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Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape

Xia-Fang Sheng*, Juan-Juan Xia, Chun-Yu Jiang, Lin-Yan He, Meng Qian

MOA Key Laboratory of Microbiological Engineering of Agricultural Environment, College of Life Science, Nanjing Agricultural University, Nanjing 210095, PR China

Heavy metal-resistant endophytic bacteria from rape have the potential of promoting the growth and lead uptake of rape.

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ABSTRACT

Two lead (Pb)-resistant endophytic bacteria were isolated from rape roots grown in heavy metal-contaminated soils and characterized. A pot experiment was conducted for investigating the capability of the two isolates to promote the growth and Pb uptake of rape from Pb-amended soil. The two isolates were identified as *Pseudomonas fluorescens* G10 and *Microbacterium* sp. G16 based on the 16S rDNA gene sequence analysis. Strains G10 and G16 exhibited different multiple heavy metal and antibiotic resistance characteristics and increased water-soluble Pb in solution and in Pb-added soil. Root elongation assays demonstrated increases in root elongation of inoculated rape seedlings compared to the control plants. Strain G16 produced indole acetic acid, siderophores and 1-aminocyclopropane-1-carboxylate deaminase. Increases in biomass production and total Pb uptake in the bacteria-inoculated plants were obtained compared to the control. The two strains could colonize the root interior and rhizosphere soil of rape after root inoculation.

1. Introduction

Heavy metal contamination in soils is one of the world's major environmental problems, posing significant risks to public health and ecosystems. Therefore, the development of a remediation strategy for metal-contaminated soils is urgent for environmental conservation and human health (Abou-Shanab et al., 2006). Phytoremediation offers significantly more benefits than conventional technology to accumulate heavy metals from the soil due to its less expensive and safer for humans and the environment (Sekhar et al., 2005; Fischerová et al., 2006). But slow growth and low biomass of plants in heavy metal-contaminated soil may limit the efficiency of phytoremediation (Kumar et al., 1995; Burd et al., 2000). This has prompted us to explore the possibilities of enhancing the biomass of metal accumulators using bacteria as plant growth-promoting bioinoculants (Sheng and Xia, 2006). Bacteria that can produce IAA, siderophores and ACC deaminase are capable of stimulating plant growth, lowering the level of ethylene by consuming ACC, the immediate precursor of ethylene in plants growing in the presence of heavy metals and helping plants acquire sufficient iron for optimal growth (Glick et al., 1995; Wang et al., 2000; Rajkumar et al., 2006). Most of the heavy metals have low mobility in soil (Garbisu and Alkorta, 2001; Chen et al., 2004) and are not easily absorbed by plant roots. Plant roots and soil microbes and their interaction can improve metal bioavailability in rhizosphere (Yang et al., 2005; Saravanan et al., 2007). Plant-associated bacteria play a key role in host adaptation to a changing environment (Sturz and Nowak, 2000). Although soil bacteria-assisted phytoremediation has been studied (Whiting et al., 2001; Zaidi et al., 2006; Dell'Amico et al., 2008), there is little information on the potential of endophytic bacteria isolated from plants grown in heavy metal-contaminated soils on the phytoremediation of heavy metal-contaminated soils. 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 (Sturz et al., 2000). Endophytic microorganisms can have the capacity to control pathogens and promote plant establishment under adverse conditions and enhance plant growth and development. A better understanding of the characteristics of heavy metal-resistant endophytic bacteria is a critical prerequisite for the development of effective phytoremediation of heavy metal-contaminated soils.

The objectives of this study were to isolate and characterize Pb-resistant endophytic bacteria from rape grown in heavy metal-contaminated soils, and to select plant growth-promoting and

* Corresponding author. Tel.: +86 25 84396484; fax: +86 25 84396326.
E-mail address: xfsheng604@sohu.com (X.-F. Sheng).

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Pb-solubilizing bacteria which might be useful to increase plant biomass production and Pb uptake by plant under unfavourable environmental conditions for improving the efficiency of phytoremediation of Pb-polluted soils.

