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Characterization of lead-resistant and ACC deaminase-producing endophytic bacteria and their potential in promoting lead accumulation of rape

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ABSTRACT

Forty-nine lead (Pb)-resistant endophytic bacteria were isolated from metal-tolerant *Commelina communis* plants grown on lead and zinc mine tailing, of which, seven 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing endophytic bacteria were initially obtained and characterized with respect to heavy metal resistance and production of ACC deaminase, indole-3-acetic acid (IAA) as well as siderophores. Two isolates (Q2BJ2 and Q2BG1) showing higher ACC deaminase activity were evaluated for promoting plant growth and Pb uptake of rape grown in quartz sand containing 0 and 100 mg kg^{-1} of Pb in pot experiments. The seven Pb-resistant and ACC deaminase-producing endophytic bacterial isolates were found to exhibit different multiple heavy metal resistance characteristics and to show different levels of ACC deaminase activity (ranging from 12.8 μM α-KB mg^{-1} h^{-1} to 121 μM α-KB mg^{-1} h^{-1}). Among the seven isolates, six isolates produced indole acetic acid, whilst five isolates produced siderophores. In experiments involving rape plants grown in quartz sand containing 100 mg kg^{-1} of Pb, inoculation with the isolates resulted in the increased dry weights of above-ground tissues (ranging from 39% to 71%) and roots (ranging from 35% to 123%) compared to the uninoculated control. Increases in above-ground tissue Pb contents of rape cultivated in 100 mg kg^{-1} of Pb-contaminated substrates varied from 58% to 62% in inoculated-rape plants compared to the uninoculated control.

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1. Introduction

Pb is one of the most common heavy metal contaminant in the environment and it cannot be biologically degraded to harmless products. Excessive Pb concentrations in the contaminated soils can pose significant risks to human health and ecosystems. Therefore, the development of a remediation strategy for metal-contaminated soils is urgent for environmental conservation and human health [1]. Phytoremediation to clean up heavy metal soil has gained more attention than conventional technology as environmental friendly and cost effective [2–4]. The effective phytoextraction process is dependent on an adequate plant yield and high heavy metal concentrations in the above-ground tissues of plants [5]. However, most hyperaccumulators identified so far are not suitable for field phytoremediation applications due to their small biomass and slow growth [6]. This has prompted us to explore the possibilities of enhancing the biomass and metal uptake of metal accumulators using plant growth-promoting bacteria (PGPB) as bioinoculants [1,5–7]. Recently, the benefits of combining heavy metal resistant endophytic bacteria with plants for increased remediation of pollutants have been successfully tried for toxic metal removal from metal contaminated soils [8]. Endophytic bacteria can have the capacity to promote plant growth and development under adverse conditions by various mechanisms such as nitrogen fixation, production of indole acetic acid (IAA), siderophore, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase [9–11]. ACC deaminase can metabolize ACC (an immediate precursor of ethylene in plants) into α-ketobutyric acid and ammonia. Some ACC deaminase-producing PGPB promote plant growth by lowering the level of ethylene in plants growing in the presence of heavy metals [12,13]. Although bacterial-assisted phytoremediation has been studied [8,14–16], to date, no attempt has been made to screen Pb-resistant endophytic bacteria from metal-tolerant *Commelina communis* plants grown in lead and zinc mine tailing for their potential to produce ACC deaminase and to enhance plant growth and Pb accumulation under Pb-contaminated conditions. Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil [17]. A better understanding of the characteristics of Pb-resistant and ACC deaminase-producing endophytic bacteria is needed for the development of efficient phytoremediation systems.

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Table 1
Characteristics of the isolated Pb-resistant and ACC-producing endophytic bacterial strains.

