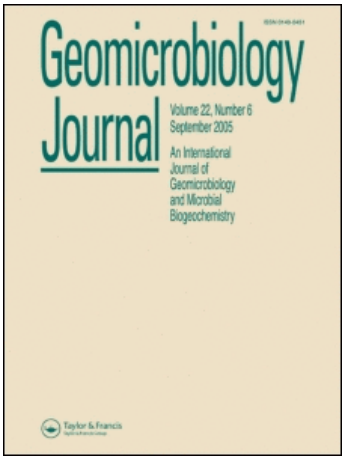

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Bacterial Communities Inside and Surrounding Soil Iron-Manganese Nodules

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Bacterial Communities Inside and Surrounding Soil Iron-Manganese Nodules

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Bacterial community structures of a Fe-Mn nodule sample and its surrounding soil were investigated using PCR, amplified ribosomal DNA restriction analysis, cloning and sequencing methods. Result showed that phylogenetically diverse bacteria were present in the nodule and soil samples, and *Acidobacteria*- and *Proteobacteria*-affiliated bacteria dominated in both samples. However, *Firmicutes* were only found in the nodules, while the soil had much more *Acidobacteria* and *Verrucomicrobia* than the nodules. Many clones retrieved in this study closely resembled the clones previously obtained from environments with high metal contents. These findings may shed light on the biological formation of Mn oxides in soil environment.

Keywords Soil Fe-Mn nodule, bacterial community, 16S rRNA gene clone library, ARDRA, biogenic Mn oxidization

INTRODUCTION

Iron (Fe) and manganese (Mn) oxides (including hydroxides and oxyhydroxides), ubiquitous in soils and sediments, play a key role in the biogeochemical cycles of metals and organic carbon while influencing significantly the transport and fate of both contaminants and nutrients in the environments through sorptive, catalytic and oxidative processes (Tebo et al. 2004). The formation of Fe-Mn nodules in soil is thought to be the result of drying-wetting alternations of soils and the corresponding oxidation and reduction cycles (Burns and Burns 1975). Under reducing conditions, Fe and Mn oxides could release Fe (II) and Mn (II) ions into the soil solution; and when the soil dries, Fe (II) and Mn (II) are oxidized and precipitated, forming new metal oxides.

Increasing evidence is showing that microorganisms, especially bacteria, play a dominant role in the oxidation of dissolved Mn (II) in natural aqueous systems (Nealson et al. 1988; Tebo et al. 1997, 2004). Bacterially mediated (biological) Mn (II) oxidation is generally much faster than abiotic Mn (II) oxidation processes (by up to 10^5 times), 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 (Tebo and Emerson, 1985; Cowen et al. 1986; Wehrli et al. 1995; Harvey and Fuller, 1998; van Cappellen et al. 1998; Kay et al. 2001). Hence, 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 transformation of the biogenic oxides (Tebo et al. 2004). To date, many phylogenetically diverse Mn(II)-oxidizing bacteria (MnOB) have been described and three model MnOB, representing different aqueous environmental settings, have been studied extensively using molecular biological techniques: 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).

The biochemical and underlying genetic characteristics of these three model MnOBs have been elucidated (Brouwers et al. 1999; Francis et al. 2001; Tebo et al. 2004). Analysis of the 16S rRNA clone libraries derived from the environmental sites rich in Mn oxides and from Mn(II)-oxidizing enrichment cultures, providing indirect evidence of MnOB, has been used to examine the microbial community associated with Lechuguilla and Spider Caves (Northup et al. 2003; Spilde et al. 2005). However, very little information is available on biological Mn oxidation in the soil environment. Douka (1977) isolated 2 MnOB from manganese concretions of an alfisol 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

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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 the soil.

Fe-Mn nodules represent a relatively isolated niche in the soil environment and may reflect the soil microbial community composition when the nodules were formed. One aim of this study was to examine the differences in microbial community structure and diversity between the Fe-Mn nodules and the surrounding soil. Another aim was to understand the possible metabolic characteristics and ecological role of those microbes inhabiting the nodules.

MATERIALS AND METHODS

Sample Collection

The sampling site was located at Wuhan, Hubei Province, Central China. Fe-Mn nodules and surrounding soil were collected at 20–40 cm depth of a subacid orthic agrudalf developed from Quaternary siliceous and alluvial sediments. Samples from 5 points (500 g each) were mixed thoroughly and nodules (5–9 mm in diameter) were picked up by autoclaved tweezers and the soils attached to the nodules were separated from the nodules. Soil and nodules were transported to laboratory on ice, stored at 4°C and used for DNA extraction in 1 week. The morphological properties, mineralogy, and chemical composition of the nodules and soil have been reported previously (Liu et al. 2002; Tan et al. 2006). The soil pH was 5.9, and organic matter, clay, Fe and Mn contents were 38, 405, 63 and 0.9 g kg^{-1}, respectively; while the nodule pH was 7.8, and Fe and Mn contents were 79 and 44 g kg^{-1}, respectively.