2. Methods and methods

2.1. Isolation of Pb-resistant endophytic bacteria from rape root interior

Healthy rape plants were collected from heavy metal-contaminated site in the suburbs of Nanjing, China. The properties and heavy metal contents of the soil sample of the site were: pH (1:1 w/v water) 6.71; organic matter 21.25 g kg^{-1}; total N 2.10 g kg^{-1}; available P 10.7 mg kg^{-1}; available K 85.0 mg kg^{-1}; lead 216.5 mg kg^{-1}; cadmium 47.6 mg kg^{-1}; Zinc 204.5 mg kg^{-1}; copper 36.4 mg kg^{-1}; nickel 12.8 mg kg^{-1}. Plant samples were washed with tap water followed by three rinses with deionized water and then separated into roots and stems. Healthy root samples were sterilized by sequential immersion in 75% (v/v) ethanol for 2 min, and 1% mercuric chloride for 1 min, and then surface-sterilized root samples were washed in sterile water for three times to remove surface sterilization agents. To confirm that the surface disinfection process was successful, root impressions were taken and water from the final rinse was plated out on Petri plates of Luria–Bertani's (LB) agar. No contamination was found. Root materials (0.2 g) were ground in a mortar containing 10 mL sterile distilled water with a pestle. Serial dilutions were prepared and spread on plates containing sucrose-minimal salts low-phosphate (SLP) medium (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) with 20 mg L^{-1} of Pb (as $Pb(NO_3)_2$). To prevent the growth of endophytic fungi, the media were supplemented with 10 mg L^{-1} of fungicidin (USP, Amresco, USA) after autoclaving. Plates were incubated for 72 h at 28 °C. Heavy metal-resistant colonies were purified on the same media by streaking 3–4 times in the fresh media. Sixteen bacterial isolates showing and having different morphological appearance on agar media were selected and stored on slants for studying the solubilization of Pb (as $PbCO_3$).

2.2. Solubilization of poorly soluble lead by the bacteria in solution culture

Inocula of bacterial isolates were prepared by using 20 h logarithmic phase cells. The composition of the incubation medium was the same as the above selection medium supplemented with 200 mg L^{-1} of $PbCO_3$. Triplicate 250-mL Erlenmeyer flasks containing 100 mL of media (autoclaved at 121 °C for 30 min) were inoculated with 1 mL (ca. 10^8 cfu mL^{-1}) of the inoculum. Uninoculated Pb-amended media or inoculated Pb-free media were made as the controls to determine the abiotic influences on Pb solubility or the presence of Pb on the pH in the inoculated media. Control flasks, previously autoclaved at 121 °C for 30 min, and experimental flasks were incubated at 28 °C on the rotary shaker at 150 rpm. After 0, 12, 24, 36, 48 and 60 h, the size of the viable bacterial populations in the flasks was estimated by plate counts. One half of spent cultures was filtered through a 0.22-μm Millipore filter for pH determinations. The other half of the spent cultures was used for water-soluble Pb determination. The harvested spent culture media were centrifuged at 925 *g* for 20 min at 6 °C and filtered through a 0.22-μm Millipore filter. The Pb concentrations in the supernatants were determined with Atomic absorption spectrometer (AAS) (TAS-986, Beijing, China). The greatest Pb ($p < 0.05$) release was obtained with strain G10 and G16 (data not shown). We therefore studied the effect of the two endophytic bacteria on the mobility of Pb in soil and the characterization of the two endophytic bacteria.

2.3. Preparation of Pb-contaminated soil

The soil for the experiments was collected from a nonfertilized field site in Nanjing, in East China (Sheng, 2005). The soil was supplemented with 0, 400 and 800 mg Pb kg^{-1} soil as $Pb(NO_3)_2$. The $Pb(NO_3)_2$ was mixed thoroughly with the soil in a plastic bag before use. The amended soils were allowed to equilibrate for a period of 3 months in the greenhouse undergoing three cycles of saturation with water and air drying, before being remixed (Blaylock et al., 1997).

