

# Microbial DNA extraction and analyses of soil iron–manganese nodules

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## Abstract

Iron–manganese (Fe–Mn) nodules and concretions are soil new growth, reflecting soil environmental conditions during their formation. Bacteria play a dominant role in the oxidation of dissolved Mn(II) in aqueous systems and the formation of marine and freshwater Fe–Mn nodules. However, the role and significance of bacteria in soil Fe–Mn nodule formation have not been well recognized. In this paper, microbial DNA was directly extracted from two Fe–Mn nodule samples collected from Wuhan and Guiyang in central China. The extracted DNA was amplified by polymerase chain reaction (PCR) and cloned. The clones were then screened by amplified ribosomal DNA restriction analysis (ARDRA). Twenty patterns were obtained for Wuhan sample and Guiyang sample, respectively. DNA sequencing and phylogenetic analyses revealed that the bacterial compositions of the Fe–Mn nodules were mainly belonged to Firmicutes,  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria branches of the domain bacteria. These divisions had close relativeness with Mn(II)-oxidizing bacteria identified from marine Fe–Mn nodules, implying the possible contributions of these bacteria to soil Fe–Mn nodule formation.

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## 1. Introduction

Fe and Mn oxides (including hydroxides and oxyhydroxides), widespread in soils and sediments, play a critical role in the fate and transport of heavy metals and organic pollutants through sorptive, catalytic and oxidative processes. The formation of Fe–Mn nodules and concretions in soil is regarded as the result of drying–wetting alternations of soil moisture and the corresponding oxidation and reduction cycles (Burns and Burns, 1975). Fe and Mn oxides release Fe(II) and Mn(II) ions into the soil solution under the reducing conditions, and the Fe(II) and Mn(II) are oxidized into Fe(III) and Mn(III, IV) and precipitated when the soil is dried out (McKenzie, 1989). Manganese is more mobile than Fe and requires a higher oxidation potential than Fe(II), resulting in the formation of alternate layers of Fe-rich and Mn-rich materials

observed in many nodules (White and Dixon, 1996). The elemental composition and distribution characteristics within the nodules may reflect their forming redox history of the pedoenvironment (McKenzie, 1989; Liu et al., 2002).

Microorganisms, especially bacteria, are known to catalyze the oxidation of Mn(II) and the formation of Mn(III, IV) oxide minerals. Biological Mn(II) oxidation is generally much faster (by up to  $10^5$  times) than abiotic Mn(II) oxidation processes, suggesting that biological Mn(II) oxidation dominates in the environment (Kim et al., 2003; Tebo et al., 2004). A number of investigations at specific field sites have shown that the biological processes are responsible for Mn(II) oxidation at those locations (e.g., Tebo and Emerson, 1985, 1986; Cowen et al., 1986, 1990; Tebo, 1991; Wehrli et al., 1995; Harvey and Fuller, 1998; van Cappellen et al., 1998; Fuller and Harvey, 2000; Kay et al., 2001). For these reasons, the majority of naturally occurring environmental Mn oxides are believed to be derived either directly from biogenic Mn(II) oxidation processes or from the subsequent

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alteration of the biogenic oxides (Tebo et al., 2004). Significant advances have been made in the last 10 years in the molecular biology of Mn(II) oxidation by three phylogenetically distinct bacteria representatives of different aqueous environmental settings: a marine *Bacillus* sp. strain SG-1; *Leptothrix discophora* strains SS-1 and SP-6, common in wetlands and in iron seeps and springs; and *Pseudomonas putida* strain MnB1, representative of freshwaters (Tebo et al., 1997). However, very little information is available about biological Mn oxidation in the soil environment. Douka (1977) isolated two Mn(II) oxidizing bacteria from manganese concretions of an alfisol soil of West Peloponnese in Greece. The bacteria were identified as *Pseudomonas* sp. nov. and *Citrobacter freundii* and their cell and the cell-free extracts could catalyze the formation of Mn precipitates. Sullivan and Koppi (1992) observed cell-like substances on the surface of manganese oxide coatings of a black earth (Typic Pellustert) in Australia using light microscopy and electron microscopy, suggesting microbial oxidation of Mn(II) contributed to the formation of the manganese oxide coatings in this soil.

