use added ferric iron oxides as terminal electron acceptor (that is, ferrihydrite and goethite). To this end, we performed a combined analysis of geochemical processes and RNA-based SIP.

Materials and methods

Soil slurry incubation and geochemical analyses

Two soils were used, both sampled near the Italian Rice Research Institute near Vercelli, Italy (for general soil characteristics see Holzapfel-Pschorn and Seiler, 1986). For the main experiment, 'recycled soil' was used, a soil that had been used earlier for growing rice in large microcosms at our greenhouse in Marburg and, thus, had been depleted in organics and nutrients (for details see Conrad and Klose, 1999). In addition, a 'fresh soil' was used, which was taken from the Italian field in March 2006, air-dried and stored as dry lumps until the beginning of the experiment. Rice field soil slurry was prepared as described earlier (Hori *et al.*, 2007). To activate the soil microorganisms and reduce endogenous electron acceptors such as available sulfate and ferric iron oxides, the slurry was pre-incubated for 21 days in the dark at 25 °C (Lueders and Friedrich, 2000, 2002; Lueders *et al.*, 2004b). Aliquots (10 ml) of the homogenized slurry were transferred into 25-ml serum vials. Different treatments were amended with (i) ferrihydrite, which was synthesized according to Schwertmann and Cornell (1991), (ii) goethite (Algoethite, Fluka, Buchs, Switzerland), and (iii) control without amendment of ferric iron minerals. The iron oxides were supplemented at a final concentration of $\sim$140 μmol g^1 soil dry weight for 'recycled' soil and of 40 μmol g^1 soil dry weight for 'fresh' soil. The μmol amount of ferrihydrite added to incubations was calculated using the formula $Fe_5HO_8 \times 4H_2O$ (Kappler and Straub, 2005); depending on the individual preparation of ferrihydrite, the water content may be lower resulting in a higher μmol amount added. Vials were sealed with butyl rubber septa, and headspaces were flushed with N_2. Labeling experiments ($n = 3$, each) were started by adding [U-^{13}C]-labeled acetate (99 atom%, Sigma, Taufkirchen, Germany) at a final concentration of 2 mM (20 μmol in total), and incubated statically for 72 h at 25 °C. Samples of headspace, pore water, and soil slurry were removed at 0, 8, 16, 24, 48, and 72 h from each vial. Volatile fatty acids from pore water samples were measured by high-pressure liquid chromatography (Krumböck and Conrad, 1991). Fe(II) and Fe(III) were determined as described earlier (Achtnich *et al.*, 1995a). Briefly, 0.5 g of soil slurry sample were extracted for 24 h using 0.5 M HCl, and extracted Fe(II) and hydroxylamine reducible Fe(III) were determined using the ferrozine method. Total CH_4 and CO_2 in headspace samples were analyzed by gas chromatography (Roy *et al.*, 1997). The ^{13}C atoms percent of CH_4 and CO_2 was determined by GC-isotope ratio mass spectrometry (Conrad *et al.*, 2000). Soil samples were stored at −80 °C for subsequent molecular analyses.

RNA extraction and isopycnic centrifugation

RNA was extracted from 0.5 ml of soil slurry sample from one set of each treatment after 48 h (fresh soil)

and 72 h of the incubation as described earlier (Noll *et al.*, 2005) and quantified using the Ribogreen RNA-quantification kit (Invitrogen, Karlsruhe, Germany) according to the manufacture's instructions. Extracted RNA (500 ng) was density separated by isopycnic centrifugation in cesium trifluoroacetate (Amersham Biosciences, Freiburg, Germany) (Lueders *et al.*, 2004a). Gradients of density-resolved RNA were fractionated, the cesium trifluoroacetate buoyant density (BD) of each fraction determined, and RNA precipitated from fractions as described earlier (Lueders *et al.*, 2004a).

Terminal restriction fragment length polymorphism analysis

RNA from each density fraction of each treatment was used as a template for reverse transcription–PCR (RT-PCR) using a single step RT-PCR system (Access Quick, Promega, Mannheim, Germany) for terminal restriction fragment length polymorphism (T-RFLP) profiling. Thermal cycling of RT-PCR (Hori *et al.*, 2007) and PCR primers used (Lueders and Friedrich, 2002; Lueders *et al.*, 2004c) were as described earlier; 20 and 23 cycles were used for amplification of bacterial and archaeal templates, respectively. Amplicons were digested using *Msp*I and *Taq*I for *Bacteria* and *Archaea*, respectively. Digested amplicons were desalted using AutoSeq G-50 columns (Amersham Biosciences). Before electrophoresis, 1 µl of the digest was suspended in 12 µl of Hi-Di formamide (Applied Biosystems, Weiterstadt, Germany) and 0.25 µl of carboxy-X-rhodamine (ROX)-labeled MapMarker 1000 ladder (Bio-Ventures, Murfreesboro, TN, USA). The mixture was denatured at 95 °C for 3 min and immediately cooled on ice. Size separation of terminal restriction fragments (T-RFs) was performed using an ABI 310 genetic analyzer (Applied Biosystems).

Cloning and sequencing of 16S rRNA, and phylogenetic analysis

Selected density fractions of bacterial RNA were subjected to cloning and sequencing. The conditions of RT-PCR were as described above. RT-PCR products were ligated into the plasmid vector pGEM-T Easy (Promega), and the ligation mixture was used to transform *Escherichia coli* JM109 cells (Promega) according to the manufacturer's instructions. The 16S rRNA inserts of randomly selected clones were sequenced at the Automatic DNA Isolation and Sequencing facility (ADIS, Max-Planck-Institute for Plant Breeding Research, Cologne Germany) using BigDye terminator cycle sequencing chemistry (Applied Biosystems) (Lueders *et al.*, 2004c). Sequences of 16S rRNA clones were compared with the DDBJ nucleotide sequence database by BLASTN searches. Phylogenetic analyses were performed using the software package ARB (http://www.arb-home.de; Ludwig *et al.*, 2004). Phylogenetic core trees were constructed from reference 16S rRNA gene sequences (>1400 nucleotides) using neighbor-joining, maximum-likelihood, and maximum-parsimony algorithms. Treeing methods did not have a significant effect on dendrogram topology. Partial 16S rRNA clone sequences (~850 bp) were added to core trees using the ARB-parsimony tool. Chimeric sequences were detected by separately determining tree positions of terminal 5′ and 3′ stretches of each clone (~300 bp, 'fractional treeing'; Ludwig *et al.*, 1998, 2004).The nucleotide sequences data have been deposited in the DDBJ nucleotide sequence database under the accession numbers AB293247–AB293418.