2.4. Mobility of Pb in soil by the bacteria

Batch studies on the effects of the two bacteria on the mobility of Pb in soil were carried out by using 50-mL Erlenmeyer flasks containing 20 g of above sterilized (autoclaved at 121 °C for 1 h) Pb-contaminated soil. The soil was moistened with sterile water and maintained at 60% of its holding capacity. Inocula of bacterial isolates were prepared as before and 1 mL (ca. 10^8 cfu mL^{-1}) of the inoculum was added to the 20 g of autoclaved soil in the Erlenmeyer flasks. The same amount of sterilized (autoclaved at 121 °C for 40 min) inoculum was added to the 20 g of autoclaved soil as a control. Three replicates were used for each treatment. The flasks were incubated at 28 °C for 10 days. After 10 days, the water-soluble Pb concentrations in the soil extracted by sterile distilled water were determined by AAS (McBride et al., 2004). For determination of soil colonization, 1 g soil was shaken with 10 mL sterile water for 30 min. The resulting suspensions were evaluated for colony forming units according to the dilution-plate method on LB agar with 20 mg L^{-1} of Pb (as $Pb(NO_3)_2$). The pH of the soil (1:1 w/v water) was determined with pH meter.

2.5. Root elongation assay on filter paper culture

The plant root elongation promoting activity of the isolated strains 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. Bacterial suspensions or sterile water (uninoculated control) (6 mL) were added to glass Petri dishes with filter paper. Bacterial suspensions and water were supplemented or not with 5 mg L^{-1} Pb (final concentration) as $Pb(NO_3)_2$. 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, washed with sterile water and the seed sterility was monitored by incubating the seeds on LB agar at 30 °C 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. The assay was repeated two times with four dishes (with 10 seeds per dish) for each treatment.

2.6. IAA, siderophore and ACC deaminase production

The tested bacterial strains 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 developed after 25 min incubation was read at 530 nm. The indole acetic acid (IAA) concentration 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 1-aminocyclopropane-1-carboxylate (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. After reaction of mixtures, containing 100 μl of cell suspension, 10 μl of 0.5 M ACC and 100 μl of 0.1 M Tris–HCl buffer (pH 8.5), for 30 min at 30 °C, 1 mL of 0.56 N 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 2 N HCl was added to 1 mL of the supernatant. The mixtures were reacted for 30 min at 30 °C, supplemented with 1 mL of 2 N NaOH and assayed for α-ketobutyrate via determination of the optical density at 540 nm.

2.7. Heavy metal and antibiotic resistance of the isolates

Heavy metal resistance of the isolates was tested using SLP agar medium with the addition of 20 mg L^{-1} Cd ($CdSO_4$), 200 mg L^{-1} Pb ($Pb(NO_3)_2$), 50 mg L^{-1} Cu ($CuSO_4$), 20 mg L^{-1} Ni ($NiCl_2$), or 100 mg L^{-1} Zn ($ZnSO_4$). Antibiotic resistance was tested using LB agar containing kanamycin (20 μg mL^{-1}), streptomycin (20 μg mL^{-1}), ampicillin (100 μg mL^{-1}) or spectinomycin (20 μg mL^{-1}), which were added aseptically to the medium after autoclaving. Cultures were incubated at 30 °C for 7 days.

2.8. Identification of the bacteria

For the 16S rDNA analysis, genomic DNA was extracted and 16S rDNA was amplified in PCR using the genomic DNA as template and bacterial universal primers, 27f (5′-GAGTTTGATCACTGGCTCAG-3′) and 1492r (5′-TACGGCTACCTTGT TACGACTT-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 unit Taq DNA polymerase, 3.75 pmol primers (each), and 0.5 μl of 2.5 unit Taq polymerase. The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) with a hot start 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 Boya 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 strains G10 and G16 have been deposited in GenBank under accession no. EF091840 and EF091839, respectively.

2.9. Increased Pb uptake by rape

Experiments for studying the effects of the strains on plant growth and Pb uptake of rape were carried out in pots containing above nonsterilized Pb-contaminated soil. Each pot contained 1.0 kg of the soil. Three replicates were used for each treatment. Seeds of rape were surface-sterilized with a mixture of ethanol and 30% H_2O_2 (1:1) for 10 min and washed with sterile water. Five surface-sterilized seeds were placed in each pot. After germination (14 days), plants were thinned to two plants per pot.

To determine the survival rate of the inoculated bacteria in rhizosphere soil and tissue interior, mutants of the heavy metal-resistant and Pb-solubilizing endophytic bacterial strains marked with antibiotic resistance were obtained after plating of the parental strains onto LB agar amended with rifampicin (150 mg L^{-1}). After incubation for 4 days at 28 °C, the rifampicin resistant strains were selected based on similarities in colony morphology, heavy metal resistance, Pb-solubilizing abilities and plant growth promotion with the parent strains and were recultured on rifampicin free medium to check stability of the antibiotic resistance marker.

