J Soils Sediments (2015) 15:1191–1199
DOI 10.1007/s11368-015-1067-9

SOILS, SEC 5 • SOIL AND LANDSCAPE ECOLOGY • RESEARCH ARTICLE

Plant growth-promoting rhizobacteria enhance the growth and Cd uptake of *Sedum plumbizincicola* in a Cd-contaminated soil

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Received: 15 October 2014 / Accepted: 13 January 2015 / Published online: 28 January 2015

Abstract
Purpose This study aimed to isolate plant growth-promoting rhizobacteria (PGPR) that exhibit heavy metal resistance to examine their influence on Cd uptake and soil microbial community structure during phytoremediation.
Materials and methods Heavy metal-tolerant PGPR were isolated from the roots of possible hyperaccumulators using plates with 1-aminocyclopropane-1-carboxylate (ACC) as sole nitrogen source. Minimal inhibitory concentrations (MICs) of each isolate were determined by the plate dilution method. The impacts of isolated PGPR on the growth and Cd accumulation of *Sedium plumbizincicola* were conducted in a pot experiment. In addition, the effect of PGPR inoculation on the microbial community during phytoextraction by *S. plumbizincicola* was studied by *454* pyrosequencing.
Results and discussion A total of nine Cd-resistant strains were isolated from the roots of Cd accumulators, and their plant growth-promoting activities were characterized. Isolates were able to produce indole-3-acetic acid (IAA) (28–133 mg L^{-1}) and solubilize phosphate (65–148 mg L^{-1}). In a pot experiment, the inoculation of isolates NSX2 and LCR1 significantly enhanced the growth of and uptake of Cd by the Cd hyperaccumulator *S. plumbizincicola*. *454* pyrosequencing revealed that the inoculation of the PGPR lead to a decrease in microbial community diversity in the rhizopshere during phytoextraction. Specifically, indigenous heavy metal-tolerant PGPR such as *Actinospica*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*, and *Mycobacterium* were selectively enriched in the treatments in which PGPR were added. It is suggested that a unique constitution of microbial communities in inoculated treatments plays a key role in enhancing Cd phytoremediation.
Conclusion Inoculation of strains *Rhodococcus erythropolis* NSX2 and *Cedecea davisae* LCR1 could promote *S. plumbizincicola* growth and enhance the remediation efficiency. The introduced PGPR could also affect the indigenous microbial community structure and the diversity in Cd-contaminated soil during phytoremediation.

Keywords Cd contamination · Hyperaccumulator · Microbial community · PGPR · Phytoextraction · *Sedum*

Responsible editor: Jizheng He

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1 Introduction

Heavy metal contamination of soil and water poses a serious threat to both ecosystems and human health worldwide. Among heavy metals, cadmium (Cd) is considered as one of the most toxic contaminants in soils due to its high mobility and low permissible exposure limit (Sheng et al. 2008). Conventional remediation methods for metal remediation such as soil washing, stabilization, acid leaching, ion

exchange, or electrochemical processes are costly, inefficient, and often have a negative impact on soil biological system (Lasat 2002; Wu et al. 2012). Over the past two decades, phytoremediation has been used as a cost-effective and environmentally friendly method for the remediation of slightly or moderately contaminated soils (Wuana and Okieimen 2011). The success of phytoremediation depends on the potential of the plants to produce high biomass yield while withstanding the metal stress (Rajkumar et al. 2012). However, many heavy metal-accumulating species used in phytoremediation are limited by slow growth and small biomass yields which restricts large-scale remediation. To date, more than 400 species of metal hyperaccumulator plants have been extensively studied for metal phytoextraction (Boularbah et al. 2006). *Sedum plumbizincicola* has been confirmed as a hyperaccumulator plant because of its remarkable capacity to withstand the metal stress in polluted soils and cadmium (Cd) and extract zinc (Zn) from polluted soils in south and east China (Wu et al. 2008).

Apart from heavy metal toxicity, plant growth may be limited in contaminated soils due to environmental stresses including drought, salinity, and nutrient deficiency. Overcoming such environmental stresses and the promotion of plant growth is essential for the optimum performance of phytoremediation. Plant growth-promoting rhizobacteria (PGPR) are a group of beneficial rhizosphere bacteria which can enhance the tolerance of plants against toxicity and promote plant growth through the production of plant growth-promoting (PGP) factors such as siderophores, indole-3-acetic acid (IAA), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007). Therefore, the application of PGPR is a promising approach to improving the growth of heavy metal hyperaccumulators and the efficiency of phytoremediation.

