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Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants

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ABSTRACT

Thirteen copper-resistant bacteria were isolated from copper-tolerant plant species growing on a copper mine wasteland. The isolates were identified by 16S rRNA gene sequence analysis and characterized by their resistance to heavy metals and plant growth-promoting characteristics. The assessment of the bacterial communities in the rhizosphere soils of copper-tolerant plants was measured as bands in denaturing gradient gel electrophoresis (DGGE) obtained directly from rhizosphere soil DNA extracts. The isolates were found to exhibit different multiple heavy metal resistance characteristics. Strains SZY6, YJ7 and JYC17 were found to produce indole acetic acid (IAA), siderophore, 1-aminocyclopropane-1-carboxylate (ACC) deaminase or to solubilize phosphate. Root elongation assay conducted on rape under gnotobiotic conditions with strains MT16, JYC17, SZY6, GZC24, and YJ7 demonstrated increase (from 16 to 41%) in root elongation of inoculated rape seedlings compared to the control plants. In the rhizosphere soil samples the DGGE profiles of the direct DNA extracts were similar. The DGGE profiles indicated that there was no significant correlation between the concentration of available copper in the rhizosphere soils and the number of the visible bands in the DGGE pattern.

1. Introduction

Copper (Cu) tailings, produced from extraction and processing of copper ores, may cause severe damage to ecosystems including plants, animals, microorganisms and human health (Kim et al., 2003; Wong, 2003; Haque et al., 2008). Ecological remediation of polluted sites has received much attention around the world because it provides an ecologically sound and safe method for restoration and remediation (Wu et al., 2006). Improvement of the beneficial associations between microorganisms and plants, particularly in the rhizosphere, is an area of research of global interest. Currently, there are a number of reports available which describe metal-accumulating plants that are used in the removal of toxic metals from soil (Belimov et al., 2005; Rajkumar et al., 2006; Zaidi et al., 2006). Elevated levels of heavy metals, however, lead to impaired metabolic activity and result in reduced plant growth. The interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals, rendering microorganisms an important component of phytoremediation technology (Glick, 2003). Rhizosphere microorganisms can enhance biomass production and tolerance of plants to heavy metals in stress environment (Sheng and Xia, 2006; Dell'Amico et al., 2008). Bacteria may promote plant growth by producing siderophore, synthesizing phytohormones and enzymes or solubilizing phosphate (Verma et al., 2001). The production of ACC deaminase, an enzyme that modulates ethylene levels in plants (Glick et al., 1998), may further contribute to the heavy metal tolerance of plants. It has been reported that ACC-utilizing bacteria could promote plant growth and protect plants against heavy metals toxicity in heavy metal-contaminated soils (Burd et al., 2000; Belimov et al., 2005; Madhaiyan et al., 2007). Although microbial communities in metal-polluted bulk soils have been studied (Ellis et al., 2003; Piotrowska-Seget et al., 2005; Lorenz et al., 2006; Liao and Xie, 2007), little attention has been paid to the microbial community of rhizosphere soils of plants growing on metal-contaminated sites (Khan, 2005; Dell'Amico et al., 2005).

In recent years, new technologies developed in molecular biology have overcome the culturing process and not only can analyze the cultured microorganisms but also can analyze the uncultured ones. Those methods employ PCR amplification of microbial 16S rRNA and then analyze the microflora multiformity by denaturing gradient gel electrophoresis (DGGE) technology. At present, PCR-DGGE is widely used on samples from soil (Muyzer et al., 1993; Ellis et al., 2003; de Oliveira et al., 2006). Analysis of the genotypic and phenotypic characteristics of indigenous rhizosphere bacteria can help to clarify the mechanism of interactions between them and plant roots.

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In this study with culture-dependent and culture-independent techniques, we investigated Cu-resistant bacteria from rhizosphere soils of Cu-tolerant plants growing on copper mine wasteland. The objectives were to isolate and characterize the isolates, with regard to their metal resistance and their plant growth-promoting characteristics and to analyse the bacterial communities in the rhizosphere soils of Cu-tolerant plants by direct PCR-DGGE analysis on the basis of DNA extracts. The isolated bacteria with multiple heavy metal resistance and plant growth-promoting characteristics may be used for plant growth promotion and Cu phytoremediation in Cu-contaminated soils.

