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ORIGINAL PAPER

Changes in the microbial community during microbial microaerophilic Fe(II) oxidation at circumneutral pH enriched from paddy soil

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Abstract Fe(II)-oxidizing bacteria (FeOB) are important catalysts for iron cycling in iron-rich marine, groundwater, and freshwater environments. However, few studies have reported the distribution and diversity of these bacteria in flooded paddy soils. This study investigates the microbial structure and diversity of microaerophilic Fe(II)-oxidizing bacteria (mFeOB) and their possible role in Fe(II) oxidation in iron-rich paddy soils. Using enrichment experiments that employed serial transfers, the changes in microaerophilic microbial community were examined via 16S rRNA gene high-throughput sequencing. During enrichments, the Fe(II) oxidation rate decreased as transfers increased, and the maximum rate of Fe(II) oxidation was observed in the first transfer (0.197 mM day^{-1}). Results from X-ray diffraction of minerals and scanning electron microscopy of the cell-mineral aggregates revealed that cell surfaces in all transfers were partly covered with amorphous iron oxide formed by FeOB. After four transfers, the phyla of *Proteobacteria* had a dominant presence that reached up to 95%. Compared with the original soil, the relative abundances of *Cupriavidus*, *Massilia*, *Pseudomonas*, *Ralstonia*, *Sphingomonas*, and *Variovorax* increased in FeS gradient tubes and became dominant genera after transfers. *Cupriavidus*, *Pseudomonas*, and *Ralstonia* have been identified as FeOB previously. Furthermore, the structure of the microbial community tended to be stable as transfers increased, indicating that other bacterial species might perform important roles in Fe(II) oxidation. These results suggest the potential involvement of mFeOB and these other microorganisms in the Fe(II)-oxidizing process of soils. It will be helpful for future studies to consider their role in related biogeochemical processes, such as transformation of organic matters and heavy metals.

Keywords Fe(II)-oxidizing bacteria · Microaerophile · Paddy soil · Iron oxidation · Microbial community

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Introduction

In south China, the iron redox cycle is a crucial biogeochemical process of paddy soils, and it strongly influences the transformation of organic matters and trace metals in paddy soils due to the presence of highly active iron (Liesack et al. 2000; Li et al. 2019a; Naruse et al. 2019). In the biogeochemical iron cycle, Fe is transformed between oxidized Fe(III) and reduced Fe(II) states via several biotic processes that occur during cycles of flooding and draining (Melton et al. 2014). At circumneutral pH, the microbiology of iron cycling includes Fe(II) oxidation by microaerophilic (Emerson and Moyer 1997), phototrophic (Widdel et al. 1993), and nitrate-reducing Fe(II)-oxidizing bacteria (FeOB) (Larese-Casanova et al. 2010), as well as Fe(III) reduction via Fe(III)-reducing bacteria (FeRB) (Lovley et al. 1989). FeRB have been studied extensively (Hori et al. 2010; Li et al. 2011), but most work on the oxidative side has focused on anaerobic microbial Fe(II) oxidation by phototrophic and nitrate-reducing FeOB that Fe(II) can be stable (Bryce et al. 2018). However, mounting evidence shows aerobic Fe(II) oxidation can be performed by microaerophilic FeOB (mFeOB) at the redox interface despite rapid chemical Fe(II) oxidation by oxygen (Emerson et al. 2010; Chan et al. 2016). Due to the restrictions on the ecological niche of mFeOB, such as oxygen concentration and energy source (Druschel et al. 2008), much less is known about the distribution, diversity, and eco-physiology of mFeOB in circumneutral pH paddy soils. Thus, investigating microbial Fe(II) oxidation is crucial to understand biogeochemical iron cycles in paddy soils.

Paddy soils are unique agroecosystems. These soils range between micro-oxic and oxic during draining and transition to anoxic after flooding. Rice is traditionally cultivated under waterlogged conditions, which can form micro-oxic zones at the redox interface, such as the water-soil interface or rhizosphere. The oxygen concentration decreases from the water surface to the soil below, and oxygen is released into the rhizosphere of paddy soils during flooding (Weiss et al. 2007; Maisch et al. 2019a; Naruse et al. 2019). Usually, the abiotic Fe(II) oxidation is considered as the main contribution to Fe(II) oxidation due to the fast chemical oxidation in the presence of oxygen (half-life < 1 min) under circumneutral pH (Emerson et al. 2010; Faivre 2016). However, microbial Fe(II) oxidation can outcompete abiotic oxidation in micro-oxic zones (Edwards et al. 2003; Wang et al. 2009; Fleming et al. 2014; Laufer et al. 2017). This process is directly and indirectly involved in the behavior of organic and inorganic substances in paddy field soils, therefore affecting rice production (Kyuma 2004). In addition, FeRB can produce Fe(II) and thereby enhance the growth of FeOB in iron-rich soils during flooding (Otte et al. 2018). Therefore, the elucidation of microbial Fe(II) oxidation and bacterial diversity in paddy soils will provide insight into the cycling of other elements, and this understanding will help maintain sustainable rice production.