Several studies have demonstrated that the inoculation of PGPR can improve the growth and heavy metal uptake of hyperaccumulators (Guo and Chi 2014). The survival of introduced PGPR and subsequent changes to the rhizopshere-associated microbial community structure during phytoremediation of heavy metal-contaminated soils are considered to be key factors for successful phytoremediation (Jeong et al. 2013; Piromyou et al. 2011). Recently, molecular biology techniques (e.g., T-RFLP and PCR-denaturing gradient gel electrophoresis (DGGE)) have been applied to investigate the interactions between the heavy metal-resistant PGPR and the plant in heavy metal-contaminated soils (Chen et al. 2013). However, these techniques do not provide high-resolution taxonomic information and focus on the most abundant members of the microbial population. Without high-resolution taxonomic insight, important small-scale community components may be missed. Recent advances in sequencing technology, such as pyrosequencing, and its application in evaluating bacterial diversity allows greater levels of in-depth analysis of the microbial community that overcomes some of these problems. However, the response of a rhizosphere-associated microbial community of a heavy metal hyperaccumulator to an introduced PGPR during phytoremediation of heavy metals, to our knowledge, has not been investigated using the pyrosequencing method.

The objectives of the present study were to (1) isolate heavy metal-tolerant PGPR which can utilize ACC as sole nitrogen source; (2) evaluate PGP traits of the isolated strains; (3) evaluate the impacts of isolated PGPR on the growth and Cd accumulation of *S. plumbizincicola*, a Cd hyperaccumulator in a pot experiment; and (4) compare the microbial community structure in soil with and without inoculation of PGPR during Cd phytoextraction.

2 Materials and methods

2.1 Isolation of Cd-tolerant PGPR

Fresh root samples (1 g) were taken from *Sedum arboretum*, *Sedum X Graptosedum*, *Crassula multicava*, and *Carpobrotus rossii* that had been grown for 120 days in 20 mg kg^{-1} Cd-contaminated soils in a glasshouse. These plants are Australian native plant species which had greater shoot biomass production, and *C. rossii* can hyperaccumulate Cd (Zhang et al. 2014). Roots were washed in sterile water before homogenization in 1 mL of sterile water using sterile mortar and pestle. Serial dilutions of this suspension were prepared (10^{-1}–10^{-3}), and 100 μL spread plated onto the SMN medium agar (Belimov et al. 2005) supplemented with 20 mg L^{-1} of $CdCl_2$. The SMN medium pH was adjusted to 7.0 before autoclaving and ACC (0.5 g L^{-1}) was added as the sole nitrogen source after autoclaving. After incubation for 7 days at 30 °C, individual colonies of distinct morphology were isolated through streak plating on the LB medium (Bertani 1954) supplemented with 20 mg L^{-1} of $CdCl_2$.

2.2 Heavy metal resistance of isolates

Minimal inhibitory concentrations (MICs) for each isolate were determined by the plate dilution method as adopted by Malik and Jaiswal (2000). Isolates, capable of utilizing ACC as sole nitrogen source, were grown in sucrose-minimal salt low-phosphate (SLP) medium (Jiang et al. 2008) comprising (g L^{-1}) the following: sucrose 10; $(NH_4)_2SO_4$ 1; K_2HPO_4, 0.5; $MgSO_4$, 0.5; NaCl, 0.1; yeast extract, 0.5; pH 7.2, supplemented with the metal cations: Cd^{2+} ($CdCl_2$); Cu^{2+} ($CuCl_2$); and Zn^{2+} ($ZnCl_2$) at a range of concentrations (0–1000 mg kg^{-1}) when required. Stock solutions of the metal salts were prepared in double deionized water and sterilized by 0.22 μm filter. The SLP agar plates without metals were used as controls. All experiments were conducted in triplicate. Cultures were incubated at 28 °C for 7 days.

2.3 Identification of isolated PGPR

The 16S ribosomal RNA (rRNA) gene was extracted and amplified by PCR as described by Lee et al. (2010) and sequenced at Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). The obtained sequences were compared to the 16S rDNA nucleotide sequences in the GenBank database by BLASTN. Multiple sequence alignment was done using CLUSTAL X software (Thompson et al. 1997).

2.4 IAA and phosphate solubilization assay

Quantitative estimation of IAA was conducted according to Salkowski colorimetric assay (Glickmann and Dessaux 1995). The ability to produce IAA of the strains with and without L-tryptophan was studied for the reason that L-tryptophan is generally considered as an IAA precursor (Mohite 2013). The bacterial isolates were screened for phosphate solubilization ability using Pikovskaya (PVK) broth (Nautiyal 1999) with 5 g L^{-1} Ca_3 $(PO_4)_2$ as the only P source. The solubilized phosphate in the culture was quantified by the ammonium molybdate spectrophotometric method as described by Payne (1994).