Strains	Closest relative sequence	% identity	ACCDA[a] (μM α-KB mg^{-1} h^{-1})	IAA (mg L^{-1})	Siderophore[b]	MIC (mM)			
						Pb	Cu	Cd	Ni
Q2BJ3	*Agrobacterium tumefaciens*	100	21.8 ± 1.8	3.9 ± 0.8	+ +	1.21	0.16	0.89	0.85
Q2BJ1	*Bacillus* sp.	99	30.8 ± 3.7	16.9 ± 1.4	+	2.41	0.16	0.22	0.43
Q2CJ3	*Bacillus* sp.	100	15.6 ± 4.9	22.8 ± 6.3	ND	1.21	1.57	0.44	0.09
Q2BJ2	*Acinetobacter* sp.	96	120 ± 22	1.6 ± 0.2	+ +	3.62	1.57	0.89	0.85
Q2CJ5	*Bacillus subtilis*	100	12.8 ± 1.0	1.9 ± 0.7	+	2.41	0.16	0.09	8.52
Q2BG4	*Bacillus megaterium*	99	13.7 ± 2.7	10.9 ± 1.8	ND	2.41	0.79	0.44	0.85
Q2BG1	*Bacillus* sp.	100	121 ± 47	ND	+ + + +	3.62	1.57	0.22	1.70

Values of absorbancy/absorbancy reference at 630 nm: +, 0.8–1.0; ++, 0.6–0.8; +++, 0.4–0.6; ++++, 0.2–0.4. ND: no detection.
[a] ACCDA—ACC deaminase activity.
[b] Siderophore production: +, little; ++, low; +++, moderate; ++++, high.

The objectives of this study were to isolate and characterize Pb-resistant and ACC deaminase-producing endophytic bacteria from Pb-tolerant plants grown in lead and zinc mine tailing, select plant growth-promoting bacteria (PGPB) and test the potential of metal-resistant and ACC deaminase-producing endophytic bacteria to promote plant growth and Pb uptake of rape for improving the efficiency of phytoextraction of Pb-polluted environment.

2. Materials and methods

2.1. Isolation of Pb-resistant and ACC deaminase-producing endophytic bacteria

Three healthy *C. communis* plants including the rhizosphere soils were collected from a lead and zinc mine tailing in the suburbs of Nanjing, China. Roots, stems and leaves of *C. communis* plants were separated and washed extensively, first in several changes of 0.01 M EDTA and then in distilled water to remove any nonspecifically bound Pb and dried to constant weight. Metal concentrations of plant tissues and rhizosphere soils were determined using an inductively coupled-plasma optical emission spectrometer (ICP-OES) (Optima 2100 DV, Perkin Elmer) according to the method of Sun et al. [18]. The isolation of Pb (1 mM Pb)-resistant endophytic bacteria from the plant tissues was made according to the method of Sun et al. [18]. Pb-resistant colonies were picked randomly and purified by streaking three to four times on the 1/5-strength Luria–Bertani's (LB) media. Forty-nine endophytic bacterial isolates (14 isolates from roots, 10 isolates from stems, and 25 isolates from leaves) growing well on subculturing were selected for ACC deaminase-producing ability.

In order to evaluate the ACC deaminase production of the Pb-resistant endophytic strains, the method of Glick et al. [19] was used. The isolates being able to utilize ACC as the sole N-source were indicated as ACC deaminase-producing isolates. Seven ACC deaminase-producing endophytic strains were finally selected and stored on slants for further study.

2.2. ACC deaminase enzyme activity

The ACC deaminase activity of cell-free extracts was determined by estimating the amount of α-ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC [20] according to the procedure of Honma and Shimomura [21]. The calibration curve was determined using α-ketobutyric acid and the protein concentration of cellular suspension in the toluenized cells [22]. To produce the protein calibration curve, bovine serum albumin (BSA) was used [23]. After determining the amount of protein and α-ketobutyrate, the enzyme activity was calculated based on the μM of released α-ketobutyrate per mg protein per h [24].

2.3. IAA and siderophore production

The production of IAA by the tested endophytic bacteria was determined according to the methods of Gordon and Weber [25] and Sheng et al. [8]. The production of siderophores by the bacteria was determined according to the chrome azurol-S (CAS) analytical method [17,26,27].