2. Materials and methods

2.1. Sampling of rhizosphere soils

Due to the heavily Cu-contamination in the yellow brown soil (Alfisols), plant growth was severely inhibited, which led to sparse vegetation in this copper mine wasteland (32°04′N, 119°05′E) located in the suburb of Nanjing, China. Eleven healthy heavy metal-tolerant plants including the rhizosphere soils were randomly collected from the copper mine wasteland and the soil adhering loosely to the roots was removed by shaking the plants. The soil firmly adhering to the root, designated as rhizosphere soil, was collected by brushing. The fresh moist soil samples were passed through a 2 mm sieve and well dispersed. The properties of the soils were shown in Table 1. A portion of the soil samples was air dried for chemical analyses. Soil pH was measured in distilled water with a ratio of soil-to-solution of 1:2.5. The concentrations of total and NH_4OAc-extractable-copper, lead, and cadmium in the rhizosphere soils of the plants were shown in Table 2. Soil total heavy metals were extracted with HF–$HClO_4$ (SSICA, 1980). Extractable heavy metals were extracted with 1 M NH_4OAc (SSICA, 1980). The above heavy metal concentrations in the extracts were determined with atomic absorption spectrometer (AAS) (TAS-986, Beijing, China). The sampling time was October 2006 at the flowering or bearing stage of the plants. The plants including the rhizosphere soils were immediately transferred in polyethylene bags and transported to the laboratory for the analysis of the bacterial communities in the rhizosphere soils.

Table 1
The basic properties of the rhizosphere soils.

Soil sample no.	Organic matter ($g\,kg^{-1}$)	pH	Total N ($g\,kg^{-1}$)	Available P ($mg\,kg^{-1}$)	Available K ($mg\,kg^{-1}$)
1	4.53	6.57	0.95	13.05	12.43
2	2.21	6.66	0.31	5.78	9.05
3	4.12	6.12	1.09	18.53	32.66
4	10.10	6.46	0.22	17.93	18.80
5	1.58	6.74	0.43	6.76	18.95
6	1.99	6.65	0.97	4.64	13.91
7	8.55	6.31	0.93	12.33	17.66
8	4.74	6.44	0.64	18.72	20.47
9	2.26	6.46	0.52	8.62	19.92
10	3.36	6.66	1.01	8.66	25.11
11	1.37	6.73	0.75	9.64	24.12

The values in table are means of values of three replications.

2.2. Culturable bacterial counts and isolation of copper-resistant bacteria from rhizosphere soils

Rhizosphere soils (1.0 g) were added to Erlenmeyer flasks containing 100 ml of sterile physiological salt solution and shaken at 180 rpm for 30 min. Then, serial 10-fold dilutions of soil suspensions were plated onto sucrose-minimal salts low-phosphate (SLP) agar (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.05%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) to determine total culturable bacteria. SLP agar amended with 0.8 mM of Cu as $CuSO_4{\cdot}5H_2O$ was used for counts of Cu-resistant bacteria. To inhibit fungal growth, the media were supplemented with 10 mg fungicidin (USP, Amresco, USA) per litre after autoclaving. Plates were incubated for 7 days at 28 °C prior to counting. Cu-resistant colonies were purified on the same media by streaking three to four times in the fresh media. Thirteen bacterial isolates showing different morphological appearance and Cu-resistance (3.1 mM) on agar media were selected and stored on slants.

2.3. Characteristics of the isolated bacterial strains

2.3.1. IAA, siderophore and ACC deaminase production

The tested culturable bacterial strains were grown in Luria–Bertani's (LB) medium. Cells in the exponential phase were collected by centrifugation at 925 × *g* for 15 min at 6 °C, washed with sterile physiological salt solution, and recentrifuged. Bacterial inoculum was prepared by resuspending pelleted cells in sterile physiological salt solution to get an inoculum density of ca. 10^8 colony forming units (cfu) ml^{-1}. Bacterial suspensions (1 ml) were cultured for 4 days in flasks containing 20 ml of sucrose-minimal salts (SMS) medium (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.2%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) supplemented with 0.5 mg ml^{-1} of tryptophan. After incubation, a 1 ml cell suspension was transferred into a tube and mixed vigorously with 2 ml of Salkowski's reagent (Gordon and Weber, 1951) and allowed to stand at room temperature for 20 min, after which time a pink color developed in the cell suspensions. The absorbance of pink color was read at 530 nm. The IAA concentra-

Table 2
Concentrations ($mg\,kg^{-1}$) of total and NH_4OAc-extractable heavy metals, total and Cu-resistant bacteria (CFU × $10^5\,g^{-1}$) in rhizosphere soils of plants grown in copper mining wasteland.

