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Phylogenetic diversity of Fe(III)-reducing microorganisms in rice paddy soil: Enrichment cultures with different short-chain fatty acids as electron donors

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Abstract

Purpose — Microbial ferric iron reduction is an important biogeochemical process in nonsulfidogenic anoxic environments, yet the structure of microbial communities involved is poorly understood because of the lack of a functional gene marker. Here, with ferrihydrite as the iron source, we characterized the potential Fe(III)-reducing bacteria from the paddy soil in the presence of different short-chain fatty acids, formate, acetate, propionate, pyruvate, succinate, and citrate.

Materials and methods — Enrichment culture was used to characterize the potential Fe(III)-reducing bacteria in the present study. Clone library and terminal restriction fragment length polymorphism (T-RFLP) analyses of bacterial 16S rRNA gene sequence fragments were conducted to reveal the bacterial community structure.

Results and discussion — T-RFLP and cloning/sequence analysis showed that Firmicutes were the predominant phylotype in all the enrichment cultures (more than 81% of total peak height, more than 75% of all clones), whereas *Geobacter* spp. represented a small fraction of the bacterial community. Specifically, distinct bacterial families in the phylum Firmicutes were enriched depending on the short-chain fatty acid amended. *Clostridium* spp. were the predominant microorganisms in pyruvate treatment, while both *Bacillus* and *Clostridium* were enriched in formate, acetate, and propionate treatments. Sequences related to Veillonellaceae and *Alkaliphilus* were predominant in succinate and citrate treatment, respectively.

Conclusions — The results indicated Fe(III)-reducing microorganisms in rice paddy soil are phylogenetically diverse. Besides the well-known *Geobacter* species, Firmicutes-related Fe(III)-reducing bacteria might also be an important group of Fe(III) reducers in paddy soil. To confirm the populations which play an important role in situ, further studies with culture-independent mRNA-based analysis are needed.

Keywords: Enrichment culture, Fe(III)-reducing bacteria, Paddy soil, Terminal restriction fragment length polymorphism (T-RFLP)

1 Introduction

Rice is a staple food for more than 50% of the world's populations — http://beta.irri.org/index.php . In Asia, nearly 140,000,000 ha are cultivated paddy soils in 2008 — http://beta.irri.org/solutions — accounting for more than 90% of global rice production — http://irri.org/about-rice . However, heavy metals such as arsenic are significant pollutants in the paddy soils within South Asia (Dittmar et al. 2010). This can greatly influence human health because rice is particularly susceptible to arsenic (As) accumulation and that

food consumption is a primary source through which humans are exposed to arsenic contamination (Zhu et al. 2008). Anaerobic iron reduction, which can result in the release of heavy metals, including arsenic (Islam et al. 2004), may play a key role in As accumulation in rice.

Microbially mediated rather than chemical iron reduction plays a significant role in the iron redox cycling in nonsulfidogenic anoxic environments. Ferric iron can be reduced through respiratory (ferric iron as the electron acceptor) as well as fermentation (ferric iron as the electron sink) by Fe(III)-reducing bacteria. Dissimilatory (respiratory) Fe(III) reduction has been established to be an important biogeochemical process in many freshwater and marine sediments for its contribution to the oxidation of both natural and contaminant organic carbon, as well as its influences on the fate of heavy metals and radionuclides (Lovley et al. 1994; Borch et al. 2010). A wide diversity of iron-reducing bacteria have been isolated, characterized, and identified from anoxic environments (see Lovley et al. 2004 for review). However, these studies have mainly been investigated in sediments and groundwater. Unlike naturally saturated sediments and wetland soils, paddy soils, especially in Asia, experience regular specific management practices, including submergence and drainage. Organic manuring such as animal manure, rice straw, and other crop residues is another management practice (Kögel-Knabner et al. 2010). These amendments introduce a significant amount of organic matter and can drive iron reduction in these environments.

Acetate is a terminal fermentation product in the degradation of complex organic matter. Thus, it is commonly used as the electron donor in experiments for enrichment and isolation of Fe(III)-reducing bacteria. However, plant-derived organic matter is decomposed to glucose, lactate, monoaromatic compounds, and long-chain fatty acids as well as acetate. Besides further degradation via fermentation, these forms of organic carbon also serve as electron donors and in some cases are completely mineralized to CO_2 by Fe(III)-reducing microorganisms (Chaudhuri and Lovley 2003; Lovley et al. 2004). Consequently, the carbon which have the potential to drive Fe(III) reduction could be the complex organic matter deposited within soils and sediments.

In paddy soils, acetate, formate, and propionate are the primary short-chain fatty acids produced during residue decomposition (Krylova et al. 1997; Yao and Conrad 2001; Rui et al. 2009; He et al. 2010). Citrate and succinate are exuded from rice roots (Aulakh et al. 2001; Hoffland et al. 2006). Therefore, in this study, we chose acetate, formate, propionate as well as succinate and citrate as the electron donor for Fe(III)-reducing microorganism enrichment cultures. Pyruvate was also chosen because it is a key intersection in the network of metabolic pathways.

The lack of a universal functional gene marker makes it still rather difficult to track the Fe(III)-reducing bacteria in the environment, thus, enrichment cultures were used in this study to identify their diversity in paddy soil. Given the physiological diversity of Fe(III)-reducing microorganisms, we hypothesize that distinct members of Fe(III)-reducing microbial community would be enriched with different organic carbon amendments.

2 Materials and methods

2.1 Soil sampling

Soil samples (top ~20 cm) were collected in 2009 from a submerged paddy field located in Lengshuijiang, China. Soils samples were stored in polyvinylchloride bottles, submerged in water, and then transported back to the laboratory. The soil had a pH 6.7 (±0.2), organic C of 29.2 g kg^{-1} (±0.2), and total Fe of 22.3 g kg^{-1} (±0.9) as measured by the standard methods (Lu 1999).