2.5 Soil and *S. plumbizincicola* propagation

Soil was collected from the top 0–20 cm of soil from Xiangtan city, Hunan province, China (27° 44′ 10″ N, 112° 56′ 23″ E). The soil was air-dried and passed through a 2-mm sieve and had soil pH (1:2.5 soil/water) 4.70, organic matter 32.1 g kg^{-1}, total N 1.75 g kg^{-1}, total K 9.15 g kg^{-1}, total P 0.37 g kg^{-1}, available N 106 mg kg^{-1}, available P 19.2 mg kg^{-1}, available K 57 mg kg^{-1}, total Cd 0.53 mg kg^{-1}, and total Zn 132 mg kg^{-1}, determined using the routine methods (Lu 1999). Stem cuttings of 10 cm in height of *S. plumbizincicola* were prepared from middle section of vigorous shoots of 1-year-old plants grown in non-contaminated soil.

2.6 Pot experiment

To evaluate the effect of the isolated PGPR on the growth and Cd phytoremediation potential of *S. plumbizincicola*, a pot experiment was conducted in growth chambers and consisted of 10 treatments (nine isolated PGPR inoculated treatments and an uninoculated control) in four replicates. Individual pots were planted with five cuttings of *S. plumbizincicola* in 1.5 kg air-dry soil supplemented with $(NH_4)_2SO_4$, K_2HPO_4, and KCl giving final rates (mg kg^{-1}) of N 250, P 100, and K 150. The soil water content was adjusted to 20 % (*w*/*w*) during the experiment.

For the PGPR-inoculated treatments, the strains were pelleted by centrifugation at 9000 rpm for 15 min at 4 °C from cultures at the exponential growth phase in the LB medium. Cell pellet was washed with sterile distilled water, and an inoculum was prepared by re-suspending pelleted cells in sterile distilled water to obtain a density of 10^9 colony-forming units (cfus) mL^{-1}. Bacterial suspensions (15 mL pot^{-1}) were mixed with the soil before planting. Plants were grown for 3 months in a glasshouse.

At harvest, the plants were cut at the soil surface and washed with deionized water. Soil from each pot was sampled and stored at −4 °C. The fresh and dry weights of the shoot were then measured. Dry plant samples (~0.5 g) were digested using a mixture of 6 mL HNO_3 and 4 mL $HClO_4$, and concentrations of Cd was determined using AAS (Varian SpectrAA 220 FS). A certified reference material (GBW07603, provided by the Institute of Geophysical and Geochemical Exploration, Langfang, Hebei Province, China) was used for quality control (Wu et al. 2012).

2.7 Soil DNA extraction and PCR amplification

Soil DNA was extracted using a soil DNA kit (Fast DNA SPIN for soils, MP Biomedicals, Solon, OH) and checked the DNA on agarose gels (Moreira 1998). The primer pair 515f and 907r (515 F 5′-GTGCCAGCMGCCGCGG-3′, 907R 5′-CCGTCAATTCMTTTRAGTTT-3′) were utilized to amplify a 392 base pair fragment of the 16S rRNA gene for *454* pyrosequencing (Xu et al. 2013). Sequencing was conducted by Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China).

2.8 Pyrosequencing data processing

The 16S rRNA gene sequence data were analyzed by the pyrosequencing pipeline tools available from the Ribosomal Database Project (RDP) and MOTHUR version 1.24.1 (Schloss et al. 2011). Counts of the number of the sequences of each cluster within each sample were converted to frequencies by dividing the number of counts of each cluster by total number of sequences generated within each sample. The 16S rRNA sequences were first trimmed, and then, sequences with <200 bases were removed from the data sets with MOTHUR. Sequences from each data set, ranging from 6381 to 10,865 individual sequences, were submitted to the RDP aligner tool for species identification. Further processing and operational taxonomic unit (OTU)-based analyses were then carried out using the MOTHUR v.1.24.1 suite of algorithms for sequence processing and diversity analysis, including commands for identifying, unique sequences, filtering, sequence alignment, generating distance matrices, clustering of sequences into OTUs with a confidence threshold of 80 %. The resulting clusters were assessed at 97 % similarity to provide the data needed for diversity analysis. Several indices (OTUs, Chao,

and Ace) for sample size of 6000 sequence were calculated using the MOTHUR program at the cutoff of 97 %.

