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ORIGINAL PAPER

Phylogenetic positions of Mn^{2+}-oxidizing bacteria and fungi isolated from Mn nodules in rice field subsoils

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Abstract We isolated manganous ion (Mn^{2+}) oxidizing bacteria and fungi from Mn nodules collected from two Japanese rice fields. The phylogenetic position of the Mn-oxidizing bacteria and fungi was determined based on their 16S rDNA and 18S rDNA sequences, respectively. Among 39 bacterial and 25 fungal isolates, *Burkholderia* and *Acremonium* strains were the most common and dominant Mn^{2+}-oxidizing bacteria and fungi, respectively. Majority of the Mn-oxidizing bacteria and fungi isolated from the Mn nodules belonged to the genera that had been isolated earlier from various environments. Manganese oxide depositions on Mn^{2+}-containing agar media by these microorganisms proceeded after their colony developments, indicating that the energy produced from Mn^{2+} oxidation is poorly used for microbial growth.

Keywords *Acremonium* · Bacteria · *Burkholderia* · Fungi · Manganese nodule · Manganese oxidation · Rice field

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Introduction

The rice field is a unique agro-ecosystem, where the field is maintained under flooded conditions during most of the period of rice cultivation and left under drained conditions after rice harvesting. Flooding the field results in soil reduction of the plow layer and Fe^{2+} and Mn^{2+} ions produced in the plow layer under flooded conditions are leached downward by water percolation and accumulated in the subsoil as exchangeable or oxidized forms at the specific soil depths, named Fe and Mn illuvial horizons, all of which are finally oxidized after the field is drained (Kimura 2000). They are the horizons characteristic of irrigated rice fields, imprinting the process of paddy soil formation. The morphology of illuviated Fe and Mn horizons is different from each other with Fe oxides forming cloud-like ferruginous mottles and Mn oxides forming spot-like manganiferrous mottles (Kyuma 2004).

Several phylogenetically wide varieties of Mn^{2+}-oxidizing bacteria have been isolated from Mn nodules in the ocean (Ehrlich 1963), Mn concretions in soils (Douka 1977), bulk soils (Bromfield and Skerman 1950), lake waters (Gregory and Staley 1982), groundwater (Katsoyiannis and Zouboulis 2004), and bay sediments (Francis and Tebo 2002). However, microbial communities associated with Mn^{2+} oxidation/deposition were only studied in freshwater sediments and caves (Northup et al. 2003; Stein et al. 2001). Therefore, we studied the bacterial communities inhabiting Mn nodules in subsoils of two Japanese rice fields by using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method with subsequent 16S rDNA sequencing of many characteristic DGGE bands (Cahyani et al. 2007). In addition, fungi have been observed to play an important role in Mn^{2+} oxidation in aquatic and soil environments (Miyata et al. 2004, 2006a, b; Takano et al.

2006; Thompson et al. 2005; Timonin et al. 1972), but their role in rice fields has not been studied. As there is no primer set specific to Mn^{2+}-oxidizing bacteria and fungi at present, we isolated Mn^{2+}-oxidizing bacteria and fungi from Mn nodules collected from two Japanese rice fields and determined their phylogenetic positions from their 16S rDNA and 18S rDNA sequences. This study also examined Mn oxides formation by Mn^{2+}-oxidizing microorganisms on agar media to estimate the contribution of energy produced by Mn^{2+} oxidation to their growth.

Materials and methods

Mn nodules

Several kilograms of soil blocks were collected from Mn illuvial horizons (at the 20–35 cm depth) in two Japanese rice fields that were located at Kojima Bay reclamation area, Okayama Prefecture, on November 14, 2006, when the fields were drained after harvest. Sites A and B were located at Fujita Omagari (34°34′ N, 133°51′ E) and Fujita Nishiki-rokku (34°35′ N, 133°54′ E), respectively. At the laboratory, large Mn nodules (several millimeters in diameter) were collected carefully with forceps and small spatula by breaking soil blocks into small pieces. They were immersed into sterile 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, which were stored at 4°C until use.

Isolation of Mn^{2+}-oxidizing bacteria and fungi

Isolation of Mn^{2+}-oxidizing microorganisms was carried out by treating 30 mg of Mn nodules with 5 mL of sterile 0.1 M $MnSO_4$ solution in a 50-mL flask. The pH of the suspension was adjusted to 6.0 with 0.1 N NaOH. The flask was then incubated at 22°C for 12 days. Then, the suspension was stirred thoroughly, transferred to an Eppendorf tube, and centrifuged at 1,900×*g* for 5 min. The supernatant was discarded, and the pellet was used for isolating Mn^{2+}-oxidizing bacteria and fungi by the spread plate method with a series of tenfold dilutions. The dilution was carried out in duplicate. Ten plates at appropriate dilutions were incubated at 22°C for 21 days.

The following five media were used for isolating Mn^{2+}-oxidizing bacteria: (1) Gerretsen medium (Bromfield and Skerman 1950) containing calcium citrate 20 g, $(NH_4)_2SO_4$ 2 g, NH_4MgPO_4 0.01 g, $MnSO_4{\cdot}4H_2O$ 5 g, agar 20 g, tap water 1 L (pH 6.0); (2) Beijerinck medium (Bromfield and Skerman 1950) containing NH_4Cl 0.5 g, K_2HPO_4 0.5 g, $MnCO_3$ 10 g, agar 20 g, tap water 1 L (pH 7.9); (3) Bromfield medium (Bromfield 1956) containing KH_2PO_4 0.05 g, $MgSO_4{\cdot}7H_2O$ 0.02 g, $(NH_4)_2SO_4$ 0.1 g, $Ca_3(PO_4)_2$ 0.1 g, "Difco" yeast extract 0.05 g, $MnSO_4{\cdot}4H_2O$ 0.05 g, agar 20 g, distilled water 1 L (pH 6.0); (4) Motomura medium (Wada et al. 1978b) containing $(NH_4)_2SO_4$ 0.1 g, KH_2PO_4 0.05 g, $MgSO_4{\cdot}7H_2O$ 0.02 g, $Ca_3(PO_4)_2$ 0.1 g, peptone 1 g, $MnSO_4{\cdot}nH_2O$ 1 g, agar 20 g, distilled water 1 L (pH 6.0); and (5) NB+Mn+HEPES medium (Emerson and Ghiorse 1992) containing nutrient broth 0.8 g, HEPES 2.383 g, $MnCl_2{\cdot}4H_2O$ 0.1 g, agar 15 g, distilled water 1 L (pH 6.0). In contrast, only Gerretsen medium (Bromfield and Skerman 1950) and Beijerinck medium (Bromfield and Skerman 1950) were used for the isolation of Mn^{2+}-oxidizing fungi, because no fungal growth was observed on the other three media.