In the present study, we improved soil DNA extraction methods and successfully extracted microbial DNA from soil Fe–Mn nodules. The extracted DNA was polymerase chain reaction (PCR) amplified and cloned. The clones were then screened and sequenced. Phylogenetic analyses revealed that the bacterial compositions of the Fe–Mn nodules were mostly belonged to Firmicutes,  $\beta$ -proteobacteria,  $\gamma$ -proteobacteria branches of the domain bacteria. Some of them had close relativeness with Mn(II)-oxidizing bacteria identified from the marine Fe–Mn nodules, implying the possible contributions of these bacteria to the formation of soil Fe–Mn nodules.

## 2. Materials and methods

### 2.1. Sample collection

Fe–Mn nodules were collected from two different locations of Wuhan, Hubei Province and Guiyang, Hunan Province in central China. Wuhan sample (WH) was collected at a 40-cm depth from the subsoil horizon of a subacid orthic agrudalf developed from Quaternary siliceous and alluvial sediments, and Guiyang sample (GY) was collected at 20–100 cm depth of an alt-udic ferrisol developed from rammell. Nodules were separated from the soil by wet sieving and stored at room temperature. The morphological properties, mineralogy, and chemical composition of the nodules have reported previously (Tan et al., 2000; Liu et al., 2002).

### 2.2. DNA extraction from the Fe–Mn nodules

Before DNA extraction, 50 g of each sample was surface sterilized by rinsing in sterile distilled water for five times, immersing in 0.1% NaClO for 1 min, and then followed by

five rinses in sterile distilled water. The nodules were ground to powder using a pestle and a mortar under asepsis condition. The control powder was autoclaved at 121 °C for 30 min twice and dried at 100 °C for 6 h. DNA extraction was carried out with a combination of physical bead beating, chemical and biological lyses as described by Zhang et al. (2005) and He et al. (2005). Fe–Mn nodule powder was suspended in 143 ml extraction buffer (200 mM NaCl, 200 mM Tris, 2 mM sodium citrate, 10 mM CaCl<sub>2</sub>, 50 mM EDTA, adjusted to pH 8.0), and then 1 ml poly(A) (10 mg ml<sup>-1</sup>) and 4.5 ml 10% pyrophosphate were added. The suspensions were treated with bead beater (Biospec Products, Bartlesville, OK) for 3 min in an ice bath after mixing with 40 g of 1-mm-diameter silica beads and 5 g of 0.1-mm-diameter glass beads. They were incubated at 37 °C for 2 h after 2 ml lysozyme (50 mg ml<sup>-1</sup>) was added, and then 1 ml protease K (20 mg ml<sup>-1</sup>) and 10 ml 20% SDS were added into. Subsequently, the suspensions were shaken at 250 rpm for 1 h at 37 °C and then incubated in a 65 °C water bath for 1 h with end-over-end inversions every 10 min and centrifuged at 6000g for 15 min. The supernatants were extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, vol:vol:vol) and precipitated by adding 0.1 volume of 3 mol l<sup>-1</sup> sodium acetate (pH 5.2) and equal volume of cold isopropanol, washed with 75% ethanol, and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The resuspended solution was re-extracted with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitated with ethanol again (He et al., 2005). The control sample was subjected to the same DNA extraction procedures.

### 2.3. PCR amplification, cloning, and amplified ribosomal DNA restriction analysis (ARDRA)

For amplification of 16S rRNA gene of bacteria, the primers 27F (5'-AGA GTT TGA TCM TGG CTC AG) and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T) (Lane, 1991) were used in a standard 35-cycles PCR with an annealing temperature of 50 °C. The DNA extracts were 10-fold diluted and used as a template. The 50- $\mu$ l reaction mixtures contained 1  $\times$  PCR buffer, 400 nM each primer, 2 mM MgCl<sub>2</sub>, 250  $\mu$ M each dNTP, 2.5 U *Taq* DNA polymerase, 1  $\mu$ l of 20 mg ml<sup>-1</sup> bovine serum albumin (BSA), and 2  $\mu$ l of DNA template. The amplified 16S rRNA gene fragments were ligated into the pGEM-T Easy Vector (Promega, Madison, WI), and the resulting ligation products were used for transforming into *E. coli* JM109 competent cells following the instructions of the manufacturer. 16S rRNA gene libraries were constructed and 40 randomly chosen colonies per sample were PCR re-amplified using the primers of T7 and SP6. The amplicons were analyzed by restriction digestion with *Hae* III, *Rsa* I, and *Hha* I (New England Biolabs). The digested DNA fragments were run in 2% agarose gel electrophoresis. The ARDRA patterns were grouped by similarity and 1 or 2 representative clones from each group were sequenced.

