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Responses of Soil Bacteria to Long-Term and Short-Term Cadmium Stress as Revealed by Microbial Community Analysis

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Abstract Soil pollution by cadmium has been a long standing ecological problem in Zhangshi Irrigation Area, Shenyang, China, as a result of the 30-year practice of irrigation with wastewater containing high levels of heavy metals. To evaluate the adverse impact of cadmium contamination on soil ecosystems, the responses of soil microbiota to both long-term and short-term cadmium stress were studied by molecular microbial community profiling with denaturating gradient gel electrophoresis (DGGE) analysis. Our results show that soil characteristics and nutrient conditions were likely more important than cadmium toxicity in shaping the soil bacterial community structure in the long term. In comparison, soil microbial genetic diversity was shown to be more closely correlated to cadmium levels under short-term cadmium stress, with the highest microbial genetic diversity occurring at mild cadmium stress conditions, which might be attributed to the enrichment of metal-resistant microbial populations through mechanisms of competitive selection and genetic adaptation. In contrast, severe cadmium stress likely presented a condition that fewer microbial populations could survive, thus leading to reduced microbial genetic diversity.

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Heavy metal pollution has been shown to adversely affect soil ecosystems, particularly the normal microbiota, which is critical for the agricultural productivity of soil. Therefore, a number of microbial indicators, such as respiration rate, enzymatic activity, and biomass content, have been developed to evaluate the impact of heavy metals on soil quality (Renella et al. 2004). The culture-dependent nature of these indicators, however, may introduce potential experimental biases and lead to inconsistent assessment of the soil microbial responses to heavy metals (Giller et al. 1998). The development of culture-independent molecular techniques capable of characterizing the dynamics of microbial community structures, such as Denaturing Gradient Gel Electrophoresis (DGGE), has made it possible to monitor the responses of soil microorganisms to heavy metals at the community level.

Zhangshi Irrigation Area in the west suburb of Shenyang, Northeast China, had been irrigated with heavy metals-containing wastewater for 30 years, making it an ideal location to study the long-term impact of heavy metals on soil ecosystems. In the past two decades, a number of studies have been conducted in this area on the toxicity of soil cadmium on plants, livestock, and human beings (Wu et al. 1989; Xiong et al. 2004). However, the effects of long-term Cd exposure on soil microbes remain largely unknown. In this study, microbial community structures in soil samples collected from the Zhangshi Irrigation Area along a soil Cd gradient were characterized using molecular microbial ecology tools to examine soil microbial responses to both long-term and short-term

cadmium exposure. Furthermore, the influence of soil characteristics on the microbial community response to cadmium stress was also investigated to evaluate other factors important to microbial heavy metal stress response.

Materials and Methods

Soil samples were taken from four sites designated as Site 1 (41°45′06″N, 123°14′59″E), Site 2 (41°44′31″N, 123°12′51″E), Site 3 (41°47′56″N, 123°14′17″E), and Site 4 (41°46′23″N, 123°10′54″E) along a soil Cd gradient from high to low in Zhangshi Irrigation Area, Shenyang, Northeast China, where wastewater containing heavy metals had been used for irrigation for a period of 30 years (1962–1992) before switching to groundwater irrigation in 1993. Triplicate samples of soil (Gleyic Cambisols, FAO) were collected at three different depths (0–10 cm, 10–20 cm, and 20–30 cm) for each sampling site on April 22, 2005. Analysis of total and available cadmium was conducted using methods recommended in China Environmental Quality Standards (GB15618-1995). Briefly, total soil Cd was determined by atomic absorption spectrometry (AAS) (Unicam Solaar32, Unicam Atomic Absorption, Cambridge, UK) following the digestion of soil samples with HCl–HF–HNO_3–$HClO_4$ and subsequent extraction with KI–MIBK (potassium iodine–methyl isobutyl ketone). Similarily, available Cd in soil was quantified by AAS following extraction by a solution containing 0.005 M diethylenetriamine pentaacetate (DTPA), 0.01 M $CaCl_2$, and 0.1 M trietanolamine at pH 7.3 (Lindsay and Norwell 1978). The AAS detection limit for Cd is 0.05 mg/kg and the recovery rates for soil Cd were found to be 90 ± 5% using standards. The means and standard deviations were calculated from triplicate measurements.