In previous studies, FeOB located proximate to redox boundaries with opposing gradients of oxygen and Fe(II) were identified as mFeOB (Emerson et al. 2010). These bacteria are active and contribute up to 40% of Fe(II) oxidation in batch culture experiments under micro-oxic conditions and account for up to 60% of total Fe(III) precipitations around the rhizosphere of wetland plants (Neubauer et al. 2002; Maisch et al. 2019b). To date, almost all mFeOB have been isolated from freshwater, groundwater, and marine environments. These bacteria are identified by their distinctive morphologies, such as the Fe-mineralized twisted stalks and sheaths formed by *Gallionella ferruginea*, *Leptothrix ochracea*, and *Mariprofundus ferrooxydans* (Emerson et al. 2010; Chan et al. 2011; Faivre 2016). However, this typical characteristic does not seem to appear in paddy soils during enrichment or isolation of these bacteria (Khalifa et al. 2018; Li et al. 2019a). This pattern might be caused by different FeOB in paddy soil. However, the structure (i.e., abundance and composition) and characteristics of mFeOB in paddy soils have yet to be investigated in detail.

In this study, iron-rich paddy soils from south China were used to enrich and isolate mFeOB with a gradient and dilution method. The abundance and composition of the microbial community of different transfers and dilutions were examined via 16S rRNA gene high-throughput sequencing. Cell morphologies and biominerals were then characterized by scanning electron microscopy (SEM) and X-ray diffraction (XRD). The results will expand our knowledge on the ecology of mFeOB in paddy soils and advance our understanding of their roles in the iron cycle.

Materials and methods

Sampling source and chemicals

Paddy soil samples containing brick-red flocs Fe(III) (oxyhydr)oxides were collected from Guanduling village, South China (26° 5′ 13.21″ N, 112° 42′ 25.19″ E) under flooded soil conditions in September 2015 when rice was at the tillering stage. The basic chemical properties of the soil sample were as follows: pH of 5.11, organic matter level of 4.99 g kg^{-1}, dithionite-citrate-bicarbonate Fe (DCB-Fe) level of 26.10 g kg^{-1}, complex-Fe level of 4.25 g kg^{-1}, and amorphous-Fe level of 8.13 g kg^{-1}. The method for paddy soil sample collection was described previously (Tong et al. 2019). In this area of paddy soils, the pH ranges from 4.91 to 5.74. The soil sample for cultivation was stored at 4 °C for less than 3 months before use. DNA of soil sample for sequencing was stored at − 80 °C (need to keep DNA for more than a year after sequencing). The analytical grade chemicals in experiments were obtained from the Guangzhou Chemical Reagent Factory, with a few exceptions as indicated.

Batch experiments for isolation and enrichment

A gradient tube approach was utilized to cultivate FeOB (Emerson and Moyer 1997; Emerson and Floyd 2005), in which opposing oxygen and Fe(II) gradients were developed within semi-solid agar overlaying an agar-stabilized mineral Fe(II) source. This produces an interface of anoxic and Fe(II)-bearing media with micro-oxic conditions that allows for the growth of mFeOB. In the gradient tubes, the media consisted of modified Wolfe's Mineral Medium (MWMM), which was bubbled with filter-sterilized CO_2, and an FeS plug was used as an iron source. Screw-cap 15 × 100 mm (13 ml) disposable tubes were applied to enrich mFeOB from paddy soil. In gradient tubes, the bottom layer consisted of 1 ml FeS per tube, with equal volumes of the FeS suspension and MWMM medium, stabilized with 1% (w/v) agarose. The top layer was 6 mL of MWMM medium with 5 mM $NaHCO_3$ and 1 ml of trace minerals and vitamins solution per liter of MWMM medium, made semi-solid by adding 0.15% agarose (w/v) (Edwards et al. 2003). Final pH of top layer was bubbled with sterile CO_2 gas to 6.2 (Edwards et al. 2003). To inoculate gradient tubes, equal sterile water and qualities of soil were mixed, and 10–15 µl of the resulting mixtures was inoculated into the top layer of gradient tubes. This process was identified as first transfer. Enrichment experiments were performed at a constant temperature of 25 ± 1 °C in a dark incubator. The growth band appeared as rust-colored Fe(III) (oxyhydr)oxides at the oxic-anoxic interface, and the cultures were subsampled and diluted into a new gradient tube every 3–4 weeks (Emerson and Floyd 2005). The cells from the tube with the highest dilution were used as inoculum for the next transfer. A total of four transfers were performed in this study. In this study and all figures, the original soil, first transfer, second transfer, third transfer, and fourth transfer are termed S0, S1, S2, S3, and S4, respectively.

Analytical methods

Iron determination

The cell growth band in gradient tubes was named as the iron oxide layer. Total iron concentrations in the growth band, which formed within the overlay, were measured using the Ferrozine method after reduction with 0.25 M hydroxylamine hydrochloride (Emerson and Moyer 1997). The rates of Fe(II) oxidation were calculated by first-order kinetics within discrete time points. For all of the gradient tubes, including the control and inoculated treatments, the samples obtained at the same depth intervals within the tubes were used for total iron determination. Methods for removing iron oxide layer and determining total iron concentration were described previously (Emerson and Moyer 1997; Emerson and Floyd 2005; Tong et al. 2019).