Sample no.	Corresponding plants	Total			NH_4OAc-extractable			TBC[a]	CRBC[a]
		Cu	Pb	Cd	Cu	Pb	Cd		
1	*Echinochloa crusgalli*	1548 ± 175	32 ± 4.1	6.5 ± 0.7	35 ± 7	0.9 ± 0.1	0.4 ± 0.03	350 ± 38	14 ± 4
2	*Murdannia triquetra*	1457 ± 151	28 ± 2.0	5.0 ± 0.6	28 ± 3	0.9 ± 0.1	0.3 ± 0.02	1000 ± 184	51 ± 14
3	*Mosla chinensis*	2259 ± 151	41 ± 3.8	4.6 ± 0.4	71 ± 9	0.8 ± 0.1	0.3 ± 0.01	600 ± 98	17 ± 5
4	*Dendranthema indicum*	2014 ± 120	34 ± 3.0	4.2 ± 0.3	44 ± 5	1.0 ± 0.2	0.3 ± 0.02	1700 ± 179	94 ± 16
5	*Digitaria sanguinalis*	2100 ± 308	39 ± 5.1	5.3 ± 0.6	63 ± 10	1.1 ± 0.2	0.3 ± 0.02	260 ± 56	54 ± 6
6	*Rumex acetosa*	1656 ± 150	30 ± 2.6	4.5 ± 0.5	46 ± 5	0.9 ± 0.1	0.4 ± 0.05	850 ± 76	30 ± 9
7	*Kummerowia striata*	2205 ± 255	43 ± 3.6	5.6 ± 0.3	63 ± 8	1.2 ± 0.1	0.4 ± 0.03	700 ± 106	45 ± 8
8	*Lactuca indica*	1436 ± 134	21 ± 1.3	4.4 ± 0.3	28 ± 6	1.1 ± 0.2	0.4 ± 0.01	1000 ± 349	7 ± 3
9	*Bidens pilosa*	2016 ± 371	47 ± 4.2	5.1 ± 0.6	74 ± 9	1.2 ± 0.1	0.4 ± 0.03	140 ± 26	17 ± 6
10	*Artemisia lavandulaefolia*	2411 ± 233	37 ± 2.8	4.9 ± 0.4	51 ± 6	1.1 ± 0.2	0.4 ± 0.02	1200 ± 190	41 ± 9
11	*Arthraxon lanceolatus*	1542 ± 293	30 ± 3.3	5.0 ± 0.4	33 ± 5	0.9 ± 0.1	0.4 ± 0.04	1000 ± 126	33 ± 7

[a] TBC: total bacterial counts; CRBC: Cu-resistant bacterial counts.

tion in culture was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

Siderophore secretion by the strains was detected by the "universal" method of Schwyn and Neilands (1987) using blue agar plates containing the dye Chrome azurol S (CAS). Orange halos around the colonies on blue agar were indicative of siderophore excretion.

The ACC deaminase activity of cell-free extracts was determined by monitoring the amount of α-ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC (Saleh and Glick, 2001). The bacterial strains were grown in test tubes containing 10 ml of a liquid BPF medium (Belimov et al., 2005) for 24 h at 30 °C and harvested by centrifugation at 9000 × *g* for 10 min at room temperature. Cell pellets were washed twice with 5 ml of 0.1 M Tris–HCl buffer (pH 7.5), resuspended in 1 ml of SM medium (Belimov et al., 2005) and then 0.5 ml of each suspension was added to 2.5 ml of liquid SMN medium containing 3 mM ACC or 2 mM $(NH_4)_2SO_4$ as a sole source of N. Bacteria were incubated for 24 h at 30 °C, centrifuged as indicated above, resuspended in 1 ml of 0.1 M Tris–HCl buffer (pH 7.5) and centrifuged at 9000 × *g* for 10 min. The pellets were resuspended in 600 μl of 0.1 M Tris–HCl buffer (pH 8.5) and cells were disrupted by the addition of 30 μl of toluene and vigorous vortexing. Cell-free extract (100 μl) was added into 1.5 ml sterile centrifuge tube and add 10 μl of 0.5 M ACC and 100 μl of 0.1 M Tris–HCl buffer (pH 8.5). After reaction of mixtures for 30 min at 30 °C, 1 ml of 0.56N HCl was added, and the mixtures were centrifuged at 14,000 × *g* for 5 min. The mixtures containing no cell suspension or no ACC were used as controls. Then, 150 μl of 0.1% 2,4-dinitrophenylhydrazine in 2N HCl were added to 1 ml of the supernatant. The mixtures were reacted for 30 min at 30 °C, supplemented with 1 ml of 2N NaOH and assayed for α-ketobutyrate via determination of the optical density at 540 nm.

2.3.2. Mineral phosphate solubilizing activity

Bacterial strains were plated onto Petri plates of Pikovskays's agar medium (Zaidi et al., 2006) with 0.5% of tricalcium phosphate as the inorganic phosphate source. The plates were incubated at 28 °C for 72 h, a clear halo around the bacterial colonies indicating the phosphate solubilization capacity of the bacterial strains.

2.3.3. Heavy metal resistance and minimum inhibitory concentrations

Another heavy metal resistance of the isolates was tested using SLP agar medium with the addition of 1 mM Pb ($Pb(NO_3)_2$), 0.2 mM Cd ($CdSO_4$), 0.9 mM ($NiCl_2$), or 3 mM Zn ($ZnSO_4$). SLP agar medium without the metal ions was used as controls. The minimal inhibitory concentration (MIC) for each bacterial isolate for five heavy metals was determined using SLP agar containing Cu, Pb, Cd, Ni, and Zn at concentrations ranging from 0.04 to 6.15 mM. Cultures were incubated at 30 °C for 7 days.