Manganese-oxidizing bacteria and fungi developed on isolation media were picked and subcultured on new agar media. The growth of Mn^{2+}-oxidizing bacteria and fungi was evaluated by observing color changes from dark brown to black around the colonies. In addition, all bacterial and fungal isolates were considered to have carried out Mn^{2+} oxidation, when the media became dark violet with 0.5% (*w*/*v*) tetra methyl diamino diphenyl methane (TDDM) solution in 10% (*v*/*v*) acetic acid. No darkening of the media by TDDM solution was checked after incubation without Mn^{2+}-oxidizing microorganisms. As the present study aimed at assessing the phylogenetic positions of various Mn^{2+}-oxidizing microorganisms in Mn nodules, we chose bacteria and fungi that developed different morphological colonies.

Growth of Mn^{2+}-oxidizing bacteria and fungi on media

Mn^{2+}-oxidizing bacteria and fungi were grown on the above-mentioned media, and the development of the dark color in the media was observed for 1 month and related to the colony development.

DNA extraction from bacterial isolates, PCR amplification, and sequencing of bacterial 16S rDNA

PCR amplification of Mn^{2+}-oxidizing bacterial colonies was conducted by using the primer set of 27f (*Escherichia coli* position: 8–27, 5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (*E. coli* position: 1510–1492, 5′-GGC TAC CTT GTT ACG ACT T-3′; Lane 1991). DNA template used in the PCR reaction was prepared by suspending a loopful of bacteria into autoclaved ultra-pure water (50 μL). PCR amplification was performed in 50 μL, which contained in a 200-μL microtube 1 μL of each primer (50 pmol each), 5 μL of 2.5 mM dNTP mixture, 5 μL of 10× *Ex Taq* buffer (20 mM Mg^{2+}; TaKaRa, Otsu, Japan), 0.25 μL of *Ex Taq* DNA polymerase (TaKaRa, Otsu, Japan), and 37.75 μL of DNA template suspension.

Cycle conditions for the amplification were initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 8 min with TaKaRa PCR Thermal Cycler Model TP 240 (TaKaRa, Otsu, Japan). The PCR product was analyzed on 2% (*w*/*v*) agarose gels containing 2% of 50× TAE buffer as described previously (Cahyani et al. 2003). The PCR products then were purified by using QIA quick PCR Purification Kit (QIAGEN, Tokyo, Japan). DNA was also extracted from bacteria that failed to form colony PCR. Bacteria growing on media were picked up and subjected to DNA extraction by using FastDNA® SPIN Kit for Soil (BIO 101, Qbiogene, Carlsbad, CA, USA) using bead beating treatment for 90 s and successive centrifugation at 1,900×*g* according to the manufacturer's protocols. The DNA was eluted from the binding matrix with 100 μL of TE buffer. PCR amplification of extracted DNA and purification of PCR products were performed as reported above. The purified PCR products were sequenced by using 27f and 1492r primers with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. After ethanol/EDTA precipitation, sequencing was run with ABI 3130 Genetic Analyzer (Applied Biosystem). Additional primers such as 357f (5′-CCT ACG GGA GGC AGC AG-3′; Muyzer et al. 1993), 517r (5′-ATT ACC GCG GCT GCT GG-3′; Muyzer et al. 1993), r2L (5′-CAT CGT TTA CGG CGT GGA C-3′; Poulsen et al. 2001), and r3L (5′-TTG CGC TCG TTG CGG GAC T-3′; Nonaka et al. 2005) were used to obtain reliable sequences.

DNA extraction from fungal isolates, PCR amplification, and sequencing of fungal 18S rDNA

Some fungi grew on the surface and inside agar media, while others only grew inside agar media. Fungal mycelia or agar media that contained fungal mycelia were picked up and subjected to extraction of fungal DNA. The extraction of fungal DNA was that reported above for bacteria. The fungal specific primers were EF4f (5′-GGA AGG G[G/A]T GTA TTT ATT AG-3′) and Fung5r (5′-GTA AAA GTC CTG GTT CCC C-3′; Smit et al. 1999). Each PCR reaction mixture contained 1 μL of DNA template from DNA extracts, 0.5 μL of each primer (50 pmol each), 5 μL of 2.5 mM dNTP mixture, 5 μL of 10× *Ex Taq* buffer (20 mM Mg^{2+}; TaKaRa, Otsu, Japan), 0.25 μL of *Ex Taq* DNA polymerase (TaKaRa, Otsu, Japan), and 37.75 μL of autoclaved ultra-pure water. The PCR thermal cycling pattern included an initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 2 min, with the final extension at 72°C for 10 min. The PCR products were purified by using QIA quick PCR Purification Kit (QIAGEN, Tokyo, Japan). Sequencing was then performed with the EF4f and Fung5r primers using the purified PCR products with the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. An additional 516r (5′-ACC AGA CTT GCC CTC C-3′) primer (Casamayor et al. 2002) was used to obtain reliable sequences.

Phylogenetic analysis and nucleotide sequence accession numbers

Sequences of the DNA fragments of isolated Mn^{2+}-oxidizing bacteria and fungi were compared with the available 16S rDNA and 18S rDNA sequences, respectively, of the database of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/E-mail/homology.html) with the BLAST search. Then, the phylogenetic trees of 16S rDNA and 18S rDNA sequences of the isolates with their closest relatives were constructed by the neighbor-joining method (Saitou and Nei 1987) with the CLUSTAL X 1.81 software packages (Thompson et al. 1997). The 16S rDNA and 18S rDNA partial sequences of isolated Mn^{2+}-oxidizing bacteria and fungi determined in the present study were deposited in the DDBJ database under the accession numbers of AB354137–AB354175 and AB354545–AB354569, respectively.

Results

Phylogeny of Mn^{2+}-oxidizing bacteria in Mn nodules

Nineteen and 20 bacteria were isolated from Mn nodules at site A and site B, respectively. More than half of bacterial isolates were obtained from both sites by using Gerretsen medium (Table 1). Sixteen bacteria from site A and 13 bacteria from site B were obtained by excluding the isolates with the same nucleotide sequences. In addition, 1b-G3 and 1b-Mt14 isolates, 1a-G12 isolate, and 1a-G5, 1a-G8, and 1a-Mt10 isolates from site A were identical in nucleotide sequences to 2a-G1, 2b-G1, 2b-G2, 2b-G3, 2b-G4, 2b-G10, 2b-Br17 and 2b-Mt19 isolates, 2a-Bj4 isolate, and 2b-G13 isolate from site B, respectively (Table 1). The closest relatives and phylogenetic positions of the isolated Mn^{2+}-oxidizing bacteria from Mn nodules are shown in Table 1 and Fig. 1. They were clustered with the members of Alphaproteobacteria (three strains), Betaproteobacteria (26 strains), Gammaproteobacteria (one strain), Firmicutes (one strain), and Actinobacteria (eight strains). The genus *Burkholderia* in Betaproteobacteria dominated in Mn nodules from two rice fields followed by members of