Results

Iron reduction and acetate turnover in slurry incubations

Ferrous iron was formed in the ferrihydrite treatment, but was not detectable in goethite and control treatments (Figures 1a–c). In the ferrihydrite treatment, ferrous iron concentration increased gradually from 75 µmol g^{-1} at the beginning of the incubation to approximately 100 µmol g^{-1} at 72 h. Total extractable iron remained constant at ~120 µmol g^{-1} during incubation. Thus, part of the ferric iron, mostly consisting of the supplemented ferrihydrite, was reduced to ferrous iron. In the goethite treatment, both total iron and ferrous iron remained at a low level (that is 70–80 µmol g^{-1}) throughout the incubation.

Acetate consumption in the ferrihydrite treatment was much more vigorous compared with goethite and control treatments (Figures 1a–c). After a short lag phase, acetate was almost completely degraded (~95%, 0.2 µmol g^{-1} residual acetate) in the ferrihydrite treatment within 72 h of incubation. The stoichiometry of acetate:Fe(II) formed (1:7.6) closely matched the expected ratio of 1:8 for acetate-dependent ferric iron reduction. Acetate was degraded to a smaller extent in goethite (65% degraded, 1.5 µmol g^{-1} residual acetate) and control treatments (44% degraded; 2.1 µmol g^{-1} residual acetate) after 72 h.

CH_4 production was largely suppressed in the presence of ferrihydrite, but not by goethite (Figures 1d–f). The concentration of CH_4 in the ferrihydrite treatment increased only moderately to 0.4 µmol g^{-1} after 72 h. In goethite and control treatments, CH_4 initially (that is 24 h) increased gradually and thereafter, rapidly to 1.3 µmol g^{-1} after 72 h. Headspace CO_2 accumulation was apparently lower in the presence of ferrihydrite than in goethite and control treatments (Figures 1d–f); the formation of siderite ($FeCO_3$) from ferrous iron and carbonate might have masked the formation of gaseous CO_2 (Yao *et al.*, 1999), and therefore, less CO_2 formation was detected.

The fate of ^{13}C-acetate was traced by following the ^{13}C-atom percentage of gaseous products CH_4 and

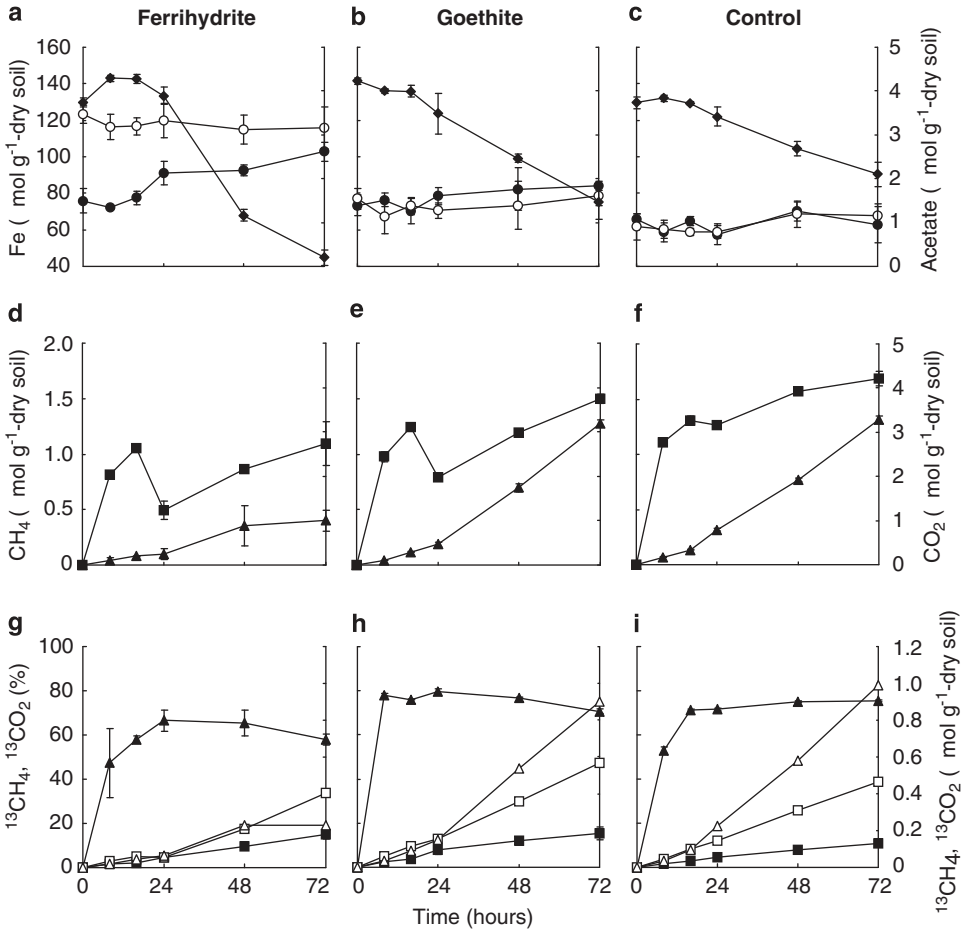

Figure 1 Kinetics of biogeochemical parameters during the anoxic incubations of rice field slurries in treatments with ferrihydrite (left panels **a**, **d**, **g**), goethite (middle panels **b**, **e**, **h**), control (no iron oxide added; right panels **c**, **f**, **i**). (**a–c**) total iron (○), ferrous iron (●), and acetate (◆). (**d–f**). CH_4 (▲) and CO_2 (■). (**g–i**). Change in ^{13}C atom percent of CH_4 (▲) and CO_2 (■) in the headspace and time course of $^{13}CH_4$ (△) and $^{13}CO_2$ (□) concentrations. The error bars indicate the standard deviations of three replications.