Characterization of enriched cell and iron minerals

Scanning electron microscopy (SEM) photographs were taken on a Zeiss Ultra 55 SEM equipped with a field emission electron gun. Sample preparation for SEM analysis was the same as described by Kato et al. (2013). The growth bands were centrifuged at 8000 r min^{-1} for 10 min, and samples were then fixed in 2.5% glutaraldehyde. After being fixed, samples were washed three times with 0.1 M phosphate buffer (15 min each). Fixed cells were dehydrated with an ethanol series (25%, 50%, 70%, 90%, and 100%) and

tert-butyl alcohol. Finally, the samples were freeze-dried and then coated with gold for SEM analysis. After several days of incubation, the band of iron oxides formed and was concentrated via centrifugation. It was then freeze-dried for X-ray diffraction (XRD) analysis. The XRD patterns of the freeze-dried samples were analyzed with a Rigaku D/Max-IIIA diffractometer at room temperature. The diffractometer was operated with Cu Kα radiation (wavelength = 0.15418 nm) at 30 mA and 30 kV. The phases were confirmed via comparison with the standard powder XRD card patterns from the Joint Committee on Powder Diffraction Standards (JCPDS) (Cornell and Schwertmann 2003).

DNA extraction and computational analysis of microbial community

Approximately 0.3 g iron minerals were collected by centrifugation in different gradient tubes. The DNA around the iron minerals was extracted by a PowerSoil™ DNA isolation kit according the instructions (Mo Bio, Inc., USA). To reduce the effect of agarose on DNA extraction, the medium including the growth band, iron oxides, and agarose, was heated up to 70 °C to melt the agarose in a water bath (Emerson and Moyer 1997). Then, cells for DNA extraction were collected by centrifugation (10,000 × *g*; 5 min) and washed five times with PBS buffer at pH 7.0. The sequencing of the 16S rRNA gene in the V4 region was performed with Illumina fusion primers according to previous report (F515 and R806) (Liu et al. 2014). The polymerase chain reaction (PCR) products were purified by UltraClean PCR clean-up kit according the instructions (Mo Bio, Inc., USA) and then quantified with the Qubit 2.0 Fluorometer by the dsDNA assay kit (Invitrogen, USA). Miseq Illumina high-throughput sequencing (Illumina Miseq platform, PE 250) was used to analyze the 16S rRNA gene amplicons by Magigene Biotechnology (Guangzhou, China). The bioinformatics analysis was processed using QIIME 2 (Caporaso et al. 2010) and the details were described in our previous report (Tong et al. 2017).*The following BioProject ID and GenBank accession number for the genomic datasets in NCBI are PRJNA381404 and SRR5408992, respectively.

Results and discussion

Simulation of micro-oxic conditions in gradient tubes

In paddy soil environments, oxygen from the atmosphere or oxygen originating from photosynthesis can be transported to the oxic-anoxic interface to form micro-oxic zones. In addition, radial oxygen loss (ROL) transports oxygen from stems to the roots and releases oxygen into the rhizosphere. This process can lead to low oxygen zones (Colmer 2002; Maisch et al. 2019a). At these micro-oxic zones, FeOB can compete with chemical processes for available oxygen and Fe(II). Fe(II) is mobile and transported via diffusion from the surrounding anoxic environment. The formation of Fe(III) (oxyhydr)oxides could lead to acidification of paddy soil around rice roots (Kappler and Newman 2005; Maisch et al. 2019a, b). Therefore, the pH of the medium in this study could not affect the enrichment and isolation of mFeOB from the paddy soil with a pH of 5.11 (Emerson et al. 1999; Picardal et al. 2011; Lin et al. 2012; Khalifa et al. 2018). In this study, the gradient tube was used to simulate micro-oxic conditions (Fig. 1). These tubes are characterized by opposing diffusion gradients of oxygen in the headspace of the tube and Fe(II) at the bottom (Li et al. 2019a). Tubes inoculated with paddy soils exhibited a steep oxygen gradient at 1 cm from the top. The oxide layer (cell band) appeared 1–2 cm below the medium surface, where oxygen concentrations were lower than

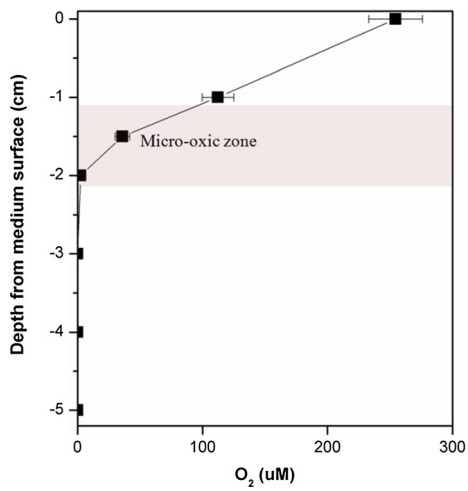

Fig. 1 The oxygen concentration in gradient tubes during inoculation

50 μM. This layer is defined as the oxic-anoxic interface in gradient tubes (Emerson and Floyd 2005; Chan et al. 2016) (Fig. 1). The oxygen concentration and appearance of precipitates suggest that the enriched microorganisms are microaerophiles with the capacity to oxidize Fe(II).

