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ORIGINAL ARTICLE

Bacterial communities in manganese nodules in rice field subsoils: Estimation using PCR-DGGE and sequencing analyses

Vita Ratri CAHYANI[1,3], Jun MURASE[1], Eiji ISHIBASHI[2], Susumu ASAKAWA[1] and Makoto KIMURA[1]

[1]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601,* [2]*Agricultural Experiment Station, Okayama Prefectural General Agricultural Center, Okayama 709-0801, Japan; and* [3]*Faculty of Agriculture, Sebelas Maret University, Surakarta 57126, Indonesia*

Abstract

The phylogenetic positions of bacterial communities in manganese (Mn) nodules from subsoils of two Japanese rice fields were estimated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis followed by sequencing of 16S rDNA. The DGGE band patterns and sequencing analysis of characteristic DGGE bands revealed that the bacterial communities in Mn nodules were markedly different from those in the plow layer and subsoils. Three out of four common bands found in Mn nodules from two sites corresponded to Deltaproteobacteria and were characterized as sulfate-reducing and iron-reducing bacteria. The other DGGE bands of Mn nodules corresponded to sulfate and iron reducers (Deltaproteobacteria), methane-oxidizing bacteria (Gamma and Alphaproteobacteria), nitrite-oxidizing bacteria (Nitrospirae) and Actinobacteria. In addition, some DGGE bands of Mn nodules showed no clear affiliation to any known bacteria. The present study indicates that members involved in the reduction of Mn nodules dominate the bacterial communities in Mn nodules in rice field subsoils.

Key words: bacteria, manganese nodule, manganese reduction, phylogeny, rice field.

INTRODUCTION

Rice is a staple crop in Asian countries. Irrigated rice fields, which account for more than 50% of the total areas managed for rice production (Halwart and Gupta 2004), provide a unique environment for soil microorganisms because the soils are maintained under flooded conditions during the main stages of rice growth and left to drain after rice harvesting. Flooding the fields results in soil reduction of the plow layer, and the resultant Fe^{2+} and Mn^{2+} ions in the plow layer are leached by water percolation and accumulate in the subsoil as exchangeable or oxidized forms at specific soil depths, all of which are finally oxidized after the rice fields are drained (Kimura 2000).

Correspondence: V. R. CAHYANI, Laboratory of Soil Biology and Chemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan. Email: cahyani@nuagr1.agr.nagoya-u.ac.jp

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The sites of Fe and Mn accumulation in the subsoil layer are termed Fe and Mn illuvial horizons, and the accumulation sites of Fe oxides are slightly shallower than those of Mn oxides as predicted from the redox behavior of these two elements (Kyuma 2004). They are horizons specific to irrigated rice fields, and usually recorded of their depths in the soil classification of rice fields. The morphology of illuviated Fe and Mn differs: Fe oxides form cloud-like ferruginous mottles at the horizon, whereas Mn oxides form spot-like manganiferous mottles at the Mn illuvial horizon.

Phylogenetically wide varieties of bacteria have been isolated from versatile environments, including those environments involving in Mn oxidation and reduction processes. To the best of our knowledge, these varieties are *Aerobacter* sp., *Arthrobacter* spp., *Bacillus* spp., *Caulobacter* sp., *Chromobacterium* spp., *Citrobacter* sp., *Corynebacterium* sp., *Cytophaga* sp., *Gallionella* sp., *Hyphomicrobium* sp., *Leptothrix* spp., *Pedomicrobium* sp., *Pseudomonas* spp., *Proteus* sp. and *Streptomyces* sp. as Mn-oxidizing bacteria from Mn nodules (the Atlantic Ocean), Mn concretions (soil), lake waters, groundwater,

bay sediments and soils (e.g. Bromfield and Skerman 1950; Ehrlich 1968; Francis and Tebo 2001; Gregory and Staley 1982; Wada *et al.* 1978b), and *Acinetobacter* spp., *Bacillus* spp., *Carnobacterium* sp., *Corynebacterium* sp., *Desulfovibrio* sp., *Desulfotomaculum* spp., *Geobacter* sp., *Pseudomonas* spp., *Pyrobaculum* sp. and *Shewanella* sp. as Mn-reducing bacteria from freshwater sediments, marine sediments, Mn nodules (the Atlantic Ocean) and aquifers (e.g. Di-Ruggiero and Gounot 1990; Ghiorse and Ehrlich 1974; Lovley *et al.* 1989; Sass and Cypionka 2004). However, there are only two papers reporting microbial communities associated with ferromanganese nodules/deposits in Green Bay (Stein *et al.* 2001) and Lechuguilla and Spider Caves (Northup *et al.* 2003). And no reports on bacterial communities inhabiting Mn nodules in paddy fields have been published to date.