2.4. Heavy metal resistance of the isolates

For the determination of minimal inhibitory concentrations of the metal ions, seven ACC deaminase-producing isolates were tested for the ability to grow in increasing concentrations of the metal cations added to the sucrose-minimal salts low-phosphate medium (SLP) [28]. Metals used were Pb as $Pb(NO_4)_2$, Cu as $CuSO_4$, Cd as $CdSO_4$, and Ni as $NiSO_4$. Stock solutions of the metal salts were prepared in double distilled water and sterilized. SLP agar plates without metals were used as controls. The experiments were carried out in triplicate. Cultures were incubated at 28 °C for 7 days.

2.5. Identification of the ACC deaminase-producing isolates

The identification of the seven Pb-resistant and ACC deaminase-producing bacteria was made according to the method of Jiang et al. [28]. The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program [29]. The nucleotide sequences determined in this study have been deposited in the NCBI database under accession numbers GU471197, GU471200 to GU471203, GU471206, and GU471208.

2.6. Plant growth and Pb uptake of rape by the ACC deaminase-producing strains

Based on the ACC deaminase activities, two isolates (Q2BJ2 and Q2BG1) with higher ACC deaminase activity (Table 1) were selected for studying the effects of the strains on plant growth and Pb uptake of rape. Rape was used in the inoculation experiment due to its fast growth, large biomass production. Experiments were conducted in plastic pots filled with 200 g sterilized quartz sand (1 mm in diameter) with 0, and 100 mg kg^{-1} of Pb^{2+} added as $Pb(NO_4)_2$. 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 and washed with sterile water. Seed sterility was verified by incubating 10 seeds on LB agar at 28 °C for 4 days. Bacteria were grown in liquid LB medium for 24 h at 28 °C, centrifuged, washed, and resuspended to 5×10^8 cells ml^{-1} in sterile distilled water. After the seeds were germinated in the dark for 2 days, seeds were soaked in the bacterial suspension or sterile water (uninoculated control) for 2 h, and then placed in each pot. Five seeds were planted in each pot and three replicates were used for each treatment. The pots were

Table 2
Heavy metal concentrations of rhizosphere soils and plants growing on Pb-contaminated soils.

Plant	Metal concentrations in rhizosphere soil (mg kg^{-1})				Pb concentrations in plant tissue (mg kg^{-1})		
	Pb	Zn	Cu	Cd	Root	Stem	Leaf
Commelina communis	1636 ± 144	3077 ± 464	157 ± 29	27 ±	261 ± 161	42 ± 9	66 ± 26

Table 3
The influence of the endophytic isolates on the dry weight (mg pot^{-1}) of rape on a quartz sand added with 0, and 100 mg kg^{-1} of Pb.

Treatment	Above-ground tissue		Root	
	0	100	0	100
Control	352 ± 50	322 ± 34	31 ± 3.8	56.5 ± 9.2
+ Strain Q2BJ2	490 ± 40*	503 ± 62*	43 ± 7.2*	76 ± 3*
+ Strain Q2BG1	596 ± 7*	550 ± 45*	69 ± 12*	89 ± 10*

* Value significantly greater than the corresponding control value ($p < 0.05$).

placed outdoors and were moved to indoors in order to protect them from the rainfall [28]. During cultivation, minimal and maximal temperature ranged from 14 to 18 °C and from 22 to 27 °C, respectively. Hoagland's nutrient solution (Hoagland's composition (mg L^{-1}): KNO_3 607, $Ca(NO_3)_2{\cdot}4H_2O$ 945, $MgSO_4{\cdot}7H_2O$ 493, $NH_4H_2PO_4$ 115, H_3BO_3 2.86, $MnCl_2{\cdot}4H_2O$ 2.13, $ZnSO_4{\cdot}7H_2O$ 0.22, $CuSO_4{\cdot}5H_2O$ 0.08, $H_2MoO_4{\cdot}H_2O$ 0.02, $FeSO_4{\cdot}7H_2O$ 5.57, Na_2-EDTA 7.45) was watered every day. After 45 days, plants were carefully removed from the pots, the roots and above-ground tissues were separated and washed in distilled water. To examine the introduced strains (Q2BJ2 and Q2BG1), plant tissue materials (0.2 g) were ground by a mortar and pestle in the presence of 5 ml of sterile distilled water. Sterile quartz sand was added to the mortar to improve the plant tissue disruption. Serial dilutions were spread on plates containing 1/5-strength LB agar with Pb (1.0 mM) and kanamycin (50 mg L^{-1}) (strains Q2BJ2 and Q2BG1 were resistant to Pb and kanamycin). After incubation for 7 days at 28 °C, the reisolated, Pb and kanamycin resistant strains were identified for colony characteristics, Pb-resistance and ACC deaminase activity against the parent strains. Plant tissue materials were dried to constant weight for determining dry weight. Metal concentrations of all subsamples were determined by the method of Sun et al. [18].