#### 2.4. Sequence analysis and GenBank accession numbers

Double-stranded DNA sequencing was performed by Applied Biosystems 3730 automated sequencers with the primers T7 and SP6 to obtain nearly full-length (about 1500 bp) sequences of bacterial 16S rRNA gene. The bidirectional gene sequences were compiled using DNASTar software 5.0 (DNASTar Inc., USA) and edited and aligned using Bio-Edit (Hall, 1999). Operational taxonomic units (OTUs) were defined as clones that shared 97% or greater sequence similarity. The sequences were then analyzed with the NCBI Blast program and RDP Chimera Check program. The most similar GenBank sequences to the clones were extracted from the GenBank for including in the phylogenetic tree construction. Phylogenetic analyzes were conducted using MEGA version 3.0 (Kumar et al., 2004) and a neighbor-joining (NJ) tree was constructed using Kimura 2-parameter distance with 1000 replicates to produce Bootstrap values.

The sequences determined in this study were submitted to Genbank and assigned accession numbers from DQ351907 to DQ351929 and from DQ537525 to DQ537535.

### 3. Results

#### 3.1. DNA extraction and PCR amplification

DNA extracts were run in 1% agarose gel electrophoresis but resulted in no detectable DNA band even if

50 g samples were used, indicating very small amount of extractable DNA in the soil Fe–Mn nodules. The 10-fold diluted DNA extracts were successfully amplified with the primers 27F and 1492R and the products produced strong and sharp bands at about 1500 bp on the subsequent agarose gel electrophoresis. No PCR product was detected from the diluted or undiluted control DNA template, and neither from undiluted DNA extracts.

#### 3.2. ARDRA of 16s rDNA clones

Each 40 clones randomly chosen from WH and GY clone libraries were re-amplified and digested with *Hae* III. The clones from WH and GY sample were grouped to 15 and 16 ARDRA patterns, respectively. Most of patterns contained 2–4 clones and partial patterns contained 1 or 6 clones. Fig. 1A shows the different restriction digestion patterns of WH clones with *Hae* III. Two or more clones that shared the same *Hae* III ARDRA pattern were digested further with *Rsa* I and *Hha* I, and were re-grouped to different ARDRA patterns (Fig. 1B). Similar banding patterns obtained after combination of the three independent digestions were grouped to finally obtain 20 groups for each sample. There were no significant differences in the ARDRA patterns between the WH and GY samples.