The responses of soil bacterial to long-term Cd stress were evaluated with community DNA extracted directly from the original soil samples with a 30-year history of Cd contamination as a result of wastewater irrigation. In comparison, short-term Cd stress responses were evaluated using laboratory incubation of soil samples immediately following amendment of various levels of cadmium. Specifically, 5.0 g soil (0–10 cm) each from Site 1, Site 3, or Site 4 was inoculated into 20 mL of the Meat Peptone Broth medium (1% Beef Extract, 0.5% Peptone, 0.5% NaCl, pH 7.0–7.2) containing 0, 1, 2 or 4 mM Cd^{2+}. After a 48-h incubation at 30°C, 1 mL homogeneous suspension from each incubation was analyzed for bacterial community structure under short-term Cd exposure.

For soil microbial community analysis, total soil DNA was extracted as described by Zhou et al. (1996). The V3 region and the V3–V5 region of 16S rDNA genes in the DNA extract were amplified with the primer pairs 341f-GC/518r and 341f-GC/907r, respectively, as previously described (Muyzer et al. 1993; Lyautey et al. 2003). DNA amplification with the polymerase chain reaction (PCR) was carried out with 1 μL of template DNA and 50 μL of a reaction mixture containing 20 pM of each primer, 200 μM dNTP, 2.5 U of Taq DNA polymerase, and a PCR buffer supplied with the Taq DNA polymerase (Takara, Shiga, Japan). PCR amplification of the V3 region was carried out in the following procedure: a single denaturation step (94°C for 4 min) followed by a 10-cycle program (denaturation at 94°C for 1 min, annealing at 60°C for 30 s and extension at 72°C for 2 min), then a 20-cycle touchdown program of 0.5°C decrease per cycle and a final extension step at 72°C for 10 min. For the amplification of the V3–V5 region, a single denaturation step (95°C for 5 min) was followed by a 35-cycle program (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min) and a final extension step at 72°C for 10 min.

Denaturing Gradient Gel Elecrophoresis (DGGE) analysis of microbial community structure was performed using the D-Code system (Bio-Rad Laboratories, Hercules, CA, USA) by loading PCR products of the V3 region onto 8% (w/v) polyacrylamide gels. The denaturing gradient was established with 50%–70% denaturant (100% denaturant corresponding to 7 M urea and 40% (v/v) deionized formamide). Similarly, PCR products of the V3–V5 region were loaded onto 6% (w/v) polyacrylamide gels with a denaturing gradient of 30%–50%. Electrophoresis was performed at 200 V for 5 h at 60°C. Dendrogram analysis of DGGE patterns was performed using Quantity One 4.2.3 (Bio-Rad Laboratories, Hercules, CA, USA) by unweighted pair-group method with arithmetic averages (UPGMA). The Shannon–Weaver diversity index was calculated from the number of bands present and their intensities in each lane (Shannon and Weaver, 1949).

Bands of interest were excised from the DGGE gel for further re-amplification with primers 341f/907r as described by Hernandez-Raquet et al. (2006). PCR products were subsequently sequenced by Shanghai Sangon Biological Engineering Technology and Services Co., Shanghai, China and sequence similarities in GenBank were searched using NCBI BLASTN (http://www.ncbi.nlm.gov/blast/). Alignment of similar 16S rDNA gene sequences were performed with ClustalX 1.83 (Thompson et al. 1997) and phylogentic trees were constructed with the neighbor-jointing method using Mega 3.1 with the bootstrap confidence values obtained with 1,000 resamplings (Kumar et al. 2004). The Fisher's least-significant difference (LSD) test and correlation analysis were conducted with the software package SPSS 13.0 for Windows (SPSS Inc. 2004). The significance level of all statistical analysis was accepted at $\alpha = 0.05$.

Sequences obtained in this study were deposited into the GenBank database under the following accession

numbers: EF590209-EF590215, EF590216-EF590223, and EF590228-EF590247.

Results and Discussion

The chemical properties and Cd content of all soil samples were summarized in Table 1, showing that total Cd content in soil samples ranged from 1.75 to 3.89 mg/kg, much higher than the background soil level in Shenyang (0.17 mg/kg) and exceeding the Secondary Standard of Chinese Soil Environmental Quality (0.30 mg/kg) (Wu 1994). Apparently, soil microbial communities at the sampling sites were subjected to long-term Cd stress given the high levels of soil Cd detected 10 years after heavy metal-containing wastewater was no longer used for irrigation.