For inoculation, the strains marked with antibiotic resistance were grown in LB medium. Cells in the exponential phase were collected by centrifugation at 925 *g* for 15 min at 6 °C, washed with sterile distilled water, and recentrifuged. Bacterial inoculum was prepared by resuspending pelleted cells in sterile distilled water to get an inoculum density of ca. 10^8 colony forming units (cfu) mL^{-1}. Bacterial suspensions (10 mL pot^{-1}) were sprayed on the soil surface 3 weeks after seedling emergence. Dead bacteria (autoclaved at 121 °C for 40 min) inoculated samples were prepared as a control. Bacteria-inoculated and dead bacteria-inoculated plants were grown in pots under greenhouse conditions with a temperature of 24–30 °C during the day and 16–21 °C at night. The soil was moistened with water and maintained at 60% of its holding capacity. The plants were harvested 3 weeks after the inoculation treatments. Shoots and roots 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 at 80 °C before determining the shoot and root dry weight. The oven-dried samples (shoot and root) were ground using a stainless steel mill (FZ102, Tianjing, China) to 0.5 mm for analysis. Then subsamples of ground shoot samples (200 mg) and root samples (30 mg) were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, v/v) (Chen et al., 2004). The volume of each sample was adjusted to 10 mL using double deionized water. The concentrations of Pb in the samples were determined by AAS. The value of Pb uptake was contents of reagent blank and analytical duplicates were used where appropriate to ensure accuracy and precision in the analysis. The water-soluble Pb concentrations in the rhizosphere soil of rape extracted by sterile distilled water were also determined by AAS (McBride et al., 2004).

For determination of rhizosphere soil colonization, 0.1 g adhering soil removed from the plant roots was shaken with 10 mL sterile water and 1% fungicidin solution for 30 min. The plant interior colonization was determined according to the method of the above endophytic bacterial selection. The resulting suspensions were evaluated for colony forming units according to the dilution-plate method on LB agar with addition of 150 mg L^{-1} rifampicin. By adding fungicidin and rifampicin, the native fungal and bacterial floras were excluded from the plates. After incubation for 4 days at 28 °C, the reisolated, rifampicin and Pb-resistant strains were identified for colony characteristics, Pb-resistance and plant growth promotion against the parent strains.

2.10. 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 and discussion

3.1. Isolation and identification of heavy metal-resistant endophytic bacteria

We have isolated Pb-resistant endophytic bacteria from root interior of rapes growing 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 20 mg L^{-1}. The endophytic bacterial strain G10 and G16 were the best at Pb-solubilizing in solution culture and were identified as *Pseudomonas fluorescens* G10 (99% similarity) and *Microbacterium* sp. G16 (99% similarity) based on the 16S rDNA gene sequence analysis, respectively.

The surface sterilization protocol was a critical prerequisite for isolating plant endophytic bacteria. This study showed that the surface sterilization protocol was effective in removing epiphytic microorganism, and that the bacterial isolates can be considered to be true endophytic bacteria. This made it possible to isolate and characterize endophytic bacteria associated with healthy rape roots.

3.2. Bacterial lead mobilization

Table 1 showed that inoculation with the *P. fluorescens* G10 and *Microbacterium* sp. G16 significantly ($p < 0.05$) increased the water-soluble Pb in solution, compared to the uninoculated control after 24 h incubation. After 60 h incubation in liquid medium, the pH of the medium in control was 6.90. A decrease in the pH (4.05–4.78) of the medium was observed with the Pb-resistant endophytic bacterial strains (Table 1). In the inoculation with the *P. fluorescens* G10 and *Microbacterium* sp. G16, water-soluble Pb in solution reached its peak values (6250–7540 μg Pb L^{-1}) after 60 h. The concentrations of water-soluble Pb in the presence of strain G10 and G16 were increased 19.7-fold and 16.4-fold compared to the control, respectively. In addition, the associated pH drop results from normal bacterial metabolism as shown in Table 1. The high quantities of biomass produced by the *P. fluorescens* G10 and *Microbacterium* sp. G16 were accompanied with high levels of soluble Pb in the medium after 24 h incubation. The results showed that insoluble Pb was gradually solubilized during the growth of the *P. fluorescens* G10 and *Microbacterium* sp. G16 as shown in Table 1, indicating that the strains have the potential to solubilize Pb. When grown in the presence of the $PbCO_3$, the strains decreased the pH of the medium which may account for the increase in Pb release by the strains in the culture filtrates. In addition, the concentrations of water-soluble Pb in solution in inoculated treatments were lower than uninoculated treatments before 12 h incubation. The decrease in the concentrations of water-soluble Pb in inoculated treatments may be due to the adsorption of Pb^{2+} by the cells of the two endophytic bacteria (Ansari and Malik, 2007).

