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ORIGINAL ARTICLE/SHORT PAPER

Bacterial communities in iron mottles in the plow pan layer in a Japanese rice field: Estimation using PCR-DGGE and sequencing analyses

Vita Ratri CAHYANI[1,3], Jun MURASE[1], Akihiro IKEDA[2], Katsutoshi TAKI[2], Susumu ASAKAWA[1] and Makoto KIMURA[1]

[1]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601,* [2]*Aichi-ken Agricultural Research Center, Aichigun 480-1193, Japan; and* [3]*Faculty of Agriculture, Sebelas Maret University, Surakarta 57126, Indonesia*

Abstract

Bacterial communities in iron (Fe) mottles in the plow pan layer in a Japanese rice field were estimated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis targeting 16S rDNA genes. The DGGE band patterns indicate that distinct bacterial communities with lower diversity inhabit Fe mottles compared with the reference soil matrix. Many of the DGGE bands of the Fe mottles that were sequenced (12 of 29 DGGE bands) were closely related to bacteria involved with Fe oxidation and reduction: *Sideroooxidans ghiorsii*, *Azoarcus* sp., *Azovibrio* sp., *Dechloromonas* sp., *Acidimicrobium ferrooxidans*, *Geobacter psychrophilus*, *Clostridium* sp., *Desulfovibrio* sp. and *Desulfonatronum cooperativum*. The results suggest that specific bacteria inhabit Fe mottles and may play a role in the Fe cycle.

Key words: bacterial community, iron mottle, iron oxidation, iron reduction, rice field.

INTRODUCTION

Floodwater plays a decisive role in soil formation in rice fields and the common field management practice of flooding and drainage in irrigated rice fields results in leaching and the accumulation and oxidation of manganese (Mn) and iron (Fe), leading to the formation of Mn nodules and Fe mottles at the horizons, which are termed Mn and Fe illuvial horizons, the two diagnostic horizons in rice fields (Kimura 2000). Iron mottles also occur in the plow pan layer of ill-drained paddy fields. It is because of the establishment of an oxidation–reduction interface between the plow layer and the plow pan layer due to the immediate intrusion of the atmospheric oxygen into the porous plow layer after drainage and the stagnation of soil water in the plow pan layer from ill permeation of the layer. Iron mottles are filmy in the layer.

In contrast to the predominance of microbe-mediated Mn oxidation in rice fields, Fe oxidation is considered mainly to proceed chemically after drainage without microbial mediation in rice fields. However, Weiss *et al*. (2003) and Weber *et al*. (2006) found that both Fe-oxidizing and Fe-reducing bacteria were involved in the aerobic Fe cycle in the rhizosphere of wetland plants and in the anaerobic Fe cycle in freshwater sediments, respectively. Phylogenetically different bacteria have been isolated from various environments as Fe oxidizers at circumneutral pH, including freshwater, salt water, marine bays and thermal springs (Hanert 1992), iron-containing ditch, river and pond waters (Mulder and Deinema 1992), groundwater (Emerson and Moyer 1997) and hydrothermal vent sites (Emerson and Moyer 2002).

Although these studies indicate a contribution of Fe oxidizers and reducers to the formation of Fe mottles in rice fields, no reports on the bacterial communities in Fe mottles in rice fields have been published to date. The present study aimed to elucidate the bacterial communities inhabiting Fe mottles in the plow pan layer in a Japanese rice field using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis and sequencing. We chose Fe mottles not in the Fe illuvial horizon but rather in the plow pan layer because Fe mottling is filmy and more concentrated in Fe in the plow pan layer compared with the Fe illuvial horizon where the mottling is cloudy-like. This is the

Correspondence: V. R. CAHYANI, Laboratory of Soil Biology, Department of Soil Science, Faculty of Agriculture, Sebelas Maret University, Jalan Ir. Sutami No.36A Kentingan, Surakarta, Jawa Tengah 57126, Indonesia. Email: vitaraturi@yahoo.com

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first study to examine the bacterial communities in Fe mottles in a rice field.