Despite the considerable variations in Cd content in soil samples (Table 1), the bacterial community structures at the four sample sites shared significant similarity (>61%)

Table 1 Chemical properties and Cd content of test soil[a]

Sample Sites		Total Organic C (g/kg)	Total N (g/kg)	Available N (mg/kg)	Total Cd (mg/kg)	DTPA-Cd (mg/kg)
0–10 cm	Site 1	13.51 ± 2.53bcd	1.37 ± 0.27a	72.35 ± 1.12b	2.66 ± 0.32c	1.05 ± 0.08ab
	Site 2	15.86 ± 2.29ab	1.28 ± 0.05ab	48.92 ± 5.76fg	2.12 ± 0.15de	0.52 ± 0.02c
	Site 3	17.02 ± 1.45a	1.19 ± 0.06ab	75.42 ± 3.37ab	2.06 ± 0.17def	0.48 ± 0.08cd
	Site 4	13.35 ± 0.22bcd	1.14 ± 0.14bc	80.80 ± 3.76a	1.85 ± 0.23ef	0.48 ± 0.11c
10–20 cm	Site 1	13.38 ± 0.71bcd	1.20 ± 0.22ab	64.73 ± 2.24c	3.22 ± 0.08b	1.00 ± 0.16b
	Site 2	13.93 ± 3.11bc	1.26 ± 0.01ab	44.36 ± 2.66gh	2.28 ± 0.21d	0.50 ± 0.07c
	Site 3	13.02 ± 1.13bcde	0.98 ± 0.05cd	57.22 ± 6.05de	2.37 ± 0.18cd	0.39 ± 0.03de
	Site 4	10.92 ± 1.18defg	1.11 ± 0.09bc	59.06 ± 0.54cd	1.86 ± 0.29ef	0.45 ± 0.10cd
20–30 cm	Site 1	11.20 ± 0.64cdef	0.87 ± 0.07def	51.56 ± 5.59ef	3.89 ± 0.26a	1.15 ± 0.15a
	Site 2	10.16 ± 3.17efg	0.96 ± 0.01cde	32.02 ± 1.93i	2.39 ± 0.29cd	0.33 ± 0.04de
	Site 3	9.71 ± 0.38fg	0.77 ± 0.07ef	47.11 ± 1.59fg	1.75 ± 0.02f	0.31 ± 0.08e
	Site 4	8.23 ± 0.19g	0.73 ± 0.03f	40.22 ± 2.86h	1.83 ± 0.15ef	0.29 ± 0.01e

[a] Means ± standard deviation (n = 3). Different letters following means ± standard deviation in the column indicate significant differences by Fisher's LSD ($p < 0.05$)

