Original Research Article

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Analysis of Bacterial Community in the Rhizosphere of Rice subjected to Heavy Metal Pollution

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A B S T R A C T

PCR amplification of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE) analysis were used to examine the compositions and dynamics of bacterial communities in the rhizosphere of rice subjected to heavy metal pollution. DGGE showed many common bands under conditions of Cd and Cr pollution, suggesting the presence of similar of bacterial communities. Principal component analysis showed different microbial community structure changes during the study period, for Cd and Cr pollution respectively. Major bands of DGGE patterns were sequenced. Phylogenetic analysis indicated that the majority of the sequences obtained were affiliated with members of Acidobacteria (18.75 %), Myxococcales (12.50 %), Gemmatimonas (12.50 %), and Thiothrix (9.38 %). When the rice plants reached the heading stage, the relative abundance of members of Gemmatimon as increased with Cd concentration, while abundance of members of Gallionellaceae increased with Cr concentration. At the maturation stage, the relative abundance of members of Chlorobiales increased with Cd concentration. These results illuminate the effect on diversity of heavy metal pollution in soil. Further study of combinations of other environmental parameters will clarify heavy metal effects on the activity of microbial communities in rice paddy fields.

Introduction

Heavy metal pollution can persist for a long time in soil, potentially migrating to crops and entering the food chain, leading to public health concerns. As one of the world's main planted crops, rice plays a huge part in the migration of heavy metals into the food chain. In recent years, heavy metal pollution has been widely reported in the arable fields of South China, due to the rapid development of mining activities, the metallurgical industry, sewage irrigation, and the application of pesticides and fertilizers (Chen *et al.,* 2014; Gong *et al.,* 2009; Zeng *et al.,* 2013a). Heavy metal pollution in soil has been paid extensive attention worldwide, especially in countries undergoing rapid industrialization and urbanization. Human-spread metals

including lead, mercury, and copper have been detected in snow samples from both Greenland and Antarctica, in areas remote from human habitation (Lobinski *et al.,* 1994; Van de Velde *et al.,* 2005). The most common heavy metal contaminants include mercury, cadmium, lead, chromium, arsenic, zinc, copper, nickel, and cobalt, each of which is potentially toxic to humans (Tang *et al.,* 2014; Xu *et al.,* 2012). While soil is a fundamentally important biome that is crucial for terrestrial life, it also acts as a reservoir for toxic substances that are deleterious to soil microbiota, the food chain, and human health (Akcay *et al.,* 2003; Khan *et al.,* 2010; Wang *et al.,* 2007).

In topsoil ecosystems, bacteria and fungi generally constitute more than 90 % of the total soil microbial biomass, and are the main regulators of soil organic matter dynamics and nutrient availability (Chen *et al.,* 2014). Soil microorganisms play an important role in energy flow, nutrient cycling, and organic matter turnover (Eguchi *et al.,* 2001; Frische and Hoper, 2003; Sabater *et al.,* 2003; Tonkovic, 1998). They are sensitive to toxic substances such as heavy metals, which affect their community structure and function.

This paper examines the effect of heavy metal pollution on microbial community structure indifferent concentrations and different heavy metal pollution, using PCR and denaturing gradient gel electrophoresis (DGGE) to describe the community and its changes in response to environmental variation. Relationships between the rhizosphere microbial community and environmental parameters are described. This study is intended to highlight the effects of heavy metal pollution, particularly its toxicity to soil microorganism near metallurgical plant districts in China.

Materials and Methods

Natural profile of the study site

The study was conducted at the experimental field station of Huazhong Agricultural University in Hubei Province, China (30°N, 114°E, 50 m a.s.l.), where the average temperature is between 15.8 ℃ and 17.5 ℃. The maximum and minimum recorded temperatures are 44.5 ℃ and −18 ℃, respectively. The average annual precipitation is 1,269 mm, and the climate is sub-tropical monsoon.