To test the effects of the *P. fluorescens* G10 and *Microbacterium* sp. G16 on soil water-soluble Pb, we examined Pb concentrations in

Table 1
Time courses of water-soluble Pb (μg L^{-1}) content, cell counts ($\times 10^6$ cfu mL^{-1}) and pH under lead carbonate condition

Time (h)	Uninoculated control		Inoculated with strain G10			Inoculated with strain G16		
	Water-soluble Pb	pH	Water-soluble Pb	pH	Cell counts	Water-soluble Pb	pH	Cell counts
0	305 ± 21	7.15	155 ± 35	7.12	4.5	160 ± 37	7.10	7.2
12	364 ± 44	7.10	304 ± 46	7.10	66 ± 7	315 ± 54	7.11	73 ± 8
24	358 ± 53	7.02	2716 ± 89	5.16	138 ± 5	2150 ± 108	6.10	151 ± 19
36	368 ± 40	6.96	4985 ± 128	4.90	368 ± 34	3774 ± 126	5.45	395 ± 32
48	375 ± 57	6.95	6788 ± 146	4.15	449 ± 26	5910 ± 167	5.12	495 ± 40
60	381 ± 64	6.90	7540 ± 178	4.05	435 ± 19	6250 ± 152	4.78	481 ± 27

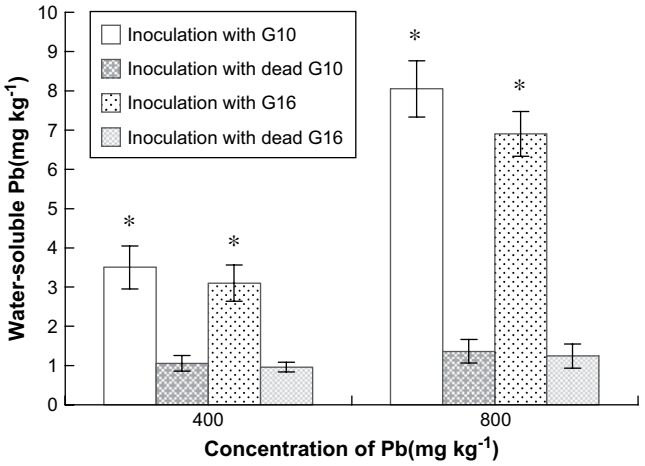

Fig. 1. The influence of the endophytic bacterial strains on water-soluble Pb (mg kg^{-1}) in a sterile yellow brown soil added with 400 and 800 mg kg^{-1} of Pb. Error bars are ±standard deviation ($n = 3$). An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$).

soil solution in response to the endophytic bacteria inoculated in the sterile Pb-contaminated soil. Fig. 1 showed that inoculation with the *P. fluorescens* G10 and *Microbacterium* sp. G16 significantly ($p < 0.05$) increased the water-soluble Pb in soil, compared to the control after 10 days incubation. In the bacteria-inoculated soils, the concentrations of water-soluble Pb were increased from 3.3- to 5.9-fold (strain G10) and from 3.2- to 5.6-fold (strain G16) compared to the dead bacteria-inoculated control, respectively. This study showed that the two endophytic bacteria isolated from the roots of rape plants growing in heavy metal-contaminated site had a pronounced effect on increasing the bioavailability of Pb in the soil. The microorganism-induced increase in Pb concentration of soil solution appears to be directly associated with bacterial activity. The cell numbers of the strains G10 and G16 were increased from 5.0×10^6 cfu g^{-1} fresh soil to 3.6×10^7 cfu g^{-1} fresh soil (G10) and 1.2×10^7 cfu g^{-1} fresh soil (G16) after 10 days incubation. The pH in the inoculated soils was not significantly decreased compared to the control (data not shown), low molecular weight organic acids excreted by bacteria might facilitate increased heavy metal availability (Saravanan et al., 2007).