DNA Extraction from the Fe-Mn Nodules and Soil

Before DNA was extracted, the nodules were intensively washed with distilled water until the soil particles were removed and the soil sample was mixed thoroughly. Nodules were surface sterilized with a 0.1% NaClO solution for one minute and rinsed with sterile distilled water, and then ground to powder using a pestle and a mortar under aseptic conditions. DNA extraction was carried out with a combination of physical (bead beating), chemical and biological lyses as described by Zhang et al. (2005, 2007). Briefly, 50 g Fe-Mn nodule powder was suspended in 143 ml extraction buffer (200 mM NaCl, 200 mM Tris, 2 mM sodium citrate, 10 mM $CaCl_2$, 50 mM EDTA, pH 8.0), and treated using a Bead Beater (Biospec Products, Bartlesville, OK) for 3 min in a solution containing 1 ml poly(A) (10 mg ml^{-1}), 4.5 ml 10% pyrophosphate, 1 mm-diameter silica beads and 0.1 mm-diameter glass beads.

The suspension was then treated with lysozyme, protease K and SDS. The extracted solution was purified with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated again with ethanol (He et al. 2005). A 10 g subsample of soil was subjected to the same DNA extraction procedures with the exception of surface sterilization. The nodule powder and soil were autoclaved twice at 121°C for 30 min and included as controls. The extracts were run on 1% agarose gels and the DNA concentration was determined using a Nanodrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies).

Polymerase Chain Reaction (PCR) and Cloning

Bacterial 16S rRNA genes were PCR-amplified using bacterial-specific primers 27F (5′-AGA GTT TGA TCM TGG CTC AG) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T) (Lane 1991). PCR amplification was carried out in a standard 35-cycle PCR program with an annealing temperature of 50°C. The final volume of the reaction mixtures was 50 μl, containing 1 × PCR buffer, 2 mM $MgCl_2$, 400 nM each primer, 250 μM each dNTP, 2.5 U *Taq* DNA polymerase, 0.4 μg μl^{-1} bovine serum albumin (BSA) and 2 μl of 10-fold diluted DNA extract. The PCR products were gel-purified with a TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa Bio Inc., Shiga, Japan) and ligated into the pGEM-T Easy Vector (Promega, Madison, USA), and the resulting ligation products were transformed into *E. coli* JM109 competent cells following the manufacturer's instructions.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

From each of the soil and nodule clone libraries, 150 clones were chosen randomly and re-amplified with the vector-specific primers T7 and SP6. The amplicons were digested with 5 U of restriction endonucleases Hae III (New England Biolabs, Canada) in a 20-μl-reaction mixture for 1 h at 37°C. Digested DNA fragments were separated by electrophoresis on a 2% agarose gel and imaged using a GBOX-HR Gel Documentation System (Syngene, UK) after ethidium bromide staining. The ARDRA patterns were grouped and then the clones with similar patterns were digested further and regrouped with Rsa I and Hha I (New England Biolabs). One clone representing each group was sequenced.

Sequencing and Phylogenetic Analysis

Double-stranded DNA sequencing was performed using an Applied Biosystems 3730 automated sequencer with the primers T7 and SP6 to obtain nearly full-length (about 1500 bp) sequences of bacterial 16S rRNA genes. The bidirectional gene sequences were compiled using DNAStar software 5.0 (DNA–star Inc, USA) and edited using Bio-Edit (Hall 1999). The sequences were then analyzed with the Chimera Check program of the RDP database (Cole et al. 2007) to exclude chimeric artifacts and searched in the NCBI GenBank database using the BLAST program. The GenBank sequences most similar to our clone sequences were included in the phylogenetic tree construction. A sequence with less than 2% dissimilarity to the adjacent sequence was defined as an operational taxonomic unit (OTU). Phylogenetic analyses 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 1,000 replicates to produce Bootstrap values.

The sequences determined in this study were deposited in the GenBank database and assigned accession numbers from DQ351907 to DQ351929, DQ537525 to DQ537535, and from EF492887 to EF492982.

RESULTS

DNA Extraction from the Fe-Mn Nodule and Soil Samples and PCR Amplification

The concentrations of DNA extracted from the soil and Fe-Mn nodules were 262 ng μl^{-1} and 5 ng μl^{-1} with A260/A280 ratios of 1.73 and 1.50, respectively. The extracted DNA size of the soil sample was about 20 kb and formed a sharp band on the 1% agarose gel, but no detectable DNA band was observed for the nodule and the two control samples, indicating much lower DNA yield in the Fe-Mn nodules than in the surrounding soil. PCR amplification with the 16S rRNA genes obtained products of about 1500 bp. No PCR product was detected from the control samples.

Screening of 16S rRNA Gene Clones by ARDRA

Amplified ribosomal DNA restriction analysis (ARDRA) was performed on each of the 150 clones randomly chosen from the soil and the nodule clone libraries and about 70 groups for each clone library were obtained. Most of the groups were represented by a single clone, with some groups containing 2-3 clones. One clone representing each group was sequenced. After removing the sequences with 98% similarity, nearly full-length 16S rRNA gene sequences from the soil clones and the nodule clones were assigned to 57 and 54 OTUs, respectively.