2.2 Enrichment conditions

Enrichment cultures were conducted in 50-ml anaerobic serum bottles sealed with thick butyl rubber stoppers and aluminum caps in triplicate. Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES)-buffered freshwater culture medium (10 mM, pH 6.8) containing 22 mM of synthetic amorphous ferrihydrite (FeOOH), 0.11 mM $MgCl_2$, 0.61 mM $CaCl_2$, 2 mM $NaHCO_3$, 0.5 mM NH_4Cl, 0.05 mM KH_2PO_4, and vitamin and trace element solutions (Lovley 2000) was prepared, autoclaved, and cooled to room temperature under an anoxic atmosphere of N_2 (99.999% purity). Synthetic FeOOH was prepared using $FeCl_3$ and NaOH as described previously (Schwertmann and Cornell 1996) and identified by X-ray diffraction (data not shown). Six different organic carbon substrates, formate, acetate, propionate, pyruvate, succinate, and citrate, were added to various treatments from sterile, anoxic stock solutions (final concentration, 25 mM). The enrichment was initiated by adding sediment slurry (1 ml) to sterile PIPES-buffered medium (9 ml) with resazurin as the redox indicator under a stream of N_2 (99.999% purity). The sediment slurry was prepared by adding 10 g of paddy soil into 90 ml of 0.1% sodium pyrophosphate solution and vigorous shaking for 30 min. In addition to the experimental treatments, a control was amended with sterile, anoxic deionized water instead of organic carbon substrates. The cultures were incubated statically in the dark at 30°C for 24 days.

2.3 Chemical analysis

Samples were periodically collected from triplicate cultures. Prior to sampling, the culture medium was homogenized by vigorously shaking for 10 s. Liquid samples (200 μL) were taken using sterile syringes and a 100-μL aliquot was immediately added to a micro-centrifuge tube containing 1.5 ml 0.5 N HCl for Fe(II) analysis. After 15 min, the extracts were centrifuged (12, 000 ×*g*) and 100 μL was added to phenanthroline in HEPES buffer according to a method modified from Lu (1999). Fe(II) was measured after 30 min. The reduction of Fe(III) was measured as the accumulation of Fe(II) in 0.5 N HCl extracts. The solution of HCl (0.5 N) is considered to be an effective dissolving agent for most biogenic solid phase Fe(II), including magnetite (Fredrickson et al. 1998; Weber et al. 2001). Therefore, we consider the measurement of 0.5 N HCl extractable Fe(II) to be an indicator of the production of Fe(II). The other 100 μL aliquot was centrifuged and filtered through a 0.22-μm nylon filter, then stored at −20°C for analysis of short-chain fatty acids by ion chromatography (IonPac® AS11 analytical column, Dionex ICS-3000 system, Dionex, Sunnyvale, CA).

2.4 DNA extraction

Samples for DNA extraction were collected when iron reduction ceased as indicated by a plateau in Fe(II) accumulation. Collected samples as well as inoculums which were stored at −20°C were centrifuged at 16, 000 ×*g* for 3 min. The pellet was resuspended in a sterilized solution of ammonium oxalate (200 mM) and oxalic acid (120 mM) and incubated in the dark (about 2 h) to dissolve the solid iron oxides. After all the iron oxides were dissolved, the mixture was centrifuged at 16, 000 ×*g* for 10 min. In order to eliminate the influence of ammonium oxalate and oxalic acid, the pellet was washed with DNA extraction buffer prior to extraction. Total genomic DNA was extracted from the pellet using a CTAB (hexadecyltrimethyl ammonium bromide) protocol as previously described (Su et al. 2007).

2.5 Terminal restriction fragment length polymorphism (T-RFLP) fingerprinting

PCR amplification and T-RFLP analyses of bacterial 16S rRNA gene sequence fragments were conducted as described previously (Rui et al. 2009). In brief, PCR amplification was performed using primers 27f and 1492r (Lane 1991). The 5′ end of the forward primer was labeled with 6-carboxyfluorescein (FAM). A touchdown PCR protocol was used with an initial denaturation (4 min, 94°C), 10 cycles of denaturation (45 s, 94°C), annealing (30 s, 60–0.5°C), elongation (60 s, 72°C), followed by another 22 cycles of denaturation (60 s, 94°C), annealing (60 s, 55°C), elongation (60 s, 72°C), and a final elongation of 72°C for 10 min. The FAM-labeled PCR products were purified using an agarose gel DNA extraction kit (TaKaRa #DV805A) and digested at 37°C for 5 h by *Msp* I (TaKaRa #D1053A). Fifty microliter of 100% ethanol and 2.5 μL 3 M sodium acetate (pH 5.2) were added to the digestion products and stored at −20°C overnight. DNA precipitate was collected by centrifugation at 18, 000 ×*g* for 30 min. The products were then washed using 70% ethanol and centrifuged at 18,000×*g* for 15 min. Finally, the purified products were air-dried and then were size-separated using the 3130xl Genetic Analyzer (Applied Biosystems).

2.6 Cloning, sequencing, and phylogenic analysis

Clone libraries were constructed from bacterial 16S rRNA gene sequences recovered from cultures and the soil as inoculum. The primers for PCR amplification were the same as those indicated above but without a label. Products (equal concentrations) from three replicates PCR amplification were combined, purified (TaKaRa #DV805A), and ligated into the pMD 19-T vector (TaKaRa #D102A) according to the manufacturer's instructions. Plasmids were transformed into *Escherichia coli* JM 109 cells (BioTeKe #DP7502), and clones were randomly selected and checked for correct insert size via standard-targeted PCR and agarose gel electrophoresis. Sequencing was performed with an ABI 3730xl sequencer using BigDye terminator cycle sequence chemistry (Applied Biosystem).

Sequences were phylogenetically identified using RDP-II Classifier and Sequence Match (Cole et al. 2005) in combination with NCBI BLASTN search program. The closest relatives to the clone sequences were aligned with Molecular Evolutionary Genetics Analysis 4.0 software for phylogenetic analyses (Tamura et al. 2007). Phylogenetic trees were constructed using the neighbor-joining method. Robustness of derived groupings was tested by bootstrap using 1,000 replications.

2.7 Nucleotide sequence accession number

Nucleotide sequences generated in this study were submitted to GenBank under the following accession numbers FR668304 to FR668397.

