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Full Length Research Paper

Diversity of isolates performing Fe(III) reduction from paddy soil fed by different organic carbon sources

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This study examined microbial diversity of Fe(III)-reducing bacteria isolates in paddy soils amended with ferrihydrite and different organic carbon (C) sources. Results show that Fe(III) reduction rate and Fe(II) accumulation in soil enrichments amended with glucose were the greatest followed by pyruvate, lactate, acetate and control. ARDRA (amplified ribosomal deoxyribonucleic acid restriction analysis) analysis showed 161 Fe(III)-reducing bacteria strains isolated from associated soil enrichment cultures fell into seven groups. Dominant populations in soil enrichments shifted according to amended organic C substrate. *Paenibacillus* spp. and *Clostridum* spp. occurred in all soil enrichment cultures. *Solibacillus* spp. and *Lysinibacillus* spp. were enriched by acetate, while *Bacillus* spp. was dominant in pyruvate-amended soil enrichments. *Azotobacter* spp. was detected in all soil enrichments except those amended with glucose, which were mainly consisted of *Pseudomonas* spp. Data analysis indicated the highest Fe(III)-reducing bacterial diversity occurring in acetate and lactate-amended soil enrichments, and glucose-amended enrichments characterized with most predominant species. In summary, this study demonstrates a high diversity of microbial Fe(III)-reducing bacterial populations and their response to different organic C sources in paddy soils. Their versatile C metabolism plays an important role in Fe-C cycling.

Key words: Microbial iron reduction, organic carbon, Fe(III)-reducing bacteria isolates, paddy soil.

INTRODUCTION

Iron (Fe) is the fourth most abundant element in the Earth's crust (after oxygen, silicon, and aluminum) and by mass makes up about 5.1% of the crust. In subsurface environments, Fe(III) reduction coupled to organic carbon (C) oxidation exerts a strong influence on terrestrial and aquatic geochemistry (Lovley, 1996, 1997; Dong et al., 2006; Lehours et al., 2009). Evidences showed reduction of Fe(III) oxide under anoxic conditions is largely controlled by microbial processes. During Fe(III) reduction, FeRB (Fe(III)-reducing bacteria, including bacteria and archaea) gain electrons from organic substrates, and then transfer to Fe(III) to generate Fe(II) rather than assimilate Fe into cell component. Due to dynamic Fe redox chemistry in circumneutral environments, microbial Fe(III) reduction in anoxic systems has a profound influence on global biogeochemical cycling of organic and inorganic species such as C, nitrogen (N), phosphate (P), sulfur (S) and trace metals (Scott and Nealson, 1994; Ivanov et al., 2005; Sawaynma, 2006; Zhao et al., 2007). At present, FeRB have been isolated from a variety of anoxic systems, such as freshwater, marine sediments, wetland soils, biofilms and microbial mats and contaminated aquifers (Anderson et al., 1998; Todorova et al., 2005; Weber et al., 2006a, b; Omoregie et al., 2008). Representative FeRB include nine phyla, fifteen classes, twenty-seven orders, thirty-seven families and sixty-five genera (Kappler and Straub, 2005; Weber

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Abbreviations: FeRB, Fe (III)-reducing bacteria; **DGGE**, denaturing gradient gel electrophoresis; **T-RFLP**, terminal restriction fragment length polymorphism; **SSCP**, single-strand conformation polymorphism; **ARDRA**, amplified ribosomal DNA restriction analysis; **TOC**, total organic carbon; **CFUs**, colony forming units; **OTU**, operational taxonomic unit; **UPGMA**, the unweighted pair group clustering method with arithmetic averages.

et al., 2006a). However, phylogenetic diversity of representative FeRB leads to difficulty in understanding microbial function in natural environment.

In recent decades, molecular phylogenetic techniques such as PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism) and SSCP (single-strand conformation polymorphism) have been developed and widely used for studying microbial ecology of FeRB (Dassonvillea et al., 2004; Scheid et al., 2004; Lin et al., 2005). Compared with microbiological cultivation techniques, molecular biological methods have shown great superiority in revealing *in situ* microbial diversity. However, research based on Fe(III)-reducing functional genes is still limited (Gralnick and Newman, 2007). This is because highly diverse FeRB evolved different reduction strategies and enzymatic Fe(III) reduction mechanism has only been studied with few representative species such as *Geobacter* and *Shewanella* (Dobbin et al., 1995; Nevin and Lovley, 2002; Straub and Schink, 2004; Bond et al., 2005; Ding et al., 2006; Qian et al., 2007; Holmes et al., 2008). As no universal primer(s)/probe(s) has been available to target all known FeRB species from environmental samples, extensive studies employing group-specific primers were done to target several representative FeRB genera and/or species such as *Geobacter*, *Shewanella* and *Goethrix* (Frank et al., 1994; Lin et al., 2005). Alternatively, clone library has been constructed to study bacteria in Fe(III)-reducing environment, but this commonly involved laborious work (Blothe et al., 2008; Wang et al., 2009). Associated gene information might also be discarded due to incomplete or missing sequence data. Therefore, culture-based study of FeRB involving isolation and characterization is of importance to obtain required physiological information of specific FeRB. This has been an efficient way to combine knowledge between biochemical and gene levels.