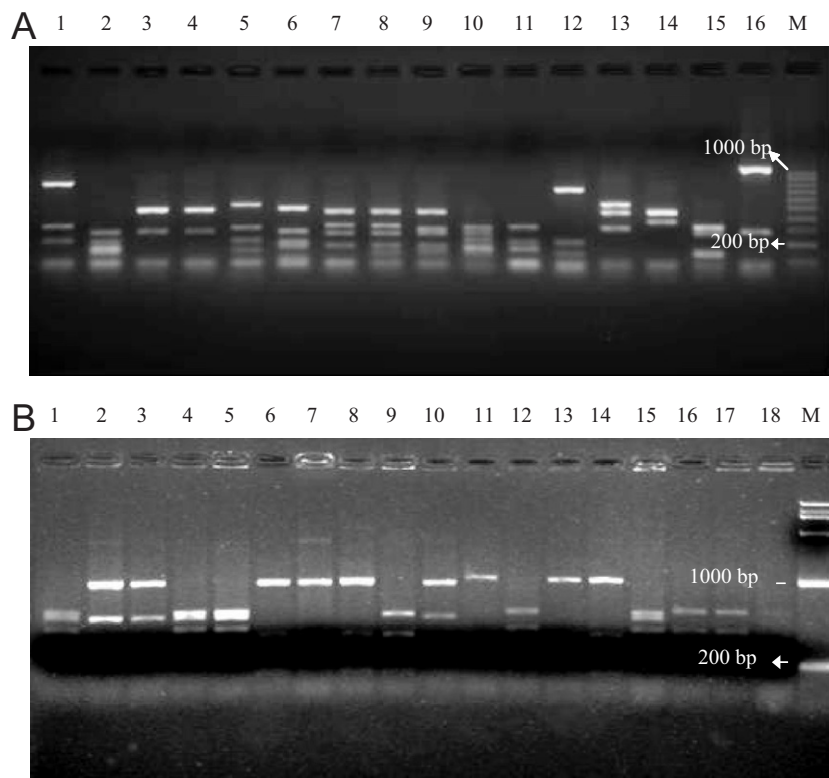


Fig. 1. Restriction patterns of 16S rDNA of clones digested with *Hae* III and *Rsa* I from the Wuhan Fe–Mn nodule sample. A 1–16: Different digestion patterns of WH clones with *Hae* III. B 1–19: Clones with the same *Hae* III ARDRA pattern were further digested to different patterns with *Rsa* I; 1–4, 5–6, 7–9, 10–11, 12–17 represented the same *Hae* III ARDRA pattern, respectively. M: DNA marker.