2.7. Statistical analysis

Analysis of variance and the Student–Newman–Keuls test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 13.0.

3. Results

3.1. Isolation of Pb-resistant and ACC deaminase-producing bacteria

The lead and zinc mine tailing contains high level of heavy metals (Pb, Zn, Cu, and Cd) (Table 2). Pb concentrations of root parts were significantly higher than that of other parts (Table 2). We have isolated Pb-resistant endophytic bacteria from the root, stem, and leaf tissue interiors of Pb-tolerant *C. communis* plants growing in lead and zinc mine tailing by using a spread plate procedure with 1/5-strength LB medium. This medium is designed to avoid the precipitation of Pb as Pb $(NO_3)_2$ at 1.0 mM. Of the 49 Pb-resistant isolates, seven isolates are able to use ACC as the sole N-source, indicating that the seven isolates can produce ACC deaminase. The ratio of ACC deaminase-producing isolates to Pb-resistant isolates was 14%. The seven Pb-resistant and ACC deaminase-producing isolates were identified as *Agrobacterium tumefaciens* Q2BJ3, *Bacillus* spp. Q2BJ1, Q2CJ3, and Q2BG1, *Acinetobacter* sp. Q2BJ2, *Bacillus subtilis* Q2CJ5, and *Bacillus megaterium* Q2BG4 based on the 16S rDNA gene sequence analysis, respectively (Table 1).

3.2. ACC deaminase activity of the endophytic strains

Among the seven Pb-resistant endophytic strains which could utilize ACC as the sole N-source, different strains showed different level of ACC deaminase activity (ranging from 12.8 ± 1.0 μM α-KB mg^{-1} h^{-1} to 121 ± 47 μM α-KB mg^{-1} h^{-1}) (Table 1). Strains Q2BJ2 and Q2BG1 showed higher levels of ACC deaminase activity than the other strains (Table 1). The strains with higher levels of ACC deaminase activity belonged to two different genera (*Acinetobacter* and *Bacillus*).

3.3. IAA and siderophore production

IAA production at levels of 1.6 ± 0.2 μg ml^{-1} to 22.8 ± 6.3 μg ml^{-1} was observed for the seven Pb-resistant and ACC deaminase-producing endophytic bacterial strains (Table 1). Only one strain (Q2BG1) did not produce detectable level of IAA. Strain Q2CJ3 produced more IAA. Of the seven ACC deaminase-producing endophytic bacterial strains, five strains were able to produce siderophores (Table 1). In particular, strains Q2BG1, Q2BJ3, and Q2BJ2 produced higher siderophores. Only two strains (Q2CJ3 and Q2BG4) did not produce siderophores.

3.4. Heavy metal resistance of ACC deaminase-producing strains

The seven ACC deaminase-producing strains showed different and a very high degree of resistance to heavy metals, especially to Pb (Table 1). *Acinetobacter* sp. Q2BJ2 was found to exhibit the highest degree of resistance to Pb, Cu, and Cd, whilst *Bacillus* sp. Q2BG1 was found to exhibit the highest degree of resistance to Pb, Cu, and Ni (Table 1). The order of the toxicity of the metals to the ACC deaminase-producing *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 was found to be Ni > Cd > Cu > Pb and Cd > Cu > Ni > Pb, respectively.