Table 1 Phylogenetic positions of Mn-oxidizing bacteria isolated from Mn nodules in rice fields

Isolates	Closest relatives (accession number)	Similarity (%)
Site A		
1a-G1 (1,377 bp)	*Methylobacterium* sp. PB176 (AB220098)	99
1a-G2 (1,377 bp)	*Mycobacterium* sp. TH-2003 (AY266138)	100
	M. fluoranthenivorans (AJ617741)	100
1a-G5 (1,401 bp), 1a-G8 (1,394 bp), 1a-Mt10 (1,369 bp)	*Burkholderia vietnamiensis* strain Ja2 (EF667353)	99
1a-G6 (1,384 bp)	*Pseudonocardia* sp. AB632 (AY974793)	99
1a-G12 (1,396 bp)	*Burkholderia gladioli* strain PA17.2 (EF193645)	100
1a-Br9 (1,364 bp)	*Lapillicoccus jejuensis* (AM398397)	99
1a-Mt11 (1,383 bp)	*Pseudonocardia* sp. AB632 (AY974793)	99
1b-G1 (1,391 bp)	Bacterium str. 96444 (AF227856)	99
	Burkholderia ferrariae strain FeGl01(DQ514537)	99
1b-G2 (1,351 bp)	*Methylobacterium* sp. CBMB19 (EF126754)	100
1b-G3 (1,400 bp)	*Burkholderia vietnamiensis* (U96929)	99
	B. cepacia strain 2Pe4 (EF178443)	99
1b-G5 (1,374 bp)	Bacterium str. 96444 (AF227856)	99
	Burkholderia ferrariae strain FeGl01 (DQ514537)	99
1b-H7 (1,394 bp), 1b-Mt8 (1,393 bp)	Burkholderiaceae bacterium KVD-1894–10 (DQ490307)	99
1b-Mt10 (1,393 bp)	*Burkholderia* sp. 45250588–5 (AY353696)	99
1b-Mt12 (1,376 bp)	*Leifsonia* sp. PTX1 (DQ901014)	99
1b-Mt13 (1,412 bp)	*Bacillus megaterium* strain PAB1C7 (EU221344)	100
	B. flexus strain L2S2 (EU221413)	100
1b-Mt14 (1,396 bp)	*Burkholderia vietnamiensis* (U96929)	100
	B. cepacia strain 2Pe4 (EF178443)	100
Site B		
2a-G1 (1,395), 2b-G1 (1,373 bp), 2b-G2 (1,374 bp), 2b-G3 (1,382 bp), 2b-G4 (1,390 bp), 2b-G10	*Burkholderia vietnamiensis* (U96929)	100
(1,327 bp), 2b-Br17 (1,389 bp), 2b-Mt19 (1,378 bp)	*B. cepacia* strain 2Pe4 (EF178443)	100
2a-G5 (1,390 bp)	*Burkholderia cepacia* strain 2Pe38 (EF178442)	100
2a-Bj4 (1,403 bp)	*Burkholderia gladioli* strain S10 (EF088208)	100
2b-G5 (1,386 bp)	*Mycobacterium* sp. K328W (DQ372732)	99
2b-G6 (1,371 bp)	*Mycobacterium* sp. K328W (DQ372732)	99
2b-G7 (1,392 bp)	*Burkholderia gladioli* strain S10 (EF088208)	99
2b-G11 (1,397 bp)	*Burkholderia vietnamiensis* strain Ja2 (EF667353)	99
2b-G12 (1,446 bp)	*Pantoea ananatis* strain BD 588 (DQ133548)	99
2b-G13 (1,386 bp)	*Burkholderia vietnamiensis* strain Ja2 (EF667353)	99
2b-G14 (1,390 bp)	*Burkholderia vietnamiensis* strain Ja2 (EF667353)	99
2b-G15 (1,397 bp)	*Burkholderia gladioli* strain PA17.2 (EF193645)	99
2b-Br16 (1,375 bp)	*Terrabacter aerolatum* strain 5516J-36 (EF212039)	98
2b-G20 (1,348 bp)	*Methylobacterium* sp. CBMB19 (EF126754)	100

Nomenclature of isolates: 1a = site A, first replicate; 1b = site A, second replicate; 2a = site B, first replicate; 2b = site B, second replicate. G = Gerretsen medium; Bj = Beijerinck medium. Example: 1a-Bj3 (site A, first replicate, Beijerinck medium, plate no. 3)

Actinobacteria. Members closely related to *Methylobacterium* were also obtained in two sites. The bacterium corresponding to *Bacillus* was only isolated at site A, whereas that corresponding to *Pantoea* was only isolated at site B (Table 1).

Phylogeny of Mn^{2+}-oxidizing fungi in Mn nodules

Seven and six fungi were isolated from site A using Beijerinck medium and Gerretsen medium, respectively, while from site B, seven and five fungi were isolated from these media. Although we isolated fungi developing different morphological colonies, seven from site A and six from site B had the same nucleotide sequences (Table 2). The closest relatives and phylogenetic positions of isolated fungi are shown in Table 2 and Fig. 2. They were members of Ascomycota belonging to Hypocreales, Pleosporales, Chaetothyriales, and Chaetothyriomycetes incertae sedis (Fig. 2), and the closest relatives of nine and six isolates from site A and site B, respectively, were *Acremonium*-like KR21–2 (AB108787), a member of the order Hypocreales (Miyata et al. 2004). *Cladosporium* strains, members of Chaetothyriomycetes incertae sedis, were also isolated as the closest relatives from both rice fields (Fig. 2). In addition,