Phylogenetic Profiles and Taxonomic Distribution of the Bacterial Community in the Fe-Mn Nodules and the Surrounding Soil

The phylogenetic trees of the clone sequences and their most similar GenBank sequences are shown in Figures 1 and 2. All of the 111 clone sequences clustered into 10 groups (phyla or classes). Forty-one sequences were affiliated with the phylum *Acidobacteria*, and further clustered into 7 subdivisions, groups 1, 2, 3, 4, 5, 6, and 7 according to the classification proposed by Hugenholtz et al. (1998) (Figure 1). The majority of the sequences were affiliated with group 1 and group 3 and were present in both the nodule and soil samples, while group 2, 5 and 7 sequences were found only in the soil. Twenty-eight sequences affiliated with the *Proteobacteria* were grouped into α-, β-, γ-, and δ-*Proteobacteria*. β-*Proteobacteria* were the dominant component of both soil and nodule samples and accounted for 53.6% of *Proteobacteria* sequences, followed by γ-*Proteobacteria* (21.4%). The other 42 sequences clustered with the *Firmicutes*, *Nitrospira*, Unclassified-bacteria, *Actinobacteria*, *Gemmatimonadetes*, *Verrucomicrobia*, *Planctomycetes* and *Bacteroidetes*.

The distribution of the taxonomic groups differed between the soil and the nodule sample (Table 1). The *Acidobacteria* group constituted 45.6% of the clone sequences from the soil sample, but only 27.8% from the nodule sample. *Proteobacteria* accounted for 21.0% and 29.7% of the sequences from the soil and the nodule sample, respectively. *Firmicutes* constituted 18.5% of the nodule sample clones but were not detected in the soil clone library. *Verrucomicrobia* sequences represented 14.0% of the soil sample clones and 7.4% of the nodule sample clones.

To characterize the microbial communities in the Fe-Mn nodules and the surrounding soil, we examined the similarity of the sequences to the sequences of previously cultured bacteria or environmental clones. Results showed that 26% of the soil sample sequences and 32% of the nodule sequences retrieved in this study closely resembled (>95% sequence similarity) those uncultured clones from environments with high levels of uranium (U), Mn and Fe elements, and known MnOB from aquatic environments. These sequences were mainly distributed in the phyla *Acidobacteria* and *Proteobacteria* (Tables 2 and 3). Five soil clone sequences (EF492954, EF492949, EF492960, EF492946, EF492950) and 6 nodule clone sequences (EF492914, EF492906, EF492908, EF492901, EF492907, EF492910) affiliated with the phylum *Acidobacteria* shared high sequence similarity (95%–98%) with clones from various U-contaminated environments (Selenska-Pobell et al. 2001; Abulencia et al. 2006) (Tables 2 and 3). Twelve of the 28 *Proteobacteria*-affiliated clone sequences from the soil and nodule sample also shared high sequence similarity (95% - 99%) with clones from diverse environmental sites rich in Fe, Mn, U and Au (Tables 2 and 3).

Two β-*Proteobacteria* clones (EF492894 and EF492895) from the nodule sample were closely related to *Leptothrix* sp. and *Chromobacterium* sp., respectively. Two γ-*Proteobacteria* clones (DQ351910 and DQ537525) from the nodule sample also showed high sequence similarity (98%–99%) to *Halomonas* sp. and *Acinetobacter lwoffii*, respectively. Two *Actinobacteria* sequences (EF492926 and EF492887) and one unclassifed bacteria sequence EF492972 were closely related (96%–97% sequence similarity) to clones from U-contaminated sediment and U-mining waste piles (Tables 2 and 3).

DISCUSSION

Recovery of DNA from the Fe-Mn Nodule and Soil Samples

Using the same DNA extraction protocol, significantly higher concentrations of DNA were extracted from the soil surrounding the Fe-Mn nodules than from the nodules. Our initial study showed that DNA extraction from the Fe-Mn nodules was difficult, possibly due to the adsorption of DNA by the Fe and Mn oxides and the low bacterial biomass of the sample. Dilution