3.5. Plant growth promotion and Pb uptake in rape

The effects of the two metal-resistant and ACC deaminase-producing endophytic bacteria on the growth of *B. napus* variety Qinyou-7 in the absence or presence of Pb are shown in Table 3. Inoculation with metal-resistant and ACC deaminase-producing endophytic bacteria in the absence of Pb significantly ($p < 0.05$) increased the dry weights of above-ground tissues (ranging from 39% to 69%) and roots (ranging from 39% to 123%). In the presence of Pb, inoculation with the ACC deaminase-producing endophytic

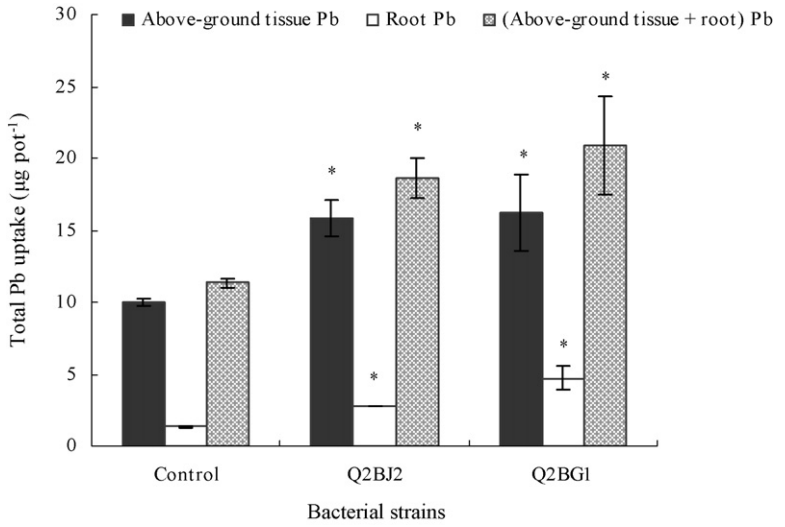

Fig. 1. The influence of the Pb-resistant and ACC deaminase-producing isolates on the total Pb uptake of rape treated with 100 mg kg^{-1} of Pb. Error bars are ±standard deviation ($n = 3$). An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$).

bacteria was found to increase the dry weights of above-ground tissues (ranging from 56% to 71%) and roots (ranging from 34.5% to 57.5%).

The metal-resistant and ACC deaminase-producing endophytic bacteria (*Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1) significantly ($p < 0.05$) increased total Pb uptake of rape in pot experiment (Fig. 1). In the presence of 100 mg kg^{-1} of Pb, inoculation with strains Q2BJ2 and Q2BG1 was found to significantly ($p < 0.05$) increase the above-ground tissue (ranging from 58% to 62%) and root (ranging from 2.1-fold to 3.5-fold) total Pb uptake of rape compared to the uninoculated control. Significant increases ($p < 0.05$) of total plant (above-ground tissue plus root) Pb uptake of bacterial inoculated-rape plants were observed compared to the uninoculated control in the presence of 100 mg kg^{-1} of Pb (Fig. 1). Increases of above-ground tissue total Pb uptake (ranging from 3.4-fold to 5.6-fold) of bacterial inoculated-rape plants were obtained compared to root total Pb uptake. In addition, the two metal-resistant and ACC deaminase-producing endophytic bacteria showed similar ability of promoting total Pb uptake of rape plants.

4. Discussion

Even metals exert toxic effects on bacteria through various mechanisms, metal-resistant bacteria could survive in these habitats and could be isolated and selected for their potential application in plant growth promotion and bacteria-assisted phytoremediation of metal-contaminated sites [6,8,28,30]. The surface sterilization protocol for the isolation of endophytic bacteria was effective in removing epiphytic microorganism, and that the bacterial isolates can be considered to be true endophytic bacteria [18]. This made it possible to isolate and characterize endophytic bacteria associated with Pb-tolerant plant tissues.