3.3. Root length promotion

The effects of two Pb-resistant endophytic bacterial strains on root elongation of *B. napus* variety Qinyou-7 in the absence or presence of Pb are shown in Table 2. The addition of 5 mg L^{-1} Pb to the filter paper culture inhibited root elongation of uninoculated seedlings by 12% ($p < 0.05$). Inoculations with Pb-resistant strains in the presence of Pb also significantly increased the root length of Pb-treated seedlings by 21–35%. The maximum root length-promoting effect on Pb-treated plants was observed after inoculation

Table 2
Root length of *Brassica napus* variety Qinyou-7 seedlings inoculated with heavy metal-resistant endophytic bacteria and grown in the absence or presence of Pb

Bacterial strains	Untreated seedlings		Treated with 5 mg L^{-1} Pb	
	Root length (mm)	Bacterial effect[a] (%)	Root length (mm)	Bacterial effect (%)
Uninoculated control	48 ± 2.05		43 ± 1.53	
Strain G10	64 ± 4.36*	+33	52 ± 4.00*	+21
Strain G16	65 ± 3.61*	+35	58 ± 4.58*	+35

An asterisk (*) denotes a value significantly greater than the control value ($p < 0.05$).
[a] Means the effect of heavy metal-resistant endophytic bacteria on the root growth of the rape.

Table 3
Characteristics of the isolated endophytic heavy metal-resistant bacterial strains

Strain	Indole production (mg L^{-1})	Siderophore production	ACC deaminase activity (μM α KB mg^{-1} h^{-1})
Strain G10	15.8 ± 2.1	+	ND
Strain G16	27.9 ± 3.6	+	28.5 ± 2.3

+, Positive; ND, no detection.

with *Microbacterium* sp. G16. The production of IAA in the two endophytic metal-resistant *P. fluorescens* G10 and *Microbacterium* sp. G16 may facilitate rape growth. An increase in root growth and root length, generally promoted by IAA-producing rhizobacteria (Patten and Glick, 1996), was also observed under toxic Pb concentration in rape inoculation experiments using *P. fluorescens* G10 and *Microbacterium* sp. G16 (IAA producers). Bacterial endophytes are able to promote plant growth and health (Reiter et al., 2002).

3.4. Plant growth-promoting characteristics of the strains

The endophytic bacterial strains had the capacity to produce siderophore and IAA in culture when the medium was supplemented with L-tryptophan. *Microbacterium* sp. G16 showed a higher production of IAA (27.9 ± 3.6 μg mL^{-1}) than *P. fluorescens* G10 (15.8 ± 2.1 μg mL^{-1}) (Table 3). In addition, the endophytic bacterial strain G16 shows ACC deaminase activity (28.5 ± 2.3 μM α KB mg^{-1} h^{-1}).

3.5. Heavy metal and antibiotic resistance of the strains

The two strains were found to exhibit different multiple heavy metal and antibiotic resistance characteristics. The *P. fluorescens* G10 was resistant to heavy metals such as Pb (200 mg L^{-1}), Cd (20 mg L^{-1}) and Zn (100 mg L^{-1}), and the *Microbacterium* sp. G16 was resistant to Pb (200 mg L^{-1}), Cd (20 mg L^{-1}), Zn (100 mg L^{-1}), Cu (50 mg L^{-1}) and Ni (20 mg L^{-1}). The two strains exhibited antibiotic resistance characteristics to kanamycin (20 μg mL^{-1}), streptomycin (20 μg mL^{-1}), ampicillin (100 μg mL^{-1}) and spectinomycin (20 μg mL^{-1}) for strain G10 and to kanamycin (20 μg mL^{-1}), ampicillin (100 μg mL^{-1}) and spectinomycin (20 μg mL^{-1}) for strain G16.