As an important substrate for of FeRB cell components, organic C also functions as an electron donor for microbial Fe(III) reduction. According to the FeRB phylogenetic diversity, a variety of FeRB species preferentially utilize different organic matters. Generally, acetate is thought to be the universal C source for FeRB, especially for *Geobacer* spp. (Lear et al., 2007). However, acidophilic FeRB *Acidiphilium cryptum* JF-5 isolated from coal mine water was found incapable of Fe(III) reduction coupled to acetate oxidation, and growth on glucose, ethanol and H_2 was inhibited in the presence of acetate (Kusel et al., 1999). Coates et al. (1999) reported *Clostridia* of Firmicutes phylum dominated sediment enrichments amended with glucose. Together such findings indicate different C metabolism among FeRB and nature of organic C source affects FeRB population diversity in subsurface environments. A better understanding of organic C metabolism and C source- induced FeRB population shifts is required to facilitate bacterial cultivation and isolation.

Being an intermediate of arid land and aquatic systems, paddy soil is a representative of studying bacterial Fe(III) reduction and associated FeRB populations. Evidences showed periodical alternations between flooded-anoxic and drained-oxic conditions support dynamic redox reactions in paddy soil discrete zones (Lüdemann et al., 2000). High activity of FeRB has been demonstrated in the paddy soil 4 to 8 mm depth (Ratering and Schnell, 2000). Root exudates and plant residues are the primary organic C source in paddy soils, where proportional contribution of Fe(III) reduction and methanogenesis to organic C oxidation are largely controlled by content and nature of organic substrates (Frenzel et al., 1999; Roden, 2003). Research indicated Fe(III) reduction may account for 50% of total C metabolism in anoxic paddy soils (Jaeckel and Schnell, 2000). Achtnich et al. (1995) demonstrated bacterial Fe(III) reduction suppresses methanogenesis by outcompeting for H_2. Different organic C composition can affect degradation rates and associated microbial communities (He and Qu, 2008). However, few studies have documented FeRB population diversity and their influencing factors during soil organic carbon turnover.

This study aimed to characterize composition and diversity of FeRB populations capable of reducing solid Fe(III)-oxyhydroxide to aqueous Fe(II) in a paddy soil. To this end, we performed a combined analysis of geochemical processes and a culture-dependent approach in soil enrichment cultures amended with different C sources (glucose, pyruvate, lactate and acetate). In each C sources condition, pure isolates were obtained to analyze cultivable FeRB diversity.

MATERIALS AND METHODS

Study area and sample handling

Soils were sampled from a post-harvest paddy soil in Xiangning County (112.57°E, 28.26°N), Hunan Province, China, which is one of the major countries for rice production. It is a reddish clayey paddy soil at annual rainfall of 1 358.4 mm and mean temperature of 17.2°C; with amorphous ferric oxide (Lovley and Phillips, 1986) 3.75 g kg^{-1} dry soil and free iron oxide (Mehra and Jachson, 1960) 27.05 g kg^{-1} dry soil. Samples were taken from the top 20 cm layer of the paddy soil. The soil was then air-dried, mechanically crushed, sieved (<1 mm), and stored until the beginning of the experiment.

To activate the microbial sensitivity to amended C sources and reduce endogenous electron acceptors such as available nitrate, sulfate and ferric iron oxides, 'recycled soil', a soil that had been flooded earlier to deplete labile organics and nutrients (Conrad and Klose, 1999), was used. For experiments with the 'recycled soil', 150 g air-dried soil (1 mm fraction) was placed into a 500 ml sterile polyethylene container, mixed with sterile water at a ratio of 1:2 (*w*/*v*) and pre-incubated for five months in the dark at ambient temperature. We did not investigate the content of sulfate in the soil, since endogenous sulfate concentration could progressively decrease to undetectable levels in depleted anoxic paddy soils within 10 days anoxic incubation (Achtnich et al., 1995). Samples were determined for total organic carbon (TOC), nitrogen (N),

Table 1. Comparison of soil nutrients before and after laboratory incubation.

Nutrient	TOC (g kg^{-1})	Total N (g kg^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})
Before flooding	14.73	1.66	0.72	33.60
After flooding	12.10	1.15	0.30	14.67

phosphorus (P) and potassium (K) levels before and after pre-incubation (Table 1). On the average, TOC level decreased by 18% after incubation, suggesting a large proportion of labile organic carbon was microbial-depleted. Residual organic carbon presumably comprised humus, which was unavailable for microbial metabolism. Total N level decreased by 31%, whereas P and K levels showed a lack of strong variation. 'Recycled soil' was transferred to sterile tubes and centrifuged at 3 000 rpm for 15 min, with supernatant discard. Sterile water was then added to soil pellet at a ratio of 1:1 (*w/v*) to produce homogenized slurry.

Soil enrichments

Aliquots (3 ml) of the homogenized slurry were transferred into 7 ml serum vials. Mixed with 1 ml synthetic ferrihydrite FeOOH, which was synthesized according to Cornell and Schwertmann (2003) and 1 ml sterile mineral solution containing 1.5 mol L^{-1} C source and 0.1 mol L^{-1} NH_4Cl. The iron oxides were supplemented at a final concentration of ca 20 mg g^{-1} soil dry weight. Stock solutions of glucose ($C_6H_{12}O_6 \cdot H_2O$, 0.25 mol L^{-1}), pyruvate ($C_3H_3O_3Na$, 0.5 mol L^{-1}), lactate ($C_3H_5O_3Na$, 0.5 mol L^{-1}) and acetate ($C_2H_3O_2Na \cdot 3H_2O$, 0.75 mol L^{-1}) were added to individual vial respectively as carbon and electron donors performed as equivalent amount of C. C-free 0.1 mol L^{-1} NH_4Cl solution was used as a blank control. Vials were capped with butyl rubber septa, sealed with aluminum lid, and headspaces were flushed with N_2 (n=3, each). All enrichments were carried out at 30°C in the dark for over 30 days. Each repetitive vial was sacrificed during sampling for Fe(II) analysis at 1, 5, 10, 15, 20, and 30 days. Dissolved Fe(II) concentrations were determined as described previously (Kalver and Pitts, 1966). Briefly, 0.4 ml sub-sample was extracted for 24 h (Schnell and Ratering, 1998) using 0.5 mol L^{-1} HCl, and extracted Fe(II) was determined photometrically as complexes with 1, 10-orthophenantrolin by a spectrophotometer at 510 nm. Enrichment samples were stored at -20°C for subsequent analyses.