2.9 Statistical analysis

Statistical analysis was conducted with SPSS 19.0 software. Duncan's multiple range tests were used to compare the means of treatments; variability in the data was expressed as the standard errors. All analyses were performed at the $p<0.05$ level.

3 Results

3.1 Isolation and identification of Cd-tolerant PGPR

Nine Cd-tolerant isolates which could use ACC as the sole nitrogen source were obtained from the root of healthy plants grown in a Cd-contaminated soil. The nine strains showed a range of tolerance to the heavy metals Cd, Zn, and Cu. MICs varied from 0 to 1000 mg L^{-1} on plate assays against Cd, Cu, and Zn, respectively (Table 1). Among the nine strains, CCM2 exhibited the highest broad range tolerance to heavy metals with growth observed on plates containing 1000 mg Zn L^{-1}, 200 mg Cd L^{-1} and 150 mg Cu L^{-1}. 16S rRNA gene sequence of isolates NSX1 (from *S. X Graptosedum*), NCR3 (from *C. rossii*), and LCR9 (from *C. rossii*) showed the highest homology to *Entobacter ludwigii*, NSX2 (from *S. X Graptosedum*) and CCM2 (from *C. multicava*) to *Rhodococcus erythropolis*, NSE2 (*S. arboretum*) to *Enterobacter cancerogenus*, NCR1 and LCR 1 (from *C. rossii)* to *Cedecea davisae*, and NCR4 (from *C. rossii*) to *Arthrobacter* sp. (Table 1).

Table 1 Host plant and taxa of bacterial isolates and their minimum inhibitory concentrations (MIC) of heavy metals

Isolate	Host plant of origin	Taxa	MIC of heavy metals (mg L^{-1})		
			Cd	Cu	Zn
NSX1	*S. X Graptosedum*	*Enterobacter ludwigii*	50	0	200
NSX2	*S. X Graptosedum*	*Rhodococcus erythropolis*	20	100	600
NSE2	*S. arboretum*	*Enterobacter cancerogenus*	150	0	200
NCR1	*C. rossii*	*Cedecea davisae*	100	200	800
NCR3	*C. rossii*	*Enterobacter ludwigii*	50	150	800
NCR4	*C. rossii*	*Arthrobacter* sp.	50	50	800
CCM2	*C. multicava*	*Rhodococcus erythropolis*	200	150	1000
LCR1	*C. rossii*	*Cedecea davisae*	50	200	200
LCR9	*C. rossii*	*Enterobacter ludwigii*	20	100	150

3.2 IAA production and phosphate solubilization

All nine isolates were able to produce IAA in the absence of L-tryptophan (Fig. 1). Production of IAA varied from 28 mg L^{-1} (NSE2) to 144 mg L^{-1} (NCR3). All nine isolated strains displayed the potential for phosphate solubilization (Fig. 2). The maximum phosphate solubilization was achieved at 148 mg L^{-1} by strain NSE2. All the strains were shown to decrease the medium pH ranging from 4.93 to 6.10, compared to the uninoculated control (pH 7.6). The maximum pH decrease was also achieved by isolate NSE2.

3.3 Influence of bacterial inoculation on plant growth and cadmium uptake

The effects of isolated PGPR on the growth and Cd uptake of *S. plumbizincicola* are summarized in Table 2. Compared to the control, the inoculation of NSX2 and LCR1 significantly increased the dry weight of plant shoots (by 22.0 and 26.9 %, respectively). However, Cd concentrations in shoots of all inoculated treatments were not significantly different from that of the control. The total amount of Cd phytoextracted (mg pot^{-1}) was calculated by multiplying the shoot dry weight by shoot Cd concentration. The inoculation of NSX2 and LCR1 significantly increased total Cd in the shoot (by 14 and 18 %, respectively), compared with the control. These two soil samples and control were selected for further *454* pyrosequencing to determine their microorganism communities.