MATERIALS AND METHODS

Collection of Fe mottles from the plow pan layer

Iron mottles were sampled from soil blocks weighing several kilograms that were collected from the plow pan layer at a depth of 13_20 cm at two sites in a rice field located at the Aichi-ken Agricultural Research Center, central Japan (35°10′N, 137°03′E) on 27 November 2006. The two sites (designated as Sites A and B) were located approximately 100 m apart from each other, and the soils were classified as fine-textured Gray Upland Soil (Epiaquept). The sites were similar in soil properties, with total C and N contents of 12.5 and 1.13 g kg^{-1}, 15.5 $cmol_c$ kg^{-1} cation exchange capacity (CEC), and exchangeable Ca, Mg and K of 11.9, 3.41 and 0.67 $cmol_c$ kg^{-1}, respectively. The soil texture was light clay. Filmy Fe mottles were scraped off the soil blocks with a small spatula. Soil samples from the soil matrix were also taken as a reference by carefully excluding Fe mottles. The Fe mottles and reference soil samples were stored at –80°C until analysis.

Fe and Mn concentrations in the Fe mottles

Iron and Mn were extracted from approximately 100 mg of the samples by reducing them with 50 g kg^{-1} hydroxylamine solution in 1 mol L^{-1} HCl. The concentrations of Fe and Mn were determined with an inductively coupled plasma (ICP) spectrophotometer (Model IRIS AP; Nippon Jarrell-Ash, Kyoto, Japan) as previously described in detail (Cahyani *et al.* 2007).

Molecular analysis of the bacterial communities

The DNA was extracted from the Fe mottles and the reference soil samples using a FastDNA SPIN Kit for Soil (BIO 101; Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocols. The PCR-DGGE analysis targeting eubacterial 16S rDNA was carried out as previously described (Cahyani *et al.* 2003, 2007). In brief, the 16S rDNA fragments were amplified using the 357f-GC clamp and 517r primers (Muyzer *et al.* 1993) and 0.4 μg of the PCR products were subjected to DGGE analysis. The PCR amplification and DGGE were carried out in triplicate with satisfactory reproducibility of the DGGE image for every sample (data not shown). The DGGE bands were excised from the gel, re-amplified using the same primers and checked for mobility. Respective bands were excised from two replicates for the determination of their sequences. The PCR products of the two replicates of each band were then cloned into pT Blue T-vector (Novagen, Darmstadt, Germany). Colony PCR was carried out to several clones using the same primers. One clone from each replicate, whose position matched the target band, was sequenced using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) with the ABI PRISMTM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

Sequences of the DGGE bands were compared with the available 16S rDNA sequences from the database of the DNA Data Bank of Japan ([DDBJ] http://www.ddbj.nig.ac.jp/E-mail/homology.html) using a BLAST search, and a phylogenetic tree of the DGGE bands with their closest relatives was constructed using the neighbor-joining method (Saito and Nei 1987) in the CLUSTAL X 1.81 software packages (Thompson *et al.* 1997).

Nucleotide sequence accession numbers

The 16S rDNA partial sequences determined in the present study were deposited in the DDBJ database under the accession numbers AB368721–AB368765.

RESULTS AND DISCUSSION

Concentrations of Fe and Mn

The Fe mottles contained higher amounts of Fe (25 and 36 g Fe kg^{-1}) than the reference soils (11 and 9 g Fe kg^{-1}) at Sites A and B, respectively, verifying the accumulation of Fe in Fe mottles. The Mn concentrations in the Fe mottles (0.1 g Mn kg^{-1}) were less than in the reference soils (0.2 g Mn kg^{-1}) at both sites, which was in contrast to the Mn oxide mottles where the concentration of Fe was higher in the Mn mottles than in the reference soils (Cahyani *et al.* 2007).

Denaturing gradient gel electrophoresis band patterns

A difference in the bacterial communities inhabiting the Fe mottles and the reference soil samples was reflected in the DGGE band patterns shown in Fig. 1. Several prominent bands were observed in the DGGE patterns of Fe mottles. The difference was more pronounced in the Fe mottles from Site B, which accumulated more Fe in the Fe mottles from the surrounding matrix soils. In contrast, the DGGE pattern of the reference soil samples from the two sites consisted of many bands with relatively weak intensities. These findings indicate that specific bacterial communities with lower diversity inhabit Fe mottles compared with the reference soils, as we previously observed in Mn nodules (Cahyani *et al.* 2007).