2.4. Root elongation assay on filter paper culture

Based on the IAA, siderophore production and mineral phosphate solubilization or ACCD activities of the bacterial strains, the strain MT16, JYC17, SZY6, GZC24, and YJ7 were used as the test of root elongation assay. The plant root elongation promoting activity of the bacteria was determined using the modified root elongation assay of Belimov et al. (2001, 2005). Bacteria were grown on solid LB medium for 48 h at 28 °C and resuspended to 5×10^7 cells ml^{-1} in sterile distilled water. Six millilitres of the bacterial suspensions or sterile water (uninoculated control) were added to glass Petri dishes with filter paper. Bacterial suspensions and water were supplemented or not with 0.3 mM Cu (final concentration) as $CuSO_4 \cdot 5H_2O$. The seeds of *Brassica napus* variety Qinyou-7 were surface-sterilized with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min, washed with sterile water and aseptically placed on wetted filter paper. Root length of seedlings was measured after incubation of closed Petri dishes for 7 days at 28 °C in the dark. The assay was repeated two times with four dishes (with 10 seeds per dish) for each treatment.

2.5. Identification of the Cu-resistant strains

For the 16S rDNA analysis, genomic DNA was extracted and 16S rDNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, 27 f (5′-GAGTTTGATCACTGGCTCAG-3′) and 1492 r (5′-TACGGCTACCTTGTTACGACTT-3′) (Byers et al., 1998). The PCR mixture (25 μl) contained 1 μl template, 2.5 μl of 10× Taq DNA polymerase buffer, 5 mM $MgCl_2$, 1 μl of dNTP at 2.5 mM, 0.5 μl of 2.5 U Taq DNA polymerase, 3.75 pmol primers (each), and 0.5 μl of 2.5 U Taq polymerase. The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) with a hot starting performed at 94 °C for 3 min, followed by 30 cycles of 94 °C for 0.5 min, 54 °C for 0.5 min, and 72 °C for 1.5 min, followed by a final extension performed at 72 °C for 5 min. The amplification products were purified using a DNA purification kit (Beyotime, China) and sequencing was performed at Nanjing Invitrogen Biotechnology Company, Limited (Nanjing, China). The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program. The 16S rDNA sequences of the strains have been deposited in GenBank. The accession numbers were EU036696–EU036699, EU036702–EU036704, EU081849–EU081850 and EU081853–EU081856 (Fig. 1).

2.6. DNA extraction of rhizosphere soils

Rhizosphere soil DNA was extracted based upon a modification of a method of Zhou et al. (1996). Soil samples (10 g) were mixed with 13.5 ml of DNA extraction buffer (100 mM Tris–HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% hexadecylmethylammonium bromide) and 100 ml of proteinase K (10 mg ml^{-1}) in centrifuge tubes by horizontal shaking at 200 rpm for 30 min at 37 °C. After the shaking treatment, 1.5 ml of 20% SDS were added, and the samples were incubated in a 65 °C water bath for 2 h with gentle end-over-end inversions every 15–20 min. The supernatants were collected after centrifugation at 6000 × *g* for 10 min at room temperature and transferred into 50 ml centrifuge tubes. The soil pellets were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 65 °C for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform–isopentanol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at 4 °C for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 × *g* for 20 min at 4 °C, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 μl. To reduce the likelihood of chimera formation and remove the inhibitors such as heavy metals and humic acids the DNA was then size fractionated by agarose gel electrophoresis and DNA ≥ 20 kb recovered using a TIANgel Midi Purification Kit (TianGen BioTech Co., Ltd., Beijing, China) according to the manufacturer's instructions.

2.7. PCR-DGGE rhizosphere soil bacterial community analysis

Primers F357 and R518 were used in this study for the amplification of bacterial 16S rDNA genes. The GC-clamp described by Muyzer et al. (1993) was added to the forward primer to stabilize the melting behavior of the DNA fragments. PCR reaction