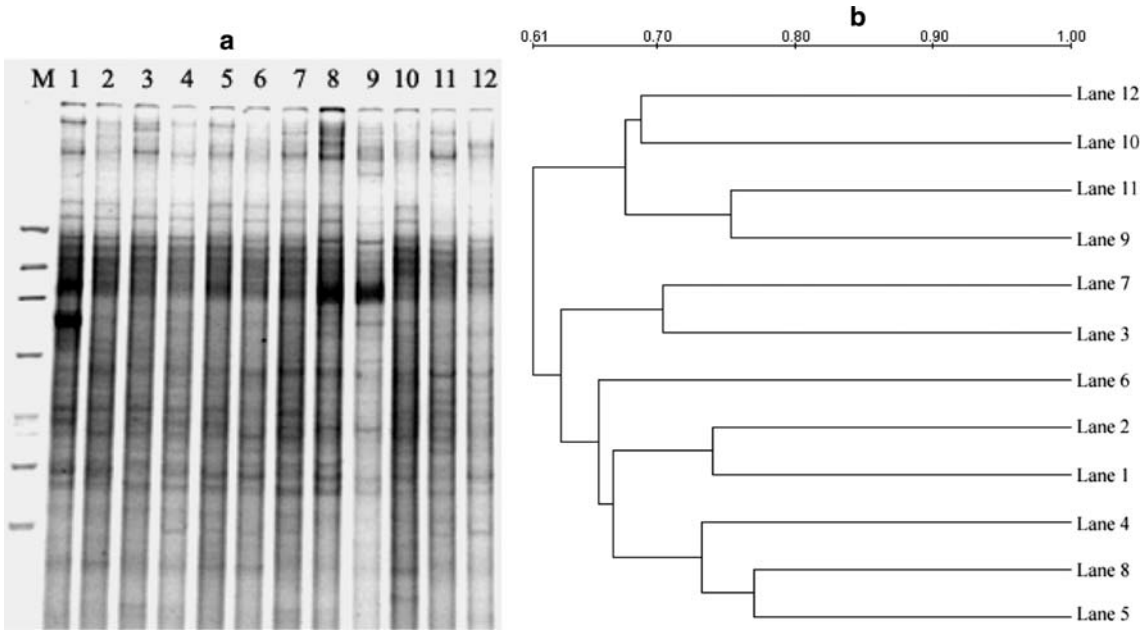

Fig. 1 DGGE fingerprints of 16S rDNA fragments amplified by 341f-GC/518r (**a**) and UPGMA dendrograms constructed from DGGE profiles (**b**) in soil samples under long-term cadmium stress. Lane 1–4: soil samples from 0–10 cm of Site 1–4; Lane 5–8: soil samples from 10–20 cm of Site1–4; Lane 9–12: soil samples from 20–30 cm of Site 1–4

at all depths as shown by the DGGE profiles (Fig. 1), suggesting that other factors might obscure the impact of long-term Cd toxicity. On the other hand, the Shannon–Weaver index, calculated from the intensities of DGGE bands as a measure of the microbial community diversity had significant positive correlation with soil total organic C (TOC) ($r = 0.622$, $p < 0.01$), total N (TN) ($r = 0.485$, $p < 0.01$), and available N ($r = 0.503$, $p < 0.05$), but no correlation with soil Cd levels (Fig. 2). Therefore, it is likely that soil characteristics and nutrient conditions might be more important than the presence of Cd in shaping the soil bacterial community structure in the long term.

The finding in this study that long-term cadmium stress had no significant influence on soil microbial community structure is supported by previous studies with similar results, which, however, is inconsistent with several other investigations showing that soil microorganisms could be negatively affected by heavy metals at concentrations close to the maximum concentrations permitted under the European Community (EC) directive (3 mg/kg) (Giller et al. 1998; Renella et al. 2004). The discrepancy may be attributed to the considerable heterogeneity of soils. Indeed, changes in soil microbial community structure by long-term heavy metal stress could be readily obscured by other soil characteristics, such as TOC and TN, which were shown in this study to be more important long-term factors determining the structure of soil microbial communities. Thus, an accurate assessment of the long-term impact of heavy metal stress on soil ecosystem has to include soil characteristics and nutrient conditions as key determinants.

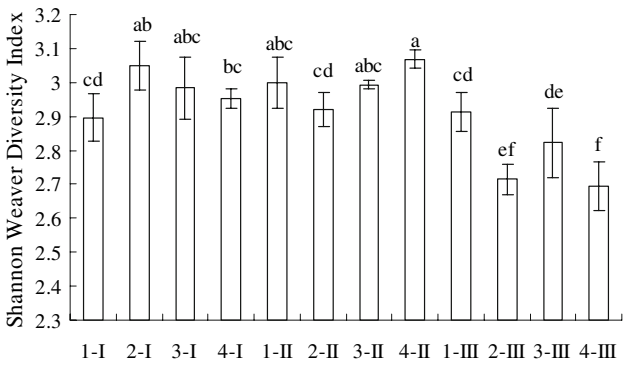

Fig. 2 Genetic diversity index of microbial communities in soil samples under long-term cadmium stress. I: 0–10 cm; II: 10–20 cm; III: 20–30 cm. Error bars (n = 3) indicate standard deviations

In short-term Cd exposure experiments, however, greater changes in soil bacterial community structure were observed with increasing Cd^{2+} concentration (Fig. 3a), suggesting the significance of cadmium toxicity in short-term shifts in microbial community. DGGE analysis showed that one bacterial population present in all sampling sites prior to cadmium addition, represented by band E, disappeared completely following the addition of Cd^{2+}. In contrast, new bacterial populations represented by bands A, C, D, and F emerged with the amendment of 1 or 2 mM Cd^{2+}. When Cd^{2+} level increased to 4 mM, additional bacterial populations (bands B and G) emerged, while other populations (bands C, D and A) in some soil samples