Experimental design

Clay loam surface (0–20 cm) soil undergoing heavy metal treatment was sampled from the experimental rice paddy field at Huazhong Agricultural University in early March 2014. The soil was air-dried, ground and passed through mesh sieves. Six-kilogram samples of soil were collected in cylindrical plastic containers (each with an upper diameter of 29 cm and a height of 30 cm). Solutions of $CdCl_2$ and $K_2Cr_2O_7$ were added to the containers. The final Cd^{2+} concentrations were 10 mg/kg (Cd 1), 20 mg/kg (Cd 2), and 50 mg/kg (Cd 3), and the final Cr^{6+} concentrations were 100 mg/kg (Cr 1), 400 mg/kg (Cr 2), and 700 mg/kg (Cr 3). The control treatment was water without heavy metal. Containers were arranged according to a randomized block design with seven treatments. Each treatment had eight replicates. Before sowing seeds, 56 randomly selected containers were filled with water to cover the soil by 2–3 cm for one month to balance the concentrations of Cd^{2+} and Cr^{6+}. Japonica rice (Wuyujing 3) was chosen as the experimental plant, because it shows different characteristics with heavy metal treatments. Seeds were sown after 5 days of germination.

Compound fertilizer (1 g) was applied at the three-leaf stage and the spike differentiation stage. Throughout preparation, sowing, germination and early seedling growth, 2–3 cm of water covered the soil. Initial characteristics of the soil samples were as follows: soil organic matter (SOM), 2.19 ± 0.07 %; available nitrogen, 51.1 ± 1.06 mg kg^{-1}; available phosphorus, 21.3 ± 2.11 mg kg^{-1}; available potassium, 74.0 ± 3.15 mg kg^{-1}; total nitrogen, 1.14 ± 1.01mg g^{-1}; total phosphorus 0.42 ± 1.21 mg g^{-1}; total Cr, 80.4 ± 1.15 mg kg^{-1}; total Pb, 25.2± 1.54 mg kg^{-1}; and total Cd, 0.18 ± 0.05 mg kg^{-1}.

Sampling of the rhizosphere of the rice plants was conducted as follows: eight soil samples per treatment were collected at random from the rice rhizosphere, and were combined. Loose soil was removed from the roots, and the remaining soil that adhered strongly to the roots was recovered as rhizosphere soil. The sampling took place at the heading stage (August 6) and maturing stage (September 18) in 2014. Part of the soil was stored at4 °C for subsequent microbial abundance determination, another part of the soil was stored at −80 °C for soil microbiological analysis, and the remainder was air-dried, ground and passed through 1-mm and 2-mm mesh sieves for chemical analysis.

DNA extraction

Total community DNA was isolated from soil samples (0.5 g) using the E.Z.N.A^{TM} Soil DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. DNA was eluted with 30 µL of elution buffer and stored at −20°C. DNA concentrations ranged from 125 to 280 ng/µL.DNA concentration was measured by NanoDrop

PCR-DGGE profiling

The V3 region of the 16S rRNA gene was amplified from the DNA samples using touchdown PCR (Van *et al.,* 1999). 16S rRNA genes were amplified using the primers 16SB-1/16SB-2; Primer sequences were as follows: 16SB-1: 5′-CGCCCGC CGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTACGGGAGGCAGCAG-3′; 16SB-2: 5′-ATTACCG CGGCTGC TGG-3′. The reaction mixture (25.0 µL) contained 1.0 µL of DNA template, 12.5 µL Takara LA-Taq Mix (TaKaRa Biotechnology Co., Ltd, Dalian, China) and 1.0 µL of each primer. PCR amplification was as follows: an initial denaturing step of 5 min at 95 °C followed by 20 cycles of 1 min at 94 °C, 1 min of annealing at 65–55 °C, and a 3 min extension at 72 °C, followed by 10 cycles of 1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C, a final extension step at 72 °C for 10 min. PCR products were checked by electrophoresis on 1 % (w/v) agarose gels. DGGE analysis was performed with the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). PCR products (20 µL) of each sample were loaded onto an 8 % (w/v) acrylamide gel with a 40–60 % denaturing gradient (urea and formamide) as described by Huang *et al.,* (2013). The DGGE profile was analyzed with Quantity One software (Bio-Rad).

Specific amplification of particular DGGE bands

DNA fragments forming particular DGGE bands were amplified using specific PCR primers. The primers were as follows: 16SS-1: 5′-AGAGTTTGATCATGGCTCAG-3′; 16SS-2: 5′-TAGGGTTACCTTGTT ACG ACTT-3′. DNA fragments were cloned in *Escherichia coli* DH5α using the pMD19-T Vector System (TaKaRa Biotechnology Co., Ltd, Dalian, China) according to the manufacturer's instructions.