Enrichment of mFeOB in paddy soils

After incubation for 5 days, a zone of Fe(II) oxidation was visible due to the appearance of thin layer of orange Fe(III) oxides at an intermediate distance between the FeS plug and the medium interface. In uninoculated gradient tubes, no iron oxides were observed at the same depth. Within inoculated treatments, microbial cell bands were observed 1–2 cm below the surface of medium in micro-oxic zones. The shape and height of orange Fe(III) oxides differed between uninoculated and inoculated treatments (Lueder et al. 2018). The uniformly orange Fe(III) oxides appeared in uninoculated treatment instead of cell bands due to the chemical oxidation by oxygen, as shown in our previous study (Tong et al. 2019). Biological Fe(II) oxidation was less diffuse than that formed by abiotic oxidation processes. These patterns confirmed the microbial Fe(II) oxidation in the inoculated treatment. The neutrophilic mFeOB growth was limited to the opposing gradients of the Fe(II)/O_2 interface. Previous reports confirmed that mFeOB could adapt to utilize energy from Fe(II) oxidation to support their growth (Emerson et al. 2010; Emerson and de Vet 2015). After a stable cell band formed, the cell band was diluted and transferred to a new gradient tube. The rates of iron oxidation over four transfers of inoculated gradient tubes, as measured by total iron accumulation (Kato et al. 2013), are shown in Fig. 2. The rates of Fe(II) oxidation in inoculated treatments were higher than the oxidation rates in the abiotic control. The Fe(II) oxidation rate decreased as transfers increased, and the maximum Fe(II) oxidation rate was observed in the first transfer (0.197 mM day^{-1}). Similarly, the incubation time for visible cell bands increased with the number of transfers. This might have been caused by a decrease in the number of non-dominant FeOB as well as a potential decrease in bacteria that obtain energy for growth from the remaining Fe(II)-oxidizing species (Wang 2011). In addition, reductions in the microbial diversity of flanking community members that play important

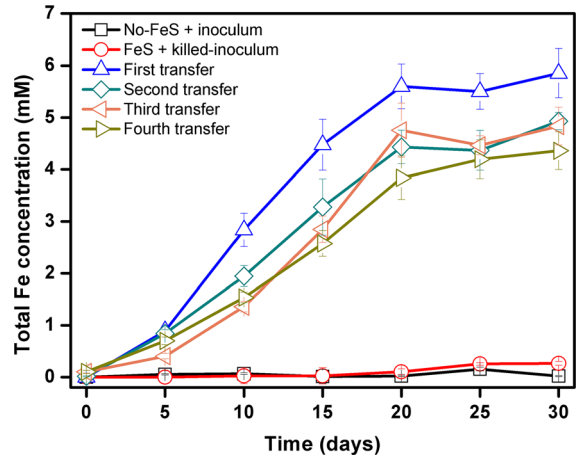

Fig. 2 Concentration of total Fe in the growth bands in different transfers. For all treatments, including the control and inoculated treatments, the samples collected at the same depth of gradient tubes were used to measure total iron concentration. Error bars show the standard errors of three replicates

roles in the physiology of the enriched FeOB may lead to decreased Fe(II) oxidation (He et al. 2016; Wang 2011). The microbial communities from different transfers were used to calculate the pairwise unweighted UniFrac distance which was able to separate all transfers from the original samples (Fig. 3). The combined first principal coordinate (PC1) and second principal coordinate (PC2) axes

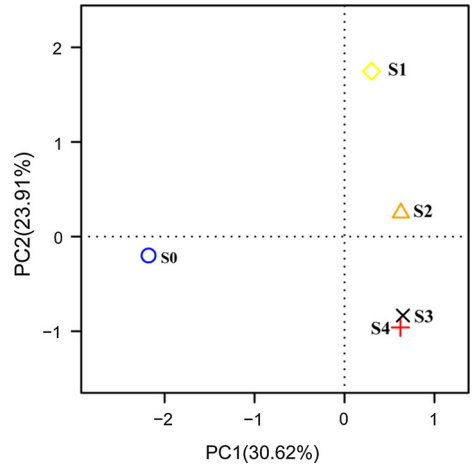

Fig. 3 Principle coordination analysis (PCoA) compares microbial community composition in original soil and different transfers. S0, S1, S2, S3, and S4 represent the original soil, first transfer, second transfer, third transfer, and fourth transfer, respectively

represented 54.53% of the total variation in the unweighted principle coordination analysis (PCoA). The details of PCoA analysis and results were described in Supporting Information. The results showed that the transfers S3 and S4 were not separated into two clusters, suggesting that they had similar microbial communities. This result indicates that microbial communities became stable and robust in the enrichments after several transfers. A complex relationship between enriched mFeOB and non-dominant FeOB might have formed to maintain the stable capacity of Fe(II) oxidation.

During the incubation process, Fe(II) oxidation occurred in a narrow band at the oxic-anoxic zone. The XRD spectrum was used to characterize the minerals that formed in the growth bands with inoculum for the first and third transfers at 20 days. No characteristic peak of crystalline minerals was detected in XRD result (Fig. 4), which suggests that the amorphous minerals were formed after microaerophilic Fe(II) oxidation (Laufer et al. 2017; Li et al. 2019a). SEM pictures of enriched Fe(II)-oxidizing bacteria showed cell surfaces partly covered with mineral particles, and the cells were mainly rod-shaped (1.5–2.2 μm in length) and oval-shaped (0.5–1.1 μm in length) (Fig. 5). The filamentous structures in SEM seem to be a small amount of unwashed agar or extracellular polymeric substances formed during Fe(II) oxidation (Kappler et al. 2005; Schädler et al. 2009). The issue of cell encrustation is prevalent in micro-oxic Fe(II) oxidation processes and is commonly thought to limit the growth of mFeOB (Emerson et al. 2010). Usually, mFeOB produce twisted stalks or sheaths for extracellular precipitation of Fe(III) to avoid cell encrustation (Schädler et al. 2009; Chan et al. 2011). However, there were no distinct twisted stalks or sheaths formed during Fe(II) oxidation in this study. Without these special structures, Fe(III) was partially precipitated on the cell surface, and this might limit the transformation of nutrients to the cell. This result agrees with previous reports of paddy soils and rhizospheres with enriched mFeOB that did not make stalks or sheaths (Weiss et al. 2007; Khalifa et al. 2018; Li et al. 2019a). Emerson (2012) speculated that the compact soil matrix probably blocked the formation of stalks or sheaths and that these microorganisms produced an exopolymer to avoid cell encrustation with Fe(III) minerals. However, these speculations require further study.