3 Results

3.1 Fe(III) reduction and short-chain fatty acids oxidation

The rate and extent of Fe(III) reductions were variable between different treatments (Figure 1). Three trends of

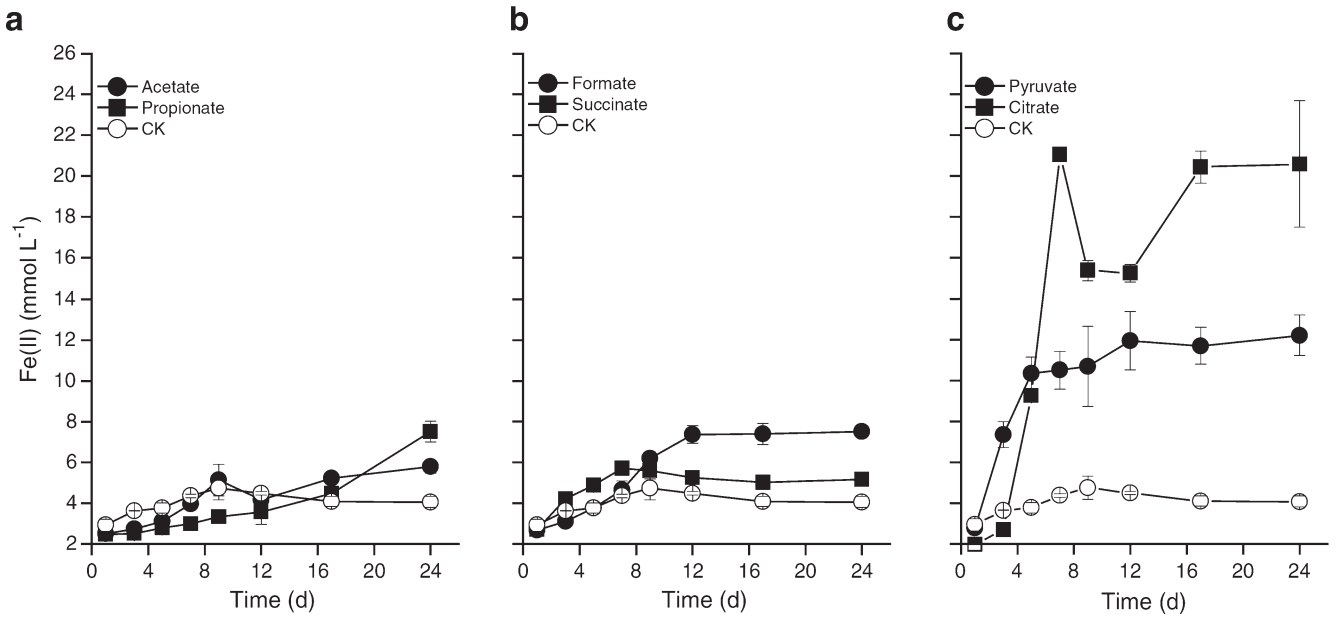

Figure 1. Fe(III) reduction in anaerobic enrichment cultures with different short-chain fatty acids (**a**, **b**, **c**). Data are means of triplicates; *error bars* indicate means ± SE

Fe(III)-reducing dynamics could be distinguished for all the treatments. In acetate and propionate treatments, Fe(III) reduction was comparatively slow, with an average rate of 308 and 264 μmol L^{-1} d^{-1} for the first 17 days of incubation, respectively (Figure 1a). Finally, about 26% of the Fe(III) added in acetate treatment and 34% in propionate treatment were reduced at the end of incubation. Fe(III) reduction in formate (615 μmol L^{-1} d^{-1}) and succinate (814 μmol L^{-1} d^{-1}) treatments were a little rapid than that in treatments amended with acetate and propionate followed by a plateau after 12 and 7 days of incubation, respectively (Figure 1b). However, the extent of Fe(III) reduced in the formate (34%) and succinate (25%) treatments was comparable to those in propionate and acetate treatments, respectively. Rapid and extensive reduction was observed in pyruvate and citrate treatments (Figure 1c). Forty-eight percent of the Fe(III) added was reduced within 5 days (2,068 μmol L^{-1} d^{-1}) when pyruvate was amended, while in citrate treatment, approximately all (94%) was reduced within 7 days (3,006 μmol L^{-1} d^{-1}). Fe(III) reduction was also observed in control, resulting in the accumulation of 4.74 mM Fe(II) and 25% of the Fe(III) was added.

Organic carbon consumption was monitored throughout the experiment. The concentration of formate decreased slightly throughout the experiment, while the decrease of acetate and propionate was undetectable (Figure 2a–c). Pyruvate rapidly decreased to detection limit in 3 days (Figure 2d), resulting in the accumulation of acetate (20 mM). Similarly, succinate and citrate were depleted within 5 and 7 days, concomitant with the increase of propionate and acetate, respectively (Figure 2e, f).

According to theoretical stoichiometry (Lovley 1995),

$$CH_2O_2 + 2\,Fe(III) + H_2O \rightarrow HCO_3^- + 2\,Fe(II) + 2\,H^+$$

$$C_2H_4O_2 + 8\,Fe(III) + 4\,H_2O \rightarrow 2\,HCO_3^- + 8\,Fe(II) + 9\,H^+$$

$$C_3H_6O_2 + 14\,Fe(II) + 7\,H_2O \rightarrow 3\,HCO_3^- + 14\,Fe(II) + 16\,H^+$$

Fe(III) reduction in the present study would only couple with the oxidation of 37, 7, and 5 μmol of formate, acetate, and propionate, respectively. This is in agreement with slight decrease of formate and undetectable loss of acetate and propionate. Though not followed over the course of the experiment, inorganic carbon was also a likely product in pyruvate, succinate, and citrate treatments. Accordingly, electrons produced and used for iron reduction was calculated from the concentration of organic matter at the end of incubation (Table 1). The total quantity of electrons produced was compared to that used to reduce Fe(III). Obviously, only 5–10% of electrons in the associated organic matter was transferred to Fe(III) oxides.