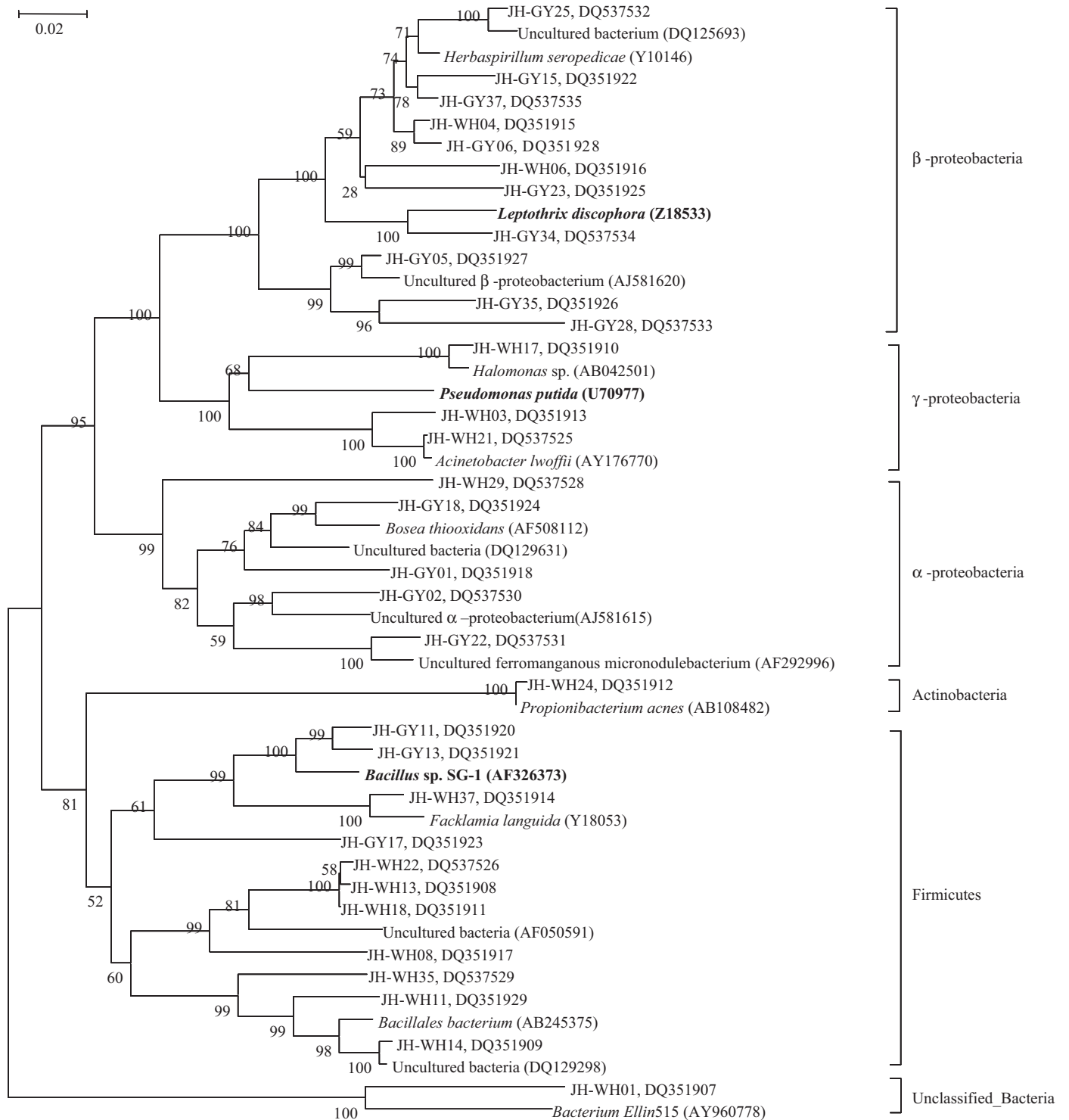


Fig. 2. Phylogenetic tree of 16S rDNA clones from the Fe–Mn nodules and their most similar GenBank sequences. Numbers at branches are bootstrap values of 1000 replications. Phylum or class names of different groups of clones are assigned based on the relationships of the clones to the known GenBank species and the NCBI Bacterial Taxonomy classification. JH-WH denotes clones of the Wuhan sample and JH-GY of the Guiyang sample. The accession number is followed after each clone. Sequences in boldface are the model Mn-oxidizing bacteria from marine Fe–Mn nodules.

### 3.3. Phylogeny of bacterial 16S rDNA clones in the Fe–Mn nodules

Each 20 clones representative of different restriction pattern from WH and GY sample were sequenced and

assigned to 16 OTUs, respectively. A total of 32 unique OTUs were recovered from the 2 samples. The NJ tree of the clone sequences is shown in Fig. 2 and their taxonomic assignments are listed in Table 1. Among the 32 clones obtained in this study, each 11 clones were grouped into

Table 1  
Distribution of bacterial rRNA gene clone types representing various phylogenetic groups in two Fe–Mn nodule samples collected from Wuhan and Guiyang of central China

Sample	Firmicutes	$\beta$ -Proteobacteria	$\gamma$ -Proteobacteria	$\alpha$ -Proteobacteria	Actinobacteria	Unclassified_bacteria
Model bacteria	<i>Bacillus</i> sp. SG-1	<i>Leptothrix discophora</i>	<i>Pseudomonas putida</i>			
Wuhan	8	2	3	1	1	1
Guiyang	3	9	0	4	0	0
Total	11	11	3	5	1	1

Firmicutes and  $\beta$ -proteobacteria respectively, three clones into  $\gamma$ -proteobacteria and five clones into  $\alpha$ -proteobacteria, each one clones into Actinobacteria and unclassified bacterium respectively.

For WH sample, 8 sequences (DQ351908, DQ351909, DQ351911, DQ351914, DQ351917, DQ351929, DQ537526, DQ537529) fell into Firmicutes, 2 sequences (DQ351915, DQ351916) into  $\beta$ -proteobacteria, 1 sequence (DQ537528) into  $\alpha$ -proteobacteria, 1 sequence (DQ351912) into Actinobacteria, 1 sequence (DQ351907) into unclassified bacterium. 3 sequences (DQ351910, DQ351913, DQ537525) were grouped into  $\gamma$ -proteobacteria and were most closely related to *Acinetobacter* sp. and *Halomonas* sp.. For GY sample, 9 sequences (DQ351922, DQ351925, DQ351926, DQ351927, DQ351928, DQ537532, DQ537533, DQ537534, DQ537535) fell into  $\beta$ -proteobacteria, 3 sequences (DQ351920, DQ351921, DQ351923) into Firmicutes, 4 sequences (DQ351918, DQ351924, DQ537530, DQ537531) into  $\alpha$ -proteobacteria. Three sequences (DQ351920, DQ351921 and DQ537534) were closely related to *Bacillus* sp. SG-1 and *Leptothrix discophora*, the model Mn(II)-oxidizing bacteria from aquatic environment. Comparatively, WH sample had more Firmicutes but less  $\beta$ -proteobacteria clones than that of GY sample.