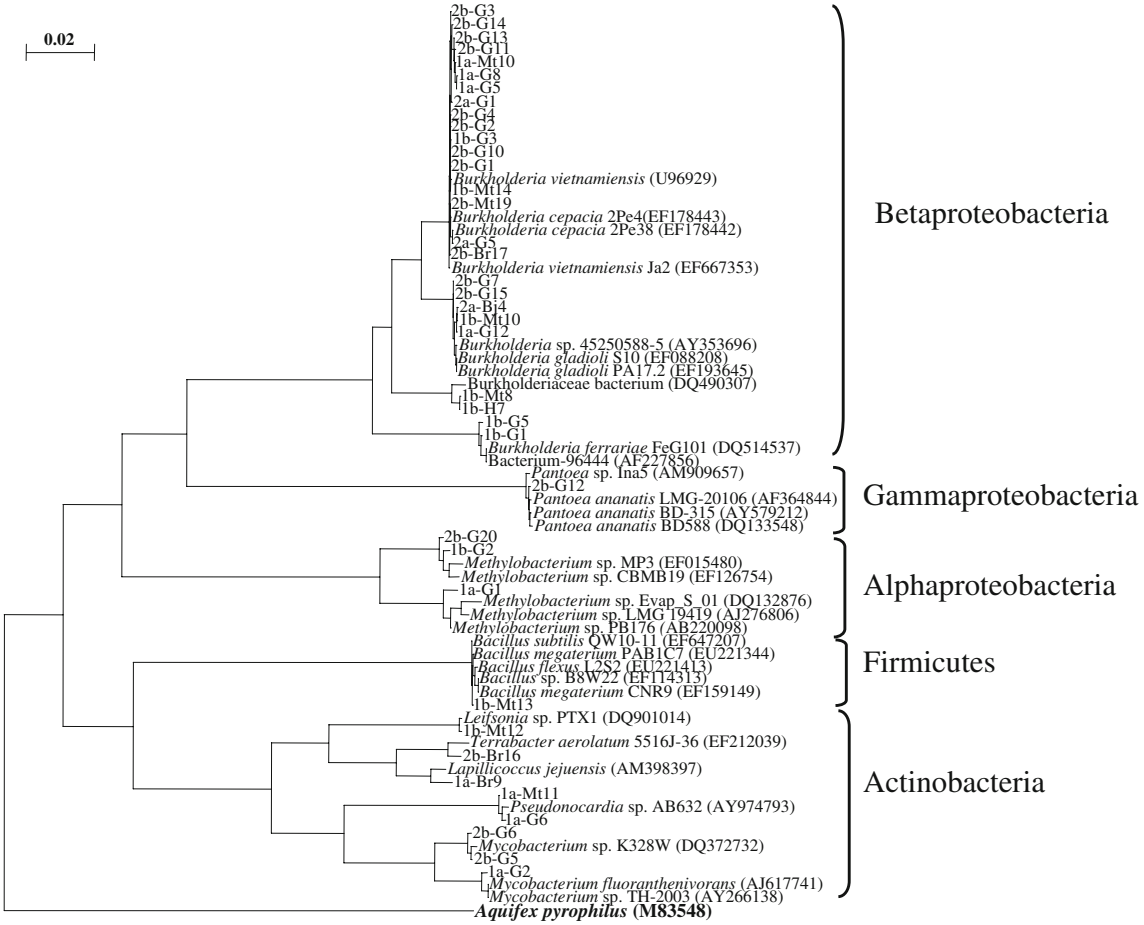

Fig. 1 The phylogenetic tree showing the relationship among the bacteria isolated from Mn nodules in rice field subsoils and their closest relatives. *Aquifex pyrophilus* was used as an outgroup bacterium

two strains of the order Pleosporales and one strain of the genus *Paecilomyces* were isolated from site A, while four strains of the order Pleosporales and one strain of the genus *Cyphellophora* were isolated from site B (Table 2).

Mn^{2+} oxidation during the growth of Mn^{2+}-oxidizing bacteria and fungi

Several types of Mn oxides were formed by Mn^{2+}-oxidizing bacteria and fungi on the Mn^{2+}-containing agar media (dark coloring of the media). There were colonies (1) with Mn oxides (Fig. 3a), (2) with dark color spreading from the colonies outward (Fig. 3b), (3) with dark color developing all over the agar plate equally and concurrently (Fig. 3c), and (4) with dark color only outside the colonies (Fig. 3d). The colonies of Mn^{2+}-oxidizing fungi could be classified as (1) those with uniform precipitation of Mn oxide (Fig. 3e), (2) those with dark color spreading from the margin toward the center of the colony (Fig. 3f), and (3) those with dark color developing all over the agar plate equally and concurrently (Fig. 3g,h). In every case, dark color appeared later after the colony development.

The type of Mn deposition depended on bacterial and fungal isolates. Different types of Mn deposition were observed by taxonomically similar bacteria and fungi. For example, 2b-G2 and 2b-G10 bacterial strains (their closest relative was *Burkholderia* sp.) showed types (3) and (4) of Mn^{2+} oxidation, respectively, whereas 1a-G13 and 1b-G7 fungal strains (the closest relative was *Acremonium* sp.) showed types (1) and (2) of Mn^{2+} oxidation, respectively. The type of Mn deposition also depended on the medium, e.g., 1a-Mt11 bacterial strain showed type (1) on Motomura medium and type (2) on Gerretsen medium, while both 1b-Bj2 and 1b-Bj5 fungal strains showed type (1) on Beijerinck medium and type (3) on Gerretsen medium (data not shown).

Discussion

Phylogeny of Mn^{2+}-oxidizing bacteria in Mn nodules

The genus *Burkholderia* was previously considered as the genus *Pseudomonas* (Palleroni 2005), which can oxidize Mn^{2+} in aquatic environments (Falamin and Pinevich 2006;

Table 2 Closest relatives of Mn-oxidizing fungi isolated from Mn nodules in rice fields

Isolates	Closest relatives (accession number)	Similarity (%)
Site A		
1a-Bj1, 1a-Bj3, 1a-G8, 1a-G13, 1b-Bj2, 1b-Bj5, 1b-G7	*Acremonium*-like hyphomycete KR21–2 (AB108787)	100
1a-Bj4	*Acremonium*-like hyphomycete KR21–2 (AB108787)	99
1a-G10, 1b-G12	*Cladosporium cladosporioides* isolate clclnf (EF114717), *C. tenuissimum* strain CBS125 (DQ780941), *Davidiella tassiana* strain ATCC 66670 (DQ780939)	100
1a-Bj5	*Ophiobolus herpotrichus* (U43453), *Leptosphaeria maculans* Unity or Thl4 (U04238), *Pyrenochaeta lycopersici* strain CBS 267.59 (DQ898288), *Coniothyrium palmarum* isolate AFTOL-ID #1379 (DQ678008), *Pleosporales* sp. GFL014 (DQ085396), *Phoma herbarum* strain ATCC 26648 (AY293775), *Neophaeosphaeria filamentosa* (AF250825)	100
1b-Bj1	*Ophiobolus herpotrichus* (U43453), *Leptosphaeria maculans* Unity or Thl4 (U04238), *Pyrenochaeta lycopersici* strain CBS 267.59 (DQ898288), *Coniothyrium palmarum* isolate AFTOL-ID #1379 (DQ678008), *Pleosporales* sp. GFL014 (DQ085396), *Phoma herbarum* strain ATCC 26648 (AY293775), *Neophaeosphaeria filamentosa* (AF250825), *Septoria nodorum* DAOM 215173 (U04236), *Phaeosphaeria avenaria* isolate AFTOL-ID 280 (AY544725), *P. obtusispora* (AF250822)	99
1a-G11	*Acremonium*-like hyphomycete KR21–2 (AB108787), *Acremonium* sp. CSSF-1 (AB167384), *Paecilomyces* sp. 080834 (DQ401104)	99
Site B		
2a-G4, 2a-G6, 2a-G7, 2a-Bj13, 2b-G1, 2b-Bj9	*Acremonium*-like hyphomycete KR21–2 (AB108787)	100
2a-G5	*Cladosporium cladosporioides* isolate clclnf (EF114717), *C. tenuissimum* strain CBS125 (DQ780941), *Davidiella tassiana* strain ATCC 66670 (DQ780939)	100
2a-Bj9, 2a-Bj10, 2b-Bj7	*Bipolaris sorokiniana* (DQ337383), *Coniothyrium minitans* strain CBS 861.71 (AY642526), *Paraconiothyrium estuarinum* strain CBS 109850 (AY642522), *Paraphaeosphaeria pilleata* (AF250821), *P. michotii* (AF250817), *Pleosporales* sp. UB32–2 (AB195634)	100
2b-Bj10	*Bipolaris sorokiniana* (DQ337383), *Coniothyrium minitans* strain CBS 861.71 (AY642526), *Paraconiothyrium estuarinum* strain CBS 109850 (AY642522), *Paraphaeosphaeria pilleata* (AF250821), *P. michotii* (AF250817), *Pleosporales* sp. UB32–2 (AB195634)	99
2a-Bj8	*Cyphellophora laciniata* (AY342010)	100