Fe(III) reduction is a microorganism-mediated biological process that can be fitted by a logistic equation which also describes the growth kinetics of microorganism (Kingsland and Sharon, 1995).

The relation is expressed by $y = \frac{a}{1+be^{-cx}}$, where, x refers to incubation time; y refers to Fe(II) contents at incubation time x; a refers to the maximum potential of Fe(III) reduction in the system (maximum accumulation of Fe(II)); b refers to the model parameter and c is the reaction rate constant. The value of maximum reaction rate (V_{max}) equals 0.25ac. The time corresponding to the maximum reaction rate (T_{Vmax}) is calculated by ln$b$/c.

Bacterial cultivation and isolation

Pyrophosphate was used as a soil dispersant, in order to increase the number of extractable microbes (Furtado and Casper, 2000). 2 mmol L^{-1} sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) was added to enrichments at a ratio of 1:9 (*w/v*) when Fe(II) accumulation rate was at logarithmic growth phase. Grading dilution of enrichment was inoculated to 5% PTY plates (peptone 0.25 g L^{-1}, tryptone 0.25 g L^{-1}, yeast extract 0.50 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.6 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.07 g L^{-1}, agar 10.00 g L^{-1}). Plates were tightly wrapped using sealing film and incubated at 30°C in an aseptic glove box. Early-formed colony forming units (CFUs) were considered as aerobic bacteria, which consumed residual O_2 in the top of plate. After O_2 was depleted, the anaerobic bacteria were formed CFUs. 100 CFUs were randomly selected from each treatment and quickly transferred to ferric citrate medium (ferric citrate 16.75 g L^{-1}, NH_4Cl 1.00 g L^{-1}, $MgCl_2 \cdot 6H_2O$ 0.20 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.01 g L^{-1}, KH_2PO_4 0.26 g L^{-1}, $K_2HPO_4 \cdot 3H_2O$ 0.70 g L^{-1}, glucose 2.48 g L^{-1}, pyruvate 2.75 g L^{-1}, lactate 2.80 g L^{-1}, acetate 5.10 g L^{-1}). When the color of ferric citrate changed from green to white or colorless, the positive cultures were performed streaking separation, as well as diluting separation for 2 to 4 times to obtain pure cultures (defined as a single colony formed by a single cell). The putatively pure strains were then transferred to ferrihydrite medium, similar to ferric citrate medium but referring to ferrihydrite used as an electron acceptor. FeRB isolates were identified as those which changed reddish-brown ferric oxide to colorless aqueous Fe(II).

ARDRA profiling and analysis

FeRB isolates were selected to proliferation with 50% LB medium. A 10 ml aliquot of enrichment culture was centrifuged at 3 500 rpm for 15 min. Cell pellet was collected and washed twice with sterile water. Total genomic DNA was extracted as reported previously (Chen and Ronald, 1999). Thermal cycling of PCR (Elifantz et al., 2010) and PCR primers (Marchesi et al., 1998) used were as described previously. Amplicons were purified using sodium acetate and ethanol and then digested using restriction enzyme Hha I (Wang et al., 2009) (37°C, 4 h). DNA digestion fragments were separated using polyacrylamide gel electrophoresis at 100 mV for 3 h and stained by silver nitrate. Each pattern was considered as an operational taxonomic unit (OTU). According to molecular fingerprints, bands of different strains sharing the same mitigation rate were marked with 1, otherwise with 0. A simple matching coefficient was use as a distance index for cluster analysis of different strains. The unweighted pair group clustering method with arithmetic averages (UPGMA) (Ling et al., 2001) was performed using NTSYSpc 2.10e statistical software.

Sequencing and phylogenetic analyses

Selected representative isolates DNA were subjected to cloning and sequencing. The conditions of PCR were as described previously. PCR amplicons were sequenced by GenScript Nanjing Inc. (Nanjing, China, http://www.genscript.com.cn/index.html). Sequence data were compared with RDP nucleotide sequence database by BLASTN searches (http://rdp.cme.msu.edu/). Phylogenetic analyses were performed using the software package MEGA 4.1. Phylogenetic core trees were constructed from reference 16S rDNA gene sequences using the Kimura 2-parameter matrix model and the neighbor-joining method. Robustness of derived groupings was tested by bootstrap using 1 000 replications.