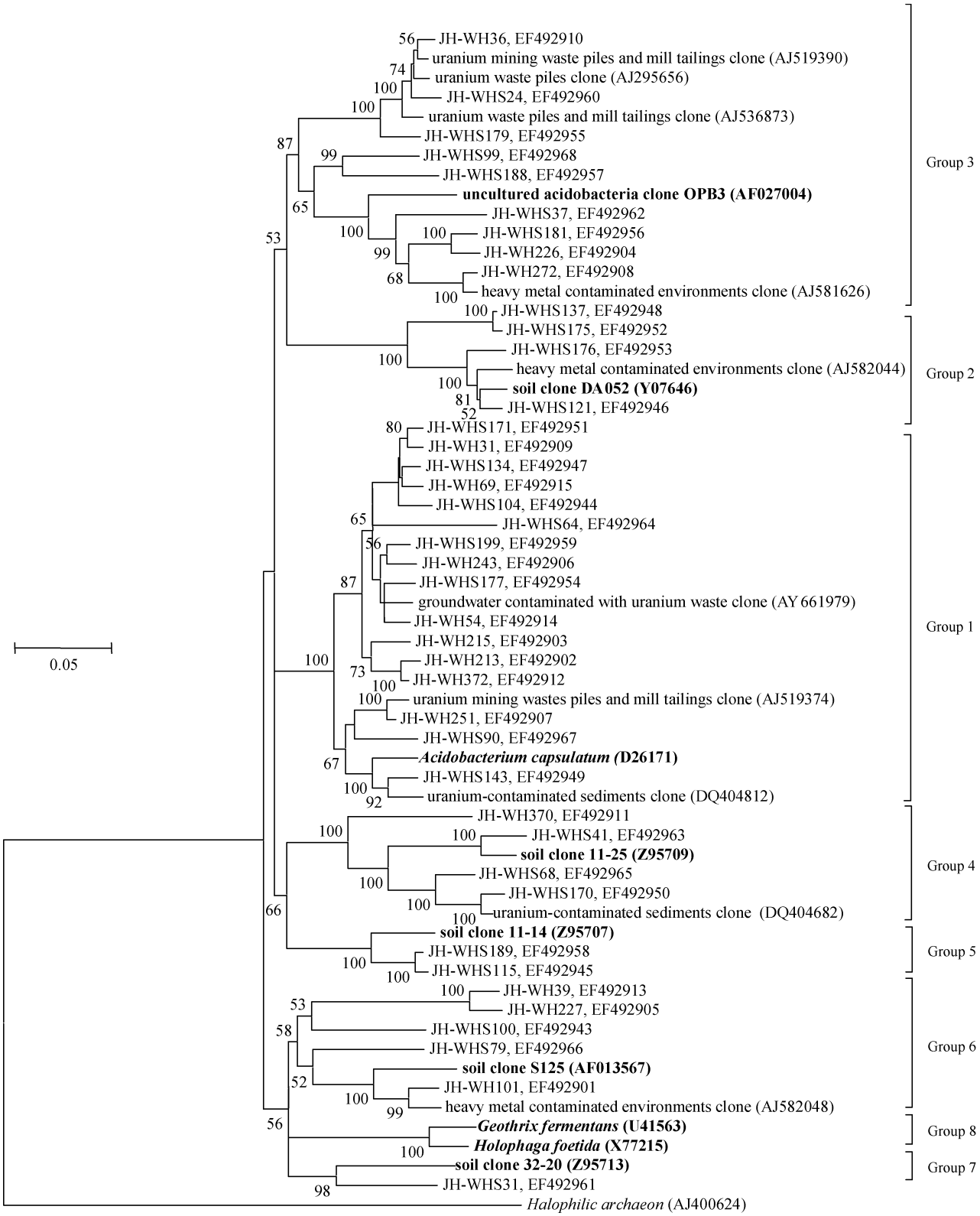

FIG. 1. Phylogenetic tree of the 16S rRNA gene sequences (~1500 bp) in the phylum *Acidobacteria* from the Fe-Mn nodules and surrounding soil at Wuhan, Central China, and their most similar GenBank sequences. Classification of the eight subdivisions is according to Hugenholtz et al. (1998). Sequences in boldface are representatives of the eight *Acidobacteria* subdivisions. JH-WH and JH-WHS denote clones from the Fe-Mn nodule sample and the soil sample, respectively. The accession number follows each clone. Bootstrap values (>50%) are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