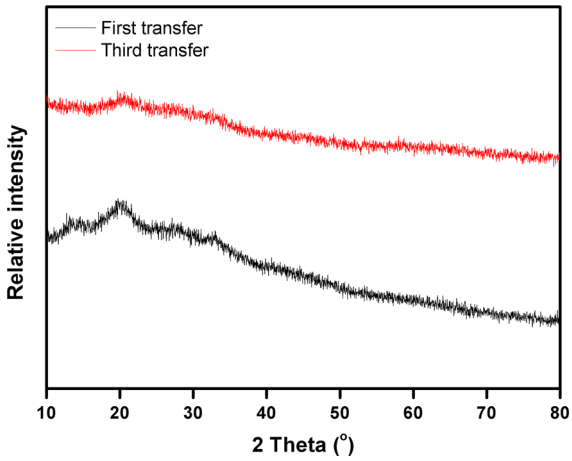

Fig. 4 X ray diffractograms of precipitates during Fe(II) oxidation in first and third transfers

Microbial community composition and diversity during Fe(II) oxidation

In total, we recovered 415,669 quality sequences from different transfers using Illumina high-throughput sequencing. Sequence frequencies for individual samples ranged from 69,660 for the first transfer to 91,883 for the third transfer. More than 99% of the sequences could be assigned to the phylum level. The microbial community composition in the original soil (S0) was complex, and the dominant phyla in S0 were the following: *Acidobacteria* (18.3%), *Actinobacteria* (19.0%), AD3 (2.0%), *Bacteroidetes* (3.1%), *Chloroflexi* (13.2%), *Proteobacteria* (35.9%), and *Verrucomicrobia* (1.7%) (Fig. 6). After four transfers, the dominant phylum changed to *Proteobacteria* (> 95.8%), which is a notable shift from S0. In the fourth transfer, the relative abundance of *Proteobacteria* reached 99.3%, consisting of 20.5% *Alphaproteobacteria*, 64.7% *Betaproteobacteria*, and 14.4% *Gammaproteobacteria*. This result is consistent with previous studies that reported almost all cultivated mFeOB as belonging to *Proteobacteria* under circumneutral pH conditions (Emerson et al. 2010). In previous reports, almost all mFeOB isolated from freshwater and sediment environments are clustered within the orders of *Betaproteobacteria* and appear to be autotrophic (Emerson et al. 2010; Chan et al. 2016). The relative abundance of *Alpha-* and *Gammaproteobacteria* accounted for 34.9% of the total in