Sequence analysis

The 16S rRNA partial sequences obtained in this study have been deposited in the NCBI. Sequences of the DGGE bands were also compared to those present in the databases using BLAST search program at the NCBI web site. Sequences were aligned using ClustalX (1.81) as implemented. The phylogenetic tree of the aligned sequences was constructed with 1,000-fold bootstrap analysis by using the neighborjoining method with the MEGA5.1 (Molecular Evolutionary Genetics Analysis, MEGA) software (Kumar, 2008).

Statistical analysis

Digitized DGGE images were analyzed with the Quantity One image analysis software (version 4.0, Bio-Rad, USA).This software identifies the bands occupying the same position in different lanes of a gel. The diversity index of the soil actinomycetes communitieswas evaluated using the Shannon–Wiener index (H) (Hill *et al.,* 2003).

Cluster analysis was performed using the unweighted pair group method with arithmetic mean. The band data were subjected to principal component analysis (PCA) using the Statistical Package for the Social Sciences version 16.0 for Windows (SPSS Incorporated 2007). The principal components were then extracted from the correlation matrix. The standardized data were then projected onto the principal axes, plotted in two dimensions, and examined for clustering behavior (Daniela *et al.,* 2012).

Nucleotide sequence accession numbers

The sequence of each clone was determined and all sequences were deposited into the GenBank database with the accession numbers KR997935.1 through KR997966.1

Results and Discussion

Community structure of bacteria in the rhizosphere of rice under pollution of heavy metals

DGGE profiles of the V3 hypervariable region of 16S rDNA genes from rice rhizosphere bacteria are shown in Table 1. Soil samples were extracted at seedling heading and maturation stages. DGGE band numbers, and the Shannon diversity index were significantly higher in the heading stage than the mature stage. Each of the distinguishable bands in the DGGE separation pattern represents an individual bacterial species (Luca *et al.,* 2002), so the species diversity of rhizosphere bacteria decreased as the rice grew. Band numbers and diversity were higher in the samples treated with different concentrations of cadmium, than in those treated with chromium, suggesting that the effects of cadmium were smaller than those of chromium. Under the heavy metal treatments, species diversity improved at the heading stage, which suggests that the rhizosphere bacteria were initially able to adapt to the heavy metal pollution. However, species diversity decreased at the mature stage, suggesting that some bacterial populations were damaged by or unable to adapt to sustained exposure to heavy metals.

Principal component analysis (PCA) on bacterial diversity data is shown in Figure 1, showing 57.24 % of the variance in the data is explained by principal component 1, and 27.98 % by principal component 2. Discrete distances were larger during heading and maturation stages with heavy metal pollution, which suggests that the rhizosphere bacterial community changed with prolonged exposure to heavy metal pollution. Discrete distances were largest for the 700 mg/kg chromium treatment, among

heavy metal treatments, suggesting it had a greater influence on the rhizosphere bacterial community than other treatments.

Sequence and phylogenetic analysis

DGGE profiles differed among treatments and among sampling times, indicating the fluctuation of the microbial community structure in response to heavy metal pollution (Figure 2). Bands differed in intensity, indicating different population densities of different species. Thirty-two representative bands were excised and sequenced. Inferred homologies to known sequences in the NCBI database, based on BLAST results, ranged in similarity from 98 % to 100 %. Phylogenetic analysis revealed that the bacterial community was separated into two major groups (Figure 3). As shown in figures 2 and 3, in the heading stage, the relative abundance of members of *Gemmatimonas* increased with Cd concentration relative to controls; and likewise the relative abundance of members of Gallionellaceae increased with Cr concentration relative to controls. In the maturation stage, the relative abundance of members of Chlorobiales increased with Cd concentration, relative to controls. The bacterial community in the rhizosphere is usually dynamic and our results show that it can be strongly affected by heavy metal pollution.

Metal pollution caused changes in the relative abundances of specific bacterial higher taxa, including *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi*, Firmicutes, Planctomycetes and Proteobacteria (Hamed *et al.,*, 2015). As shown in Figure 4, higher taxonomic affiliations of excised DGGE bands included *Acidobacteria* (18.75 %), Myxococcales (12.50 %), *Gemmatimonas* (12.50 %), *Thiothrix* (9.38 %), *Firmicutes* (6.25 %), Chlorobiales (6.25 %), *Proteobacterium* (6.25 %), Gallionellaceae (6.25 %), Bacteroidales (3.13 %), *Caulobacter* (3.13 %), Chloroflexi (3.13 %), Denitratisoma (3.13 %), *Ferribacterium* (3.13 %), *Agrobacterium* (3.13 %) and *Clostridium* (3.13 %).