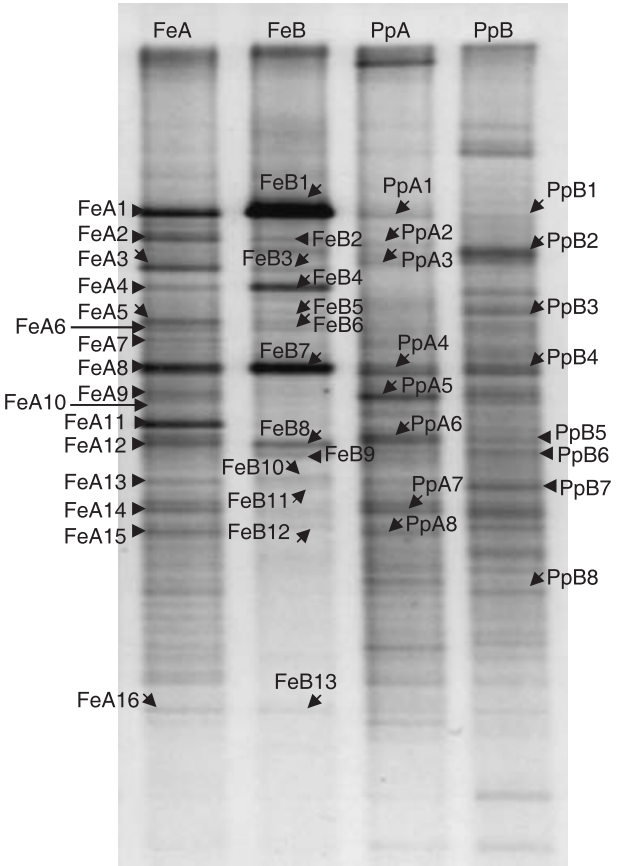

Figure 1 Denaturing gradient gel electrophoresis patterns of the bacterial communities in Fe mottles and the reference plow pan layer soils in a rice field. FeA, Fe mottles at Site A; FeB, Fe mottles at Site B; PpA, plow pan layer soil of Site A; PpB, plow pan layer soil of Site B.

Phylogenetic affiliation of representative denaturing gradient gel electrophoresis bands

Sixteen and thirteen bands from Fe mottles from Sites A and B, respectively, were successfully sequenced and their phylogenetic affiliations are listed in Table 1. Although the closest relatives of three respective band pairs (FeA1 and FeB1, FeA13 and FeB10, and FeA16 and FeB13), which were located at the same positions in the polyacrylamide gel, were the same, the other two pairs of DGGE bands, which were also located at the same positions in the polyacrylamide gel (FeA4 and FeB4, and FeA8 and FeB7), had different close relatives. These findings might indicate that the DNA bands consisted of fragments that had the same mobility on the gel, but different sequences.

As shown in Table 1, the closest relatives were different from each other between the Fe mottles and the reference soils, clearly indicating a difference in the bacterial communities in the Fe mottles and the reference soil. Thus, specific bacterial communities were considered to develop in Fe mottles in the plow pan layer. In addition, it is important to note that many of the closest relatives in the DGGE bands from the Fe mottles were bacteria potentially related to Fe oxidation and reduction. The closest relatives of the most prominent DGGE bands (FeA1 and FeB1) were *Siderooxidans ghiorsii* (Gammaproteobacteria) and *Azoarcus* sp. (Betaproteobacteria), with similarity indexes of 93%. And bands FeA6 and FeB4 and band FeB7 were closely related to *Azoarcus* spp. and *Azovibrio* sp., respectively. *Siderooxidans ghiorsii* (Accession No. DQ386859) is reported to be

Table 1 Closest relatives to the denaturing gradient gel electrophoresis bands obtained from Fe mottles and plow pan layer soils in a rice field at Sites A and B

DGGE band	Sequence bp	Closest relatives				
		Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment
Fe mottles Site A						
FeA1	160	*Siderooxidans ghiorsii* strain LD-1	Gammaproteobacteria	DQ386859	93	147/158
		Azoarcus sp. BH72	Betaproteobacteria	AF011344	93	147/158
FeA2	161	*Kribbella solani* strain YB2	Actinobacteria	EF623891	98	54/55
FeA3	159	Bacterium Ellin5102	Verrucomicrobia	AY234519	96	153/159
FeA4	156	*Pedobacter* sp. H37	Bacteroidetes	EF204468	92	144/156
FeA5	161	*Geobacter psychrophilus* strain P11	Deltaproteobacteria	AY653551	99	157/158
FeA6	160	*Azoarcus* sp. HA	Betaproteobacteria	AF482683	95	153/160
FeA7	159	Bacterium Ellin517	Verrucomicrobia	AY960780	88	140/159
FeA8	162	*Nitrospira marina*	Nitrospirae	X82559	97	81/83
FeA9	155	Bacterium strain XB45	Bacteroidetes	AJ229237	98	99/101