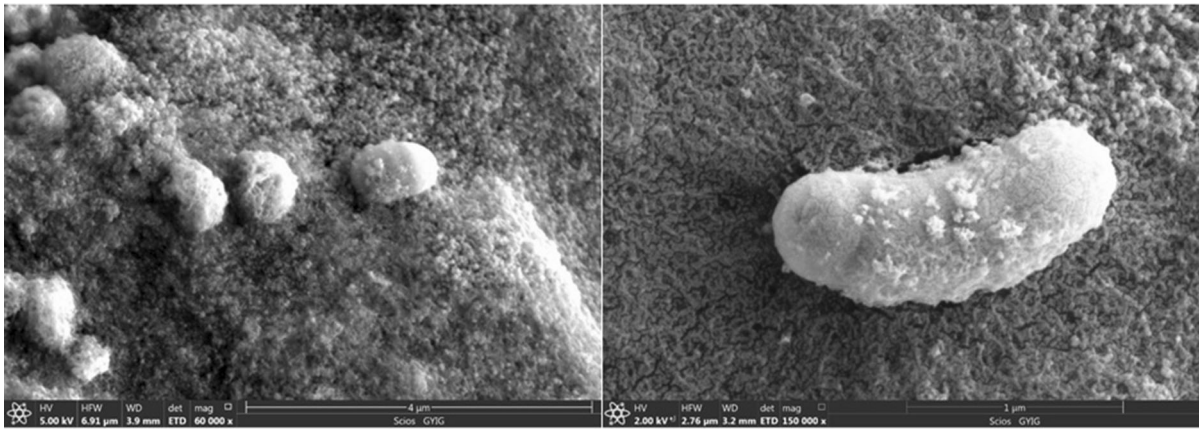

Fig. 5 SEM images of bacteria in semi-solid enrichment cultures

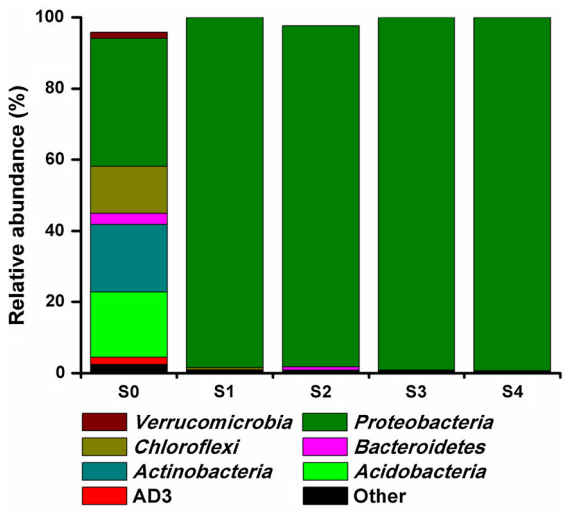

Fig. 6 Relative abundances (> 1%) of the dominant microbial phyla in different transfers revealed by 16S rRNA high-throughput sequencing. S0, S1, S2, S3, and S4 represent the original soil, first transfer, second transfer, third transfer, and fourth transfer, respectively. Others represented the sum of relative abundance of unclassified phyla that were larger than 1%

this study, indicating that some other bacteria might participate in Fe(II) oxidation in paddy soil. Some FeOB that belong to *Alphaproteobacteria* are not obligate Fe(II) oxidizers, and they can grow heterotropically with organic matters (Shelobolina et al. 2012). These results suggest that the microbial community responsible for Fe(II) oxidation in paddy soil is different from the freshwater and sediment communities. Furthermore, the diversity of the microaerophilic microorganisms with Fe(II) oxidation ability seems to be more plentiful than have been isolated from various other environments.

The dominant genera in S0 were *Acidothermus*, *Acidothermus*, *Bradyrhizobium*, *Holophaga*, *Marmoricola*, *Nevskia*, and *Nitrosomonadaceae* (Fig. 7). The maximum relative abundance of microorganisms was no more than 6.0% in S0, and the relative abundance of most dominant genera decreased to less than 0.1% after the fourth transfer (S4). During serial transfers, *Cupriavidus*, *Massilia*, *Pseudomonas*, *Ramlibacter*, *Ralstonia*, *Sphingomonas*, and *Variovorax*

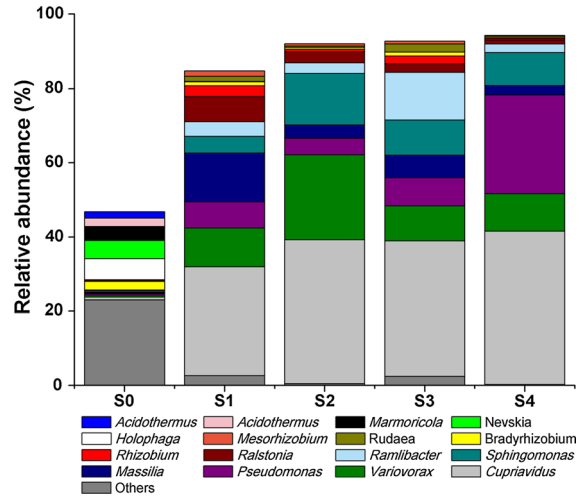

Fig. 7 Relative abundance of bacterial genera with a minimum relative abundance greater than 1% in different transfers revealed by 16S rRNA high-throughput sequencing. S0, S1, S2, S3, and S4 represent the original soil, first transfer, second transfer, third transfer, and fourth transfer, respectively. Others represented the sum of relative abundance of unclassified genera that were larger than 1%

were dominant genera, accounting for 90% of the genera (Fig. 7). The relative abundances of the top four genera were more than 6.0% after S4. For example, the relative abundances of *Cupriavidus* and *Pseudomonas* markedly increased as number of transfers increased and reached 41.3% and 26.6% after S4, respectively. The relative abundances of *Sphingomona* and *Variovorax* increased evidently during S1–S2 and then decreased to average values of 9.1% and 9.8%, respectively, during S3–S4. The changes in relative abundances of these dominant genera indicate that the mFeOB exhibit low abundance in the original soil, resulting from the limited micro-oxic conditions where microbial Fe(II) oxidation can successfully compete with the oxygen-limited abiotic oxidation (Druschel et al. 2008; Laufer et al. 2016; Maisch et al. 2019a). In gradient tubes, the opposing gradients of oxygen and Fe(II) provided favorable conditions for mFeOB bacterial growth. These gradients can be found in paddy soils, sediments, springs, and groundwater at oxic-anoxic transition zones (Emerson and Weiss 2004; Emerson et al. 2010; Maisch et al. 2019a). Although the relative abundances of dominant species reached up to 90% after four transfers, we still could not isolate one strain of mFeOB. Extensive efforts using serial dilution-to-extinction method and alternating Fe(II)-sources to isolate mFeOB indicate a significant role of the flanking community members in Fe(II) oxidation by the dominant mFeOB (He et al. 2016). This phenomenon is similar to a previous report on the enrichment of autotrophic Fe(II)-oxidizing culture KS (Straub et al. 1996). The culture in that report was dominated by a neutrophilic mFeOB, *Sideroxydans lithotrophicus* ES-1 (Emerson and Moyer 1997; Nordhoff et al. 2017). However, the primary FeOB has not yet been isolated in culture KS despite a significant amount of effort. Additionally, another study found that this primary FeOB could not be grown in pure culture with a soluble Fe(II) source (Blothe and Roden 2009). This suggests a synergistic relationship between primary FeOB and other bacteria in enrichment, which could be a potential explanation for the stable microbial communities after several transfers (Fig. 3). In further studies, the metagenomic analysis can be used to gain insight into functional genes for Fe(II) oxidation, electron transfer pathways, potential interspecies interactions, and ecophysiological characteristics of mFeOB in order to better understand the metabolism and physiology in our enrichment.