The present study aimed to elucidate the bacterial communities inhabiting Mn nodules in the subsoil layer of Japanese rice fields using molecular techniques of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and sequencing. The PCR-DGGE analysis has been found to be a suitable method for detecting microbial community structure from environmental samples (Cahyani *et al.* 2003, 2004a, b). This is the first study to examine the bacterial communities in Mn mottles/nodules in the Mn illuvial horizons of rice fields.

MATERIALS AND METHODS

Collection of manganese nodules

Several kilograms of soil blocks were collected from Mn illuvial horizons in two Japanese rice fields located in Kojima Bay reclamation area, Okayama Prefecture, on 16 March 2006. Sites A and B were located in Fujita Omagari (34°34′N, 133°51′E) and Fujita Nishiki-rokku (34°35′N, 133°54′E), respectively. In addition, plow layer soil and subsoil samples apart from Mn nodules were also taken as references. Some of the soil properties at Sites A and B are as follows: (1) plow layer soils: total C content 16 and 17.1 g kg^{-1}, respectively; total N content 1.4 and 1.4 g kg^{-1}, respectively; pH (H_2O) 6.6 and 6.4, respectively; cation exchange capacity (CEC) 20.1 and 18.8 $cmol_c$ kg^{-1}, respectively, (2) subsoils: total C content 5.6 and 12.8 g kg^{-1}, respectively; total N content 0.5 and 1.2 g kg^{-1}, respectively; pH (H_2O) 6.8 and 6.6, respectively; CEC 17.4 and 18.5 $cmol_c$ kg^{-1}, respectively.

In the laboratory, approximately 15 g of large and firm Mn nodules (several millimeters in diameter) were collected carefully with forceps and a small spatula by the breaking soil blocks into small pieces. The nodules were immersed into distilled water and sonicated for a minute to disperse clinging soil particles from the nodules. This washing was repeated several times to obtain clean Mn nodules. The nodules were then stored at –80°C until use.

Chemical analysis of manganese nodules

Concentrations of Mn and Fe in the Mn nodules and in the reference soil samples were determined using an inductively coupled plasma (ICP) spectrophotometer (Model IRIS AP, Nippon Jarrell-Ash, Kyoto, Japan). Air-dried samples were pulverized using a mortar and a pestle. Approximately 100 mg of the samples was placed in a 25-mL glass tube and 10 mL of 50 g kg^{-1} hydroxylamine in 1 mol L^{-1} HCl was added. The glass tube was incubated in a water bath at 70°C for 3 h. Five milliliters of 50 g kg^{-1} hydroxylamine in 1 mol L^{-1} HCl was added again to the tube, and incubation continued at 70°C for 3 h, when sample residues turned from dark brown to pale brown. The sample was transferred to a 50-mL volumetric flask after cooling it at room temperature and filled up to 50 mL with 1 mol L^{-1} HCl. The sample was left to stand overnight at room temperature. Then, 10 mL of supernatant was carefully removed to a 10-mL glass tube so as not to disturb settled residues and subjected to the determination of Mn and Fe concentrations using an ICP spectrophotometer.

DNA extraction

The DNA was extracted from approximately 400 mg of Mn nodule and reference samples after melting them using FastDNA SPIN Kit for Soil (BIO 101, Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocols. The DNA was eluted from the binding matrix with 100 μL of TE buffer (10 mmol L^{-1} Tris-HCl (pH 8.0), 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA)) and stored at –20°C. The DNA extraction was carried out on three replicates for each sample.