Nomenclature of isolates: 1a = site A, first replicate; 1b = site A, second replicate; 2a = site B, first replicate; 2b = site B, second replicate. G = Gerretsen medium; Bj = Beijerinck medium. Example: 1a-Bj3 (site A, first replicate, Beijerinck medium, plate no. 3)

Francis and Tebo 2001; Gregory and Staley 1982; Parikh and Chorover 2005) and soils (Bromfield and Skerman 1950; Douka 1977), including rice soil (Wada et al. 1978b). Actinobacteria were the second dominant Mn^{2+}-oxidizing bacteria in Mn nodules. Since the 16S rDNA sequence of Mn^{2+}-oxidizing Actinobacteria, exclusively *Streptomyces* spp., has not been determined in previous studies (Bromfield 1974, 1979; Bromfield and David 1976; Bromfield and Skerman 1950; Ehrlich 1963), phylogenetic relation of our Actinobacteria was unknown with Mn^{2+}-oxidizing Actinobacteria in bibliography. A *Bacillus* strain was isolated in Mn nodules only at site A. Whereas *Bacillus* strains have been isolated as Mn oxidizers in diverse environments (Francis and Tebo 2002; Gregory and Staley 1982; Northup et al. 2003; Trimble and Ehrlich 1968; Wada et al. 1978b), other *Bacillus* strains can also reduce Mn in natural environments (Gounot 1994; Nealson et al. 1988; Nealson and Ford 1980), and the role of *Bacillus* isolate on Mn nodule formation needs to be evaluated. There was no report about the involvement of *Methylobacterium* and *Pantoea* species in Mn^{2+} oxidation and reduction, although the presence of *Pantoea* in ferromanganese deposits in Lechuguilla cave was reported by Northup et al. (2003). The present study was the first to isolate *Methylobacterium* and *Pantoea* strains as Mn^{2+}-oxidizing bacteria. Thus, the majority of the Mn^{2+}-oxidizing bacteria isolated from Mn nodules belonged to the genera commonly isolated as Mn^{2+}-oxidizing bacteria (*Burkholderia*, *Streptomyces*, and *Bacillus*) in several environments. In our previous study on the determination of bacterial communities in Mn nodules by

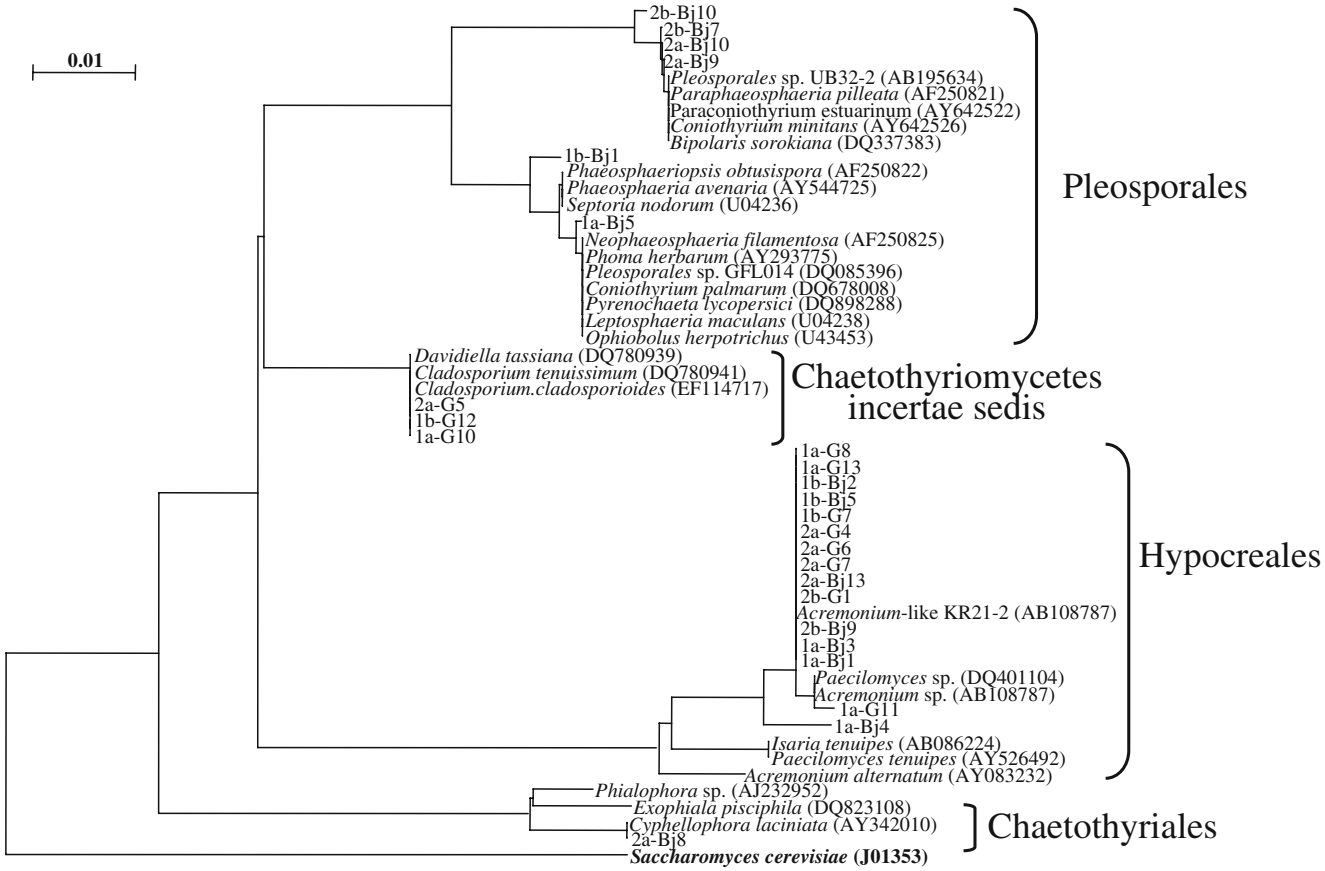

Fig. 2 The phylogenetic tree showing the relationship among the fungi isolated from Mn nodules in rice field subsoils and their closest relatives. *Saccharomyces cerevisiae* was used as an outgroup fungus