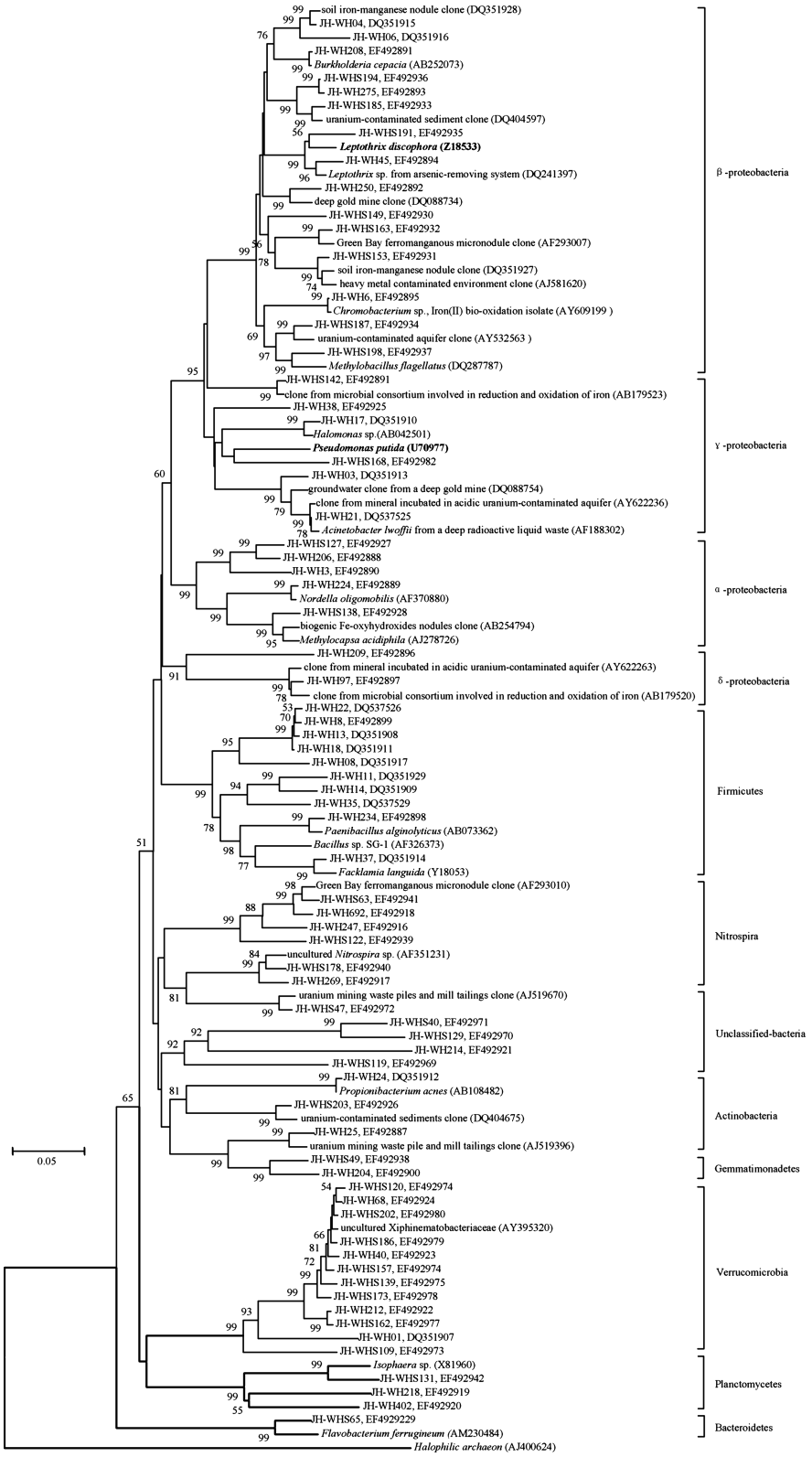

FIG. 2. Phylogenetic tree of the 16S rRNA gene sequences (∼1500 bp) of bacteria, except for members of the phylum *Acidobacteria*, retrieved from the Fe-Mn nodules and surrounding soil at Wuhan, Central China, and their most similar GenBank sequences. Phylum or class names of different groups of clones are based on the relationships of the clones to the known GenBank species and the NCBI Bacterial Taxonomy classification. Sequences in boldface are the model Mn-oxidizing bacteria from marine Fe-Mn nodules. Specification of the sequence name is as described in Fig. 1.

TABLE 1
The percentage of bacterial 16S rRNA gene clone types representing various groups in Fe-Mn nodules and their surrounding soil

	Acidobacteria	*Proteobacteria*				*Firmicutes*	*Verrucomicrobia*	*Nitrospira*	*Actinobacteria*	*Planctomycetes*	*Gemmatimonadetes*	Unclassified Bacteria	*Bacteroidetes*
		β-	γ-	α-	δ-								
Nodule sample	27.8	13.0	7.4	5.6	3.7	18.5	7.4	5.6	3.7	3.7	1.9	1.9	0.0
Soil sample	45.6	14.0	3.5	3.5	0.0	0.0	14.0	5.3	1.8	1.8	1.8	7.0	1.8

plate counts on nutrient broth also showed that the concentrations of culturable bacteria in the soil were 10^3-fold greater than in the nodules (data not shown). Compared to the surrounding soil, Fe-Mn nodules provide a much more oligotrophic and isolated habitat. Mn is present at high content (44 g kg^{-1}) in the nodules, 49-fold greater than in the bulk soil.

Generally, Mn (II) in the environment rarely exceeds 1–5 μM, and if above 10 μM, it can be toxic to some bacteria (Chapnick et al. 1982). Moreover, trace elements such as Ba, Cd, Co and Pb are highly accumulated in the nodules (Tan et al. 2006) and these are also toxic to some bacteria. These differences in the chemical characteristics between the soil and its nodules could account for the lower bacterial counts and DNA recovery from the nodules than from the soil.

Structure and Diversity of Bacterial Communities in the Fe-Mn Nodules and the Surrounding Soil

The structure and diversity of a microbial community adapted to a particular environment should reflect conditions of the surrounding milieu. In a soil environment, DNA molecules liberated from microorganisms can be adsorbed by clay minerals, humic acids and oxides, and are partially protected against degradation by nuclease for long periods (Khanna and Stotzky, 1992; Stokstad 2003; Crecchio et al. 2005; He et al. 2005). Compared to the bulk soil, Fe-Mn nodules have higher contents of Mn and Fe oxides (Liu et al. 2002; Tan et al. 2006), which can strongly bind the DNA. The DNA from dead cells in nodules may be adsorbed and conserved for years, and thus the DNA extracted from the nodules may represent the soil microbial community composition at the nodule-forming stages.