3.4 Evolution of microbial community structure and diversity by inoculations

The 16S rRNA gene survey for genetic diversity produced a total of 26,667 sequence reads. The control, LCR1, and NSX2 treatments individually produced 10,909, 7427 and 8331 sequence reads, respectively. At a 97 % similarity cutoff, the

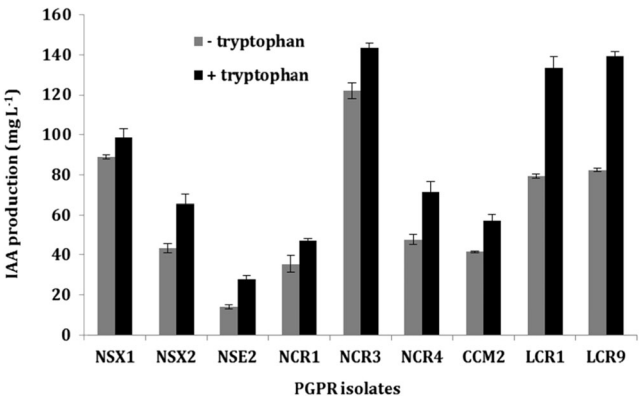

Fig. 1 Quantitative estimation of IAA production by isolates of plant growth-promoting rhizobacteria (PGPR) in the LB medium. Values are means±SD ($n=3$)

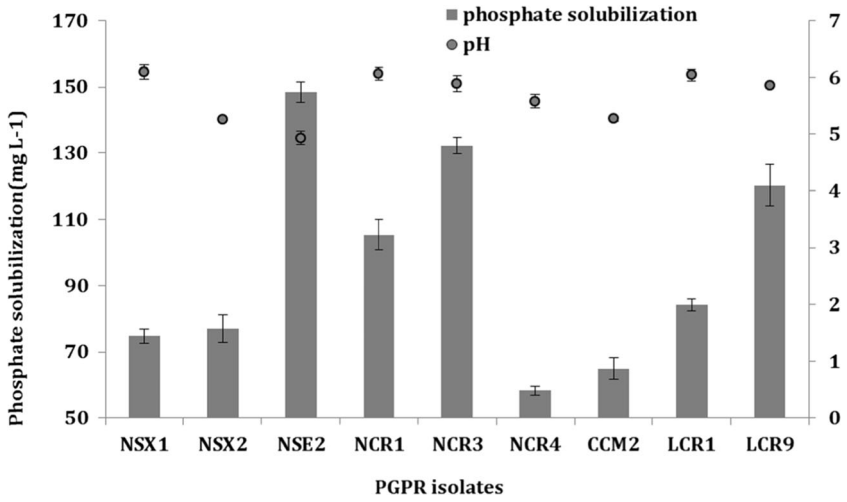

Fig. 2 Quantitative estimation of phosphate solubilization of selected isolates of plant growth-promoting rhizobacteria (PGPR) in liquid Pikovskaya's medium after 5 days of incubation. Values are means±SD (n=3)

reads from the control, LCR1, and NSX2 treatments were binned into 2197, 1521, and 1845 operational taxonomic units respectively. Within rarefaction curves, the distance value of 0.03 (97 % similarity) was used as the point at which differentiation occurs at the specie level (Bowman et al. 2012). Rarefraction curves (figure not shown) indicate that the analysis of species richness was an accurate representation of bacterial diversity due to the samples trending toward a plateau at a 0.03 cutoff at 6000 reads. The inoculation of the PGPR decreased the richness of microbial community after 3 months. The Ace and Chao index showed a clear drop compared to the control treatment (Table 3).

The phylogenetic spectrum classification of sequences from the soil samples at the phylum level accounted for 78.2, 82.9, and 82.8 % in the control, LCR1, and NSX2 treatments, respectively. *Proteobacteria*, *Actinobacteria*, and *Firmicutes* were comprised the top three phyla groups, which on average comprised 26.2, 15.8, and 10.9 % in the uninoculated soil population, respectively. The remaining phylotypes were associated with *Acidobacteria* (9.0 %), *Chloroflexi* (6.8 %), *Planctomycetes* (3.7 %), *Bacteroidetes* (3.6 %), *Gemmatimonadetes* (0.57 %), *Verrucomicrobia* (0.51 %), *Armatimonadetes* (0.39 %), *Crenarchaeota* (0.29 %), *Euryarchaeota* (0.20 %), *TM7* (0.14 %), *Nitrospirae* (0.10 %), and *BCR1* (0.05 %). The dominant phylum, *Proteobacteria*, decreased to 23.6 and 24.8 % in the LCR1 and NSX2 treatments, respectively. The next two dominant phyla *Actinobacteria* and *Firmicutes* increased to 20.1 and 16.9 %, and 11.6 and 12.2 % in the LCR1, and NSX2 treatments, respectively. *Planctomycetes* increased to 6.0 and 5.2 % in the LCR1 and NSX2 treatments, respectively, almost doubled the control. The reads for the phylum *Acidobacteria* and *Euryarchaeota* also increased to 9.84 and 10.72 %, and 0.46 and 0.30 % in the LCR1 and NSX2 treatments, separately (Fig. 3a).