PCR-DGGE and sequencing analyses, ten and five clones were affiliated to Deltaproteobacteria and Actinobacteria, respectively, and only two clones belonged to Betaproteobacteria (Cahyani et al. 2007). However, no Mn^{2+}-oxidizing bacterium belonging to Deltaproteobacteria and Betaproteobacteria (*Burkholderia* spp.) was isolated in this study. These results indicate that Mn^{+}-oxidizing bacteria are minor members in bacterial communities of Mn nodules, even though cultivation method biased the isolation of specific bacteria selectively.

Phylogeny of Mn^{2+}-oxidizing fungi in Mn nodules

Fungi grew only on Gerretsen and Beijerinck media. In addition, Mn^{2+}-oxidizing fungi grew well and oxidized manganous carbonate to manganese oxides in Beijerinck medium, although the medium did not contain any carbon source for the growth. Fungal oxidation of manganous ion to manganese oxides in Gerretsen and Beijerinck media was reported by Beijerinck (1913) and Bromfield and Skerman (1950). We also found that *Acremonium* fungi, previously known as *Cephalosporium* (Kirk et al. 2001), were Mn^{2+}-oxidizing fungi. This genus has been isolated from various soils and natural ferric and manganic deposits on streambed pebbles (Miyata et al. 2004; Timonin et al. 1972). Manganese oxide formation by *Acremonium* strain KR-21–2 was reported to be enzymatic (Miyata et al. 2004, 2006b). The closest relative of two strains from site B, *Pleosporales* sp. UB32–2, can also produce biogenic Mn oxides enzymatically (Miyata et al. 2006a). Mn^{2+} oxidation by *Cladosporium* and *Pleospora* was reported by Bromfield and Skerman (1950), who isolated the strains from Australian soils and observed the formation of Mn oxides on solid culture media by these strains. However, the phylogenetic relation of these strains with strains of the present study is unclear because of the lack of genetic information. Timonin et al. (1972) isolated 18 species of Mn^{2+}-oxidizing fungi from Canadian soil, and two were identified as *Phoma* and *Coniothyrium*. *Phoma* strains isolated from building stone were also Mn oxidizers (De La Torre and Gomez-Alarcon 1994). Recently, *Phoma* KY-1 and *Paraconiothyrium* (*Coniothyrium*) WL-1 and WL-2 strains were isolated as Mn^{2+}-oxidizing fungi from Mn-rich sediments of a stream and inflow to an artificial wetland (Takano et al. 2006). The phylogenetic positions of these fungi were close to our isolated fungi (99% similarity of