Polymerase chain reaction amplification

The eubacterial sets of two primers 357f-GC clamp and 517r were applied to amplify the 16S rDNA fragments. The DNA sequences of primers 357f-GC clamp (*Escherichia coli* position: 341-357, 5′-CGCCCGCC-GCGCGCGGCGGGCGGGGCGGGGGCACGGCC-TACGGGAGGCA GCAG-3′, the underlined sequence corresponded to the GC clamp) and 517r (5′-ATTAC-CGCGGCTGC TGG-3′, *E. coli* position: 517–534) (Muyzer *et al.* 1993) were used. The primers were specific for the V3 variable region of eubacterial 16S rDNA (Neefs *et al.* 1990). Three replicates of DNA extract from each sample were subjected to PCR amplification. The PCR was carried out with TaKaRa PCR Thermal Cycles Model TP 240 (TaKaRa, Tokyo, Japan). The total volume of the reaction mixture was 50 μL containing 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mmol L^{-1} dNTP mixture, 5 μL of 10× *Ex Taq* buffer

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(20 mmol L^{-1} Mg^{2+}; TaKaRa), 0.25 μL of *Ex Taq* DNA polymerase (TaKaRa), 1 μL of environmental DNA template and 37.75 μL of milli-Q water. Cycle conditions for the PCR amplification were as follows: the first path was one cycle of initial denaturation at 94°C for 3 min, followed by a second path of 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1.5 min, and the third path was one cycle of final extension at 72°C for 8 min.

DGGE analysis, direct sequencing and sequencing after cloning

The DGGE analysis was carried out by loading the PCR products onto an 80 g L^{-1} polyacrylamide gel with a denaturing gradient ranging from 30 to 70% in an electrophoresis cell D-code System (Bio-Rad Laboratories, Hercules, CA, USA) with 1× TAE buffer (40 mmol L^{-1} Tris, 20 mmol L^{-1} acetic acid and 1 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA] at pH 8.0) at 60°C and 100 V for 14 h. The procedure from the preparation of the gel to the visualization of the DGGE band pattern was the same as that described previously (Cahyani *et al*. 2003). Most bands with strong intensity in the lane of Mn nodule samples in the DGGE gel were readily isolated and sequenced directly. However, bands with weak intensity in the lane and all bands in the lanes of reference soils (subsoil and plow layer) were unable to be sequenced directly. Therefore, cloning was carried out for those bands before sequencing. The procedures of direct sequencing and sequencing after cloning were the same as those described previously (Cahyani *et al*. 2003). In the DGGE pattern analysis, we first compared the DGGE patterns among three replicates of each sample, and found exactly the same patterns among them. Respective bands were excised from three replicates for the determination of their sequences. However, we presented the DGGE pattern of each sample without replication in Fig. 1.

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 the BLAST search.

Nucleotide sequence accession numbers

The 16S rDNA partial sequences determined in the present study have been deposited in the DDBJ database under the accession numbers AB292482–AB292524.

RESULTS AND DISCUSSION

Chemical properties of manganese nodules

Manganese nodules from site A and site B contained 31 and 42 g Mn kg^{-1} of soil and 22 and 38 g Fe kg^{-1} of soil, respectively. The concentration of Mn in the Mn nodules from the two sites was 50–60-fold higher than the concentration in the reference soils (Table 1). The concentration of Fe in the Mn nodules also tended to be higher than in the reference subsoils (Table 1). As Murase and Kimura (1997) elucidated the chemical