Phylogenetic analyses of 16S rRNA gene sequences from the soil and nodules in this study showed that the most abundant clone sequences were affiliated with the phyla *Acidobacteria* and *Proteobacteria*. These results were consistent with some previous surveys showing that *Acidobacteria* and *Proteobacteria* were the dominant components of bacterial community in soils from different locations (Chow et al. 2002; He et al. 2006). Comparatively, the soil sample contained more *Acidobacteria*-affiliated sequences (45.6%) than the nodule sample (27.8%). *Acidobacteria* is a bacterial division with 8 monophyletic subdivisions (Hugenholtz et al. 1998). The phylum was expanded to 11 subgroups and then to 26 subgroups recently (Zimmermann et al. 2005; Barns et al. 2007).

To date, there are only 3 well-characterized representatives: *Acidobacterium capsulatum* (group 1), *Geothrix fermentans* and*Holophaga foetida* (group 8), with little physiological information available on the other 6 groups. *A. capsulatum* is a moderately acidophilic aerobic heterotroph (Hiraishi et al. 1995) and the lower pH of the bulk soil (pH5.9), compared to the nodule (pH7.8), could account for the high relative abundance of *Acidobacteria* in the soil sample. *Holophag*a and *Geothrix* are strict anaerobes. *Acidobacteria* subdivisions 1, 3, 4, and 6 are well represented by the environmental clone sequences from the other studies (Hugenholtz et al. 1998; Barns et al. 1999). In this study, the sequences from the soil and nodule samples were mainly distributed in these 4 groups while groups 2, 5 and 7 were found only in the soil sample, accounting for the higher abundance of *Acidobacteria* in the soil sample.

Firmicutes accounted for 18.5% of the sequences from the nodule sample but were absent in the soil sample. *Firmicutes*, especially Bacillus/Clostridium are spore-formers, facilitating survival in adverse environments. Furthermore, the *Firmicutes* is one of the main branches of MnOB of marine origin (Nealson et al. 1988; Tebo et al. 2004; Dick et al. 2006). Poor nutrition and high levels of metals in the Fe-Mn nodules may have selected for the *Firmicutes*-affiliated sequences. *Verrucomicrobia* were the third most abundant cluster for the soil clone sequences and accounted for 14.0% of sequences from the soil sample, greater than in the nodules (7.4%). Limited physiological information is available on *Verrucomicrobia* but culture-independent analyses indicate that the *Verrucomicrobia*, like *Acidobacteria*, are widespread in the environment and abundant, particularly in soils (Hedlund et al. 1997; He et al. 2006).

TABLE 2
The closest relative sequences of the representative bacterial clone sequences from a soil sample at Wuhan, Central China

Phylogenetic group	Clone and accession No.	Closest identified relative in GenBank	Source or physiology of the closest relative	% Sequence identity	Reference
Acidobacteria	JH-WHS177, EF492954	Uncultured bacterium clone (AY661979)	Groundwater contaminated with high levels of nitric acid-bearing uranium waste	96	Fields et al. unpublished[1]
	JH-WHS143, EF492949	Uncultured bacterium clone (DQ404812)	Uranium-contaminated sediments	96	Abulencia et al. 2006
	JH-WHS24, EF492960	Uuncultured *Holophaga* sp. (AJ519390)	Uranium mining waste piles and mill tailings	96	Geissler et al. unpublished
		Uncultured bacterium clone (AJ295656)	Uranium mining waste piles	96	Selenska-Pobell et al. 2001
	JH-WHS121, EF492946	Uncultured *Acidobacteria* bacterium (AJ582044)	Heavy metal contaminated environments	95	Sacanska and Selenska-Pobell, unpublished
	JH-WHS170, EF492950	Uncultured bacterium clone (DQ404682)	Uranium-contaminated sediments	98	Abulencia et al. 2006
α−proteobacteria	JH-WHS138, EF492928	Uncultured bacterium clone (AB254794)	Biogenic Fe-oxyhydroxides nodules	96	Yoshida et al. unpublished
β−proteobacteria	JH-WHS153, EF492931	Unclutured bacterium clone (DQ351927)	Soil iron-manganese nodule in Hunan Province, China	98	Zhang et al. 2007
		Unclutured bacterium clone (AJ581620)	Heavy metal contaminated environments	97	Sacanska and Selenska-Pobell, unpublished
	JH-WHS163, EF492932	Unclutured bacterium clone (AF293007)	Green Bay ferromanganous micronodule	97	Stein et al. 2001
	JH-WHS194, EF492936	Unclutured bacterium clone (DQ404597)	Uranium-contaminated sediments	95	Abulencia et al. 2006
	JH-WHS185, EF492933	Unclutured bacterium clone (DQ404597)	Uranium-contaminated sediments	98	Abulencia et al. 2006
	JH-WHS187, EF492934	Unclutured bacterium clone (AY532563)	Uranium-contaminated aquifer	95	Gihring et al. unpublished
γ-proteobacteria	JH-WHS142, EF492981	Unclutured bacterium clone (AB179523)	Microbial consortium involved in reduction and oxidation of iron in siliceous sedimentary rock	98	Yoshida et al. unpublished
Nitrospira	JH-WHS63, EF492941	Unclutured *Nitrospira* bacterium clone (AF293010)	Green Bay ferromanganous micronodule	96	Stein et al. 2001
Actinobacteria	JH-WHS203, EF492926	Unclutured bacterium clone (DQ404675)	Uranium-contaminated sediments	96	Abulencia et al. 2006
Unclassifed_Bacteria	JH-WHS47, EF492972	Unclutured bacterium clone (AJ519670)	Uranium mining waste piles and mill tailings	97	Geissle et al. unpublished