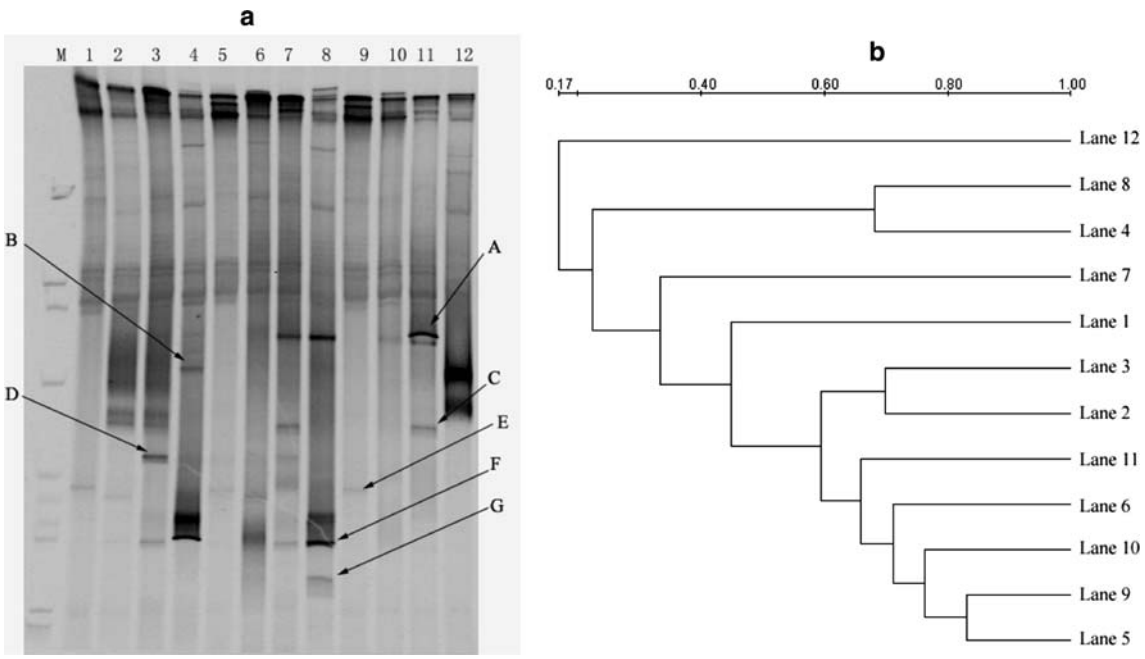

Fig. 3 DGGE fingerprints of soil bacterial community under short-term cadmium stress with different concentrations of cadmium (**a**) and UPGMA dendrograms constructed from DGGE profiles (**b**). Lane 1–4: Site 1 under the stress of 0, 1, 2 and 4 mM Cd^{2+}; Lane 5–8: Site 3 under the stress of 0, 1, 2 and 4 mM Cd^{2+}; Lane 9–12: Site 4 under the stress of 0, 1, 2 and 4 mM Cd^{2+}

diminished. Cluster analysis further indicates that soil cadmium levels controlled soil microbial community structures, as greater similarities were found in soil samples received the same level of Cd treatment (Fig. 3b). Evidently, the presence of cadmium played a key role in short-term changes in soil microbial community.

Moreover, microbial genetic diversity as measured by the Shannon–Weaver index was considerably higher in soil samples exposed to short-term cadmium stress than those of the controls received no cadmium addition (Fig. 4). More specifically, under mild Cd stress conditions (cadmium level <2 mM), higher soil bacteria diversity indices (Site 1 and 3) were found with increasing Cd^{2+} concentrations. In contrast, microbial genetic diversity was significantly reduced under severe Cd stress condition (cadmium level at ~4 mM); however still higher as compared to those in controls. Thus in general, mild cadmium stress resulted in higher soil microbial genetic diversity, which was subsequently lowered under more severe cadmium stress.

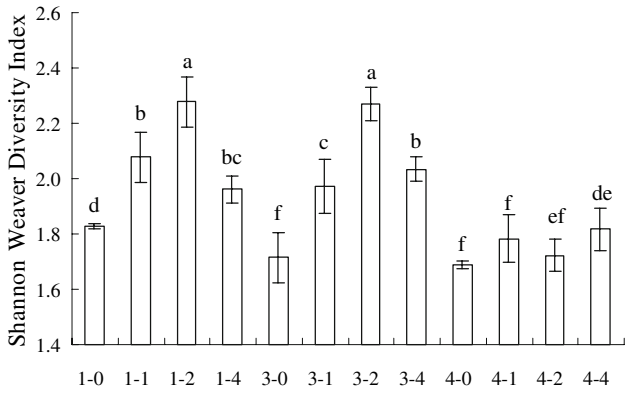

Fig. 4 Dynamic changes of bacterial genetic diversity under short-term cadmium stress with different Cd concentrations. 1-0 to 1–4: Site 1 under the stress of 0, 1, 2 and 4 mM Cd^{2+}; 3-0 to 3-4: Site 3 under the stress of 0, 1, 2 and 4 mM Cd^{2+}; 4-0 to 4-4: Site 4 under the stress of 0, 1, 2 and 4 mM Cd^{2+}. Different letters indicate significant differences by Fisher's LSD ($p < 0.05$)