CO_2 over time (Figures 1g–i). After rapid initial increases in all treatments, the ^{13}C atom percent of CH_4 varied between 55–60% (ferrihydrite) and up to 80% (goethite and control) indicating that methane was formed to a large extent from added ^{13}C-acetate. In contrast, the increase of ^{13}C atom percent of CO_2 (up to 15% in 72 h) revealed no difference among treatments. Overall, acetoclastic $^{13}CH_4$ formation was significantly suppressed in the ferrihydrite treatment (77%), but not much by goethite (9%; Figures 1g–i), whereas $^{13}CO_2$ formation was not affected.

In microcosms with 'fresh' soil ~100 μmol Fe(II) per g dry soil had been formed after 48 h. However, methane formation was suppressed only by 13%, which was probably a consequence of the lower amount of ferrihydrite added and higher organics and nitrogen content than in 'recycled' soil.

Bacterial and archaeal community dynamics in density gradient fractions of RNA

RNA-based SIP was performed to identify microorganisms capable of acetate assimilation in all treatments of recycled soil after 72 h of incubation. Sufficient amounts of supplemented ^{13}C-labeled acetate had been metabolized in ferrihydrite, goethite, and control treatments (95%, 65%, and 44%, respectively).

Bacteria-specific amplicons were obtained from fractions with highest BDs (up to 1.823 g ml^{-1}) only for the ferrihydrite treatment, which corresponded to the density of highly ^{13}C-labeled rRNA (>1.81 g ml^{-1}; Lueders *et al*., 2004a). Less label had been incorporated by bacteria in treatments with goethite (up to 1.808 g ml^{-1}) and the control (up to 1.793 g ml^{-1}) (Figure 2). The T-RFLP fingerprinting patterns of all treatments were highly similar in 'light' RNA fractions (BDs of 1.767–1.781 g ml^{-1}) (Figures 2a–c). The community profile changed with increasing BD only in treatments with ferrihydrite and goethite. In the ferrihydrite treatment, two major T-RFs (161 and 163 bp, ~85% of total peak heights) and a minor T-RF (129 bp; ~8%) predominated in 'heavy' fractions (BDs ⩾1.793 g ml^{-1}). Likewise, in the goethite treatment, these peaks were predominant; however, the 129 bp T-RF was now as abundant (~45%) as the

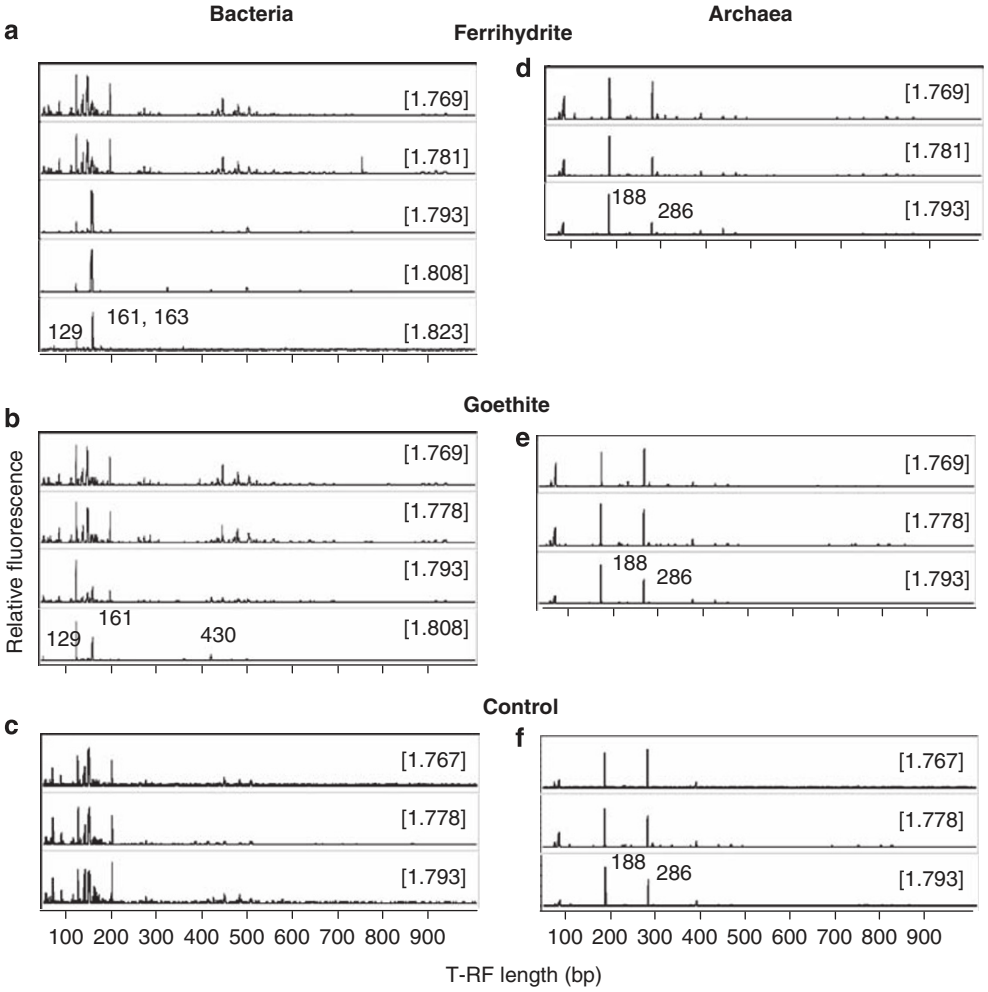

Figure 2 T-RFLP analysis of density-resolved bacterial (**a**, **c**, **e**) and archaeal (**b**, **d**, **f**) 16S rRNA derived from the ferrihydrite (**a**, **b**), goethite (**c**, **d**), and control treatments (**e**, **f**) after 72 h with recycled soil. The cesium trifluoroacetate BDs (g ml^{-1}) of the fractions are shown in brackets. The T-RF size is given in base pairs.