Table 1 *continued*

DGGE band	Sequence bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment
FeA10	136	*Dehalococcoides* sp. BHI80-15	Chloroflexi	AJ431246	89	122/136
FeA11	159	*Opitutus* sp. VeSm13	Verrucomicrobia	X99392	95	152/159
FeA12	155	Bacterium 052306	Bacteria	AB202142	86	134/155
		Flavobacterium sp. DiSf7	Bacteroidetes	EF195103	86	134/155
FeA13	136	*Methylobacterium* symbiont	Bacteria	AB112774	93	123/132
FeA14	135	*Dehalococcoides* sp. BHI80-15	Chloroflexi	AJ431246	91	124/136
FeA15	136	*Sphaerobacter thermophilus*	Chloroflexi	AJ420142	96	73/76
FeA16	136	*Coriobacterium* sp. 3WC8.1	Actinobacteria	AJ586811	89	119/133
Site B						
FeB1	160	*Siderooxidans ghiorsii* strain LD-1	Gammaproteobacteria	DQ386859	93	150/160
		Azoarcus sp. BH72	Betaproteobacteria	AF011344	93	150/160
FeB2	136	*Clostridium* sp. BA-1	Firmicutes	AB196728	97	81/83
		Firmicutes symbiont of Osedax sp.	Firmicutes	EF117250	88	121/136
FeB3	140	*Mycoplasma cheloniae* strain H3110	Firmicutes	U19768	96	73/76
FeB4	160	*Azoarcus* sp.	Betaproteobacteria	X85434	93	148/159
FeB5	160	*Desulfovibrio* sp. 49MC	Deltaproteobacteria	EF442988	89	85/95
FeB6	149	*Epulopiscium* sp. N.l._2_clone_38	Firmicutes	AY844991	94	69/73
FeB7	161	*Azovibrio restrictus*	Betaproteobacteria	AF011346	96	155/161
FeB8	160	*Dechloromonas* sp. FL9	Betaproteobacteria	AF288773	96	155/160
FeB9	136	*Caldilinea aerophila*	Chloroflexi	AB067647	89	124/138
		Acidimicrobium ferrooxidans strain TH3	Actinobacteria	EF621760	93	74/79
FeB10	136	*Methylobacterium* symbiont	Bacteria	AB112774	92	122/132
FeB11	161	*Desulfonatronum cooperativum* Z-7999	Deltaproteobacteria	AY725424	90	86/95
FeB12	136	Bacterium 2-400 C2.5	Planctomycetes	Z77532	90	77/85
FeB13	135	*Coriobacterium* sp. 3WC8.1	Actinobacteria	AJ586811	88	118/133
Plow pan layer soils						
Site A						
PpA1	159	Bacterium Ellin518	Verrucomicrobia	AY960781	95	92/96
PpA2	159	Bacterium Ellin513	Verrucomicrobia	AY960776	93	148/159
PpA3	155	Unidentified eubacterium BSV13	Bacteria	AJ229182	99	151/152
PpA4	135	*Thermus thermophilus* HB8	Deinococcus-Thermus	AP008226	94	74/78
PpA5	161	*Holophaga foetida* strain TMBS4-T	Acidobacteria	X77215	91	148/161
PpA6	155	Bacterium 052306	Bacteria	AB202142	86	134/155
		Flavobacterium sp. DiSf7	Bacteroidetes	EF195103	86	134/155
PpA7	160	Unknown Actinomycete (MC 64)	Actinobacteria	X68461	93	139/149
PpA8	135	Bacterium Ellin5258	Acidobacteria	AY234609	94	128/136
Site B						
PpB1	159	Bacterium Ellin5102	Verrucomicrobia	AY234519	93	149/159
PpB2	136	*Clostridium* sp. BA-1	Firmicutes	AB196728	97	81/83
PpB3	160	*Chrysiogenes arsenatis*	Chrysiogenetes	X81319	98	56/57
		Gemmatimonas aurantiaca	Gemmatimonadetes	AB072735	93	68/73
PpB4	135	*Magnetobacterium bavaricum*	Nitrospirae	X71838	92	122/132
PpB5	155	Bacterium strain XB45	Bacteroidetes	AJ229237	96	81/84
PpB6	160	*Desulfobulbus* sp. BG25	Deltaproteobacteria	U85473	90	145/161
PpB7	134	Gram-positive bacteria SOGA31	Bacteria	AJ244807	91	123/135
PpB8	140	Actinobacterium MH3-4	Actinobacteria	EF187355	96	125/130

DGGE, denaturing gradient gel electrophoresis.