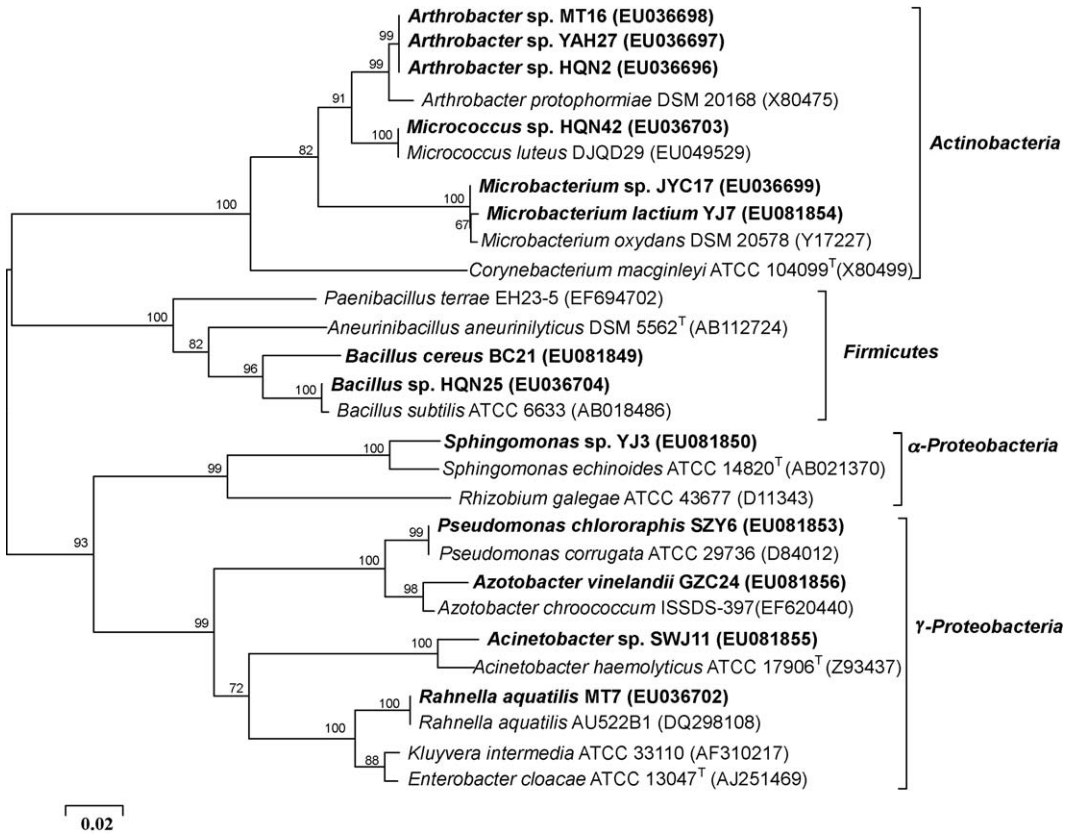

Fig. 1. Phylogenetic tree constructed by the Neighbour Joining method on the basis of partial 16S rDNA sequences of the thirteen Cu-resistant bacterial strains. Bootstrap numbers indicated the value of 1000 replicates. GenBank accession numbers are given in parentheses.

was performed in a PTC-200 DNA Engine Cycler (Bio-Rad, USA) in 0.2 ml tubes using 50 μl reaction volume. The reaction mixture contained 5 μl of 10× buffer, 5 μl of 50 mM $MgCl_2$, 4 μl of each 2.5 mM dNTP, 1 μl of 10 μM of each primer, 2.5 U Taq polymerase, and 1 μl of template. Cycling conditions used to amplify the 16S rDNA gene fragment were 94 °C for 3 min, followed by 20 cycles of 94 °C for 0.5 min, 65 °C (reduced by 0.5 °C each cycle) for 0.5 min, and 72 °C for 0.5 min; 10 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 0.5 min; and a final extension at 72 °C for 5 min. 5 μl of the PCR products were checked by electrophoresis in 1.5% agarose gels stained with ethidium bromide prior to DGGE. For DGGE analysis, PCR products generated from each sample were separated on an 8% acrylamide gel with a linear denaturant gradient range from 38 to 60% using the Bio-Rad DCode System. DGGE was performed using 20 μl of the PCR product in 1× TAE buffer at 60 °C, 200 V for 10 min, then 85 V for 12 h (Zoetendal et al., 1998). Gels were stained with silver staining (Sanguinetti et al., 1994) and the gels were scanned with gel photo GS-800 system (Bio-Rad, USA).

2.8. Analysis of DGGE patterns

Digitized DGGE images were analyzed with Quantity One image analysis software (Molecular Analyst 1.61, Bio-Rad, USA). This software identifies the bands occupying the same position in the different lanes of the gel. The numbers of bands were used as a measure of bacterial diversity of the community.

2.9. Statistical analysis

Analysis of variance (ANOVA) and the post hoc Fisher least significant difference test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 10.0.

3. Results

3.1. Soil heavy metal analysis and bacterial counts

The concentrations of total Cu, Pb and Cd ranged from 1436 to 2411, 21 to 47 and 4.2 to 6.5 mg kg^{-1}, respectively, and the NH_4OAc-extractable Cu, Pb and Cd ranged from 28 to 74, 0.8 to 1.2 and 0.3 to 0.4 mg kg^{-1}, respectively (Table 2). Positive correlations between the total and NH_4OAc-extractable Cu and Cd were found with the correlation coefficients of 0.98 and 0.66, respectively. A significant variation ($p < 0.05$, $n = 33$) in total (from 1436 to 2411 mg kg^{-1}) and NH_4OAc-extractable (from 28 to 74 mg kg^{-1}) Cu in rhizosphere soils was found. A little variation in total and NH_4OAc-extractable Pb and Cd in rhizosphere soils was also found. In Table 2 we report the averages of CFUs of heterotrophs and Cu-resistant bacteria of rhizosphere soils. The total bacterial counts were from 140×10^5 (sample 9) to 1700×10^5 (sample 4) CFU g of rhizosphere $soil^{-1}$. The Cu-resistant bacterial counts were from 7×10^5 (sample 8) to 94×10^5 (sample 4) CFU g of rhizosphere $soil^{-1}$. The Cu-resistant bacterial counts accounted for 0.7–21% of the total bacterial counts.