161 and 163 bp T-RFs (~44%; BD ⩾ 1.793 g ml^{-1}); a third peak of 430 bp T-RF (4.4%) was detectable in the highest density fraction (BD 1.808 g ml^{-1}). From treatments with 'fresh soil', bacteria-specific RT-PCR amplicons were obtained from high BD (>1.82 g ml^{-1}) fractions after 48 h of SIP (Figure 3). Three major T-RFs (129, 161, and 163 bp) were likewise predominant (14%, 23%, 23% of total peak height, respectively) not only in fractions with high (1.828 g ml^{-1}), but also in fractions with intermediate BD (>1.804 g ml^{-1}). Moreover, intermediate BD fractions had a major T-RF with 504 bp (19%) and two smaller T-RFs with 428 and 430 bp (~5%), indicating the presence of a more diverse community responsive to ^{13}C-acetate amendment than in the recycled soil treatment.

Archaeal rRNA templates from all treatments were amplified only from 'light' RNA fractions (BDs of 1.769–1.793 g ml^{-1}) despite a higher cycle number (23 cycles) used than in bacteria-targeting RT-PCR (20 cycles) indicating low archaeal abundance (Figures 2d–f). T-RFLP fingerprinting patterns were not significantly different between ferrihydrite, goethite, and control treatments, and two dominant T-RFs of 188 and 286 bp were detected throughout the density fractions. In the ferrihydrite treatments, the relative abundance of the 286 bp T-RF was lower with increasing BDs of the density fractions than in goethite and control treatments. Most likely, the 188 bp T-RF corresponds to *Methanosarcina* spp. and the 286 bp T-RF to *Methanosaeta* spp., as was earlier shown (Lueders and Friedrich, 2000; Ramakrishnan *et al.*, 2001; Weber *et al.*, 2001; Chin *et al.*, 2004).

Phylogenetic identification of microorganisms incorporating ^{13}C-acetate

Major populations represented by T-RFLP profiling in heavy gradient fractions were identified by cloning and sequencing of randomly selected clones of treatments with ferrihydrite ($n = 61$ clones), goethite ($n = 58$ clones), and control ($n = 53$ clones).

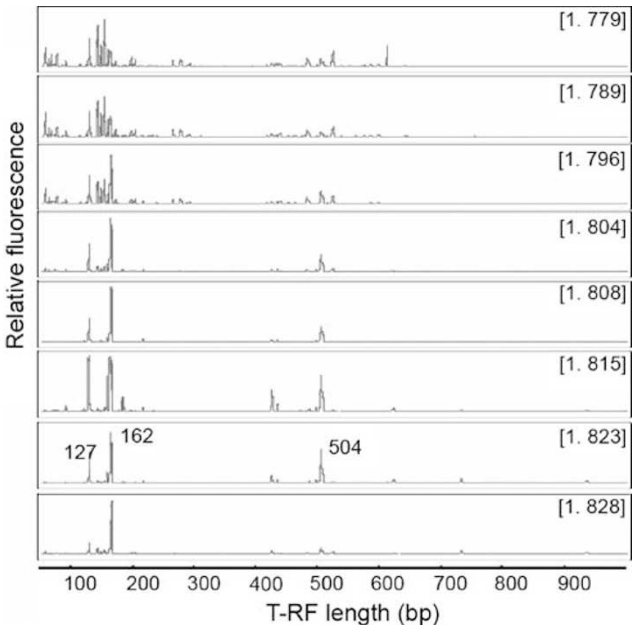

Figure 3 T-RFLP analysis of density-resolved bacterial 16S rRNA derived from the ferrihydrite treatment with fresh soil after 48 h of incubation. The cesium trifluoroacetate BDs (g ml^{-1}) of the fractions are shown in brackets. The T-RF length is given in base pairs.

Phylogenetic affiliations and numbers of the 16S rRNA clones are shown in Table 1.

Among the three clone libraries, the most diverse community structure was observed in the control library, which coincided with the highest diversity of T-RFs (Figure 2c). In ferrihydrite and goethite treatments, *Geobacter*-related sequences were predominant (that is 85% and 33% of all clones in the ferrihydrite and goethite libraries, respectively). The T-RF sizes of these sequences were 161 and 163 bp, which corresponded to dominant peaks in the T-RFLP fingerprints (Figures 4a and b). These clones were related to cultivated *Geobacter* spp. (90–98% sequence identity) and fell into specific clades partly defined by ferrihydrite usage or presence of goethite as a potential electron acceptor (Figure 4a). In the goethite library, the *Anaeromyxobacter*-related sequences were most numerous (33% of clones) and represented a major T-RF (129 bp; Figure 4b). More specifically, these clones were affiliated with novel clusters within the radiation of the genus *Anaeromyxobacter* (95–96% sequence identity; Figure 4b). Furthermore, a large part of clones in the goethite library (that is 17% of the total) was related to β-proteobacteria, representing the third T-RF (428, 430 bp) of the heavy fraction (BD 1.808 g ml^{-1}) from the goethite treatment (Figure 2c). These sequences formed a novel branch within the phylogenetic radiation of the *Rhodocyclaceae* (Figure 4c) and were related to *Azonexus fungiphilus* and *Dechloromonas* spp. (~94–97% sequence identity). In addition, single clones in the goethite library fell into the Chloroflexi, Acidobacteria, Actinobacteria, γ-Proteobacteria, Planctomycetes, Firmicutes, and Thermus groups, some of which were also represented in the ferrihydrite and control libraries (Table 1).