Table.1

Sampling period	Treatments	Band numbers	Shannon-index
Heading stage	Cd1	29±0.5a	-3.2686±0.021a
	Cd2	29±1.5a	-3.2762±0.054a
	Cd3	28±2.5ab	-3.2550±0.098a
	Cr1	28±0.5ab	-3.2452±0.019a
	Cr2	26±2.0bc	-3.1841±0.100abc
	Cr3	26±0.5abc	-3.2239±0.026ab
	Control	26±1.0bc	-3.2044±0.044ab
Maturation stage	Cd1	23±2.0de	-3.0228±0.031de
	Cd2	25±3.0cd	-3.0850±0.048cd
	Cd3	25±1.0cd	-3.1258±0.111bcd
	Cr1	22±0.5e	-3.0827±0.020cd
	Cr2	22±0.5e	-3.0782±0.026de
	Cr3	22±0.0e	-2.9759±0.077e
	Control	25±1.0cd	-3.1868±0.076bcd

Fig.1 Principal component analysis of changes in bacterial diversity in the
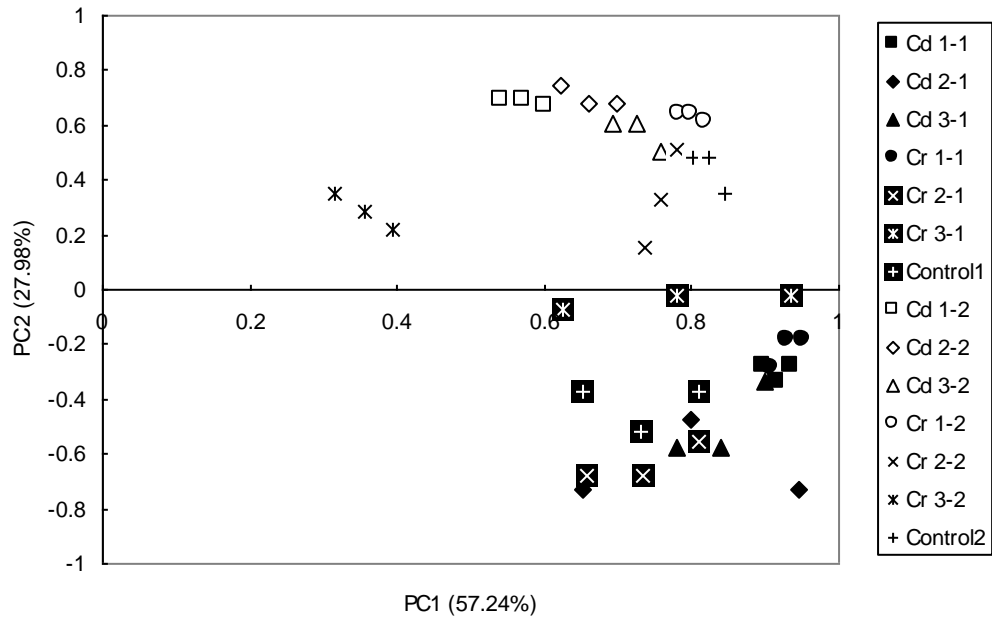rhizosphere of rice subjected to heavy metal pollution.