3.2. Isolation and identification of the copper-resistant bacteria

We have isolated Cu-resistant bacteria from rhizosphere soils of plants grown in heavy metal-contaminated soil by using a spread plate procedure with pH-neutral SLP medium. This low-phosphate medium is designed to avoid the precipitation of heavy metal salts at 0.78 mM (the concentration of water-soluble Cu in the medium

Table 3
Characteristics of the isolated Cu-resistant bacterial strains.

Strain	IP ($mg\,l^{-1}$)	SP	ACCDA ($\mu M\,\alpha$-KB $mg^{-1}\,h^{-1}$)	PS	MIC (mM)				
					Cu	Pb	Cd	Ni	Zn
HQN2	50.5 ± 1.73a	+	0	−	4.7	2.9	0.27	1.03	2.31
HQN25	0.7 ± 0.13d	+	0	−	3.9	1.2	0.09	0.34	0.77
HQN42	0	−	0	+	3.9	3.4	0.27	1.03	1.54
BC21	3.2 ± 0.36c	+	0	−	3.9	1.5	0.09	1.03	3.85
SZY6	16.7 ± 1.30b	−	30.6 ± 3.44a	+	4.7	2.9	0.27	0.34	6.15
YJ3	47.8 ± 2.08a	−	0	+	3.9	0.5	0.09	0.34	0.77
YJ7	0.9 ± 0.20d	+	27.1 ± 1.49a	+	3.9	0.5	0.04	0.34	0.77
SWJ11	4.6 ± 0.78c	+	0	−	5.5	2.9	0.45	1.03	4.62
MT7	0.9 ± 0.16d	−	0	+	3.9	0.5	0.09	0.34	3.85
MT16	2.8 ± 0.40c	+	0	+	3.9	2.4	0.27	1.03	3.85
JYC17	3.3 ± 0.96c	+	8.0 ± 1.32b	+	4.7	2.9	0.45	1.03	1.54
GZC24	0	−	29.7 ± 2.91a	+	4.7	1.9	0.27	1.03	6.15
YAH27	16.0 ± 1.73b	+	0	−	5.5	4.4	0.45	1.03	6.15

Values of IP and ACCDA are the mean ± standard deviation of three replicates. Within each vertical column, values followed by the same letter are not statistically different from each other ($p < 0.05$). +, positive; −, negative; IP, indole production; SP, siderophore production; ACCDA, ACC deaminase activity; PS, phosphate solubilization.

was 0.78 ± 0.09 mM). Thirteen Cu-resistant bacterial isolates were identified as *Arthrobacter* sp. HQN2, *Bacillus* sp. HQN25, *Micrococcus* sp. HQN42, *Bacillus cereus* BC21, *Pseudomonas chlororaphis* SZY6, *Sphingomonas* sp. YJ3, *Microbacterium lactium* YJ7, *Acinetobacter* sp. SWJ11, *Rahnella aquatilis* MT7, *Arthrobacter* sp. MT16, *Microbacterium* sp. JYC17, *Azotobacter vinelandii* GZC24, and *Arthrobacter* sp. YAH27 based on the 16S rRNA gene sequence analysis, respectively (Fig. 1). The similarity of each strain with known bacterial 16S rRNA sequences present in the database was 98% for strains HQN42 and MT7, 99% for strains HQN2, HQN25, YJ3, SWJ11, JYC17, and YAH27, and 100% for strains BC21, SZY6, YJ7, MT7, and GZC24.

3.3. Plant growth promoting and metal-resistant characteristics of the bacterial isolates

Plant growth-promoting traits were assessed on the thirteen Cu-resistant isolates from different rhizosphere soils. Salkowski's reaction of culture supernatants revealed that most isolates were able to produce IAA from tryptophan with variations in the amounts produced. The IAA produced by the cultures ranged from 0.7 $mg\,l^{-1}$ in strain HQN25 to 50.5 $mg\,l^{-1}$ in strain HQN2. Eight isolates proved positive for siderophore production and had the ability to solubilize phosphates (Table 3). In addition, the isolates (SZY6, YJ7, JYC17, and GZC24) from rhizosphere soils of *Commelina communis*, *Rumex acetosa*, *Kummerowia striata*, and *Bidens bipinnata*, respectively show ACC deaminase activity (from 8.0 to 30.6 $\mu M\ \alpha$-KB $mg^{-1}\,h^{-1}$).

The bacterial isolates show the different resistance to the tested heavy metals (Table 3). The strains YJ3 and YJ7 were only resistant to Cu, but strains SWJ11, MT16, GZC24 and YAH27 could be resistant to Cu, Pb, Cd, Ni, and Zn.