Discussion

^{13}C-acetate assimilation linked to dissimilatory iron reduction

Ferric iron reduction is a widespread trait in the microbial realm comprising many bacterial lineages and even members of the Archaea (Lovley *et al.*, 2004; Weber *et al.*, 2006a). Conversely, the phylogenetic diversity renders tracking of dissimilatory iron-reducing microorganisms in the environment rather difficult because a universal biosignature or molecular assays for detection, that is functional gene markers, are not available. The physiology of dissimilatory iron reducers as anaerobically respiring microorganisms, however, facilitates detection of these microorganisms as a unique guild by SIP when the availability of electron acceptors is known, and the labeled substrate is carefully chosen (Friedrich, 2006). Acetate, the most important metabolite of the anaerobic food chain in rice field soils and many other anoxic environments is an ideal substrate for targeting anaerobically respiring microorganisms by SIP. Provided that other electron acceptors have been depleted, anaerobic acetate oxidation to CO_2 is thermodynamically feasible only when it is coupled to a respiratory process such as iron(III)oxide reduction or is mediated by syntrophic acetate-oxidizing consortia (Schink, 1997; Kittelmann and Friedrich, 2008a); the latter can be ruled out for the Italian rice field soils studied based on careful checks of methane formation from the radiolabeled methyl group of acetate (Achtnich *et al.*, 1995b; Chidthaisong and Conrad, 2000). In fact, we have shown recently that ^{13}C-acetate assimilation in RNA-SIP experiments can be linked to specific anaerobic respiratory processes such as chloroethene dehalorespiration (Kittelmann and Friedrich, 2008a, 2008b); here we show that it can be linked to iron(III) oxide reduction.

The pre-incubation of soil slurries ensured that inorganic electron acceptors other than CO_2 were depleted before initiating SIP. Labeled acetate was added at concentrations (2 mM or 3.3 μmol g^{-1} dry soil) relevant to flooded rice paddies (Lueders and Friedrich, 2000), and isotope probing was limited to a rather short time (72 and 48 h), which are prerequisites for specific SIP (Friedrich, 2006). Under these conditions, ferric iron-reducing bacteria oxidized and assimilated ^{13}C-labeled acetate in the presence of ferrihydrite, which was corroborated by ferrous iron formation nearly matching the expected stoichiometry of iron-dependent acetate oxidation (Figure 1), and rather specific label incorporation into rRNA (Table 1; Figures 2–4).

Table 1 Phylogenetic affiliations, T-RF length, and numbers of 16S rRNA clones retrieved from the high-density fractions of bacterial RNA from the ferrihydrite, goethite, and control treatments

Phylogenetic group	*Ferrihydrite treatment*		*Goethite treatment*		*Control treatment*	
	Clones (n)	*T-RF (bp)*	*Clones* (n)	*T-RF (bp)*	*Clones* (n)	*T-RF (bp)*
δ-proteobacteria						
Geobacter	52	**161**, **163** (**85%**)[a] 130	19	**161** (**44%**)[a] **130**, 66		
Anaeromyxobacter	3	**129** (**8 %**)[a]	19	**129** (**45%**)[a]	3	**129**
Uncultured	1	141	1	167	5	67, 78, 474,
Uncultured						483, 484
β-proteobacteria						
Rhodocyclaceae	1	430	10	**428**, **430** (**4.4%**)[a]		
Uncultured			2	122, 490		
Acidobacteria			1	152	9	**201**, 73, 144, 283
Actinobacteria	1	163	1	161	2	132, 139
α-proteobacteria					3	152, 400, 443
Bacteroidetes					1	93
Chloroflexi	2	457, 468	1	164	11	**458**, **514**, 117,
						123, 157, 506,
						513, 515, 523
Cyanobacteria					1	494
Firmicutes	1	137	1	272	3	481, 512, 553
γ-proteobacteria			1	444		
Planctomycetes			1	117	1	154
Thermus/deinococcus			1	116		
Unidentified affiliation					14	**150**, **68**, **203**
						82, 97, 152,
						166, 265, 520
Total	61		58		53	

Characteristic T-RFs length (bp) for different phylogenetic groups as indicated. T-RF detected for more than one clone within the group is indicated as boldface.
[a]The relative abundance of the corresponding peaks in the T-RFLP is presented in parenthesis.

Geobacter spp. accounted for the largest part of the ^{13}C-acetate-assimilating populations (~80% of all clones, ~85% of total T-RF peak height). Members of the genus *Geobacter* are a known clade of dissimilatory iron-reducing bacteria capable of acetate oxidation and have been detected in many other anoxic environments as well (Weber *et al.*, 2006a). A second, smaller population (~8%) was closely related to the dissimilatory iron-reducing *Anaeromyxobacter* spp., a genus within the Myxococcales, species of which had been isolated from rice field soil earlier as acetate oxidizers (Treude *et al.*, 2003). Both populations were not detected in heavy gradient fractions of the control (with ^{13}C-acetate only) indicating that acetate assimilation was linked to acetate oxidation by microbial ferrihydrite reduction. T-RFs corresponding most likely to *Geobacter* spp. (161 and 163 bp T-RFs) and *Anaeromyxobacter* spp. (129 bp T-RF) were found also in ferrihydrite reduction coupled to acetate turnover in an experiment with 'fresh' flooded Italian rice field by RNA-SIP conducted just after 48 h of incubations; although a clone library was not established for the 'fresh' soil experiments, it is most parsimonious to assume that the 161 bp T-RF corresponds to *Geobacter* spp. because its clone frequency in the 'recycled' soil experiment (Table 1) was much higher than that for Actinobacteria (1 clone) with a 163 bp T-RF, and the 'fresh' soil was from the same sampling site. Notably, *Anaeromyxobacter* spp. were much more active in the fresh soil than in recycled soil (14% of T-RF peak heights).