Table 2 Influence of selected isolates on the plant growth and the uptake (mg) of Cd by *S. plumbizincicola* grown for 3 months

Treatments	Shoot dry weight (g plant^{-1})	Shoot Cd concentration (mg kg^{-1})	Shoot Cd accumulation (mg pot^{-1})
Control	11.7±1.3 ab	42.7±0.7 abc	0.50±0.02 a
NSX1	11.6±0.5 a	45.8±0.9 c	0.53±0.02 ab
NSX2	14.3±1.3 c	41.3±0.9 ab	0.59±0.02 cd
NSE2	12.7±2.6 abc	43.4±1.2 bc	0.55±0.03 abcd
NCR1	13.3±1.3 abc	42.1±1.2 ab	0.56±0.02 abcd
NCR3	12.9±2.1 bc	41.7±0.8 ab	0.54±0.01 abc
NCR4	11.6±0.8 a	43.3±0.3 bc	0.50±0.01 a
CCM2	11.6±0.6 a	43.1±0.9 abc	0.50±0.01 a
LCR1	14.9±1.3 c	41.5±0.8 ab	0.57±0.02 bcd
LCR9	12.9±1.0 bc	42.7±1.4 abc	0.55±0.03 abcd

Values are means±standard errors (n=4). Means sharing the same letter do not differ significantly from each other at $p<0.05$

Furthermore, genus level analysis was also conducted to examine functional evolution of the community (Jeong et al. 2013). The *Gp3 Acidobacteria* population was found to increase in the LCR1 and NSX2 treatments (1.57 and 1.84 %, respectively) compared to the uninnoculated soil (1.22 %). The inoculation of LCR1 increased *Oryzihumus*, *Mycobacterium*, and *Actinospica* (*Actinobacteria*) by 31.5, 30.2, and 227.5 %, respectively, while the inoculation of NSX2 increased *Burkholderia*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (*Proteobacteria*) by 27.2, 28.3, 350.0, and 550.0 %, respectively (Fig. 3b).

Although both of NSX2 and LCR1 were isolated from the roots of plants grown in a soil contaminated with high levels of heavy metals including Cd and Zn, only inoculated *R. erythropolis* NSX2 (0.18 %, relative to 0.01 % of the control) was found in soil 3 months after initial inoculation while *C. davisae* LCR1 was not detected.

Table 3 Richness estimators to predict species numbers in soil without (control) or with inoculation of LCR1 and NSX2[a]

Samples	OTUs[b]	ACE	Chao
Control	2197	10,083	5744
LCR1	1521	6224	3945
NSX2	1845	6262	3868

[a] The richness estimators and diversity indices were calculated based on equal number of sequences (6000 sequences) which were randomly selected from each sample

[b] Operational taxonomic units (OTUs) were calculated based on 97 % sequence similarity

4 Discussion

Bioremediation strategies using a combination of PGPR and plants have been demonstrated to a cost-effective way for promoting plant growth and protecting plants from heavy metal toxicity (Guo and Chi 2014). The PGPR strains must be tolerant to heavy metals constitutively or adaptively by exclusion, physical sequestration, detoxification, and complexion (Nies 2003). In this study, nine Cd-tolerant PGPRs were isolated from the roots of Cd accumulator plants (*S. arboretum*, *S. X Graptosedum*, *C. multicava*, and *C. rossii*) grown on Cd-contaminated soil (Zhang et al. 2014). On the basis of colony morphology and 16S rRNA gene sequence, the nine strains were identified as *E. ludwigii* (NSX1, NCR3, and LCR9), *R. erythropolis* (NSX2 and CCM2), *E. cancerogenus* (NSE2), *C. davisa* (NCR1 and LCR1), and *Arthrobacter* sp. (NCR4) (Table 1).

In addition to tolerance to heavy metals, these strains showed the PGP properties of ACC deaminase and IAA production as well as phosphate solubilization. The PGPR containing ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to a-ketobutyric acid and ammonia, and in this way promote plant growth (Glick et al. 1998). This present study obtained nine strains of rhizobacteria that could utilize ACC as the sole nitrogen source, indicating that these isolates could produce ACC deaminase and utilize ACC as nitrogen source. Bacterial produced IAA has previously been reported to contribute to decreasing metal stress, promoting plant growth, and increasing the total metal uptake (Dell'Amico et al. 2008). Phosphate solubilization is important for improving plant growth in P-deficient soils. All the strains were shown to decrease the medium pH compared to the uninoculated control (pH 7.6), which has previously been linked to solubilization of calcium phosphate. In this present study, all nine isolates had the capacity of producing IAA and solubilizing phosphate (Figs. 1 and 2). In soils inoculated with PGPR, a slightly decrease of soil pH (data not shown) was observed, indicating that the phytoavailable Cd would increase and thus its uptake by plants. To the best of our knowledge, among these strains, only *R. erythropolis* was reported as a Cd-tolerant species (Becerra-Castro et al. 2012), but this is the first study reporting the potential of *R. erythropolis* to increase plant growth in metal-contaminated soils.