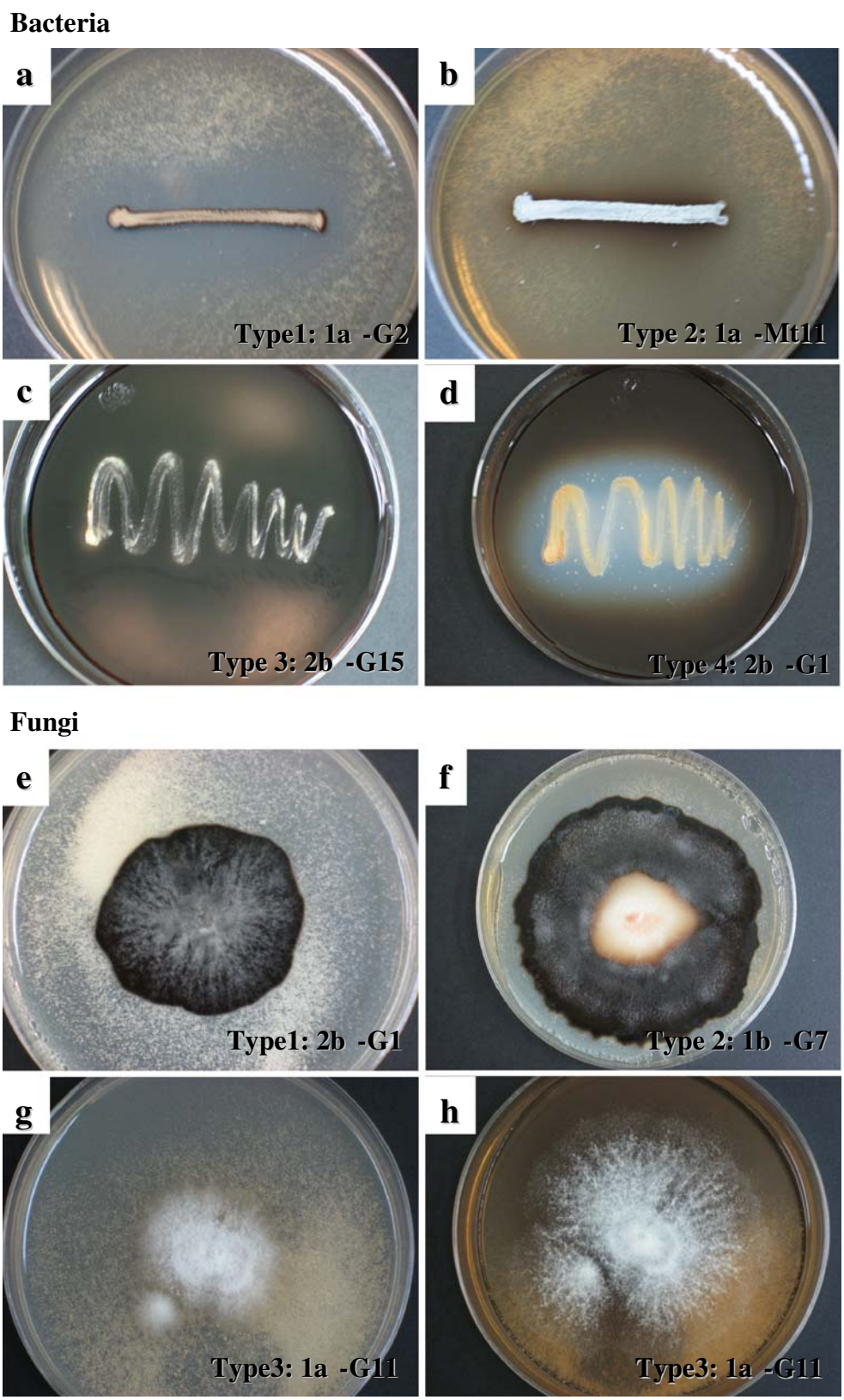

Fig. 3 Various patterns of Mn^{2+} oxidation on the Gerretsen medium. **a** Formation of Mn oxides only occurs on bacterial colony, **b** dark color from the bacterial colonies outward, **c** dark color all over the agar plate, **d** dark color only present outside bacterial colonies, **e** uniform precipitation of Mn oxide on fungal colonies, **f** dark color from the margin toward the center of fungal colonies, **g** no Mn oxide precipitation during fungal growth, and **h** dark color all over the plate in late growth phase

1a-Bj5 to KY-1 and 99% similarity of 2a-Bj9 and 2b-Bj10 to WL-1 and WL-2; Table 2). Thus, we have again shown that the majority of the fungi isolated from Mn nodules as Mn^{2+} oxidizers belonged to the genera that were commonly isolated as Mn^{2+}-oxidizing fungi in other environments.

Mn^{2+} oxidation during the growth of bacterial and fungal isolates

As already mentioned, the type of Mn deposition depended on the isolate and the medium used, which indicates that it is genetically and physiologically variable. Mn^{2+} oxidation was observed in the late phase of bacterial and fungal growth (Fig. 3), and thus, it does not provide energy for the growth of Mn^{2+}-oxidizing bacteria and fungi. This speculation is partially supported by the finding that Mn^{2+}-oxidizing bacteria were minor components of bacterial communities of Mn nodules (Cahyani et al. 2007), thus being not favored by their Mn^{2+} oxidation.

The delayed dark coloration of the media by bacterial colony formation (types 1 and 2) or fungal colony formation (type 1) during Mn^{2+} oxidation may partly support the hypothesis proposed by Wada et al. (1978a). According to this hypothesis, Mn^{2+} is oxidized by the activities of Mn^{2+}-oxidizing bacteria in the initial phase with formation of invisibly tiny dot-shaped Mn deposits, whereas Mn^{2+} is non-biologically oxidized on/around the tiny Mn deposits in the succeeding phase with the formation of large/visible Mn deposits (Mn nodules). However, this hypothesis cannot explain the uniform dark color of the medium by bacterial type 3 and fungal type 3 and the dark color only outside the colonies of bacterial type 4, since the biological formation of tiny Mn oxides outside the area of colony cannot occur. Dark color from the margin toward the center of fungal colonies (fungal type 2) was also not explained by this hypothesis. Although Mn^{2+}-oxidizing enzymes and/or pH increase around the colony could explain the Mn^{2+} oxidation of fungal type 2, unknown mechanisms of Mn nodule formation are also possible for Mn^{2+} oxidation.

Conclusions

We isolated Mn^{2+}-oxidizing bacteria and fungi from Mn nodules collected from two Japanese rice fields and determined their phylogenetic positions by their 16S and 18S rDNA partial sequences. *Burkholderia* and *Acremonium* strains were the common and dominant Mn^{2+}-oxidizing bacteria and fungi, respectively. Many Mn^{2+}-oxidizing bacteria and fungi having the identical 16S or 18S rDNA sequences were isolated between two rice fields. The majority of Mn^{2+}-oxidizing bacteria and fungi isolated from Mn nodules belonged to the genera of Mn^{2+}-oxidizers isolated from other environments. Four and three types of Mn oxide deposition were observed by Mn^{2+}-oxidizing bacteria and fungi, respectively, and some of them seemed to be due to unknown mechanism. Deposition of Mn oxides on Mn^{2+}-containing agar media by the isolated microorganisms occurred after the colony development, which indicates that the energy produced by Mn^{2+} oxidation is not used for the growth of Mn^{2+}-oxidizers. This finding partly supports the hypothesis that Mn^{2+}-oxidizers are minor members in bacterial communities inhabiting Mn nodules (Cahyani et al. 2007).

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References

Beijerinck MW (1913) Oxidation des Mangan carbonates durch Bacterien und Schimmelpilze. Folia Mikrobiol Delft 2:123–135

Bromfield SM (1956) Oxidation of manganese by soil microorganisms. Aust J Biol Sci 9:238–252

Bromfield SM (1974) Bacterial oxidation of manganeous ions as affected by organic substrate concentration and composition. Soil Biol Biochem 6:383–392 doi:10.1016/0038-0717(74)90048-0

Bromfield SM (1979) Manganous ion oxidation at pH values below 5.0 by cell-free substances from *Streptomyces* sp. cultures. Soil Biol Biochem 11:115–118 doi:10.1016/0038-0717(79)90086-5

Bromfield SM, Skerman VBD (1950) Biological oxidation of manganese in soils. Soil Sci 69:337–347 doi:10.1097/00010694-195005000-00019

Bromfield SM, David DJ (1976) Sorption and oxidation of manganous ions and reduction of manganese oxide by cell suspensions of a manganese oxidizing bacterium. Soil Biol Biochem 8:37–43 doi:10.1016/0038-0717(76)90019-5

Cahyani VR, Matsuya K, Asakawa S, Kimura M (2003) Succession and phylogenetic composition of bacterial communities responsible for the composting process of rice straw estimated by PCR-DGGE analysis. Soil Sci Plant Nutr 49:619–630

Cahyani VR, Murase J, Ishibashi E, Asakawa S, Kimura M (2007) Bacterial communities in manganese nodules in rice field subsoils: estimation by PCR-DGGE and sequencing analyses. Soil Sci Plant Nutr 53:575–584 doi:10.1111/j.1747-0765.2007.00176.x

Casamayor EO, Massana R, Benlloch S, Øvreås L, Díez B, Goddard VJ, Gasol JM, Joint I, Rodríguez-Valera F, Pedrós-Alió C (2002) Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. Environ Microbiol 4:338–348 doi:10.1046/j.1462-2920.2002.00297.x

De La Torre MA, Gomez-Alarcon G (1994) Manganese and Iron oxidation by fungi isolated from building stone. Microb Ecol 27:177–188 doi:10.1007/BF00165816

Douka CE (1977) Study of bacteria from manganese concretions, precipitation of manganese by whole cells and cell-free extracts of isolated bacteria. Soil Biol Biochem 9:89–97 doi:10.1016/0038-0717(77)90043-8