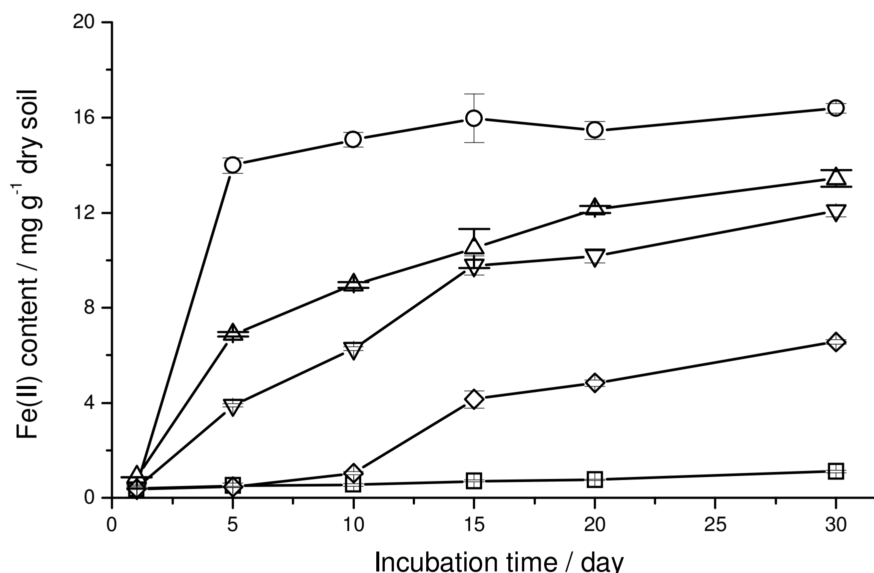

Figure 1. Changes in dissolved Fe(II) level in paddy soil enrichments amended with glucose (○),pyruvate (△), lactate (▽), acetate (◇) and blank control (□). Error bars represent the standard deviation of replicate (*n* = 3).

Table 2. The kinetics parameters of microbial Fe(III) reduction in paddy soils amended with different carbon sources (mean ± standard error, n = 3).

Carbon source	Logistic model parameter					
	a	*b*	*c*	R^2	V_{max} / mg (g d)$^{-1}$	T_{Vmax} / day
Glucose	16.77±0.23	106.40±5.98	1.25±0.01	0.969	5.26	4
Pyruvate	14.83±0.09	4.28±0.19	0.16±0.02	0.963	0.61	9
Lactate	13.74±0.14	7.21±0.33	0.17±0.01	0.980	0.59	12
Acetate	12.69±0.13	13.16±0.92	0.09±0	0.984	0.29	29

Analysis of diversity and similarity

The numbers of OTUs in profiles from different C treatments were pressed as the phylotype abundance, dominance and evenness. The *α*-diversity indices, such as Margalef index (d_{Ma}), Fisher *α* index, Shannon-Wiener index (*H'*), McIntosh index (D_{mc}), Simpson's index (*λ*), Berger-Parker index (*d*) and Pielou's index (J_{sw}) were calculated as previously described (Devaurs and Gras, 2010; Spatharis and Tsirtsis, 2010). Based on *α*-diversity indices, Euclidean distance was used to cluster isolates associated with different organic C substrates. *β*-diversity indices such as Jaccard similarity coefficient (Seifoddini and Djassemi, 1991) were also used to compare diversity of isolates. Data analysis was performed using Biodap and SPSS 13.0.

RESULTS

Bacterial Fe(III) reduction measurement

Fe(III)-reduction was determined in enrichments by measuring the production of Fe(II) (Figure 1). An increase in aqueous Fe(II) concentration was observed over the period of the experiment in enrichments containing organic C as a substrate. The reaction fitted well to the logistic model, with the coefficient of determination 0.963 to 0.984. Greatest Fe(II) accumulations were noted in enrichments amended with glucose 16.77 mg g^{-1} dry soil, followed by pyruvate, lactate and acetate (Table 2). Conversely, a lack of Fe(II) accumulation in control treatment indicated that insufficient electron donor was not effective at stimulating Fe(III) reduction. Rapid Fe(III) reduction occurred within 5 days incubation in glucose-amended soil enrichment, and V_{max} was 5.26 mg (g d)$^{-1}$. In pyruvate and lactate amended treatments, dissolved Fe(II) levels constantly increased throughout 30 days incubation, although, Fe(III) reduction rates were only one-tenth of glucose treatment. Fe(II) level was substantially low during 10 days incubation in acetate-amended soil enrichment before increasing at similar rates as those in pyruvate- and lactate-amended soil enrichments. However, the maximum Fe(III) reduction accumulation (*a*) could reach 13 mg g^{-1} dry soil, with the T_{Vmax} was 29 days. It can be inferred that there would be more enrichment of FeRB preference of acetate with the incubation time extending. The time corresponding to the

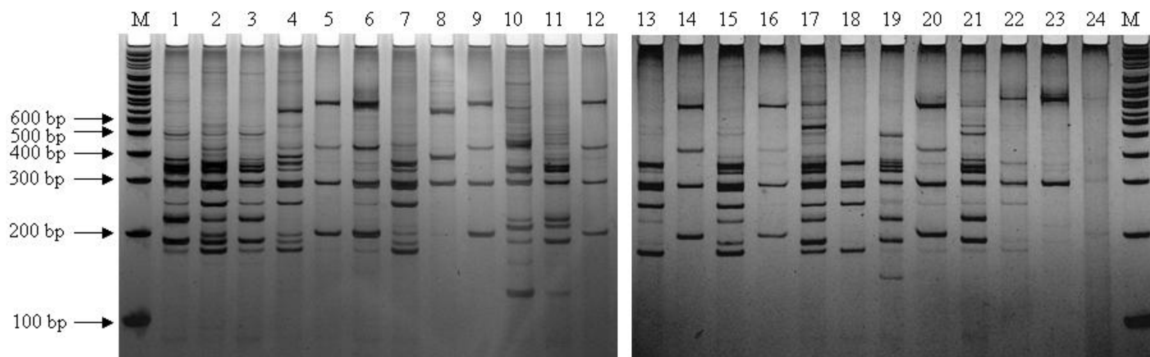

Figure 2. ARDRD fingerprinting of FeRB 16S rDNA partial sequences digested by *Hha*I (lane M: markers; lanes 1 to 24: representative samples)