Goethite as potential electron acceptor

In the presence of goethite, *Geobacter* and *Anaeromyxobacter* spp. became labeled to a similar extent (~30% of all clones, ~44% and 45% peak heights; Table 1; Figures 2 and 4). On the basis of these data, *Anaeromyxobacter* spp. were as active in the presence of goethite as *Geobacter* spp. However, iron(II) formation was not detectable in the incubations with goethite amended as a potential electron acceptor. Thus, a direct proof of goethite reduction by *Geobacter* and *Anaeromyxobacter* spp. could not be obtained. However, labeled rRNA of *Geobacter* and *Anaeromyxobacter* spp. was detected in heavy gradient fractions, but not in the control treatment without amended iron oxides. Although the geochemical background in all treatments was the same, goethite amendment had specifically stimulated *Geobacter* and *Anaeromyxobacter* spp.—which are known iron-reducing bacteria—but other populations (see below) only to a minor extent.

In the control treatment, however, *Geobacter* and *Anaeromyxobacter* spp. had not become labeled, although ^{13}C-labeled acetate was available for assimilation. On the basis of the indirect evidence presented, goethite reduction might have occurred. Iron extraction using 0.5 M HCl facilitates detection of adsorbed iron(II) or siderite precipitates, for example in rice field soil (Ratering and Schnell, 2000), but not magnetite (Fe_3O_4) (Raiswell *et al.*, 1994), which could have potentially been formed in goethite amended slurries. Magnetite is a mixed Fe(II)-Fe(III) mineral, which may be observed in closed systems upon secondary mineral transformations of iron(III) phases, especially ferrihydrite (Hansel *et al.*, 2003). Slightly higher levels of inorganic carbon (calculated from headspace CO_2 and

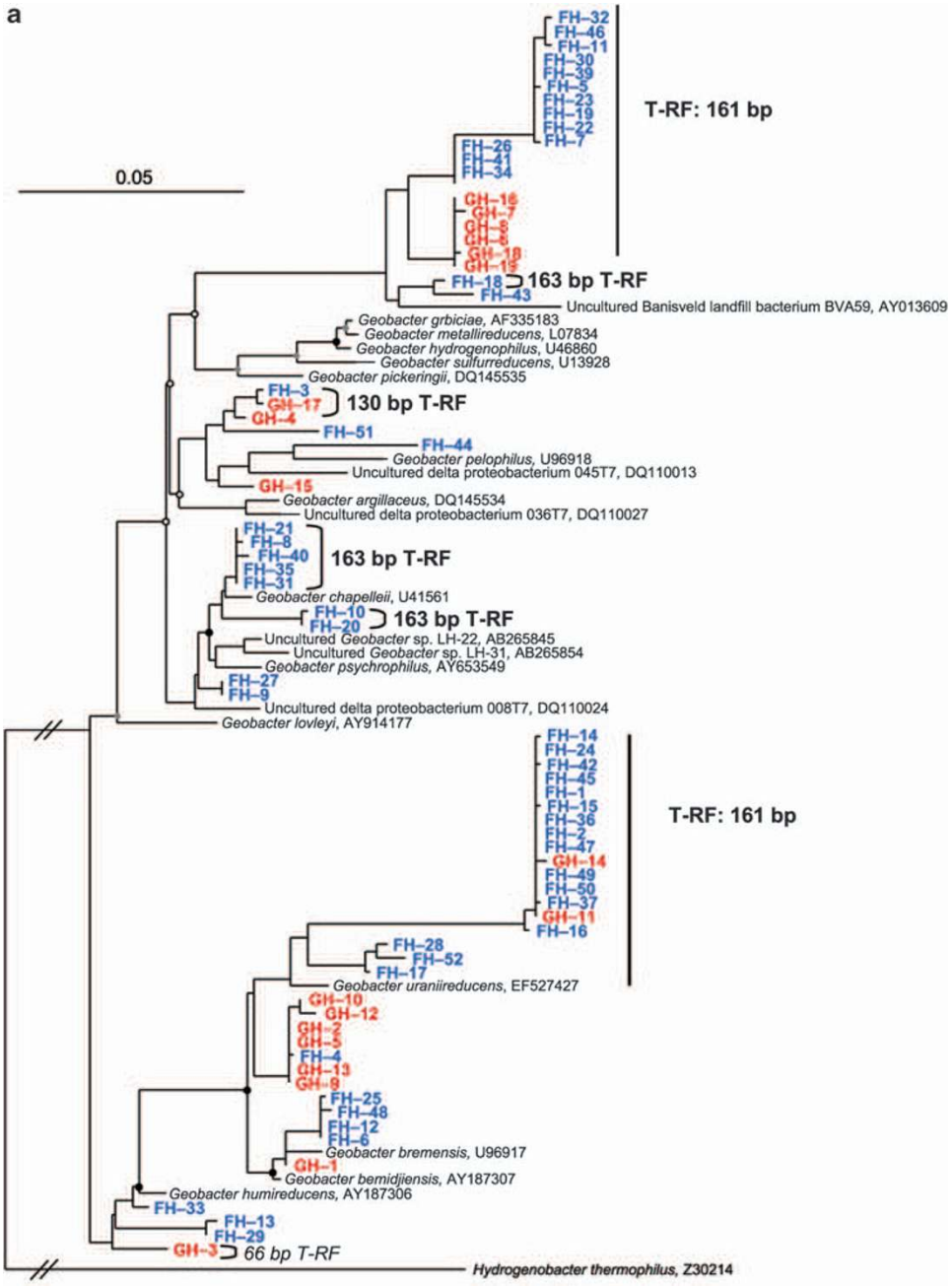

Figure 4 Phylogenetic trees showing the relationships of 16S rRNA clone sequences related to *Geobacter* spp. (**a**), *Anaeromyxobacter* spp. (**b**), and β-proteobacteria (**c**). Clones obtained in this study were indicated by ferrihydrite (heavy RNA fraction of the ferrihydrite treatment; in blue, and boldface), goethite (heavy RNA fraction of the goethite treatment; in red, and boldface), and control (heavy RNA fraction of the control treatment; in black). The T-RF sizes are as indicated in base pairs. The core trees with reference sequences (>1400 nucleotides) were calculated using the maximum-parsimony algorithm. Bootstrap values were obtained from 1000 replications, and >90%, 70–89% and <69% are shown with black, gray, and open circles, respectively. The scale bar represents 5% sequence divergence. GenBank accession numbers of reference sequences as indicated.