Fig.2 DGGE fingerprint profiles obtained with primers targeting the V3 region of 16S rRNA, run
in a denaturing 30–55 % gradient gel. Numbered bands correspond to the clones in Fig. 3. A:
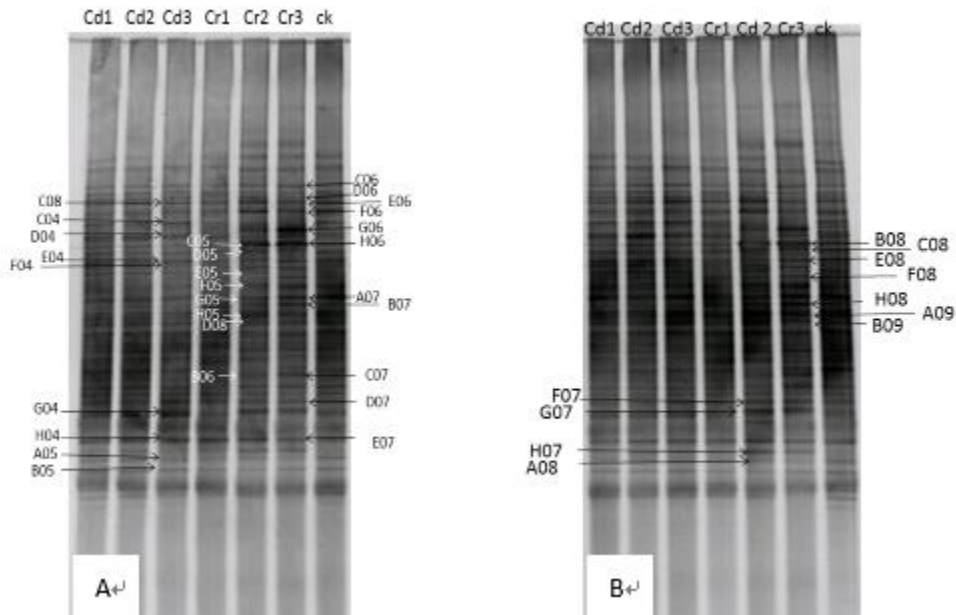Heading stage, B: Maturation stage.