Cupriavidus, as the most abundance genus in enrichments, increased from 29.2 to 41.3% after the fourth transfer. They have been reported to grow autotrophically and oxidize Fe(II) under micro-oxic conditions (Shelobolina et al. 2012). To date, several *Cupriavidus* strains had been isolated from oxic-anoxic transition zones with oxygen as the electron acceptor and soluble Fe(II) as the electron donor (Shelobolina et al. 2012; Benzine et al. 2013). In the present study, $NaHCO_3$ was the sole carbon source in the medium. These results are in line with previous reports that *Cupriavidus* could obtain energy to grow via Fe(II) oxidation coupled with carbon fixation using the Calvin-Benson carbon dioxide subsystem under micro-oxic conditions (Kato et al. 2013; Yu et al. 2013; Przybylski et al. 2015). Furthermore, *Cupriavidus* can produce siderophores to improve bacterial motility and iron scavenging, thereby potentially increasing iron uptake to overcome iron limitation (Li et al. 2019b). Microbial micro-oxic Fe(II) oxidation can produce insoluble Fe(III) that can cause cell encrustation and block the metabolic activity of FeOB due to adsorption on the cell surface with negative charge (Schädler et al. 2009). The siderophores from *Cupriavidus* might help avoid encrustation to some extent. In the present study, the relative abundance of dominant mFeOB became stable after four transfers, which is consistent with the tendency of the Fe(II) oxidation rate (Fig. 8). Previous reports showed that the pure strains might have a lower capacity for Fe(II) oxidation than original isolation conditions that can

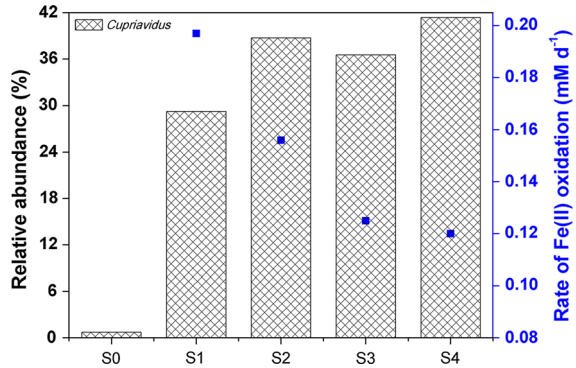

Fig. 8 The tendency of relative abundance of *Cupriavidus* and rate of Fe(II) oxidation in different transfers. S0, S1, S2, S3, and S4 represent the original soil, first transfer, second transfer, third transfer, and fourth transfer, respectively

maintain Fe(II) oxidation over several transfers (Bryce et al. 2018). Therefore, the potential *Cupriavidus*-related mFeOB might be crucial to Fe(II) oxidation in different transfers.

In addition to *Cupriavidus*, *Pseudomonas* is another dominant genus that belongs to the class *Gammaproteobacteria*. The relative abundance of *Pseudomonas* remained at an average of 6.6% during the S1-S3 transfers, and then it increased markedly to 26.6% in the S4 transfer. *Pseudomonas* have been reported as anaerobic nitrate-reducing FeOB at circumneutral pH (Muehe et al. 2009; Xiu et al. 2015; Li et al. 2018). Although no reports have identified *Pseudomonas* as Fe(II)-oxidizers under micro-oxic conditions, *Pseudomonas* strains are prevalent in the rhizosphere soil (Costa et al. 2006; Deveau et al. 2016). Oxic-anoxic zones can form in this environment due to the diffusion of oxygen into the rhizosphere via the root aerenchyma (Kumarathilaka et al. 2018). Considering the above discussion and the high relative abundance of *Pseudomonas*, nitrate-reducing FeOB like *Pseudomonas* might play a role in Fe(II) oxidation under micro-oxic environments (Duckworth et al. 2009).

The relative abundance of *Ralstonia* increased to 6.7% in S1 transfer and then decreased to 2.2% in S4 transfer. *Ralstonia* strains were confirmed to oxidize Fe(II) by isolating the strain with FeS as the iron source under micro-oxic conditions (Swanner et al. 2011; Shelobolina et al. 2012). No stalks or sheaths were produced by *Ralstonia* during Fe(II) oxidation (Swanner et al. 2011), and this result was also observed in the present study. The enrichment of *Ralstonia* and the similar characteristics of iron minerals suggest that Fe(II) oxidation by *Ralstonia* strains is active in paddy soil. Compared with the original soil, the relative abundance of *Sphingomonas* and *Variovorax* increased from 0.1 to 8.7% and from 0.5 to 10.2%, respectively. Both of these two genera were found to be enriched in environments with strong redox gradients, high concentrations of metals, and floating flocculated Fe precipitates (Stein et al. 2001; Duckworth et al. 2009; Blackwell et al. 2019). Furthermore, these genera have numerous metabolic capabilities that are associated with utilization of different carbon sources and degradation of complex chemical compounds (Männistö et al. 2001; Satola et al. 2013). The increase in the relative abundances of these two bacteria genera in the present study suggests that they probably were supported by autotrophic microorganisms that might carry out important roles in Fe(II) oxidation due to their metabolic capabilities. *Massilia* species, as aerobic heterotrophs, likely proliferated via the oxidation of sulfide present in the FeS (Zecchin et al. 2019). Usually, these sulfide-oxidizing bacteria inhabit ecosystems with steep opposing gradients of sulfur and oxygen (Dahl et al. 2008). In this study, the carbon fixation by some FeOB might have supported the growth of *Massili*a species. In addition, *Massilia* species can produce acids to lower the pH around the growth bands, and this could reduce cell encrustation (Jin et al. 2015). This is consistent with SEM observations that showed cells partly covered with iron oxides. *Ramlibacter* species have been found in other gradient tubes with FeS as the iron source (Tong et al. 2019), and they can thrive via oxyanion reduction under micro-oxic conditions (Zhen et al. 2014). However, there is no direct evidence confirming whether *Ramlibacter* species are capable of Fe(II) oxidation.