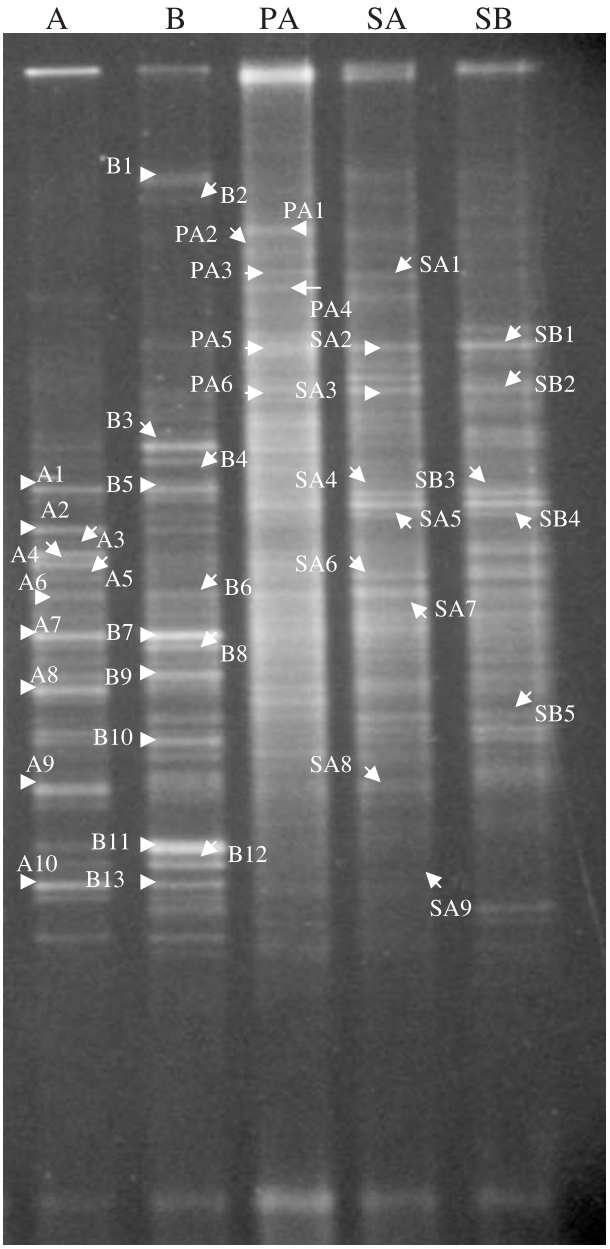

Figure 1 Denaturing gradient gel electrophoresis pattern of the bacterial communities in Mn nodules and the reference soil samples in rice field soils. A, Mn nodules at site A; B, Mn nodules at site B; PA, plow layer soil of site A; SA, subsoil of site A; SB, subsoil of site B. Sites A and B were located in Fujita Omagari and Fujita Nishiki-rokku, respectively.

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Table 1 Concentrations on a dry weight basis of Mn and Fe in Mn nodules and the reference soils in rice fields

No.	Sample	Mn (g kg^{-1})	Fe (g kg^{-1})
1	Mn nodules (Site A)	31	22
2	Mn nodules (Site B)	42	38
3	Plow layer soil (Site A)	0.9	29
4	Subsoil (Site A)	0.6	19
5	Subsoil (Site B)	0.7	22

Site A, Fujita Omagari; Site B, Fujita Nishiki-rokku.

reduction of Mn oxides by Fe^{2+} in soil ($MnO_2 + 2Fe^{2+} + H_2O \rightarrow Mn^{2+} + Fe_2O_3 + 2H^+$), Fe^{2+} leached from the upper layer might have reduced Mn oxides in Mn nodules resulting in the accumulation of Fe in Mn nodules.

Bacterial communities in Mn nodules

Bacterial communities in Mn nodules are represented by the DGGE band patterns shown in Fig. 1. The DGGE patterns of Mn nodules from both sites were characterized by several strong bands, indicating that several specific bacteria inhabit the Mn nodules. The DGGE patterns of Mn nodules from the two sites showed similar patterns, and several DGGE bands in Mn nodules from site A and site B were located at the same positions in polyacrylamide gel, indicating that common bacteria inhabit Mn nodules at these sites.

In contrast, the DGGE patterns of the reference soils at both sites consisted of many weak bands, especially for the plow layer soil at site A, indicating that microbial communities in the plow layer and subsoils consist of a wide range of bacteria without any specific bacteria as dominant members (Fig. 1). A similar finding of numerous DGGE bands was also observed in the plow layer soils of other Japanese rice fields (H. Kikuchi *et al.* 2007). There was no band of strong intensity in the plow layer and subsoil samples that showed the same mobility as the DGGE bands of strong intensity observed in the Mn nodules (Fig. 1). Therefore, we concluded that bacterial communities in Mn nodules are markedly different from those in the plow layer and subsoil.

Representative bacteria in Mn nodules

The closest relatives of some representative bands in the Mn nodules from Sites A and B are listed in Table 2. Four respective band pairs A1 and B5, A6 and B6, A7 and B7, and A10 and B13 (prefix A from site A and prefix B from site B) that showed equivalent mobility belonged to the same closest relatives, respectively. Bands A1 and B5 corresponded to Betaproteobacteria, and Bands A6, B6, A7, B7, A10 and B13 to Deltaproteobacteria. This finding also indicated that common bacteria inhabit Mn nodules at these two sites. The members of Deltaproteobacteria were also found to be common bacteria in ferromanganous nodules in Green Bay (Stein *et al.* 2001). However, no Deltapropteobacterial member was detected in ferromanganese deposits in Lechuguilla and Spider Caves (Northup *et al.* 2003). Thus, the members of Deltaproteobacteria seemed to be bacteria commonly inhabiting Mn nodules in aquatic environments where the rapid cycling of oxidation and reduction occurs. The closest relatives of the common bacteria represented by DGGE bands in the present study mainly originated from aquatic environments (freshwater sediment, groundwater, marine sediment, lake and paddy soil).