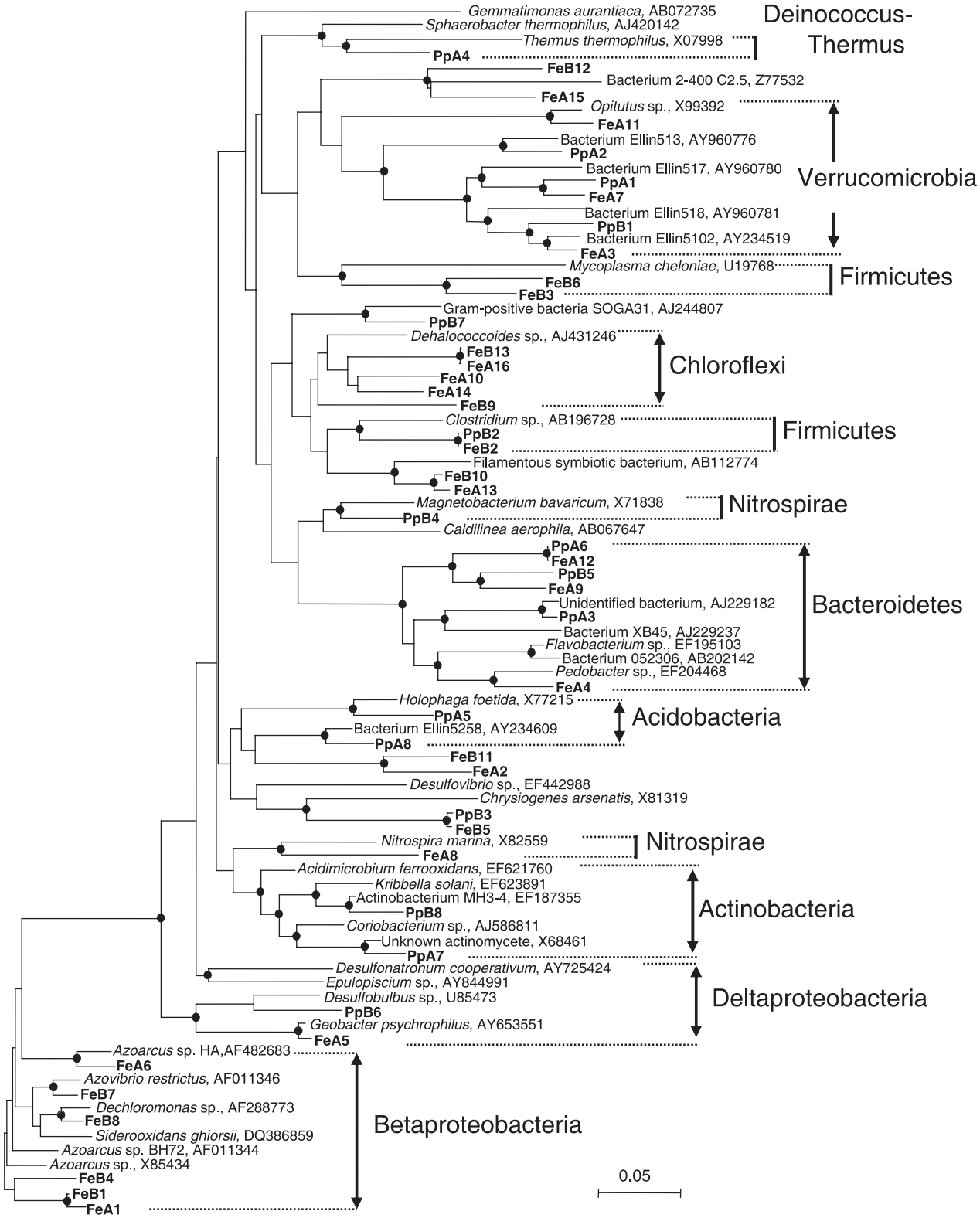

Figure 2 Neighbor-joining phylogenetic tree of the bacterial communities in Fe mottles and plow pan layer soils in a rice field. The symbol ● indicates internal nodes with at least 50% bootstrap support. The scale bar represents the abundance of nucleotide substitutions per residue. FeA, Fe mottles at Site A; FeB, Fe mottles at Site B; PpA, plow pan layer soil of Site A; PpB, plow pan layer soil of Site B.