3.4. Root length promotion

The effects of the Cu-resistant bacterial strains on root elongation of *B. napus* variety Qinyou-7 in the absence or presence of Cu is shown in Table 4. With the addition of 0.31 mM Cu to the filter paper culture inhibited root elongation of uninoculated seedlings by 14% ($p < 0.05$). The tested strains increased the root length of the seedlings in the absence or presence of Cu. Inoculations with strains MT16, JYC17, SZY6, GZC24, and YJ7 could significantly increase the root length of Cu-treated and Cu-untreated seedlings by 17–38 and 20–41%, respectively. The maximum root length-promoting effect on Cu-treated plants was observed after inoculation with strain MT16.

3.5. Molecular analysis of the bacterial communities of rhizosphere soils

Bacterial DGGE profiles generated from the universal bacterial primers (F357 and R518) revealed the structural composition of communities in soil samples (Fig. 2a). Each of the distinguishable bands in the separation pattern represents an individual bacterial group. As shown in Fig. 2, soil sample 4 showed the most complex DGGE pattern with thirteen visible bands, indicating the presence of a high number of different bacterial taxa. Soil sample 9 showed the simplest DGGE pattern with six visible bands. Five shared bands (marked with frame in Fig. 2a) were found in the DGGE profile, indicating that these bacteria may have colonized widely during the long-term deposition of heavy metals in the soil.

Clustering of the profiles revealed the differences among the eleven rhizosphere soil samples (Fig. 2b). The greatest difference was found between the profiles of sample 9 and all other samples.

Table 4
Root length of *Brassica napus* variety Qinyou-7 seedlings inoculated with heavy metal-resistant bacteria and grown in the absence or presence of Cu.

Bacterial strains	Untreated seedlings		Treated with 0.31 mM Cu	
	Root length (mm)	Bacterial effect[a] (%)	Root length (mm)	Bacterial effect (%)
Uninoculated control	50.3 ± 3.2b		44.3 ± 2.1c	
MT16	71.0 ± 4.0a	+41	61.3 ± 4.0a	+38
JYC17	60.3 ± 5.6a	+20	52.7 ± 2.1b	+19
SZY6	62.3 ± 3.5a	+24	53.0 ± 4.2b	+20
GZC24	61.3 ± 3.1a	+22	51.7 ± 2.8b	+16
YJ7	64.7 ± 3.6a	+29	60.0 ± 1.0a	+35

Values are the mean ± standard deviation of three replicates. Within each vertical column, values followed by the same letter are not statistically different from each other ($p < 0.05$).
[a] The effect of heavy metal-resistant bacteria on the root growth of the rape.

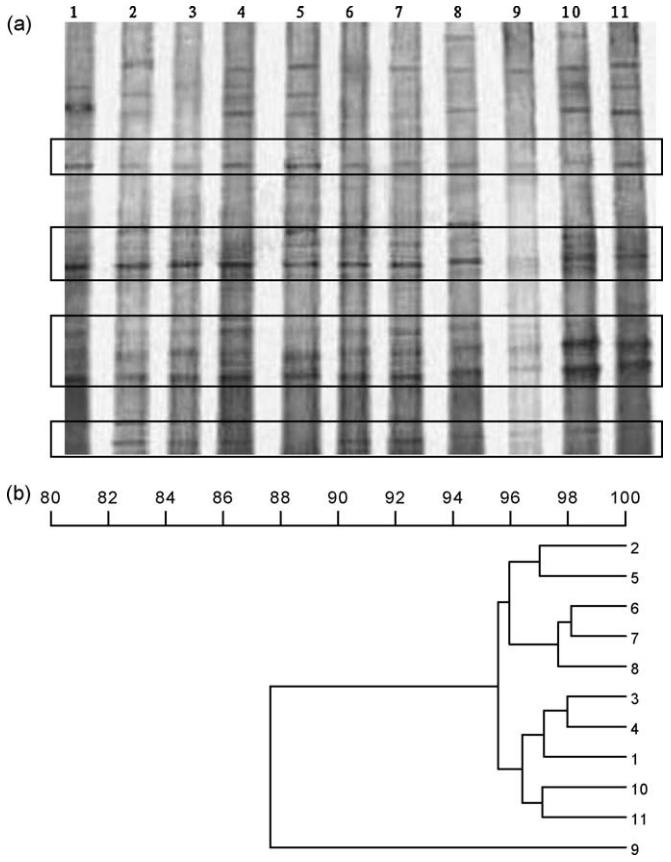

Fig. 2. Analysis of the composition of the bacterial community in eleven rhizosphere soil samples of plants grown in Cu-contaminated soil. (a) DGGE profiles of the eleven rhizosphere soil samples; (b) similarity of the eleven rhizosphere soil samples based on UPGMA clustering method.

The profile of sample 9 clustered apart from the others, with the similarity of about 88%. The profiles of the other ten samples were separated into two major clusters; profiles of samples 2, 5, 6, 7 and 8 into one and profiles 3, 4, 1, 10 and 11 into another group. However, all the rhizosphere soil samples except sample 9 showed the high similarity (over 95%).