3.2 Composition of bacterial populations

Eight clone libraries of bacterial 16S rRNA genes were constructed to determine the composition of bacterial community during enrichment culture as well as the paddy soil used as inoculum (Table 2 and Supplementary Material, Resource 1). The paddy soil inoculums were primarily composed of members within the Proteobacteria (up to 57%). However, after 24 days of incubation, members of the Firmicutes were predominant microbial community in all the enrichment cultures. These members represented more than 75% of all clones

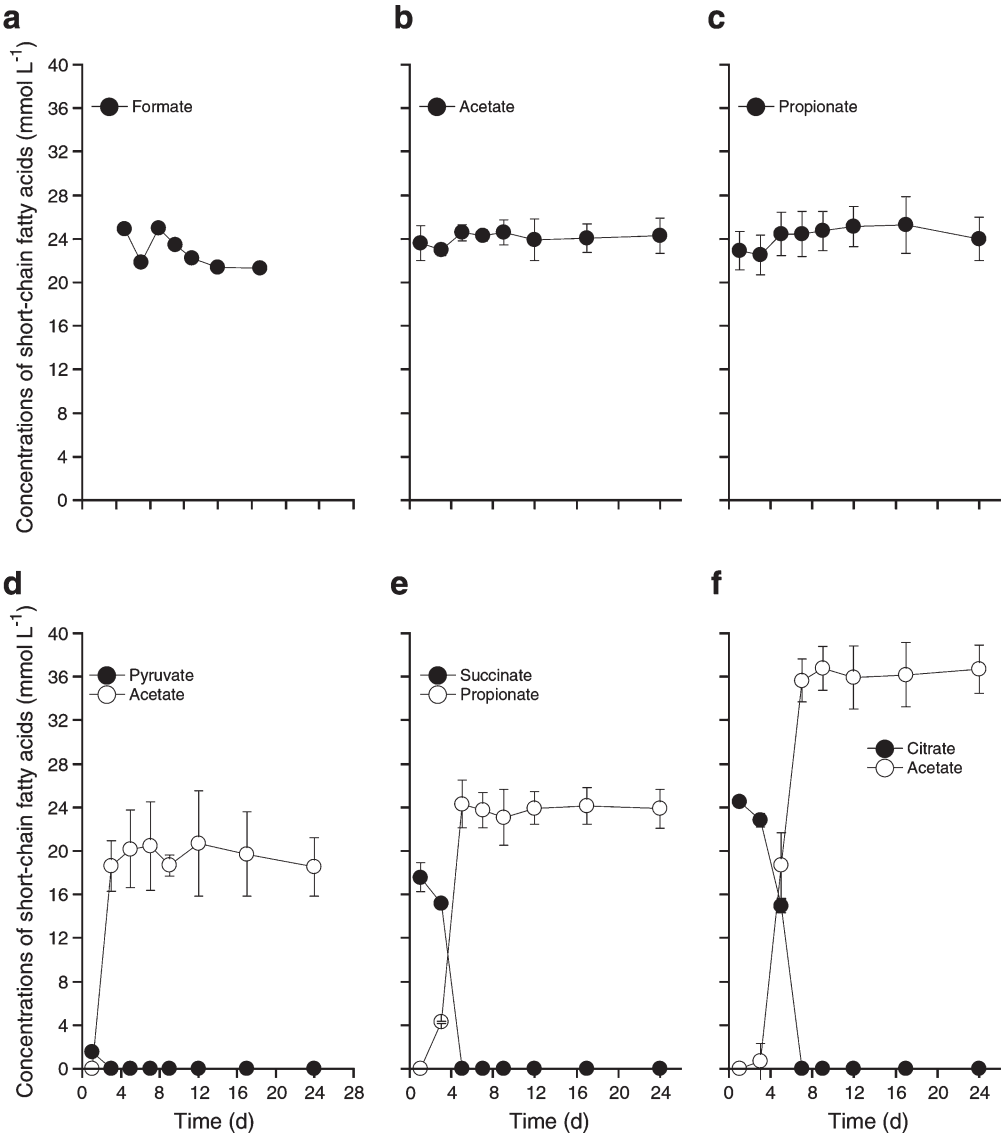

Figure 2. Dynamics of short-chain fatty acids in enrichment cultures with different short-chain fatty acids (**a**) formate, (**b**) acetate, (**c**) propionate, (**d**) pyruvate, (**e**) succinate, and (**f**) citrate. Data are means of triplicates except (**a**) because some data were missed; *error bars* indicate means ± SE

and even as high as 98% in pyruvate, succinate, and citrate treatments.

Table 1. Metabolic values measured and calculated after 24 days of enrichment incubation amended with pyruvate, succinate, and citrate

	Pyruvate	Succinate	Citrate
Fe(II) (μmol)	120	50	200
Pyruvate/succinate/citrate (μmol)	250	250	250
$C_2H_4O_2/C_3H_6O_2$ (μmol)	200	240	360
Inorganic carbon (μmol)[a]	350	280	780
Electron production (meq e^-)	1,190	980	2,340
Percentage of electron implicated in the Fe(III) reduction (%)	10	5	9

a. Inorganic carbon contents were estimated stoichiometrially, the carbon incorporated into bacterial biomass was also included.

3.3 T-RFLP fingerprinting of the bacterial community

The bacterial community was analyzed by T-RFLP in combination with sequences of a clone library constructed from bacterial 16S rRNA genes. Following Rui et al. (2009), only those with a relative abundance higher than 5% in at least one profile were selected as the signature T-RFs for analyses of bacterial community. A combination of in silico analyses of 630 sequences and the T-RFLP fingerprinting of the representative clones were used to assign the major T-RFs to individual bacterial lineages (Table 3). T-RFLP profiles revealed that short-chain fatty acids substantially influenced the structure of Fe(III)-reducing bacterial community. The T-RFs of 139, 149, 268, and 508 bp were abundant in formate, acetate, and propionate treatments (Figure 3a–c). The 159-bp T-RF was also present in formate and acetate treatments. Obviously, T-RFLP profiles in pyruvate,

Table 2. Phylogenetic affiliation and percentage of clone numbers of bacterial 16S rRNA genes retrieved from enrichment cultures amended with different short-chain fatty acids and the control (CK) after 24 days incubation, and the paddy soil used as inoculum (CS)