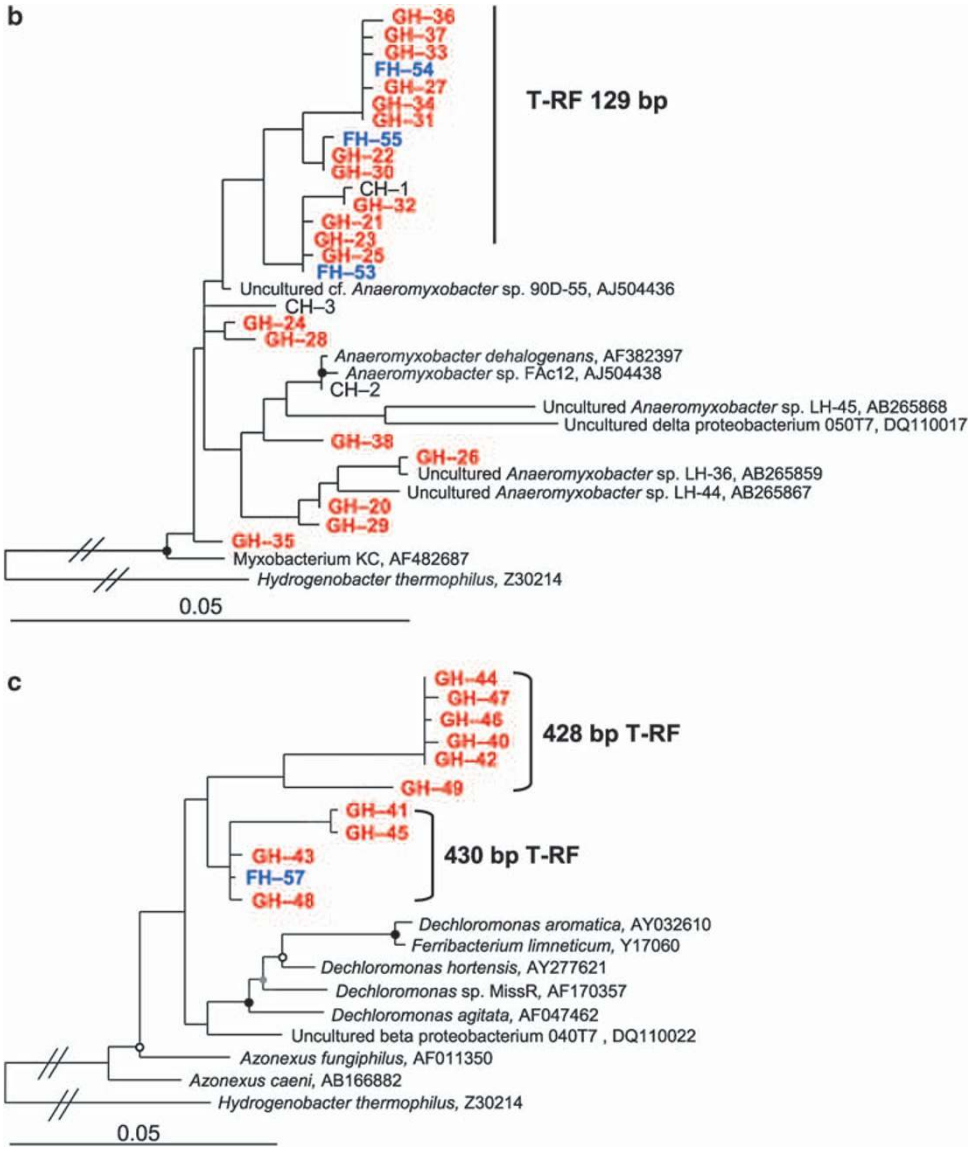

Figure 4 Continued.

slurry pH) were formed in goethite (2.9 μmol) compared with control treatments (2.2 μmol) and more acetate was degraded in goethite (65%) than in control treatments (44%). Thus, goethite reduction might have occurred potentially to some extent, but was not an important process in the first 72 h of incubation.

The extent of microbial goethite reduction and mineral dissolution in the environment varies greatly. In a number of studies, goethite reduction was either undetectable or goethite was reduced only to a small extent (Lovley and Phillips, 1986, 1987, 1988; Kukkadapu *et al.*, 2006; Komlos *et al.*, 2007), whereas goethite was reduced considerably on biostimulation in a subsurface sediment (Stucki *et al.*, 2007). In Italian rice field soil, neither goethite nor Al-goethite added to soil slurries was microbially reduced after 180 days of incubation (Qu *et al.*, 2004).

The mechanisms conveying superiority in acetate usage to *Geobacter* spp. in the presence of ferrihydrite and competiveness to *Anaeromyxobacter* spp. in the presence of goethite are presently elusive. It is tempting to speculate that the characteristics of the iron oxides (surface area, mid-redox potential, crystal phase properties) are selecting for distinct populations of iron-reducing bacteria.