Although many studies have reported Mn oxidizing or reducing bacteria belonging to Gram-negative Proteobacteria, these closest relatives were not found in the present study. As the closest relatives conduct anaerobic respiration: sulfate respiration by *Olavius crassitunicatus, Desulfocapsa* sp. and *Desulfovibrio alaskensis* (Bands A6, B6, A10, and B13) and iron oxide respiration by *Anaeromyxobacter* sp. (A7 and B7), the bacteria of those DGGE bands may contribute to the reduction of Mn oxides because produced sulfides and ferrous iron reduce Mn oxides chemically (Murase and Kimura 1997). Many studies also revealed the involvements of some sulfate-reducing and iron-reducing bacteria in biological Mn reduction. For example, *Desulfotomaculum reducens* (Tebo and Obraztsova 1998), *Desulfovibrio indonesiensis* and *Desulfotomaculum geothermicum* (Sass and Cypionka 2004) used Mn oxides, in addition to various sulfur compounds, as an electron acceptor. And *Geobacter metallireducens* (Lovley *et al.* 1993) and *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens*) (Lovley *et al.* 1989; Myers and Nealson 1990; Nealson and Myers 1992) could obtain energy for growth by coupling the oxidation of various electron donors with the dissimilatory reduction of Mn or Fe oxides. The Mn nodules fit to sulfate and iron reducers because substrates for respiration (sulfate and iron oxides) are regenerated chemically by redox reactions with Mn oxides. From these considerations, it is believed that the closest relatives of Bands B3, B4, A8 and B8 belong to sulfate and iron reducers: *Thermodesulforhabdus* sp., *Pelobacter venetianus* and *Anaeromyxobacter* sp.

Bands A2, A3 and A4 were closely related to methane-oxidizing bacteria of *Methylococcus capsulatus* (Dedysh *et al.* 2004), *Methylocystis parvus* (Hou *et al.* 1979) and *Methylosinus trichosporium* (Bowman *et al.* 1993), respectively. The presence of methanotrophs in Mn nodules might be explained by the findings reported by Zehnder and Brock (1980) that under anaerobic

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Table 2 Closest relatives to denaturing gradient gel electrophoresis bands obtained from Mn nodules at Sites A and B

DGGE band	Seq bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment	Source
Site A							
A1	162	Bacterium PE03-7G14	Bacteria	AB127733	94	147/156	Freshwater sediment
		Alcaligenaceae bacterium BL-169	Betaproteobacteria; Burkholderiales	DQ196633	93	151/162	Contaminated groundwater
A2	161	*Methylococcus capsulatus* strain Texas	Gammaproteobacteria; Methylococcales	AJ563935	91	149/162	
A3	138	*Methylocystis parvus* clone MFC-EB21	Alphaproteobacteria; Rhizobiales	AJ630289	97	134/138	Electricity-generating microbial fuel cell
		Methylosinus trichosporium	Alphaproteobacteria; Rhizobiales	AY460188	97	134/138	
A4	137	*Methylocystis parvus* clone MFC-EB21	Alphaproteobacteria; Rhizobiales	AJ630289	97	134/138	Electricity-generating microbial fuel cell
		Methylosinus trichosporium	Alphaproteobacteria; Rhizobiales	AY460188	97	134/138	
A5	156	*Nitrospira* sp. GaII	Nitrospirae; Nitrospirales	AY796334	90	93/103	Hot spring
		Nitrospira sp. Ga9-4	Nitrospirae; Nitrospirales	AY796333	90	93/103	Hot spring
A6	139	Bacterium ROME1095sa	Bacteria	AY998186	93	75/80	Cerro Negro
		Olavius crassitunicatus d1-P20-III-32	Deltaproteobacteria.	AJ620509	93	75/80	Marine sediment
		Desulfocapsa sp. Cad626	Deltaproteobacteria; Desulfobacterales	DES511275	93	75/80	Chemocline of lake
A7	161	*Anaeromyxobacter* sp. FAc12	Deltaproteobacteria; Myxococcales	AJ504438	91	87/95	Paddy soil
		Myxobacterium KC	Deltaproteobacteria; Myxococcales	AF482687	91	87/95	
		Geobacter sulfurreducens	Deltaproteobacteria; Desulfuromonadales	U13928	92	82/89	
A8	161	Deltaproteobacterium EtOHpelo	Deltaproteobacteria	AY771935	87	138/157	Marine surface sediment
		Pelobacter venetianus	Deltaproteobacteria; Desulfuromonadales	U41562	87	138/157	
A9	142	Actinomycetales str. Ellin143	Actinobacteridae; Actinomycetales	AF408985	95	136/142	
		Frankia sp.	Actinobacteridae; Actinomycetales	U60287	95	136/142	Specific host=Alnus nepalensis nodule
A10	161	*Desulfovibrio alaskensis* strain HEB223	Deltaproteobacteria; Desulfovibrionales	DQ867001	97	81/83	