an Fe-oxidizing bacteria. Some strains of the genera *Azoarcus* and *Azovibrio* are diazotrophs (Hurek *et al.* 1997; Reinhold-Hurek and Hurek 2000), and some of them are denitrifying bacteria (Anders *et al.* 1995; Heylen *et al.* 2006). Several studies elucidated anaerobic Fe(II) oxidation by mesophilic denitrifying bacteria with nitrate as an electron acceptor (Benz *et al.* 1998; Ratering and Schnell 2001; Weber *et al.* 2006). In addition, *Dechloromonas* sp. (Weber *et al.* 2006), *Acidimicrobium ferrooxidans* (Clark and Norris 1996) and *Geobacter psychrophilus* (Nevin *et al.* 2005), the closest relatives of some bands (Table 1), have been shown to be involved in Fe oxidation, Fe reduction or both. In particular, it is interesting to note that the genus *Geobacter* is a dominant community member in wetland sediments that are capable of both dissimilatory Fe(III) reduction and Fe(II) oxidation by the reduction of NO_3^- to NH_4^+ (Weber *et al.* 2006).

Bands FeB5 and FeB11 were closely related to the sulfate-reducing bacteria of *Desulfovibrio* sp. (Meyer and Kuever 2007; Suzuki *et al.* 2007b) and *Desulfonatronum cooperativum* (Zhilina *et al.* 2005), respectively. Sulfide produced by sulfate reduction reduces Fe oxides at circumneutral pH (Lovley 1991; Nealson and Myers 1992). In addition, several species of *Desulfovibrio* are also known to reduce Fe(III) directly through an enzymatic mechanism (Coleman *et al.* 1993). Clostridial members were found to be close relatives of Bands FeB2 and FeB6. Lovley (1991) noted that some clostridial members reduce Fe(III) for growth by using it as an electron acceptor. Thus, the present study indicated that many of the closest relatives of DGGE bands in Fe mottles (12 of 29 bands) are related to Fe oxidation and/or reduction, and physiological study of the bacteria in Fe mottles may be an interesting subject to understand the Fe dynamics in rice fields.

For the plow pan layer soils from Sites A and B, 16 DGGE bands were sequenced (Table 1) and 10 of these bands (PpA1, PpA2, PpA3, PpA6, PpA8, PpB1, PpB3, PpB5, PpB7 and PpB8) had a close relationship with cultured bacteria, including bacteria isolated from rice fields (PpA3, PpA6 and PpB5). In contrast to the Fe mottles, only two DGGE bands were closely related to potential Fe-reducing bacteria: *Clostridium* for Band PpB2 and *Desulfobulbus* (Sass *et al.* 2002; Suzuki *et al.* 2007a) for Band PpB6.

Phylogenetic comparison of the bacterial communities in the Fe mottles and reference soils

Figure 2 shows the phylogenetic relationships of the DGGE bands in the Fe mottles and the reference soil samples in the plow pan layers. The DGGE bands from the Fe mottles and the reference soils were similarly distributed to most clades, from Deinococcus-Thermus to Deltaproteobacteria (Fig. 2). This finding may result from considerable contamination of the Fe mottle samples by the soil matrix, notwithstanding the careful collection of Fe mottles as was observed for the Fe concentration in Fe mottles. An exception was the clades of Betaproteobacteria, where six DGGE bands from the Fe mottles (FeA1, FeA6, FeB1, FeB4, FeB7 and FeB8) belonged exclusively to these clades, and all of their closest relatives were potential Fe oxidizers and/or reducers.

In conclusion, the present study clearly demonstrated that the bacterial communities in Fe mottles in the plow pan layer in a rice field are phylogenetically different from those in the reference soil in the plow pan layer. In addition, the present study suggested that various types of bacteria, whose closest relatives can conduct Fe oxidation and reduction, inhabit the Fe mottles. Physiological study of the bacteria in Fe mottles should be an interesting subject to understand Fe mottle formation in rice fields.

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