[1]The unpublished reference was cited from NCBI GenBank database.

TABLE 3
The closest relative sequences of the representative bacterial clone sequences from a Fe-Mn nodule sample at Wuhan, Central China

Phylogenetic group	Clone and accession No.	Closest identified relative in GenBank	Source or physiology of the closest relative	% Sequence identity	Reference
Acidobacteria	JH-WH54, EF492914; JH-WH243, EF492906	Uncultured bacterium clone (AY661979)	Groundwater contaminated with high levels of nitric acid-bearing uranium waste	95	Fields et al. unpublished[1]
	JH-WH272, EF492908	Uncultured *Acidobacterium* sp. (AJ581626)	Heavy metal contaminated environments	98	Sacanska and Selenska-Pobell, unpublished
	JH-WH101, EF492901	Uncultured *Acidobacteria* bacterium (AJ582048)	Heavy metal contaminated environments	95	Sacanska and Selenska-Pobell, unpublished
	JH-WH251, EF492907	Uncultured *Holophaga* sp. (AJ519374)	Uranium mining waste piles and mill tailings	97	Geissler et al. unpublished
	JH-WH36, EF492910	Uncultured *Holophaga* sp. (AJ519390)	Uranium mining waste piles and mill tailings	97	Geissler et al. unpublished
		Uncultured bacterium clone (AJ295656)	Uranium mining waste piles	96	Selenska-Pobell et al. 2001
β−proteobacteria	JH-WH45, EF492894	*Leptothrix* sp. S1.1 (DQ241397)	Bioreactor removing arsenic from a mine drainage water	95	Battaglia-Brunet et al. 2006
	JH-WH275, EF492893	Uncultured bacterium clone (DQ404597)	Uranium-contaminated sediments	95	Abulencia et al. 2006
	JH-WH04, DQ351915	Uncultured bacterium clone (DQ351928)	Soil iron-manganese nodule in Hunan Province, China	98	Zhang et al. 2007
	JH-WH6, EF492895	*Chromobacterium* sp. 2002 (AY609199)	Anaerobic Nitrate-Dependent Iron(II) Bio-Oxidation	99	Weber et al. 2006
	JH-WH250, EF492892	Uncultured bacterium clone (DQ088734)	Groundwater from a deep gold mine of South Africa	95	Lin et al. 2006
γ-proteobacteria	JH-WH03, DQ351913	Uncultured bacterium clone (DQ088754)	Groundwater from a deep gold mine of South Africa	95	Lin et al. 2006
	JH-WH21, DQ537525	*Acinetobacter lwoffii* strain A382 (AF188302)	Groundwater from a deep radioactive liquid waste repository	99	Nazina et al. 2000
		Unclutured bacterium clone (AY622236)	Surrogate minerals incubated in an acidicacidic uranium-contaminated aquifer	99	Reardon et al. 2004
	JH-WH17, DQ351910	*Halomonas* sp. (AB042501)	Halophilic bacteria from a deep-sea hydrothermal mound and Antarctic habitats	98	Okamoto et al. 2004
δ−proteobacteria	JH-WH97, EF492897	Uncultured bacterium clone (AY622263)	Surrogate minerals incubated in an acidicacidic uranium-contaminated aquifer	96	Reardon et al. 2004
		Uncultured bacterium clone (AB179520)	Microbial consortium involved in reduction and oxidation of iron in siliceous sedimentary rock	96	Yoshida et al. unpublished
Nitrospira	JH-WH692, EF492918	Uncultured bacterium clone (AF293010)	Green Bay ferromanganous micronodule	95	Stein et al. 2001
Actinobacteria	JH-WH25, EF492887	Uncultured*Actinobacterium* clone (AJ519396)	Uranium mining waste pile and mill tailings	96	Geissle et al. unpublished

[1]The unpublished reference was cited from NCBI GenBank database.