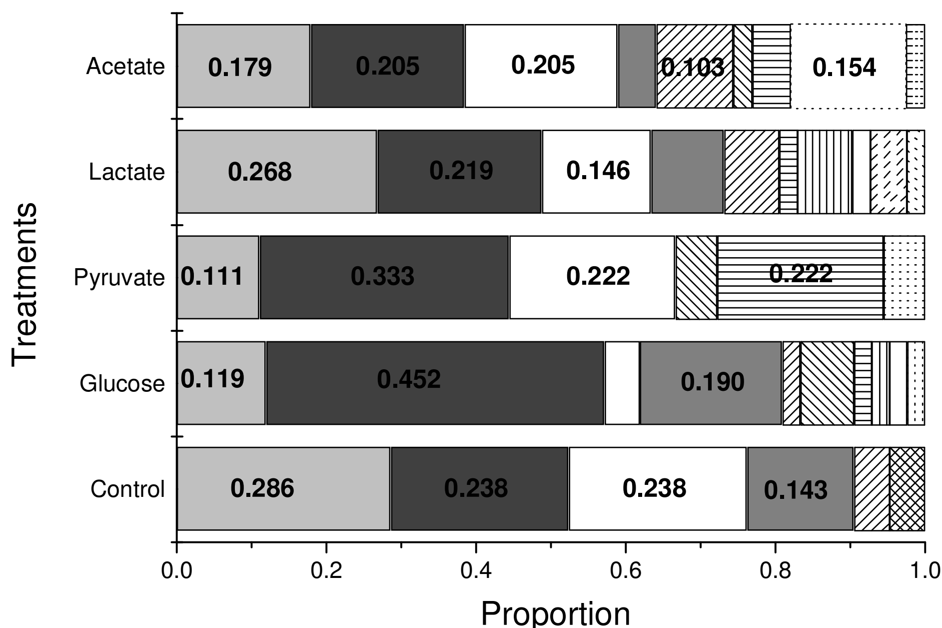

Figure 3. OTUs composition of FeRB populations associated with glucose, pyruvate, lactate, acetate and control. The same patterns out of different treatments denote that they share the same populations.

maximum reaction rate in different soil enrichments containing organic C suggested the logarithmic bacterial growth was between 4 and 29 days. Soil slurries were taken on days 15 and used for inoculation of 5% PTY plates.

Isolation and molecular biological analysis of cultivable FeRB

500 CFUs were excised randomly from laboratory enrichment cultures. 372 cultures were able to reduce completely citrate-Fe, with 17% related to glucose, 19% related to pyruvate, 22% related to lactate, 21% related to acetate and 21% related to control treatment. FeRB strains were identified as those which changed reddish-brown ferric oxide to colorless aqueous Fe(II). A total of 161 FeRB strains were obtained to amplify the corresponding 16S rDNA genes, with 26% related to glucose, 11% related to pyruvate, 25% related to lactate, 24% related to acetate and 13% related to control treatment. Fe(II) measurement indicated more than 50% of original Fe(III) was reduced (data not shown).

ARDRA was performed on 161 FeRB isolates and patterns were obtained with *Hha* I digestion (Figure 2). The distribution of the taxonomic groups differed among different C amended enrichments based on ARDRA results (Figure 3). 161 FeRB isolates were grouped into sixteen OTUs, with 10, 6, 10, 9 and 6 OTUs related to glucose-, pyruvate-, lactate-, acetate-amended samples

Table 3. Alpha diversity indices of FeRB population in soil enrichments.

Index	Control	Glucose	Pyruvate	Lactate	Acetate
Number of individuals(N)	21	42	18	41	39
Abundance (S)	6	10	6	10	9
Pielou's Index (J_{sw})	0.899	0.743	0.893	0.869	0.897
Shannon-Wiener index (H')	1.610	1.710	1.600	2.000	1.970
Margalef Index(d_{Ma})	1.642	2.408	1.730	2.424	2.184
Fisher α Index	2.806	4.152	3.152	4.214	3.668
McIntosh's index(D_{mc})	0.679	0.573	0.683	0.702	0.719
Simpson's index(λ)	0.181	0.247	0.183	0.145	0.135
Berger-Parker index(d)	0.286	0.452	0.333	0.268	0.205

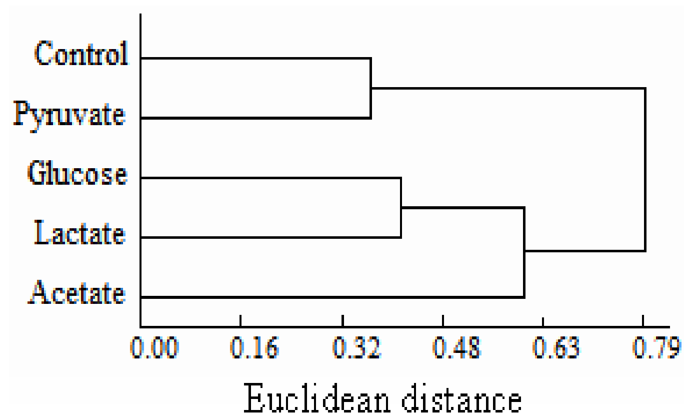

Figure 4. Cluster tree of FeRB populations in soil enrichments.

and control treatment. Among these, seven OTUs were considered dominant (>10%, Figure 3) and the resulting 20 FeRB isolates were sequenced to assess the identity of the corresponding organism.