Fig.3 Neighbor-joining phylogenetic tree of V3 region 16S rRNA sequences, showing the relationship between the sequences retrieved from DGGE bands.
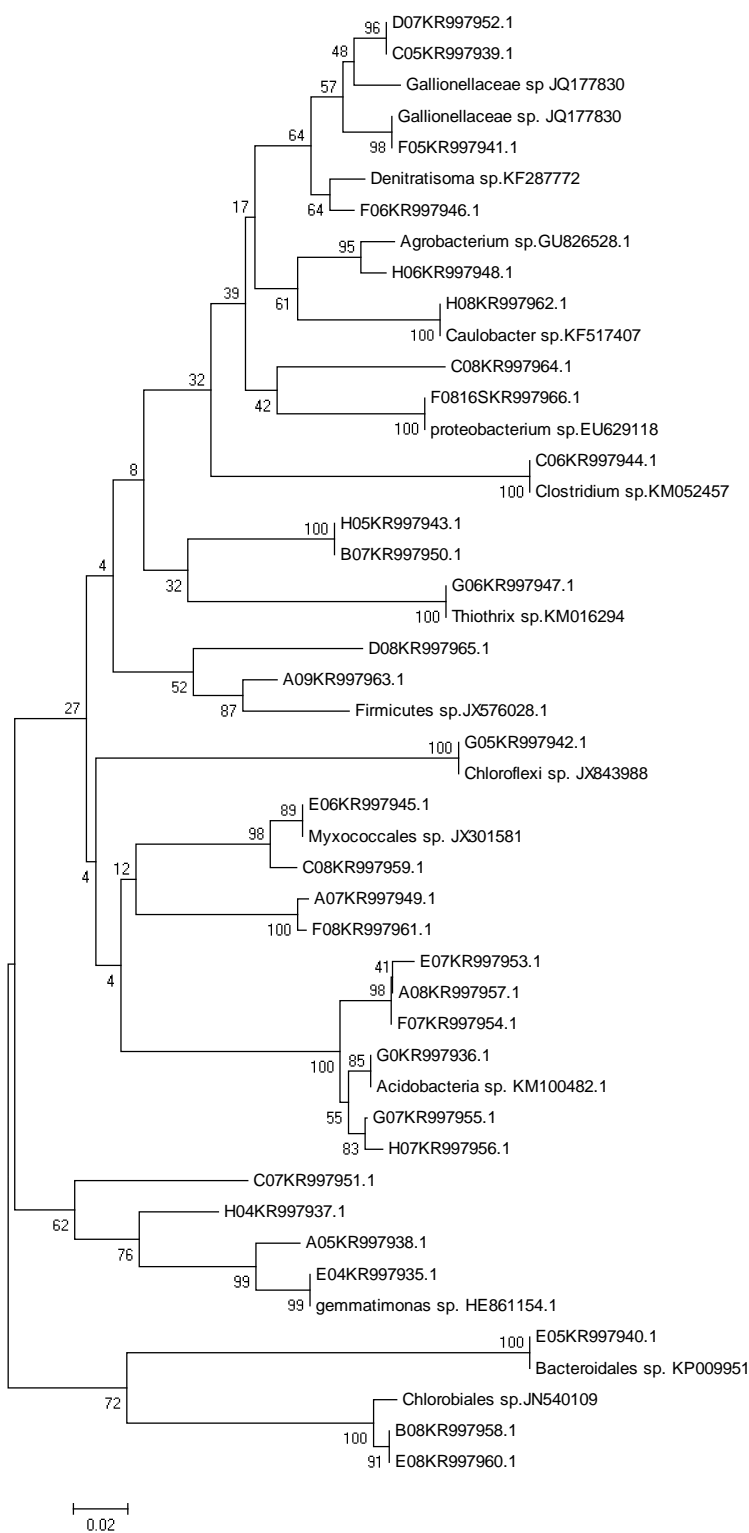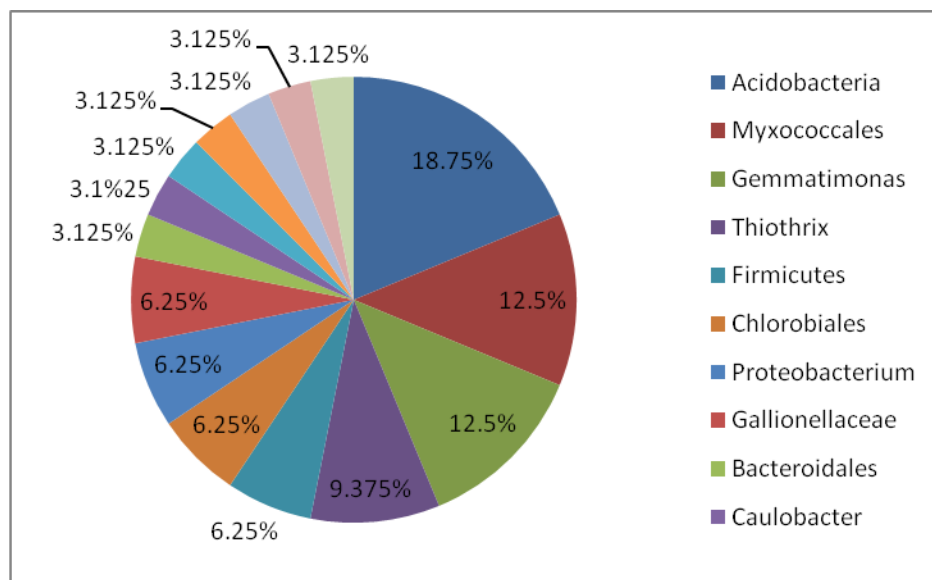
Fig.4 Taxonomic affiliations of the most-represented bacteria in the rhizosphere of rice subjected to heavy metal pollution.

In conclusion, agricultural land contamination with heavy metals is an increasing problem globally. It presents potential human health risks via direct exposure to heavy metals, as well as via indirect changes to the food chain because of the effects on soil microbiota, crops and livestock (Grimm *et al.,* 2008). In China, pollution from heavy metals, particularly chromium and cadmium, had been widely reported in major rice production areas, including the lower Yangtze River delta (Wu *et al.,* 2006; Hang *et al.,* 2009) and the river valleys in the Jiangxi and Hubei Provinces (Xu *et al.,* 2007). It is critical to reduce the bioavailability of heavy metals, and thus to prevent soil-food chain transfer of toxic metals from rice paddies (Chaney *et al.,* 2004). Results presented here of DGGE and sequence data show that heavy metal contamination can strongly affect microbial community structure and abundance. DGGE showed that as heavy metal concentration and plant growth increased, the microbial community diversity and abundance decreased. Particular haplotypes were enriched in the contaminated samples,

suggesting that particular individual species of heavy metal resistant bacteria may thrive in contaminated environments. The PCR-DGGE method can provide detailed information about microbial community structure and how it changes with particular parameters such as pollution. These results will be applicable to the assessment and remediation of rice paddies contaminated by heavy metals.

Acknowledgments

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