Ehrlich HL (1963) Bacteriology of manganese nodules. I. Bacterial action on manganese in nodule enrichments. Appl Environ Microbiol 11:15–19

Emerson D, Ghiorse WC (1992) Isolation, cultural maintenance, and taxonomy of sheath-forming strain of Leptothrix discophora and characterization of manganese-oxidizing activity associated with the sheath. Appl Environ Microbiol 58:4001–4010

Falamin AA, Pinevich AV (2006) Isolation and characterization of a unicellular manganese-oxidizing bacterium from a freshwater lake in Northwestern Russia. Microbiology 75:180–185 doi:10.1134/S0026261706020111

Francis CA, Tebo BM (2001) *cum*A multicopper oxidase genes from diverse Mn(II)-oxidizing and non-Mn(II)-oxidizing *Pseudomonas* strains. Appl Environ Microbiol 67:4272–4278 doi:10.1128/AEM.67.9.4272-4278.2001

Francis CA, Tebo BM (2002) Enzymatic manganese (II) oxidation by metabolically dormant spores of diverse *Bacillus* species. Appl Environ Microbiol 68:874–880 doi:10.1128/AEM.68.2.874-880.2002

Gounot AM (1994) Microbial oxidation and reduction of manganese: consequences in groundwater and applications. FEMS Microbiol Rev 14:339–350 doi:10.1111/j.1574-6976.1994.tb00108.x

Gregory E, Staley JT (1982) Widespread distribution of ability to oxidize manganese among freshwater bacteria. Appl Environ Microbiol 44:509–511

Katsoyiannis IA, Zouboulis AI (2004) Biological treatment of Mn (II) and Fe (II) containing groundwater: kinetic considerations and product characterization. Water Res 38:1922–1932 doi:10.1016/j.watres.2004.01.014

Kimura M (2000) Anaerobic microbiology in waterlogged rice fields. In: Bollag JM, Stotzky G (eds) Soil biochemistry, vol. 10. Marcel Dekker, New York, pp 35–138

Kirk PM, Cannon PF, David JC, Stalpers JA (2001) Ainsworth and Bisby's Dictionary of the fungi, 9th edition. CAB International, Wallingford

Kyuma K (2004) Paddy soil science. Kyoto University Press, Kyoto, p 280

Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, M Goodfellow (eds) Nucleic acid technology in bacterial systematics. Wiley, New York, pp 115–175

Miyata N, Tani Y, Iwahori K, Soma M (2004) Enzymatic formation of manganese oxides by an *Acremonium*-like hyphomycete fungus, strain KR21–2. FEMS Microbiol Ecol 47:101–109 doi:10.1016/S0168-6496(03)00251-4

Miyata N, Maruo K, Tani Y, Tsuno H, Seyama H, Soma M, Iwahori K (2006a) Production of biogenic manganese oxides by anamorphic Ascomycete fungi isolated from streambed pebbles. Geomicrobiol J 23:63–73 doi:10.1080/01490450500533809

Miyata N, Tani Y, Maruo K, Tsuno H, Sakata M, Iwahori K (2006b) Manganese (IV) oxide production by *Acremonium* sp. Strain KR21-2 and extracellular Mn (II) oxidase activity. Appl Environ Microbiol 72:6467–6473 doi:10.1128/AEM.00417-06

Muyzer G, Waal ECD, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Nealson KH, Ford J (1980) Surface enhancement of bacterial manganese oxidation: implications for aquatic environments. Geomicrobiol J 2:21–37

Nealson KH, Tebo BM, Rosson RA (1988) Occurrence and mechanisms of microbial oxidation of manganese. Adv Appl Microbiol 33:279–318 doi:10.1016/S0065-2164(08)70209-0

Nonaka L, Connel SR, Taylor DE (2005) 16S rRNA mutations that confer tetracycline resistance in *Helicobacter pylori* decrease drug binding in *Escherichia coli* ribosomes. J Bacteriol 187:3708–3712 doi:10.1128/JB.187.11.3708-3712.2005

Northup DE, Barns SM, Yu LE, Spilde MN, Schelble RT, Dano KE, Crossey LJ, Connolly CA, Boston PJ, Natvig DO, Dahm CN (2003) Diverse microbial communities inhabiting ferromanganese deposits in Lechuguilla and Spider Caves. Environ Microbiol 5:1071–1086 doi:10.1046/j.1462-2920.2003.00500.x

Palleroni NJ (2005) Genus Burkholderia. The Proteobacteria. Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. In: Garrity GM (ed) Bergey's manual of systematic bacteriology, 2nd edition. Springer, New York, pp 575–600

Parikh SJ, Chorover J (2005) FTIR spectroscopic study of biogenic Mn-oxide formation by *Pseudomonas putida* GB-1. Geomicrobiol J 22:207–218 doi:10.1080/01490450590947724

Poulsen H, Nilsson J, Damgaard CK, Egebjerg J, Kjems J (2001) CRM1 mediates the export of ADAR1 through a nuclear export signal within the Z-DNA binding domain. Mol Cell Biol 21:7862–7871 doi:10.1128/MCB.21.22.7862-7871.2001

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Smit E, Leeflang P, Glandorf B, Van Elsas JD, Wernars K (1999) Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl Environ Microbiol 65:2614–2621

Stein LY, La Duc MT, Grundl TJ, Nealson KH (2001) Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. Environ Microbiol 3:10–18 doi:10.1046/j.1462-2920.2001.00154.x

Takano K, Itoh Y, Ogino T, Kurosawa K, Sasaki K (2006) Phylogenetic analysis of manganese-oxidizing fungi isolated from manganese-rich aquatic environments in Hokkaido, Japan. Limnology 7:219–223 doi:10.1007/s10201-006-0177-x

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882 doi:10.1093/nar/25.24.4876

Thompson AI, Huber DM, Guest CA, Schulze DG (2005) Fungal manganese oxidation in a reduced soil. Environ Microbiol 7:1480–1487 doi:10.1111/j.1462-2920.2005.00842.x

Timonin MI, Illman WI, Hartgerink T (1972) Oxidation of manganous salts of manganese by soil fungi. Can J Microbiol 18:793–799

Trimble RB, Ehrlich HL (1968) Bacteriology of manganese nodules. III. Reduction of MnO_2 by two strains of nodule bacteria. Appl Microbiol 16:695–702

Wada H, Seirayosakol A, Kimura M, Takai Y (1978a) The process of manganese deposition in paddy soils. I. A hyphothesis and its verification. Soil Sci Plant Nutr 24:55–62

Wada H, Seirayosakol A, Kimura M, Takai Y (1978b) The process of manganese deposition in paddy soils. II. The microorganisms responsible for manganese deposition. Soil Sci Plant Nutr 24:319–325