Diversity and similarity analysis

Diversity analyses of FeRB 16S rDNA gene are shown in Table 3. In glucose enrichments, the bacterial diversity was low (J_{sw} = 0.743); oppositely, dominance indices λ and d were the highest, indicating that there were dominant species appearing at glucose enrichments. In lactate- and acetate-amended enrichment, a more complex bacterial community structure was observed in lactate- and acetate-amended media, with the higher bacterial abundance indices such as H', d_{Ma}, Fisher α and D_{mc}. Cluster analysis based on Euclidean distance indicated FeRB populations in control and pyruvate-amended enrichments were clustered together (Figure 4). Associated bacterial populations in glucose- and lactate-amended soil enrichments were closer to those in acetate-amended soil enrichments, which appeared to be the most distant.

Table 4 shows similarity of FeRB populations in soil enrichments amended with different carbon C substrates. Higher similarity occurred when FeRB populations share more similar OTUs. According to Jaccard similarity coefficient, two populations were considered significantly dissimilar when C_J=0.00 to 0.25; dissimilar when C_J=0.25 to 0.50; similar when C_J=0.50 to 0.75; and significantly similar when C_J=0.75 to 1.00. Similarity coefficients between FeRB populations in C-amended soil enrichments and control ranged from 0.333 to 0.500 (dissimilar), suggesting shifts in bacterial population diversity due to organic C amendment. Higher similarity coefficients (>0.500) between FeRB populations in glucose- and lactate/acetate-amended soil enrichments, suggesting similar bacterial populations occurred in associated soil enrichments.

Phylogenetic analysis of Fe(III)-reducing enrichments

Sequence analyses of the 20 isolates indicated dominant FeRB obtained from soil enrichments in this study include members of genera *Clostridium*, *Paenibacillus*, *Pseudomonas*, *Bacillus*, *Azotobacter*, *Lysinibacillus* and *Solibacillus* (Figure 5). Among these, *Paenibacillus* and *Clostridum* spp. retrieved in all soil enrichment treatments, especially genera *Clostridum*-related sequences were obtained and accounted for 45% of the glucose-amended enrichment (Figure 3). In contrast to the other enrichments, *Azotobacter* spp. were detected as dominant species in pyruvate-, lactate- and acetate-amended enrichment as well as control treatment, with the proportion 22, 15, 20 and 24%, respectively (Figure 3). *Pseudomonas* spp. related strains is retrieved from all enrichments except pyruvate, also, they were as dominant species appeared in glucose-amended (19%) and control treatment (14%, Figure 3). *Bacillus* spp. could not be retrieve from control treatment while it appeared in pyruvate-amended as dominant species (22%, Figures 3 and 5). Isolates A20 and A66 representatives of acetate-amended were affiliated to *Solibacillus* spp. and *Lysinibacillus* spp. Genera *Bacillus* is dominate species (22%, Figure 3) retrieved in pyruvate-amended enrichment.

Table 4. *β* diversity index of FeRB populations in soil enrichments.

Treatment	Jaccard similarity coefficient (C_J)				
	Control	Glucose	Pyruvate	Lactate	Acetate
Control	1.000				
Glucose	0.455	1.000			
Pyruvate	0.333	0.455	1.000		
Lactate	0.455	0.667	0.500	1.000	
Acetate	0.500	0.583	0.333	0.462	1.000

DISCUSSION

The relatively high concentrations of Fe(III) (free iron oxide) as well as the Fe(II) formation indicate potential for microbial Fe(III) reduction in the paddy soil from Hunan Province (Sun et al., 2008). In this study, a culture-dependent strategy was performed to determine the effect of carbon sources for the microbial Fe(III) reduction pathway and to assess the diversity of isolated iron-reducers in the flooded paddy soil. Despite pitfalls of isolation methods (soil extraction, medium and O_2, (Furtado and Casper, 2000)), plate isolation remains a convenient way to retrieve metabolic actively microbes from the environment, comparing with method of agar shakes or isolation under the microscope. Isolates retained in this laboratory incubation were not from strict oxygen free conditions (such as in anaerobic glove box), however, they were all highly active to reduce ferrihydrite with the ratio of reduction more than 50%. Therefore, study will discuss the diversity of putative FeRB in this laboratory condition, to show the structure of micro-aerobic bacteria during the early period of flooding paddy soil and find some typical or model FeRB.

Putative FeRB in enrichments

There were three reasons for the observations that facultative FeRB was isolated as the main population. The primary factor was the culture conditions. Isolation of strains was not under strictly anaerobic conditions, which may lead to the absence of strictly anaerobic micro-organisms. Secondly, glucose and pyruvate are typical fermentative substrates, which produce intermediate metabolites (such as H_2) that were more preferred to facultative FeRB. The last but more important reason was that the isolating time was no more than 15 days during incubation. According to the study of Yi et al. (2010), microbial communities from the soil extract during flooded 30 days could well use lactate and acetate as carbon to perform Fe (III) reduction, which presumed that members of *Shewanell* and *Geobacter* were retrieved in the late-period of flooding incubation. From ARDRA profiles, an effect of carbon sources on the structure of bacterial community in enrichments was noted suggesting that iron reducers may use different organic matters in the anoxic zone of flooding paddy soil. Our results reveal that most of the bacteria isolated in enrichments were facultative iron reducers; they belong to phylum Firmicutes (low G+C, G^+) and class *γ*-proteobacteria. Similar observations were made by Lehours et al. (2009) in the anaerobic zone of the meromictic Lake Pavin (France) and Lin et al. (2007) in sediments of the Scheldt estuary. According to 16S rDNA sequences, *Clostridium* spp. and *Paenibacillus* spp. were distributed as predominant species among all treatments amended organic carbon source including control treatment. Especially, *Clostridium* species were obtained and accounted for 45% of the whole strains isolated from glucose enrichment. Hammann and Ottow (1974) first reported reductive dissolution of Fe_2O_3 by saccharolytic Clostridia under anaerobic conditions. Several Fe(III)-reducing *Clostridium* species have been described as using Fe(III) as an electron sink like *C. beijerinckii* and *C. butyricum* in order to harvest more free energy per unit carbon dissimilated (Lin et al., 2007). The genus *Paenibacillus*, containing nitrate-reducing organisms previously isolated from sediment, can utilize acetate as a carbon source (Nakamura, 1984). However, as far as we know, there were no published pure culture on microbial Fe(III) reduction by *Paenibacillus* sp. Results from enrichment culture and quantitative PCR approaches provided strong evidence for identifying members of *Paenibacillus* catalyzing Fe(III) reduction in the uranium-contaminated areas (Petrie et al., 2003). Researchers assumed this organism could be fermenting rather than respiring glucose. Our study confirmed this speculation by isolation of such organisms and further characterization of their capacity for microbial Fe(III) reduction.