Differences in the distribution of taxonomic groups between the Fe-Mn nodules and the surrounding soil indicate that physical and chemical properties of the Fe-Mn nodules during their formation may have resulted in a change of bacterial community structure. Lack of knowledge of the physiological characteristics of uncultured organisms make it difficult to determine the microbial ecology of the Fe-Mn nodules.

It would be valuable to isolate mRNA rather than DNA from the nodules to investigate the active bacterial communities of the nodules. However, attempts to extract RNA from the nodules were not successful. The phylogenetic results provided an overview of the bacterial communities within the nodules and the surrounding soil.

Analysis of Potential Functions of the Bacterial Clones

A phylogenetic assessment of uncultivated organisms from an environment can provide insight into the metabolic potential of these organisms in the environment by comparison with related sequences from previously cultured bacteria or environmental clones (Pace, 1997). The phylogenetic analyses of clone sequences from the soil and the nodules have shown that many sequences in this study closely resembled the cultured Fe(II)-/Mn(II)-oxidizing bacteria and environmental clones from environments with high levels of U, Mn, Fe and Au elements. The most abundant clades were in *Acidobacteria* and *Proteobacteria*.

Eleven *Acidobacteria*-affiliated sequences from the soil and nodule samples shared high sequence similarity (95%–98%) with the clones from various U-contaminated environments (Selenska-Pobell et al. 2001; Abulencia et al. 2006). *Acidobacteria*-affiliated sequences have been retrieved from a wide variety of environments, including special habitats such as acid mine drainage, contaminated aquifers, hot springs, and deep-sea sediments (Hugenholtz et al. 1998; Barns et al. 1999; 2007). The ubiquity, diversity, and abundance of *Acidobacteria* phylum members in soils and sediments, and their ability to withstand metal-contaminated, acidic, and other extreme environments suggest that they are as genetically and metabolically diverse and perhaps as ecologically important as the better-characterized *Proteobacteria* (Hugenholtz et al. 1998; Barns et al. 2007).

Among all *Proteobacterium* clones, *β-Proteobacteria* dominated and accounted for 53.6% of *Proteobacteria* sequences, followed by *γ-Proteobacteria* (21.4%) in this study. One *α*-proteobacterium sequence EF492928 was 96% similar to the sequence AB254794 from biogenic Fe oxide nodules. Among the *β-Proteobacteria* sequences, EF492931 and DQ351915 displayed high sequence identity (98%) to clones DQ351927 and DQ351928 from the soil Fe-Mn nodule sample in Hunan, China (Zhang et al. 2007), and the sequence EF492931 shared 97% similarity with a heavy-metal-contaminated environment clone (AJ581620). The sequence EF492932 had a close sequence identity (97%) to the clone AF293007, which was retrieved from Green Bay ferromanganous micronodule (Stein et al. 2001). Another 5 *β-Proteobacteria* sequences (EF492936, EF492933, EF492934, EF492893, EF492892) and one *γ-Proteobacteria* sequence DQ351913 shared 95%–98% sequence identity to clones from the sites rich in U and Au (Abulencia et al. 2006; Lin et al; 2006). One *γ-Proteobacteria* sequence EF492981 and one *δ-Proteobacteria* sequence EF492897 were 98% and 96% similar to the clones AB179523 and AB179520, respectively, retrieved from a microbial consortium involved in the reduction and oxidation of iron in a siliceous sedimentary rock.

The sequence EF492897 also showed high similarity with the clone AY622263 from an acidic U-contaminated aquifer (Reardon et al. 2004). Phylogenetic analysis based on 16S rRNA gene sequences has demonstrated that *β-Proteobacteria* and *γ-Proteobacteria* contain 2 main branches of MnOB (Nealson et al. 1988; Tebo et al. 2004; Templeton et al. 2005). High relative abundance of *β-* and *γ-Proteobacteria*-affiliated clones in this study and their close relatedness to environmental clones from the sites with high levels of metal elements suggested that these clones may play a critical role in the oxidation and reduction of these metal elements.

This study represents the first study that analyzes the microbial community associated with soil Fe-Mn nodules and their surrounding soil. Phylogenetic analyses of the 16S rRNA gene sequences indicated that the different chemical properties in Fe-Mn nodules during their formation may determine the microbial community in the nodules, resulting in the difference in the distribution of the bacterial taxonomic groups between the soil and the nodules. Many of the clones were related to Fe, Mn or other heavy metal-oxidizing bacteria. The extent to which metal-oxidizing bacteria contribute to the formation of Fe-Mn nodules in soil is yet unknown. Phylogenetic analyses based on DNA extracted from environmental samples were effective in elucidating microbial community composition of Fe-Mn nodules. The nodule and soil samples were very limited in this study and the microbial communities of the Fe-Mn nodules need to be elucidated from a wider range of soil types and replicated samples, and thus deserve further investigation.

Recently, we have isolated several strains with high Mn-oxidizing activity from the nodules and soil samples used in this study, and these strains were identified as the members of *Bacillus* and *Pseudomonas*. More research on these isolates is underway, and may provide a more direct indication of the biological process of Fe-Mn nodule and Mn oxide formation in soil.

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