The mFeOB are generally found where Fe(II)/O_2 interfaces develop, including hydrothermal vents, chalybeate springs, freshwater, sediments, wetlands, and the rhizosphere (Emerson et al. 2010; Emerson 2012). The marine mFeOB communities are related to the class *Zetaproteobacteria*, whereas the mFeOB communities from soil or freshwater are mostly restricted to the class *Betaproteobacteria* (Chan et al. 2016). The mFeOB members from the class *Zetaproteobacteria* have not been found in soil or freshwater environments. Therefore, the enrichment of mFeOB in this study was mainly compared with previous reports from soil or freshwater environments, and the results showed a large difference (Emerson et al. 2010; Chan et al. 2016). In recent decades, several typical mFeOB have been isolated from these environments, including *Leptothrix*, *Gallionella*, and *Sideroxydans*, and their Fe(II) oxidation capacity was confirmed over several transfers (Emerson and Moyer 1997; Emerson and Floyd 2005; Emerson et al. 2010; Kato et al. 2013; Fleming et al. 2014). These typical mFeOB make distinct stalks or sheaths associated with Fe(III) minerals (Chan et al. 2016). However, these unique structures of Fe(III) minerals did not form in pure or enrichment cultures from paddy soils (Khalifa et al. 2018; Li et al. 2019a). For example, *Ferrigenium kumadai* isolated from paddy soil was clustered with mFeOB in the family *Gallionellaceae* and shared 95.2% sequence similarity with *Gallionella*

capsiferriformans ES-2 SSU rRNA gene, but it did not form these special structures (Khalifa et al. 2018). In different environmental niches, different types of mFeOB could play a role in Fe(II) oxidation. In the present study, although the opposing gradient tube cultivation method was the same as the one used for enriching FeOB, i.e., *Gallionella* and *Leptothrix* (Emerson et al. 2010), no sequences were assigned to these two bacteria. One possible explanation for this result might be that *Gallionella* and *Leptothrix* are overwhelmed by other microorganisms under present conditions. They might not have been recoverable in our dilution or non-dominant in circumneutral and iron-rich environments (Yu et al. 2009; Hassan et al. 2016). Another explanation is that these *Gallionella* and *Leptothrix*-related FeOB could not be amplified by the general primers in this study due to some mismatched bases (Wang et al. 2011; Naruse et al. 2019). Based on new methods, an increasing number of circumneutral FeOB species have been isolated from paddy soil. These results will hopefully help researchers understand the roles and contributions of these species in iron cycling and related biogeochemical processes, such as transformation of organic contaminants and heavy metals in paddy field soils.

Implications and conclusions

In flooded paddy soil, Fe(II) oxidation is intricately regulated by abiotic oxidation, anaerobic FeOB (i.e., nitrate-reducing and phototrophic organisms), and also mFeOB. Around the rhizosphere, root-related ROL favors the formation of low oxygen niches for mFeOB to induce Fe(II) oxidation (Maisch et al. 2019a, b). Most well-known mFeOB produce distinct morphologies with twisted stalks or sheaths in freshwater or sediments (Chan et al. 2016). Nevertheless, there were none of these typical structures in this study, indicating that other bacteria also have a major role in Fe(II) oxidation in paddy soil. The dominant genera in the enrichment included *Cupriavidus*, *Variovorax*, *Pseudomonas*, *Sphingomonas*, and *Ralstonia*, accounting for 90% of the bacteria in these microbial communities. In addition, the microbial community structure was relatively stable after several transfers and was markedly different from these enrichments from freshwater, wetlands, and marine sediments. These results suggest that neutrophilic Fe(II) oxidation by mFeOB might be more widespread than has been appreciated. The identification of these potential mFeOB can expand our knowledge of FeOB on the abundance and diversity at the redox interface. During microbial Fe(II) oxidation, the growth band formed at micro-oxic zones in gradient tubes, and amorphous iron minerals were found to be the dominant precipitates of Fe(II) oxidation. The high reactivity and surface area of these biominerals could strongly influence immobilization and transformation of heavy metals, such as arsenic (Tong et al. 2019). The microbial Fe(II) oxidation not only affected the arsenic immobilization but also stimulated the arsenic oxidation (Tong et al. 2019), which could reduce the availability of arsenic around the rhizosphere (Yu et al. 2017). Recognizing the diversity and function of these mFeOB from various environments could help researchers understand their roles in iron and heavy metals redox cycling under micro-oxic conditions.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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