Azotobacter species were dominant out of glucose-amended enrichments. Dassonvillea (2004) found that during the initial 6 days, the fermentation was accompanied by non-symbiotic N_2 fixation which promoted bacterial growth and he referred that the possible reason of Fe(III) reduction throughout the incubation period was the contribution of H_2 produced in the fermentative process. In our laboratory experiment, N_2 was used in the container instead of oxygen; some of the mechanism might be $8H^+ + 8e^- + N_2 = 2NH_3 + H_2$. *Azotobacter* spp. are ubiquitous in neutral and weakly basic soils, but not

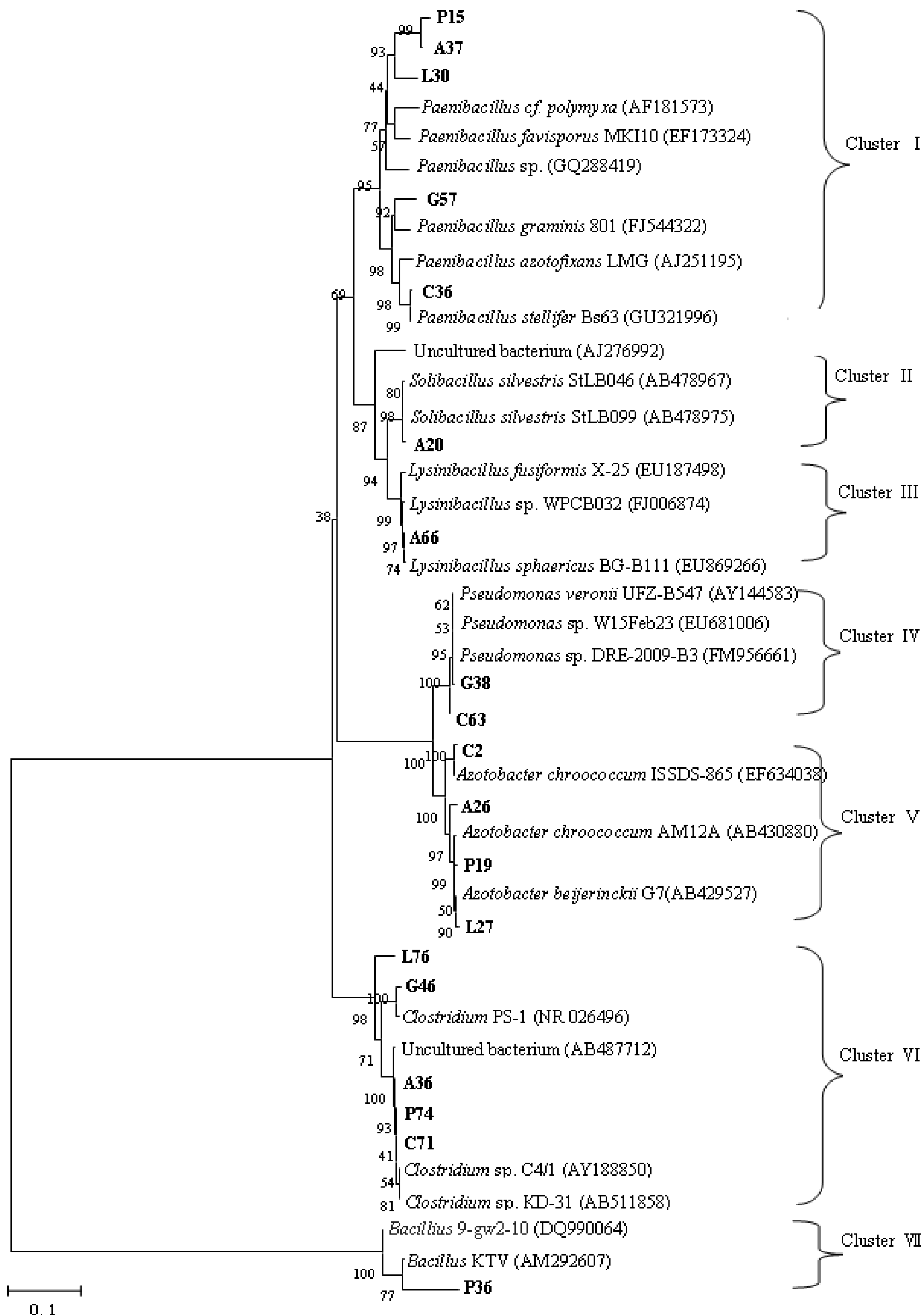

Figure 5. Phylogenetic tree of 16S rDNA gene partial sequences (*ca.* 1 500 bp) retrieved from FeRB isolates in soil enrichments (bar represents 10% variance). Bold initial letter A, strain from acetate-amended enrichment; C, strain from control enrichment; G strain from glucose-amended enrichment; L, strain from lactate-amended enrichment; P, strain from pyruvate-amended enrichment.