Affiliation	Number/percentage (%)							
	Formate	Acetate	Propionate	Pyruvate	Succinate	Citrate	CK	CS
Proteobacteria								
α-Proteobacteria	0/0	0/0	2/2.5	0/0	0/0	0/0	0/0	9/10.3
β-Proteobacteria	3/5.2	3/3.2	0/0	0/0	0/0	0/0	3/3.4	21/24.1
γ-Proteobacteria	0/0	0/0	1/1.3	0/0	0/0	0/0	0/0	4/4.6
δ-Proteobacteria	0/0	0/0	1/1.3	1/1.4	0/0	0/0	6/6.8	16/18.4
Firmicutes								
Bacillales	14/24.1	31/33.0	25/31.3	1/1.4	5/6.0	11/16.9	15/17.0	1/1.1
Clostridiales	34/58.6	49/52.1	49/61.3	72/97.3	78/92.9	53/81.5	51/58.0	8/9.2
Diverse	7/12.1	6/6.3	2/2.5	0/0	1/1.2	1/1.5	13/14.8	28/32.2
Total number of clones	58	94	80	74	84	65	88	87

CK control, *CS* inoculum

succinate and citrate treatments differed greatly from those in formate, acetate, and propionate treatments (Figure 3d–f). The 508-bp T-RF showed a high abundance in the treatment supplemented with pyruvate (up to 57% of total peak height), while in the succinate treatment, the T-RFs of 290 and 403 bp were present predominantly (43% and 57%, respectively). For the citrate treatment, the T-RF with a size of 571 bp became exclusively dominant (50%). Seven major T-RFs were detected in control, among which the T-RF of 116 bp was dominant (39%) (Figure 3g). In contrast to all these enrichment incubations, the paddy soil used as inoculum was characterized by the 430-bp T-RF, which accounted for 46% of total peak height (Figure 3h).

4 Discussion

4.1 Fe(III) reduction and short-chain fatty acids metabolism

Our results showed that the amendments of carbon substrates substantially influenced the rate and extent of Fe(III) reduction. In treatments amended with citrate, Fe(III) reduction was rapid and complete (94%), which was in line with the result when Fe(III)-citrate was used as the electron acceptor (Lovley and Phillips 1988; Wang et al. 2009). Citrate is known to mediate the dissolution of iron oxides and form Fe-citrate complexes, thus, the aqueous complexes that have formed would be available for iron-reducing bacteria. Fe(III) was also reduced rapidly when pyruvate was amended. Complexes with 1:1 ratio of Fe(III) to pyruvate were identified (Kim et al. 1972); therefore, ferrihydrite might also be dissolved by pyruvate and readily used by Fe(III) reducers in the present study.

The reduction of Fe(III) was much slower and less extensive in other treatments, which could be caused by the inability to form Fe complexes and dissolution of amorphous FeOOH. Fe(III) reduction was also observed in control. This could be due to the high content of organic carbon (29.2 g kg^{-1}) in the inoculum which could be utilized as the substrate. However, it is hard to determine if it was respiratory Fe(III) reduction since the exact substrate was unknown.

Based on the close agreement between the theoretical and actual organic carbon consumed, respiratory Fe(III)

Table 3. Assignment of dominant T-RFs to defined bacterial taxa

T-RF (bp)	Affiliation
116	*Symbiobacterium* (Incertae sedis XVIII)
139	*Bacillus*
149	Bacillaceae/Clostridiaceae
159	*Geobacter*
164	Peptococcaceae
268	*Clostridium*
278	Acidobacteria (Gp 10)
287	Acidobacteria (Gp 6)
290	Veillonellaceae
306	Gracilibacteraceae
403	Veillonellaceae
431	β-Proteobacteria
472	*Clostridium*
508	*Clostridium*
515	*Clostridium*
571	*Alkaliphilus*