#### 4. Discussion

In our preliminary experiments, SDS-lysis method for soil DNA isolation developed by Zhou et al. (1996) were used to extract DNA from the Fe–Mn nodules, but DNA extracts could not be successfully amplified under the same PCR conditions. Fe(III) and Mn(IV) were present in Fe–Mn nodules with high concentration (Tan et al., 2000). We observed some color changes during the DNA extraction process, possibly indicating intense chemical oxidation reaction during the DNA extraction. Bias in DNA recovery may be resulted from inefficient of cell lysis especially in the spore-producing Gram-positive cells, adsorption of DNA to sample particles, and chelation of DNA by metals (He et al., 2005). In the present study, a joint method combined with physical (bead-beating), chemical (SDS-lysis) and enzymatic (lysozyme and protease K) was used to DNA recovery from the nodules, which was demonstrated to be effective to break the cell. Moreover, poly(A), with similar chemical characteristics of DNA, could compete the adsorption sites on the surface of sample particles with DNA so that the adsorption of DNA

to particle can be reduced. The efficient cell lysis and the desorption of poly(A) make it possible to recover the trace DNA from the Fe–Mn nodules. Zhang et al. (2005) recovered DNA successfully from rock samples with the similar method. Because the Fe–Mn nodules may contain very small amount of DNA, a further purification step would reduce the amount of DNA. Diluting template sample could abate interference of those substances in the PCR instead of further purification.

Manganese oxidization bacteria are ubiquitous in a wide range of environments and phylogenetically diverse, which mainly belonged to low-GC Gram-positive bacteria (Firmicutes),  $\beta$ -proteobacteria and  $\gamma$ -proteobacteria branches of the domain bacteria based on the 16S rRNA sequences (Nealson et al., 1988; Tebo et al., 1984, 2004; Templeton et al., 2005). Phylogenetic analyses of 16S rDNA sequences from Fe–Mn nodule from WH and GY samples indicated that there were various bacteria inhabiting in the two soil Fe–Mn nodules and the presence of possible Mn(II)-oxidizing bacteria could be originated from a diverse community. Three (DQ351913, DQ537525 and DQ351910) of the 16 clones from WH sample were affiliated with  $\gamma$ -proteobacteria. DQ351913 and DQ537525 shared the high similarity (95% and 99%) with *A. lwoffii*, and DQ351910 was 98% similar with *Halomonas* sp.. Isolates marine Fe–Mn nodules originally in the two genera have been demonstrated bearing high Mn(II)-oxidizing activity (Templeton et al., 2005). Possible evidence for Mn(II)-oxidizing bacteria existing in the nodules also comes from clones (DQ351920, DQ351921, DQ537534 and DQ537531) of the GY sample. DQ351920 and DQ351921 showed 94% and 92% similarity with *Bacillus* sp. SG-1, respectively, and DQ537534 was 92% similar to *L. discophora*. The two bacteria, as the model Mn(II)-oxidizing bacteria belong to Firmicutes and  $\beta$ -proteobacteria lineages, have been well studied (Tebo et al., 2004). The clone DQ537531 in  $\alpha$ -proteobacteria lineage was 92% similar to an uncultured bacteria clone from Green Bay ferromanganous micronodule (Stein et al., 2001). The high sequence similarity reveals the presence of some organisms related to the known Mn(II)-oxidizing bacteria from aquatic environment and they may play significant role in the formation of soil Fe–Mn nodules. The species, their physiological function of Mn(II) oxidation, and possible mechanism involving in the biological catalyzing of Mn(II) oxidation, need further

investigation. Moreover, the comparative study of the microbial communities of Fe–Mn nodules and the surrounding soil samples may shed light on the evolution of microbial communities, because a Fe–Mn nodule is a relatively separated niche and may represent the soil microbial communities at its forming stages.

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