Table 2 *Continued*

DGGE band	Seq bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment	Source
Site B							
B1	164	Bacterial species clone 32-11	Bacteria	Z95711	95	142/148	note=Holophaga/ Acidobacterium phylum
B2	164	Bacterial species clone 32-11	Bacteria	Z95711	95	142/148	note=Holophaga/ Acidobacterium phylum
B3	164	*Thermodesulforhabdus* sp. nov. M40/2 CIV-3.2	Deltaproteobacteria; Syntrophobacterales	AF170420	85	98/115	Geothermally heated sediment
		Deltaproteobacterium EtOHpelo	Deltaproteobacteria	AY771935	83	134/160	Marine surface sediment
B4	164	*Thermodesulforhabdus* sp. nov. M40/2 CIV-3.2	Deltaproteobacteria; Syntrophobacterales	AF170420	85	98/115	Geothermally heated sediments
		Deltaproteobacterium EtOHpelo	Deltaproteobacteria	AY771935	83	134/160	Marine surface sediment
B5	160	Bacterium PE03-7G14	Bacteria	AB127733	96	148/154	Freshwater sediment
		Alcaligenaceae bacterium BL-169	Betaproteobacteria; Burkholderiales	DQ196633	95	152/160	Contaminated groundwater
B6	139	Bacterium ROME1095sa	Bacteria	AY998186	93	75/80	Cerro Negro
		Olavius crassitunicatus d1-P20-III-32	Deltaproteobacteria.	AJ620509	93	75/80	Marine sediment
		Desulfocapsa sp. Cad626	Deltaproteobacteria; Desulfobacterales	DES511275	93	75/80	Chemocline of lake
B7	162	*Anaeromyxobacter* sp. FAc12	Deltaproteobacteria; Myxococcales	AJ504438	91	87/95	Paddy soil
		Myxobacterium KC	Deltaproteobacteria; Myxococcales	AF482687	91	87/95	
		Geobacter sulfurreducens	Deltaproteobacteria; Desulfuromonadales	U13928	92	82/89	
B8	162	*Anaeromyxobacter* sp. FAc12	Deltaproteobacteria; Myxococcales	AJ504438	91	87/95	Paddy soil
		Myxobacterium KC	Deltaproteobacteria; Myxococcales	AF482687	91	87/95	
		Geobacter sulfurreducens	Deltaproteobacteria; Desulfuromonadales	U13928	92	82/89	
B9	139	Bacterial species clone 32-10	Bacteria	Z95710	98	81/82	note=Holophaga/ Acidobacterium phylum
		Acid streamer iron-oxidizing bacterium CS11	Bacteria; Actinobacteria	AY765999	91	64/70	Streamer in acidic, iron-rich spa water
B10	136	Bacterium species clone Ep_T1.185	Bacteria	Z73368	98	129/131	
		Acidobacteria bacterium Ellin7137	Bacteria; Acidobacteria	AY673303	95	130/136	Soil
B11	137	*Propionicimonas* sp. F6	Actinobacteridae; Actinomycetales	AY570689	90	126/140	Low-temperature biodegraded Canadian oil reservoir
B12	137	*Propionicimonas* sp. F6	Actinobacteridae; Actinomycetales	AY570689	90	126/140	Low-temperature biodegraded Canadian oil reservoir
B13	161	*Desulfovibrio alaskensis* strain HEB223 16S	Deltaproteobacteria; Desulfovibrionales	DQ867001	97	81/83	

Site A, Fujita Omagari; Site B, Fujita Nishiki-rokku. DGGE, denaturing gradient gel electrophoresis.