acidic soils http://en.wikipedia.org/wiki/Azotobacter (Yamagata and Itano, 1923). However, in the enrichment fed with glucose, since glucose fermentation generated a large number of acid, the pH decreased which was not conducive to the growth of *Azotobacter*. So *Azotobacter* was not the dominant population. *Pseudomonas* spp. plays an important role in Fe(III) reduction in agro-ecosystems and is frequently isolated from top-layer soil and rhizosphere in rice fields (Bowman et al., 1997; Marilley and Aragno, 1999), which was the first modern Fe(III) reducer shown to connect respiratory and reduction of Fe(III) oxide. It couples its growth to the reduction of ferric iron and accompanied by the oxidation of molecular hydrogen to the reduction of ferric iron (Balashova and Zavarzin, 1980). Straub and Schink (2004) elucidated that *Pseudomonas* sp. can lower the redox potential of a medium prior to or during growth, and then stimulated the iron reduction. *Bacillus* species were dominant in pyruvate enrichment culture (Figures 3 and 5). Microorganisms belonging to the *Bacillus* are widely distributed in natural systems, such as soils, waters, marine sediments and foods (Slepecky and Hemphill, 2006). Some of them have been reported to perform Fe(III) reduction (Boone et al., 1995) or retrieved from Fe(III)-reducing enrichment environment (Humayoun et al., 2003; Scheid et al., 2004). Here, several strains had been isolated from other carbon sources, which demonstrated that these bacteria affiliated with *Bacillus* sp. play an important role in paddy soil. *Solibacillus* sp. and *Lysinibacillus* spp. were collected under acetate enrichment; however, there was no report so far about the iron reduction function of these two populations. Although, results from our enrichment cultures and pure cultures provide some information regarding microbial community members catalyzing iron reduction, it mainly focused on the isolation of such organisms and further characterization of their capacity for microbial Fe(III) reduction is necessary.

All the sequences retrieved were affiliated to quite versatile strains with regard to their ability for anaerobic growth with a range of electron acceptor (example, *Clostridium*, *Paenibacillus*), their ability for aerobic growth (example, *Pseudomonas*, *Bacillus*) or their ability to survive as spores (example *Clostridium*, *Bacillus*) and cysts (example *Azotobacter* (Moreno et al., 1986)).

Based on pure cultures of the 16S rDNA-ARDRA found, no known obligatory FeRB (such as members of the *Geobacter* sp.) were retrieved in soil enrichment, even though amending with acetate. We nevertheless did not exclude their existence. You et al. (2011) studied the dynamic succession of Geobacteraceae community structure in paddy soil after flooding applying the RFLP and RT-PCR technology, and they found the abundance of Geobacteraceae was ranged from 1.1×10^6 to 18.4×10^6 copies of 16S rDNA g^{-1} dry soil. However, Geobacteraceae in the proportion of total bacteria was just 1.2 to 4.5%. Such a small proportion might result we missed some *Geobacter* spp. when we selected the colons randomly from solid medium plate. Our results reveal that most of the bacteria isolated in enrichments were facultative FeRB and agreed with the phenomenon that Fe(III) reduction rate and Fe(II) accumulation in soil enrichments amended with glucose were the greatest, followed by pyruvate, lactate, acetate and control.

Effects of carbon sources on FeRB

Data analysis indicated the highest Fe(III)-reducing bacterial diversity occurring in acetate and lactate-amended soil enrichments, and glucose-amended enrichments characterized with most predominant species. Higher similarity FeRB populations occurred in glucose- and lactate/acetate-amended soil enrichments. It is possible that microbial metabolism of fermentable substrates added could have yielded the actual electron donors (example, glucose would lead to the production of H_2, pyruvate, acetate and lactate). However, the repeated observations of highest Fe(III) reduction in glucose enrichments in this study and in other analyses of FeRB communities in paddy soils (Yi et al., 2010) suggest that our observations are not mainly the result of cross-feeding interactions. The predominant population in glucose-amended enrichment soils was *Clostridium* spp., which was reported most as its biological property to producing H_2. Qu (D. Qu, Northwest A and F University, China, PhD dissertation, 2000) found there was the close relationship between iron reduction and producing H_2 during the early period after flooding in paddy soils. The maximum Fe(III) reduction rate was significantly related to the consumption of H_2. Balance pressure of H_2 from 200 ppmv (parts per million by volume) in control quickly reduced to 3 to 5 ppmv in the treatment amending with ferrihydrite or lepidocrocite, and the Gibbs free energy which reducing Fe(III) by H_2 consumption was reduced from 10 to 15 kJ mol^{-1} compared to the control treatment (Qu et al., 2003). H_2 production peak usually appeared within the initial 5 days after flooding in paddy soil, and the corresponding time of most Fe(III) reduction rate was from 3 to 7 days (Qu et al., 2005). It is suggested that FeRB using H_2 as electron donors play an important role on iron reduction in the flooding paddy soils.

Conclusion

In the isolation of pure culture and 16S rDNA sequencing of selected dominant isolates, no obligatory FeRB relatives were detected in the organic carbon and ferrihydrite enrichments. The fermentative FeRB members such as *Clostridium*, *Paenibacillus* and *Bacillus* may be the important contributors to the iron reduction in paddy soils after flooding and they may use preferentially H_2 and alternative intermediate products for Fe(III) reduction.

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