Further microbial populations involved in acetate assimilation

Besides *Geobacter* spp. and *Anaeromyxobacter* spp., other populations were detected in gradient fractions with heavy rRNA. A relatively large, but so

far unidentified population with a TRF of 504 bp (~19% of total TRF heights) was detected in heavy RNA fractions from microcosm with fresh soil only (Figure 3). The duration of isotope probing (only 48 h) might determine which populations can be traced by SIP; hence, it is possible that certain populations are only stimulated intermittently before the more competitive *Geobacter* spp. gain predominance in ferrihydrite amended soil. A second further population was represented by the β-proteobacterial Rhodocyclaceae (T-RFs of 428 and 430 bp) detected in goethite treated microcosms with recycled soil (Figure 2; Table 1; Figure 4c), and ferrihydrite amended samples with 'fresh' soil (Figure 3) at relative abundances of 7% and 5%, respectively. These novel β-proteobacteria were most closely related to *Dechloromonas* spp. and *Azonexus* spp. (Figure 4c). Uncultured *Dechloromonas* spp. were also detected in anoxic enrichment cultures with freshwater sediments under a regime of iron reduction and nitrate-dependent iron(II) oxidation (see clone 040T7 in Figure 4c), but a direct involvement in ferric iron reduction had not been proven (Weber *et al*., 2006b). Our results indicate that ^{13}C-acetate was assimilated by these novel, as-yet-uncultured Rhodocyclaceae bacteria only when goethite was present, reducing conditions prevailed in the soil incubations, and no other electron acceptors were present, that is nitrate and sulfate; in the light of earlier and our own findings, it is suggestive to hypothesize that these novel *Dechloromonas* spp. might be involved in ferric iron oxide reduction. In more extensive experiments with flooded rice field soil microcosms, we found that *Dechloromonas*-related populations were involved in acetate assimilation when iron oxide mineral phases were largely reduced (Müller and Friedrich, unpublished data). Whether less bioavailable iron(III) mineral phases can be reduced by these *Dechloromonas* spp. as hypothesized earlier for *Geobacter* spp. and *Anaeromyxobacter* spp. (Hori *et al*., 2007) remains to be elucidated.

Although a number of novel populations might be involved in iron reduction in rice field soil, it is unknown so far, which mechanism of iron reduction is involved in rice field soil. Besides direct contact of cells with iron oxides, shuttle molecules such as humic acids or sulfur compounds might be involved in transferring electrons to the iron(III) oxide surface (Lovley *et al*., 1996; Straub and Schink, 2004; Weber *et al*., 2006a).

Suppression of methanogenesis

In the incubations with amended ferrihydrite (140 μmol g^{-1} dry soil) $^{13}CH_4$ formation was reduced by 77% in recycled soil (Figure 1d), corroborating an earlier study (84% suppression; (Lueders and Friedrich, 2002). SIP showed that novel and diverse members of *Geobacter* spp. became highly labeled with ^{13}C within 72 h of the incubation (Figures 2a and 4a), indicating that *Geobacter* spp. were preferentially involved in the significant suppression of methane release during the amendment. Several factors might have been involved in the suppression of methanogenesis. According to the thermodynamic theory, methanogens might be out-competed for acetate when substrate concentrations are too low to support methanogenesis. However, during the course of SIP, acetate concentrations in slurry pore waters were always above 100 μM (Figure 1a), and thus, above known thresholds for methanogenesis in rice paddy soil from acetate (>10 μM; Frenzel *et al*., 1999). Besides competition for the carbon source, methanogens might have been directly inhibited by ferric iron as suggested by van Bodegom *et al*. (2004); however, the biochemical mechanism of inhibition is still unknown. *Methanosarcina barkeri* reduced Fe(III) in pure cultures with H_2/CO_2 and methanol methanogenesis was suppressed, possibly, because hydrogen oxidation was linked to ferric iron reduction rather than CO_2 reduction (Bond and Lovley, 2002; van Bodegom *et al*., 2004). Earlier, we had found that rRNA levels attributed to rice cluster I methanogens increased in ferrihydrite amended soil incubations when methanogenesis was suppressed (that is day 05), suggesting a possible involvement of these Archaea in ferric iron reduction (Lueders and Friedrich, 2002). However, in the present SIP experiments, we did not observe a stimulation of hydrogenotrophic methanogens, for example the abundant rice cluster I methanogens, suggesting that iron reduction by methanogens was not an important process.

Smaller amounts of ferrihydrite (40 μmol g^{-1}; data not shown) as well as goethite amendment (Figure 1e) had only a minor effect on the suppression of methane formation (~13% and 9%, respectively) corroborating an earlier iron oxide amendment experiment (Qu *et al*., 2004). Apparently, the type and amount of iron oxide amended has a crucial function in controlling the suppression of methane formation. The pre-incubation of the soil had resulted in the reduction of most of the indigenous ferric iron oxides leaving a high concentration of Fe^{2+} at the beginning of the SIP incubation; under these conditions, added ferric iron oxide might have become coated by the indigenously present Fe^{2+}, and thus, reducing the effective iron(III) oxide surface available for microbial reduction (Roden and Urrutia, 2002; Roden, 2003). This might also explain why only little Fe^{2+} formation was observed in the goethite treatment.

Conclusions

The dissimilatory iron-reducing *Geobacter* spp. and *Anaeromyxobacter* spp. were identified by RNA-SIP as actively iron-reducing bacteria in rice paddy soil with ferrihydrite as electron acceptor. Thus, our study shows that these bacteria can be studied as

functional guild of iron-reducing bacteria, when they are actively metabolizing a substrate that can be metabolized by respiratory microorganisms only. This is a major advancement in cultivation-independent study of iron-reducing microorganisms enabling a less biased assessment of their function in iron reduction in the environment.

Geobacter ssp. were highly competitive with ferrihydrite as electron acceptor over other populations and were labeled to the largest extent during SIP. On the other hand, the presence of the less accessible, crystalline iron(III) oxide goethite favored *Anaeromyxobacter* spp. as well as other microrganisms not known as iron reducers so far such as *Dechloromonas*-related bacteria. Their function in iron oxide mineral phase reduction, especially goethite, is not clarified yet and requires further work.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Melanie Klose, Bianca Pommerenke, and Peter Claus for their significant technical assistance, and thank Tzu-Chiao Chao for iron oxide determination, and critical reading of the manuscript. We also thank Masaharu Ishii and Shin Haruta (University of Tokyo) for their continuous encouragement. This study was financially supported by the DFG within the special research program SFB395 and the Fonds der Chemischen Industrie. T Hori received a scholarship from the Max-Planck Society and the University of Tokyo International Academic Exchange Activities Program.

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