To further identify the microbial populations with significant changes in abundance under short-term cadmium stress, DNA in selected DGGE bands was purified and sequenced (Fig. 3a). Sequence analysis showed that the sequences of band A and B were 99% identical to those of *Burkholderia cepacia* strain ORS 3307 (EF054874) and *Ralstonia campinensis* strain WS2 (AF312020), respectively. The sequences of band C, D, G and F had 100% identity with those of *Bacillus* sp. m2-38 (DQ923226), *Bacillus* sp. 02Co-2 (DQ784775), *Arthrobacter* sp. DiSca5 (EF195091), and *Arthrobacter aurescens* strain 51 (AF388032), respectively. Interestingly, all of these microorganisms represent metal-resistant bacteria isolated from heavy metal polluted environments (Goris et al. 2001; Macur et al. 2004). The DNA sequence of B and E, which disappeared under cadmium stress, was found to be 98% identical to that of *Pontibacter* sp. z2 (DQ888331). Phylogentic analysis of sequences derived from the DGGE bands (Fig. 5) shows that the species represented by DGGE bands were clustered primarily into four groups: *β-Proteobacteria* (band A and B), low G + C Gram-positive *Firmicutes* (band C and D), *Bacteroidetes* (band E), and high G + C Gram-positive *Actinobacteria* (band F and G), suggesting the potential importance of Gram-positive bacteria and *β-Proteobacteria* in metal resistance.

Our findings in the short-term cadmium exposure study show that mild Cd stress resulted in the highest microbial genetic diversity, which is in good agreement with the "hump-back" model proposed by Giller et al. (1998) to describe the relationship between species diversity and environmental stress. In our study, the increased genetic

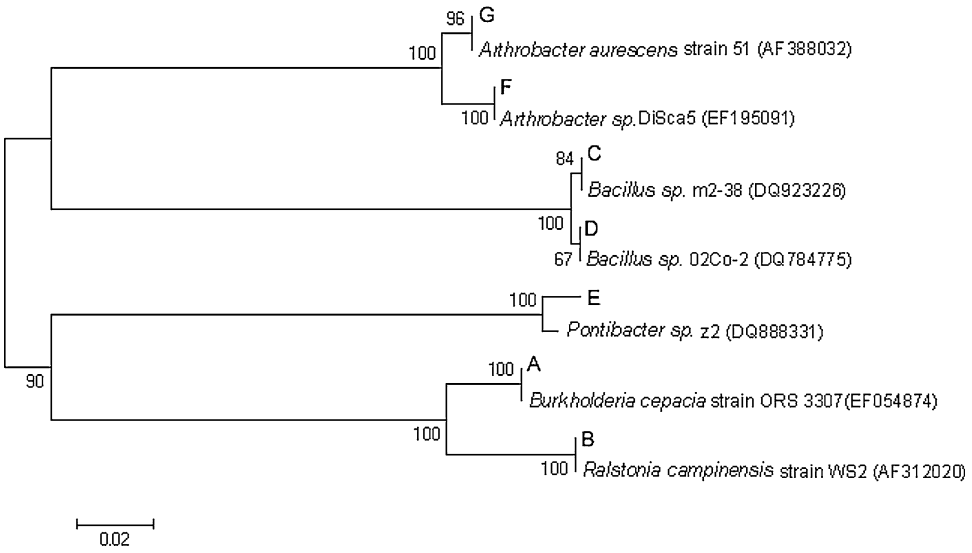

Fig. 5 Neighbour-joining phylogenetic tree of bands recovered from DGGE gel. The numbers at the nodes indicate the percentages of occurrence in 1,000 bootstrapped tree. The GenBank accession numbers of previously published sequences are given in parentheses

diversity of soil bacteria (Site 1 and 3) under mild cadmium stress might be attributed to the enrichment of metal-resistant microbial populations through mechanisms of competitive selection and genetic adaptation, such as lateral gene transfer (Coombs and Barkay 2004). In contrast, severe cadmium stress might present a condition that fewer microbial populations could survive, thus leading to reduced microbial genetic diversity.

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