It is noticed that isolate NCR3, which produced the highest IAA, did not significantly increase the growth or Cd phytoextraction of *S. plumbizincicola*. However, the greatest effect on plant growth and Cd accumulation was found for the LCR1 and NSX2-inoculated treatments (Table 2). Similarly, Belimov et al. (2001) did not find a correlation between the PGP ability and plant growth. Their experiments showed that the nine tested strains significantly varied in ACC deaminase activity of cell-free extracts while only slight or no effects were observed in quartz sand culture in the absence of $CdCl_2$, and only three strains *P. putida* Am2, *P. putida* Bm3,

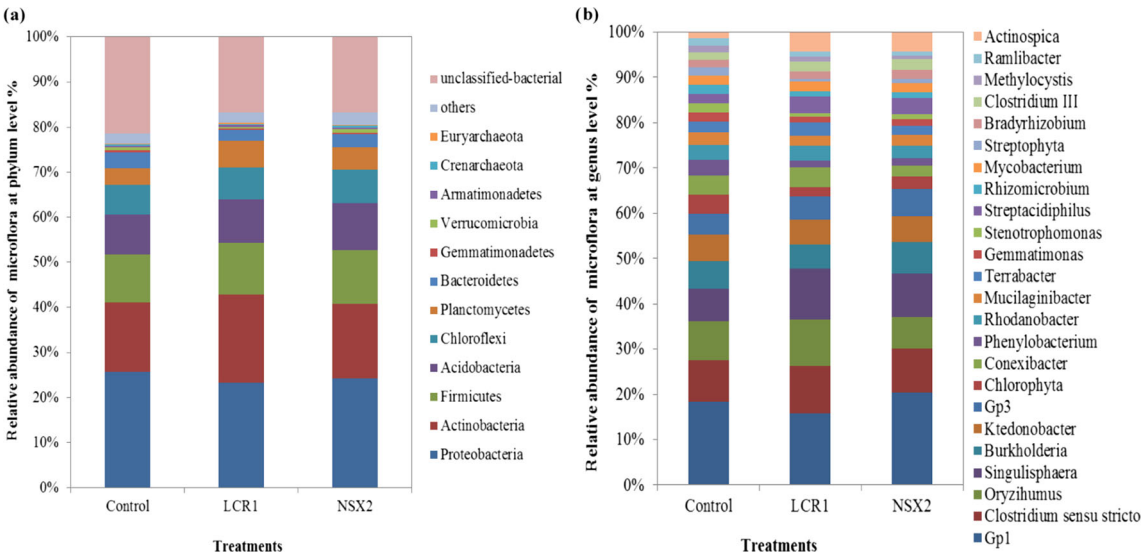

Fig. 3 Relative abundance of microflora at phylum (3a) and genus (3b) levels in soils of different inoculating treatments

and *P. brassicacearum* Am3 significantly increased root elongation (by 14–20 %) under Cd stress. The explanation for the lack of correlation between the PGP ability and plant growth might be that other bacterial properties associated with their taxonomic position could also affect the interaction of rhizobacteria with plant roots.

Apart from the inoculated PGPR, the microbial population of the soil also plays a significant role in phytoremediation of heavy metals in contaminated soils by affecting heavy metal mobility, bioavailability to the plant, and enhancement of the phytoremediation processes (Jing et al. 2007). Here, we adopted a pyrosequencing approach to elucidate the change of microbial community during phytoremediation in the Cd-contaminated soil, which is more sensitive than the previously reported PCR-DGGE method (Jeong et al. 2013). This study demonstrated that the introduced PGPR had clear impacts on the microbial community structure and diversity (Fig. 3a, b). The richness estimators (OTUs, Ace, and Chao) revealed that the inoculation of PGPR decreased the diversity of microbial community (Table 3). Furthermore, the inoculation of LCR1 and NSX2 markedly enhanced the relative abundance of the phylum *Planctomycetes* in the Cd-contaminated soil. In a previous study, the richness and diversity of *Planctomycetes* were shown to be driven by pH and to have strong spatial and seasonal variations linked to environmental conditions (Pollet et al. 2011). In the present study, the slight decrease of soil pH (date not shown) might lead to an increase of the relative abundance of *Planctomycetes* in the inoculated treatments.