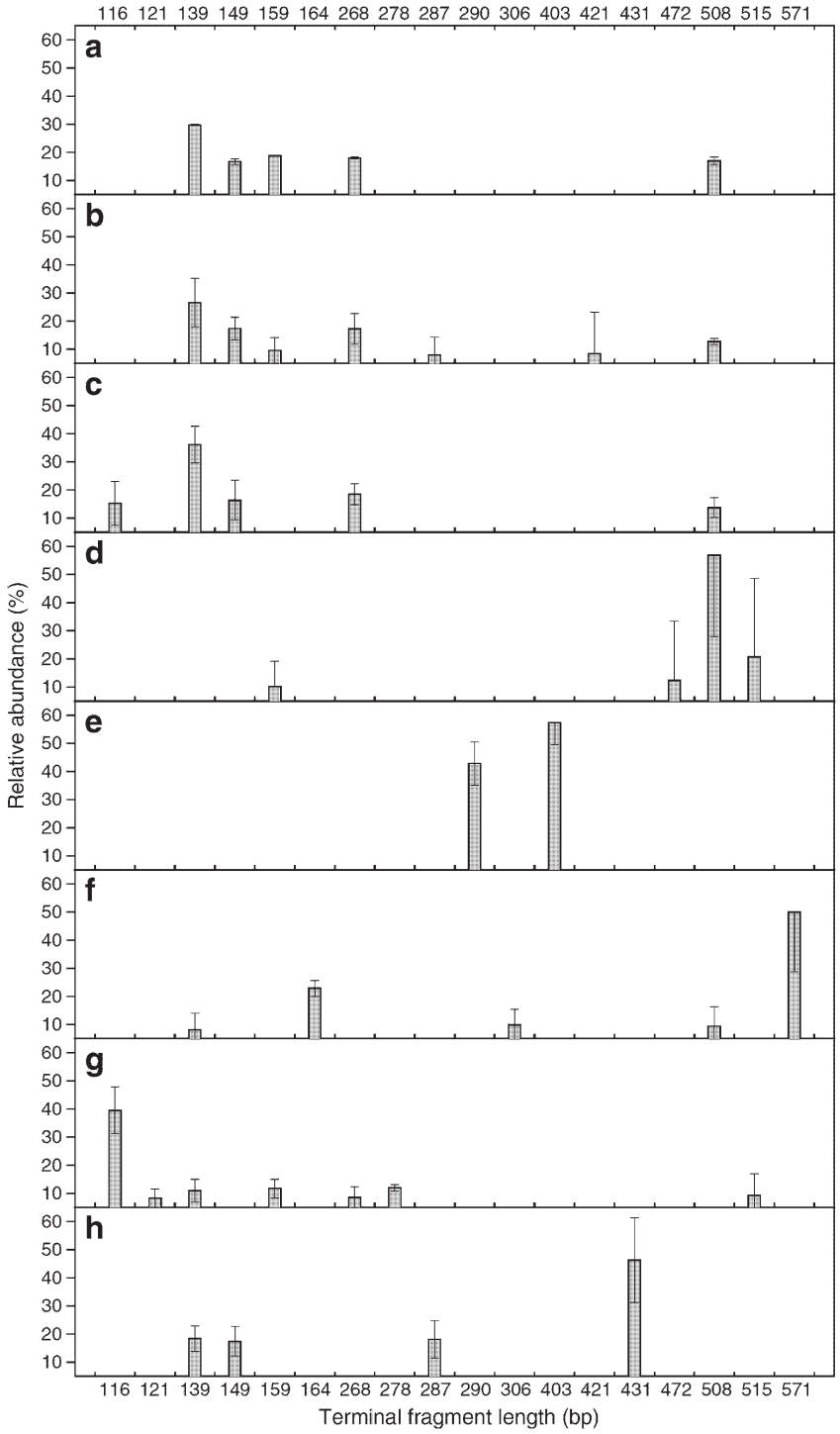

Figure 3. T-RFLP analysis of bacterial 16S rRNA genes derived from the enrichment cultures with different short-chain fatty acids after 24 days of incubation: (**a**) formate, (**b**) acetate, (**c**) propionate, (**d**) pyruvate, (**e**) succinate, (**f**) citrate, (**g**) the control, and (**h**) CS, the paddy soil used as inoculum. Data are means ± standard error (n = 3). Only major T-RFs are shown

reduction was presumed to have occurred in formate, acetate, and propionate treatments. In pyruvate, succinate, and citrate treatments, since only 5–10% of electrons in the associated organic matter was transferred to Fe(III) oxides, Fe(III) served as an the electron sink rather than electron acceptor. Thus, fermentative Fe(III) reduction likely occurred in pyruvate, succinate, and citrate treatments.

4.2 Community structure in the iron-reducing enrichments

Iron(III)-reducing microorganisms are phylogentically and physiologically diverse, thus identification of the responsible microorganisms in situ is essential. In subsurface environments where Fe(III) reduction is an important biogeochemical process, Geobacteraceae has often

been demonstrated to be the most abundant microorganism (Lovley et al. 2004). Recently, a study reported that *Geobacter* spp. accounted for about 85% of the iron-reducing communtiy in an Italian paddy soil with acetate as the electron donor and ferrihydrite as the electron acceptor (Hori et al. 2010). However, in the present study, *Geobacter* was only detected in control and the formate and acetate treatments, representing a minor fraction (12%, 10%, and 19% of total peak height, respectively). Cloning/sequence and T-RFLP analysis of the bacterial communities revealed that Firmicutes was the dominant group of Fe(III)-reducers in all the enrichment cultures. Nevertheless, it is not surprising the dominant capability of Firmicutes to reduce Fe(III), since similar results have been reported recently (Kostka et al. 2002; Scala et al. 2006; Lin et al. 2007; Boonchayaanant et al. 2009).

Specifically, the amendment of different carbon substrates selected for distinct members in the phylum of Firmicutes. *Clostridium* spp. were the predominant microorganisms in the culture amendment with pyruvate, while both *Bacillus* and *Clostridium* were enriched in the formate, acetate and propionate treatments. The capability of *Bacillus* and *Clostridium* to reduce Fe(III) has been reported as early as 1950-70 s (Bromfield 1954; Hammann and Ottow 1974), and further confirmed by the isolation of pure cultures in recent years. For example, *Bacillus* spp. have been shown to be capable of respiratory as well as fermentative Fe(III) reduction (Boone et al. 1995; Pollock et al. 2007). *Clostridium* had always been considered to reduce Fe(III) through fermentation (Dobbin et al. 1999; Park et al. 2001; Scala et al. 2006; Lin et al. 2007), however, respiratory Fe(III) reduction is also possible. A strain of *Clostridium butyricum* isolated from a Chinese paddy soil has showed the capability to reduce Fe(III) when glucose as well as the non-fermentative substrate (acetate) was amended (Guan 2007). In the present study, the abundant *Clostridium* in formate, acetate and propionate treatments also indicated the potential respiratory Fe(III) reduction by *Clostridium*.

Sequences related to Veillonellaceae and *Alkaliphilus* were predominant in succinate and citrate treatments, respectively. Clones within the cluster of Veillonellaceae shared most similarity to *Sporotalea propionica*, which was a propionigenic Firmicute; however, its capability to reduce Fe(III) has not been reported (Boga et al. 2007). Though the capability of *Alkaliphilus* species to reduce Fe(III) has been confirmed by the isolation of pure cultures (Ye et al. 2004; Lin et al. 2007), Fe(III) reduction fermentative by *Alkaliphilus* has not been reported.

Incertae sedis XVIII-related sequences which showed a relationship to *Symbiobacterium* were enriched in control. The potential for *Symbiobacterium* to be involved in nitrate respiration has been proven (Rhee et al. 2002; Ueda et al. 2004), however, *Symbiobacterium* species have so far not been directly linked to Fe(III)-reducing activity. The distinct community structure between the control and the treatments indicated that other organic carbons rather than those chosen as electron donors/substrates in the present study was used by the Fe(III) reducers in control, which suggests that the potential electron donors/substrates for Fe(III) reducers is more complicated in paddy soils in situ.