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Table 3 Closest relatives to DGGE bands obtained from reference soil samples at Sites A and B

DGGE band	Seq bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment	Source
Plow layer (Site A)							
PA1	135	*Wolinella succinogenes*	Epsilonproteobacteria; Campylobacterales	BX571660	84	114/135	
		Helicobacter muridarum	Epsilonproteobacteria; Campylobacterales	AF302104	84	114/135	
PA2	136	Anaerobic filamentous bacterium IMO-1	Bacteria; Chloroflexi	AB109437	91	125/136	Thermophilic UASB granular sludge
		Anaerolinea thermophila	Chloroflexi; Anaerolinea	AB046413	91	125/136	
PA3	160	*Spirochaeta asiatica*	Spirochaetes; Spirochaetales	X93926	88	141/160	
PA4	160	*Lysobacter* sp. 2-O-7	Gammaproteobacteria; Xanthomonadales	AB272385	91	147/160	Forest soil
PA5	136	Swine manure bacterium 37-1	Bacteria.	AY167963	89	120/134	Manure
		Actinobacterium MWH-Ta8	Actinobacteria.	AM182889	97	80/82	Freshwater lake
PA6	155	Unidentified eubacterium BSV40	Bacteria.	AJ229196	94	89/94	Anoxic bulk soil of a flooded rice microcosm
		Hippea maritima	Deltaproteobacteria; Desulfurella group	AB072402	94	93/98	
Subsoil (Site A)							
SA1	136	*Dehalococcoides* sp. BHI80-52	Chloroflexi; Dehalococcoidetes	AJ431247	85	116/136	
		Anaerolinea thermophila	Chloroflexi; Anaerolinea	AB046413	85	104/121	
SA2	164	*Wolinella succinogenes*	Epsilonproteobacteria; Campylobacterales	BX571660	95	78/82	
		Helicobacter brantae strain MIT 04-9366	Epsilonproteobacteria; Campylobacterales	DQ415546	95	78/82	Specific host=Canada goose
SA3	157	Bacterial species clone 32-11	Bacteria	Z95711	92	146/157	note=Holophaga/Acidobacterium phylum
		Roseomonas sp. 1 LOT M4	Alphaproteobacteria; Rhizobiales	AY624051	93	92/98	
SA4	136	Gram-positive bacteria SOGA31	Bacteria	AJ244807	93	127/136	From the cuttlefish Sepia officinalis accessory nidamental glands

Table 3 *Continued*

DGGE band	Seq bp	Closest relatives: Microorganisms	Phylogenetic affiliations	Accession number	Similarity (%)	Alignment	Source
SA5	136	Gram-positive bacteria SOGA31	Bacteria	AJ244807	93	127/136	From the cuttlefish Sepia officinalis accessory nidamental glands
SA6	137	*Nannocystis exedens* strain BICC 8785	Deltaproteobacteria; Myxococcales	DQ491074	98	76/77	
		Desulfonema limicola	Deltaproteobacteria; Desulfobacterales	U45990	97	78/80	Anaerobic mud
SA7	137	*Kribbella aluminata* strain HKI 0480	Actinobacteridae; Actinomycetales	EF126969	95	59/62	
		Actinobacteria strain VeSm15	Actinobacteria	AJ229243	92	64/69	From anoxic bulk soil of a flooded rice microcosm
SA8	161	*Geobacter sulfurreducens* PCA	Deltaproteobacteria; Desulfuromonadales	AE017180	87	139/159	
		Geobacter sulfurreducens	Deltaproteobacteria; Desulfuromonadales	U13928	87	139/159	
SA9	135	*Desulfosarcina variabilis*	Deltaproteobacteria; Desulfobacterales	M26632	98	76/77	
Subsoil (Site B)							
SB1	165	*Wolinella succinogenes*	Epsilonproteobacteria; Campylobacterales	BX571660	92	78/82	
		Helicobacter brantae strain MIT 04-9366	Epsilonproteobacteria; Campylobacterales	DQ415546	92	76/82	Specific host=Canada goose
SB2	135	*Mycoplasma coragypsi*	Firmicutes; Mollicutes	L08054	95	81/85	Specific host=Coragyps atratus
SB3	136	Gram-positive bacteria SOGA31	Bacteria	AJ244807	93	127/136	From the cuttlefish Sepia officinalis accessory nidamental glands
SB4	136	Gram-positive bacteria SOGA31	Bacteria	AJ244807	93	127/136	From the cuttlefish Sepia officinalis accessory nidamental glands
SB5	135	*Thermodesulfovibrio islandicus*	Nitrospirae; Nitrospirales	X96726	91	117/128	
		Thermodesulfovib rio yellowstonii	Nitrospirae; Nitrospirales	AB231858	91	117/128	

Site A, Fujita Omagari; Site B, Fujita Nishiki-rokku. DGGE, denaturing gradient gel electrophoresis.