Subsequently, DGGE gels were interpreted based on the numbers of DGGE bands. DGGE profiles indicated that there was no significant correlation ($r = -0.778$, $n = 6$) between the concentration of copper in the rhizosphere soils and the numbers of visible bands. There was significant correlation ($r = 0.834$, $n = 6$) between DGGE pattern and soil organic matter. High content of organic matter in the soil (soil samples 4 and 7) showed the presence of a high numbers of visible bands (Table 1, Fig. 2).

4. Discussion

Even metals exert their toxic effects on microorganisms through various mechanisms, metal-tolerant bacteria could survive in these habitats and could be isolated and selected for their potential application in bioremediation of contaminated sites (Piotrowska-Seget et al., 2005). Although the assessment of bacterial community composition in the rhizosphere of a Cu-accumulator, *Elsholtzia splendens*, has been made in the Cu-added soil (Wang et al., 2008), to date, no investigation has been made into the effects of Cu pollution on the rhizosphere soil bacterial communities in copper-tolerant plants. Such an investigation would be beneficial in informing remediation strategies in copper mining wasteland and remediation efforts in other copper-polluted sites. In the rhizosphere soils of the tested eleven Cu-tolerant plants, there were no significant change in the NH_4OAc-extractable Pb and Cd (Table 2), but the NH_4OAc-extractable Cu changed significantly (Table 2) which may exert main toxic effects on rhizosphere soil microorganisms. Cu-resistant bacteria could be isolated from the rhizosphere soils of the tested plants, indicating that these bacteria populations had a marked adaptation to heavy metal under constant metal stress for a long time (Table 2). In the present study, we have isolated thirteen rhizosphere bacteria which could be resistant to high level of Cu. 16S rRNA gene sequence analysis showed that the bacteria covered nine different genera. The genera *Bacillus*, *Micrococcus*, *Arthrobacter*, *Sphingomonas*, and *Microbacterium* were common metal-tolerant Gram-negative and Gram-positive bacteria (Ellis et al., 2003; Piotrowska-Seget et al., 2005; Abou-Shanab et al., 2007).

Our bacterial strains showed different degree of resistance to heavy metals, strains YJ3 and YJ7 could only be resistance to Cu, however, strains (SWJ11, MT16, GZC24 and YAH27) could be co-resistance to heavy metals such as Cu, Pb, Cd, Ni and Zn (Table 3). It has been reported that plant growth-promoting bacteria could promote the growth and heavy metal uptake of plants (Madhaiyan et al., 2007; Rajkumar and Freitas, 2008). In the study, a large portion of strains were possessed with plant growth-promoting characteristics (Table 3). Some strains could solubilize phosphate and contain ACC deaminase activity. Bacteria having the characteristics of producing IAA, siderophores, ACC deaminase, and inorganic phosphate solubilization may have the potential for the plant growth promotion and heavy metal accumulation as shown in the root elongation assay which the metal-resistant bacterial strains (MT16, JYC17, SZY6, GZC24 and YJ7) facilitates rape growth even under toxic Cu concentration (Table 4) (Dell'Amico et al., 2005, 2008; Zaidi et al., 2006; Nair et al., 2007; Jiang et al., 2008).

This study focuses on obtaining a comprehensive picture of the rhizobacterial community of the Cu-tolerant plants growing on a copper mining wasteland. The copper mining wasteland had been subjected to heavy metal pollution over a long time period. Although contamination of soil with heavy metals affects the qualitative and quantitative structure of microbial communities, resulting in decreased metabolic activity and diversity (Giller et al., 1998), both the cluster analysis and the DGGE pattern showed no significant effect of Cu in the soil on the soil bacterial diversity (Fig. 2). Using DGGE, Renella et al. (2004) also showed that long-term exposure with Cd had little effect on soil bacterial diversity. The DGGE profiles obtained directly from the DNA of rhizosphere soils of the tested Cu-tolerant plants were very similar, indicating similarity in bacterial species composition and that several dominant groups were relatively stable in the tested rhizosphere soils. As shown in Table 2, soil sample 9 contains the highest amount of extractable copper which may exert the highest toxic effects on rhizosphere soil microorganisms, resulting in the simplest DGGE pattern with only six visible bands.

5. Conclusion

This study has demonstrated that different bacterial communities live in association with rhizosphere soils and are able to withstand high Cu concentrations. Over a long-term exposure with Cu, the bacterial communities in the eleven rhizosphere soils of different plants growing in the copper mining wasteland soil had exhibited similarity. The rhizobacteria were found to have plant growth-promoting characteristics that can potentially support heavy metal uptake and reduce stress symptoms in plants. Several studies have evidenced that heavy metal-resistant and plant growth-promoting bacteria can protect plants from the toxic effects of metals, or even enhance metal uptake by hyperaccumulator plants (Burd et al., 2000; Dell'Amico et al., 2005; Jiang et al., 2008). The combination of both culture-independent and

culture-dependent techniques might provide useful and complementary information on the structure of microbial communities (Edenborn and Sexstone, 2007). Future work will address the effect of selected rhizosphere bacteria on plant growth and the revegetation and phytoremediation of Cu-contaminated soils as well as the mechanisms involved.

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