The Pb-resistant and ACC deaminase-producing endophytic bacteria isolated from the heavy metal-tolerant *C. communis* plants grown on lead and zinc mine tailing possess various plant growth promoting features (Table 1). Different strains differed in their ability to produce ACC deaminase, IAA, or siderophore (Table 1). Bacteria having the characteristics of producing ACC deaminase, IAA, and siderophores may have the potential for the promotion of plant growth and heavy metal uptake [28,31,32]. Inoculation of plants with plant growth promoting bacteria, which contained ACC deaminase and produced IAA and siderophores, protected the plants against Ni, Pb and Zn toxicity [28,33,34]. Siderophore overproducing strains were found capable of stimulating plant biomass and enhance phytoextraction of metals (Ni, Zn and Cr) [34].

In this study, *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 did no significant differences in their promotion of plant growth and total Pb uptake of rape in the Pb-contaminated environment (Table 3 and Fig. 1). Although *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 differed in their ability to produce IAA and siderophores, the two strains produced similar level of ACC deaminase, suggesting that the ability of *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 to promote plant growth and Pb uptake under Pb-contaminated condition may be related to their capacity to produce ACC deaminase. Bacterial endophytes are able to promote plant growth and health [35]. The ACC deaminase has been proposed to play a key role in microbe–plant association [36]. PGPR can significantly increase the growth of plants in the presence of heavy metals [33,37].

Studies have evidenced that heavy metal-resistant bacteria can enhance metal uptake by plants [7,14,38,39]. The same results were obtained in our experiment that the total Pb uptake by rape plants was significantly enhanced by the metal-resistant and ACC deaminase-producing endophytic bacterial strains Q2BJ2 and Q2BG1. Although a number of studies have demonstrated the importance of bacterial inoculation for plant growth and heavy metal accumulation in heavy metal-polluted environments [8,14,18,40], to the best of our knowledge, this is the first research report elucidating the role of heavy metal-resistant and ACC deaminase-producing endophytic *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 isolated from *C. communis* in Pb accumulation by rape with concurrent promotion of plant growth in a pot experiment.

Although the metal-resistant and ACC deaminase-producing endophytic *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 did not significantly influence the concentrations of Pb in root and above-ground tissue systems (data not shown), the application of strains Q2BJ2 and Q2BG1 effectively promoted the growth of rape plants, consequently increasing the total Pb uptake of the plants. Dell'Amico et al. [15] also found that cadmium-resistant rhizobacteria did not influence the specific accumulation of cadmium in the root and shoot systems, but all increased the plant biomass and consequently the total cadmium uptake of the *B. napus*. Inoculation with endophytic isolates might have significant potential

to improve phytoextraction efficiency in metal contaminated soils [6].

In addition, as successful plant growth-promoting inoculants, bacteria must be able to rapidly colonize the root system during the growing season [41]. Dilution-plate method using 1/5-strength LB agar containing Pb (1.0 mM) and kanamycin (50 mg L^{-1}) showed that the tested metal-resistant and ACC deaminase-producing endophytic bacteria colonized the root and shoot interiors of the rape plants. The numbers of the cells in roots and shoots of rape plants were 10^3–10^4 cfu g^{-1} of fresh root and 10^2–10^3 cfu g^{-1} of fresh shoot, respectively.

5. Conclusion

Our researches demonstrated that metal-resistant and ACC deaminase-producing endophytic bacteria could be isolated from plants grown in lead and zinc mine tailing and chosen as the bioinoculant for the effective phytoextraction of Pb-contaminated environment. The isolated endophytic bacteria had the innate capability of expressing multiple heavy metal resistance and plant growth-promoting characteristics. Inoculation with the two metal-resistant and ACC deaminase-producing *Acinetobacter* sp. Q2BJ2 and *Bacillus* sp. Q2BG1 was found to increase the plant biomass and total Pb uptake in the Pb-contaminated environment. A further understanding of the relationship between the plant and the metal-resistant and ACC deaminase-producing endophytic bacteria is essential for the development of effective phytoremediation of Pb-contaminated environment. This may therefore provide a new endophytic bacterial-assisted phytoremediation of Pb-contaminated environment.

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