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conditions Mn oxides are used as an electron acceptor by the bacteria for methane oxidation. As Band A5 is also autotrophic *Nitrospira* sp., Mn nodules may be a favorable habitat for members producing energy by oxidation of inorganic substances, which may reflect the substrate-poor habitat of Mn nodules. In addition, the presence of *Nitrospira* sp. in Mn nodules indicates that nitrite is present and potentially plays a role in non-enzymatic reduction of Mn oxide (Lovley 1991; Vandenabeele *et al.* 1995). Stein *et al.* (2001) and Northup *et al.* (2003) also found Nitrospira in microbial communities inhabiting Mn nodules/deposits in Green Bay and in Lechuguilla and Spider Caves.

The involvement of members of Actinobacteria in Mn oxidation has been reported in several studies. However, Bands A9, B11 and B12 belonging to Actinobacteria did not match the listed members. Some bands showed no clear affiliation to any known bacteria (Bands B1, B2, B9 and B10).

Bacteria in plow layer soil and subsoils

It was difficult to excise single DGGE bands from the DGGE gel of plow layer soil and subsoil samples because the DGGE patterns consisted of many weak bands. Furthermore, cloning of some bands from these samples revealed that a seemingly single band contained several DNA fragments with different mobilities in the polyacrylamide gel. Therefore, it was impossible to determine the phylogenetic position of all bacteria in these reference soils from the direct sequence of those DGGE bands.

The closest relatives of some representative bacteria in the plow layer soil and subsoils are listed in Table 3. A wide phylogenetic variety of bacteria inhabit these reference soils. It is interesting to note that some bacteria were found commonly as the closest relatives in subsoil samples from Site A and Site B (Bands SA2 and SB1, and Bands SA4, SA5, SB3 and SB4). These common bacteria corresponded to members of Epsilonproteobacteria and Gram-positive bacteria.

Bacteria detected in the reference soils were markedly different from those in Mn nodules. For example, the bacteria belonging to Epsilonproteobacteria (Bands PA1, SA2 and SB1), Spirochaetes (Band PA3) and Chloroflexi (Bands PA2 and SA1) were found in the reference soils, whereas they were not found in the Mn nodules.

Manganese oxidizing and reducing bacteria and microbial communities in Mn nodules

The bacterial communities in Mn nodules consisted of members potentially related to Mn reduction. The predominance of Mn-reducing bacteria and the superficial absence of Mn-oxidizing bacteria in Mn nodules might support the hypothesis of the process of Mn nodule formation proposed by Wada *et al.* (1978a), which is that the process of Mn formation is divided into two stages. In the initial stage, Mn^{2+} is oxidized by the activities of Mn-oxidizing bacteria, which form tiny dot-shaped Mn deposits. In the later stage, Mn^{2+} is oxidized non-biologically on the tiny Mn-deposits, resulting in the formation of large Mn deposits (Mn mottles or Mn nodules). As the Mn nodules observed in this study were several millimeters in diameter, the hypothesis suggests that the Mn nodules used in the present study were formed mainly by the abiotic process. And the bacterial communities dominated by members relating to the reduction of Mn nodules were, in part, a result of the utilization of Mn oxides for the regeneration of their electron acceptors.

In this study, we did not find any close relatives of DGGE bands for Mn nodule samples that match the members described previously. This unexpected result may derive from differences in methodology between the method used in the present study and that used for bacterial isolation. The bacteria reported in many previous studies were obtained using cultivation methods that allow the selective proliferation of minor members, which the PCR-DGGE method cannot detect. The present study indicates that Mn-oxidizing bacteria are minor members in abundance in Mn nodules, and that the majority of bacteria in Mn nodules relate directly or indirectly to Mn reduction.

Noteworthy was that many DGGE bands of Mn nodules matched the available DNA database only for short sequences with similarities of less than 95%. Sequences of Bands A5, A6, A7, A10, B3, B4, B6, B7, B8, B9 and B13 were matched for less than 100 bp to their closest relatives. This fact may explain why many bacterial members in Mn nodules have never been cultured or isolated to date. The phylogenetic positions of predominant bacterial community members and their roles in Mn mottle formation in the subsoils of rice fields are interesting topics for future research because the topics are interdisciplinary, bridging soil microbiology and pedology.

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