The inoculated bacterial *R. erythropolis* NSX2 increased from 0.01 % (control) to 0.18 %, which exhibited various PGP features such as ACC deaminase, indole, and siderophore production ability (Trivedi et al. 2007). In addition, diverse symbiotic microorganisms including *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* are now being used worldwide as bioinoculants to promote plant growth under stresses of various heavy metals (Wani and Khan 2010). These indigenous genera increased inordinately in soil inoculated with NSX2. All of these bacterial groups could not only increase the heavy metal mobilization, and production of IAA, siderophores, HCN, and ammonium, and P solubilization, but also increase biomass (plant and microorganism), nitrogen content, and accumulation of metals (Wani et al. 2007). Additionally, a few taxa showed a positive response to the soil even when they were present only in relative low abundance. *Mycobacterium* and *Actinospica* significantly increased to 0.69 and 1.31 %, separately, in soil inoculated with *C. davisae* LCR1 which were accounted for 0.53 and 0.40 % in the uninoculated soil, both of which were identified as PGPR (Bhattacharyya and Jha 2012; Tsavkelova et al. 2005). That is, the percentage of the species responsible for Cd phytoavailability becomes more abundant in both of the inoculated soils.

Although both of NSX2 and LCR1 were inoculated to the Cd-contaminated soil, only inoculated *R. erythropolis* NSX2 (0.18 %, relative to 0.01 % of the control) was found in soil 3 months after initial inoculation while *C. davisae* LCR1 was not detected. The reason may be that the strain NSX2 was isolated from *S. X Graptosedum* (Table 1) which was the same family with *S. plumbizincicola* used in this experiment, but *C. davisae* LCR1 was separated from *C. rossii*. Nevertheless, soil microorganisms were only monitored at the end of the remediation which was difficult to reflect the survival dynamics of introduced bacteria in the process of phytoremediation. We suspected that the PGP properties of inoculated PGPR combined with the changes of microorganism community, especially of species responsible for Cd phytoavailability, leading to the better growth of *S. plumbizincicola* and Cd phytoextraction. The survival dynamics of introduced bacteria in the process of phytoremediation needs further study.

Although above discussion highlights the effectiveness of PGPR in enhancing the growth and Cd uptake of *S. plumbizincicola*, PGPR *C. davisae* LCR1 was not detected in soil 3 months after initial inoculation. This could be attributed to competition for the inoculated PGPR with the indigenous microflora as well as various kinds of environmental stresses (Brockwell et al. 1995). Encapsulation of the bacteria may be an effective alternative to free cell dispersal. It is reported that encapsulating bacteria could survive in soil and be released into soil in a controllable manner, so that long-term effects can be optimized (Wu et al. 2011). Furthermore, the use of multi-strain inoculants could be a good strategy that enables organisms to successfully survive and maintain themselves in communities (Andrews et al. 1991). Each strain in the multi-strain consortium may compete effectively with indigenous population in the rhizosphere and enhance plant growth with its partners. It has been reported that coinoculation of *Bradyrhizobium* and PGPR microorganisms significantly improved soybean growth and its yield components as compared with the sole application of *Bradyrhizobium* (Wasule et al. 2007).

5 Conclusions

In this study, nine Cd-tolerant PGPR were isolated from the roots of heavy metal accumulators. All isolates had varying levels of IAA production and phosphate solubilization. Among the isolates, NSX2 and LCR1 were found to significantly enhance the Cd uptake of *S. plumbizincicola*. Based on 16S rRNA gene sequence, NSX2 and LCR1 were identified as *R. erythropolis* and *C. davisae*, respectively. Pyrosequencing analysis of the microbial community after inoculation of test isolates indicates that the PGPR could invoke changes of the microbial community structure and diversity during the experimental period. These findings

demonstrate that the information on the activity of introduced bacteria in soil and microbial community variation is required for effective bacteria-associated phytoextraction.

Acknowledgments We thank the National High Technology Research and Development Program of China (863 Program) (No. 2012AA06A204), Jiangsu Provincial Natural Science Foundation of China (BK2012891), the International Scientific Collaborative Program of the Ministry of Science and Technology of China (2010DFA92360), and National Natural Science Foundation of China (41001182) for financial support. Tang and Liu were also supported by an Australian Research Council Linkage Project (LP100100800).

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