Conclusively, Fe(III)-reducing bacteria in paddy soil are phylogenetically diverse. Besides the well-known *Geobacter* species, Firmicutes-related Fe(III)-reducing bacteria were also enriched in the present study. The short-chain fatty acids supplemented greatly influenced the community structure of Fe(III)-reducing bacteria in the enrichment. Though Firmicutes-related Fe(III)-reducing bacteria in the present enrichment cultures were relatively abundant, it does not mean the same will be true for natural environment. To confirm the populations which play an important role in situ, further studies with culture-independent mRNA-based analysis are needed.

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Supplementary material follows the References.

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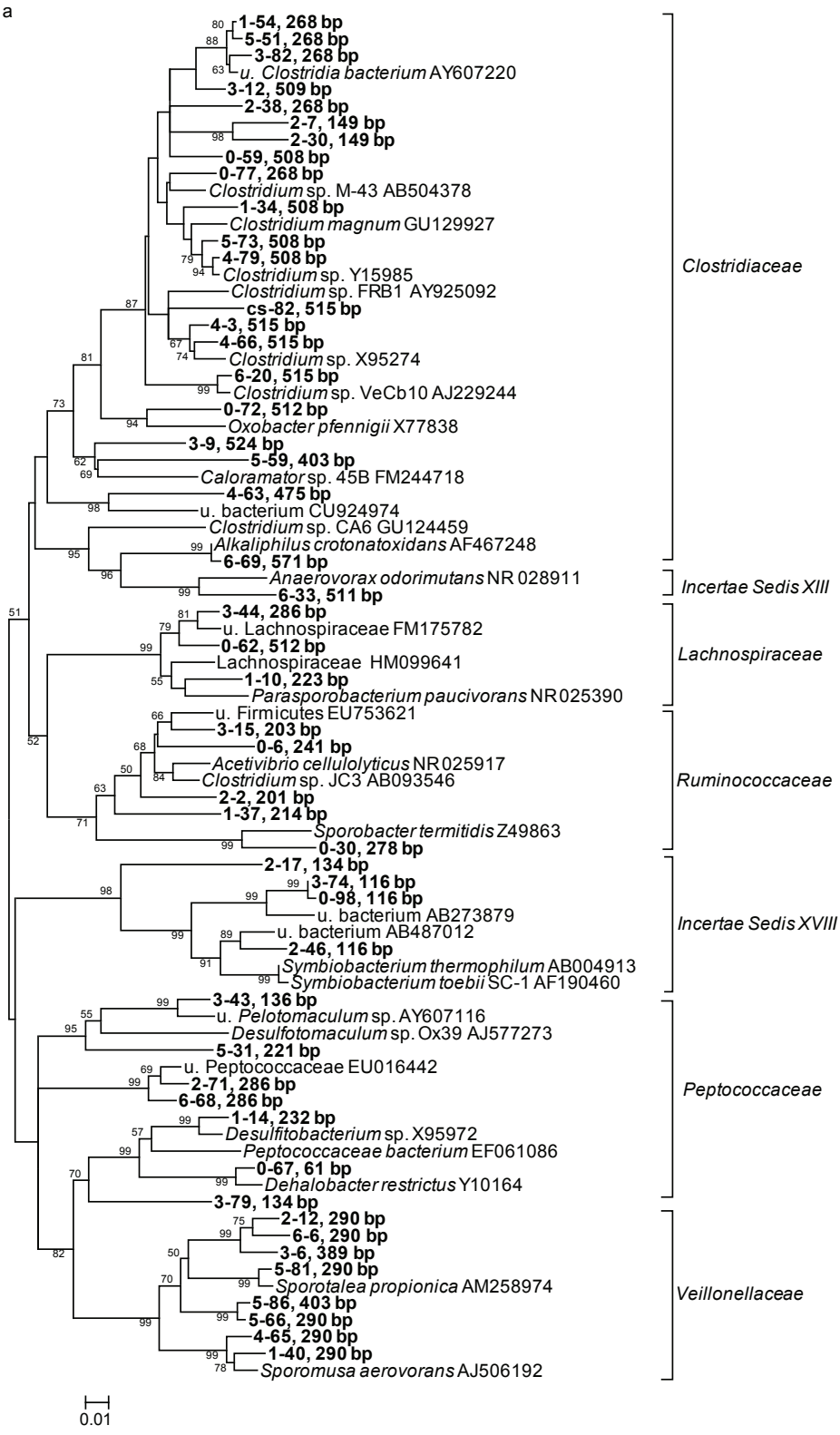
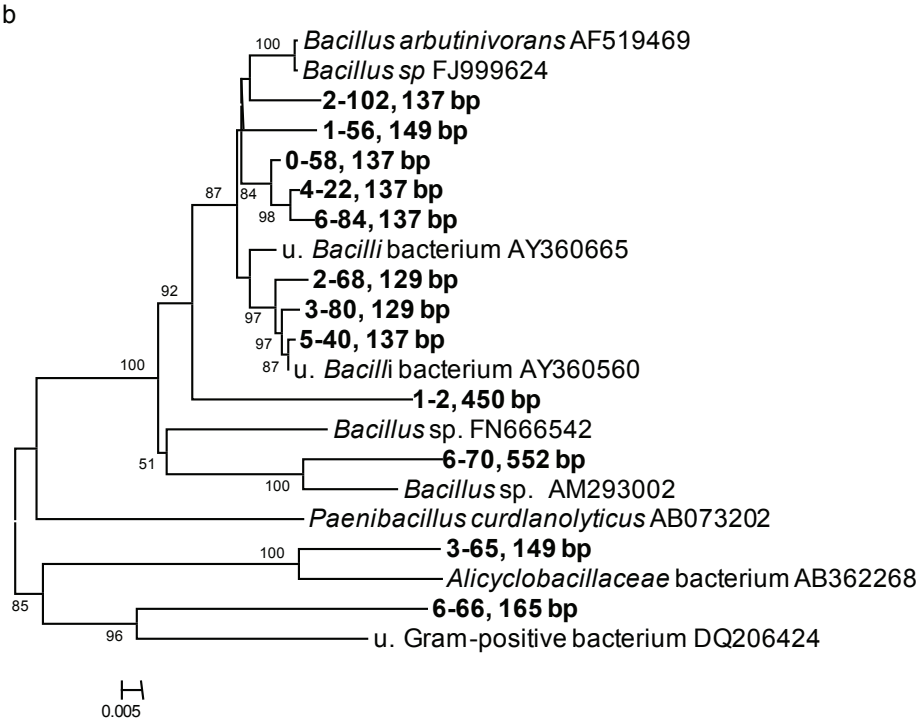
Supplementary Material

Resource 1. Phylogenetic trees showing the 16S rRNA gene clone sequences related to *Firmicutes*, including *Clostridia* (a) and *Bacilli* (b), and the other phyla (c) generated from iron-reducing enrichment cultures. Different numbers indicate different treatments (0 = the control, 1 = Formate, 2 = Acetate, 3 = Propionate, 4 = Pyruvate, 5 = Succinate, 6 = Citrate, cs = the paddy soil used as inoculum). The scale bar represents 1% (a and c) or 0.05% (b) sequence divergence; The T-RF sizes are indicated.



b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Bacillus sp FJ999624
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1-56, 149 bp
0-58, 137 bp
87
84
4-22, 137 bp
98
6-84, 137 bp
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92
2-68, 129 bp
97
3-80, 129 bp
97
5-40, 137 bp
87
u. Bacilli bacterium AY360560
100
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Bacillus sp. FN666542
51
6-70, 552 bp
100
Bacillus sp. AM293002
Paenibacillus curdlanolyticus AB073202
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Alicyclobacillaceae bacterium AB362268
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6-66, 165 bp
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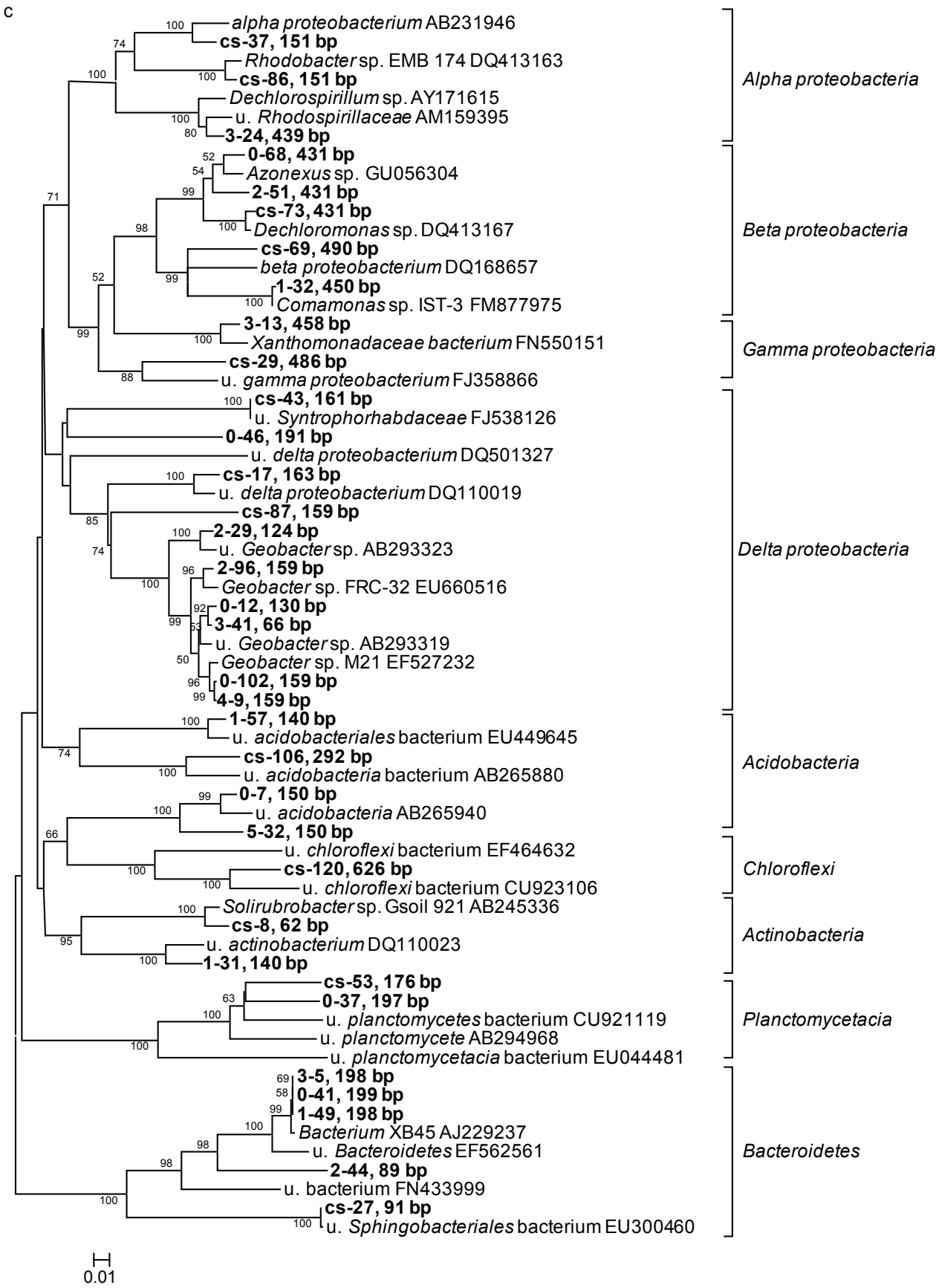
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Rhodobacter sp. EMB 174 DQ413163
cs-86, 151 bp
Dechlorospirillum sp. AY171615
u. Rhodospirillaceae AM159395
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0-68, 431 bp
Azonexus sp. GU056304
2-51, 431 bp
cs-73, 431 bp
Dechloromonas sp. DQ413167
cs-69, 490 bp
beta proteobacterium DQ168657
1-32, 450 bp
Comamonas sp. IST-3 FM877975
3-13, 458 bp
Xanthomonadaceae bacterium FN550151
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Alpha proteobacteria
Beta proteobacteria
Gamma proteobacteria
Delta proteobacteria
Acidobacteria
Chloroflexi
Actinobacteria
Planctomycetacia
Bacteroidetes
0.01