3.6. Plant growth promotion

Low and high Pb treatments significantly decreased the growth of rape. Significant increases of root and shoot dry weights were observed when the soil was inoculated with strains G10 and G16, compared to the dead bacterial-inoculation soil in rape plants (Table 4). Root dry weights were increased from 23 to 37% ($p < 0.05$) and shoot dry weights were increased from 12 to 39% ($p < 0.05$) when the soil was inoculated with the strains G10 and G16 compared to the soil with the two dead bacterial-inoculation (Table 4).

Table 4
The influence of the endophytic isolates on the dry weight (mg) of rape on a yellow brown soil added with 0, 400 and 800 mg kg^{-1} of Pb

Treatment	Shoot			Root		
	0	400	800	0	400	800
+Strain G10	390 ± 20.3*	310 ± 3.5*	260 ± 11.5*	81 ± 3.6*	59 ± 1.7*	58 ± 3.5*
+Dead strain G10	280 ± 6.6	230 ± 7.0	210 ± 6.6	62 ± 7.2	46 ± 2.7	44 ± 3.0
+Strain G16	360 ± 7.2*	300 ± 18.5*	240 ± 13.5*	78 ± 5.3*	54 ± 3.6*	53 ± 3.6*
+Dead strain G16	268 ± 6.2	220 ± 7.8	215 ± 7.6	57 ± 6.2	44 ± 2.4	41 ± 2.0

An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$).

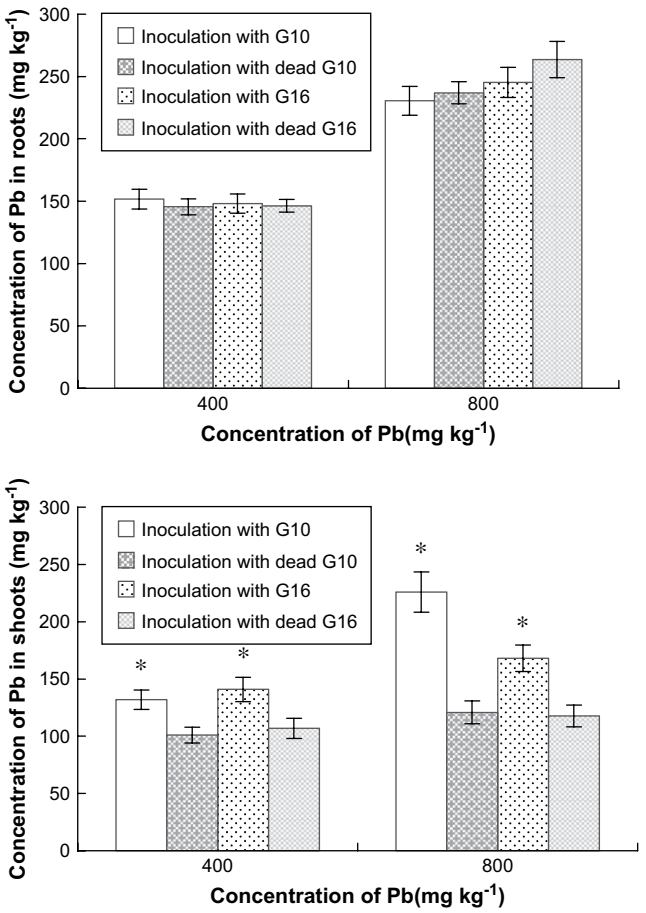

Fig. 2. The influence of the endophytic bacterial strains on root and shoot Pb concentration (mg kg^{-1}) of rape on a yellow brown soil added with 400 and 800 mg kg^{-1} of Pb. Error bars are ±standard deviation ($n = 3$). An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$).

The data from this study suggested that the ability of the endophytic bacteria to protect plants against the inhibitory effects of high concentrations of Pb may be related to their capacity to stimulate rape plant growth by synthesis of IAA, siderophore or ACC deaminase activity as has been shown for many PGPRs (Burd et al., 2000; Grichko et al., 2000; Hontzeas et al., 2004; Egamberdiyeva and Höflich, 2004). The ACC deaminase has been proposed to play a key role in microbe–plant association (Hontzeas et al., 2004). PGPR can significantly increase the growth of plants in the presence of heavy metals including nickel, lead and zinc (Burd et al., 2000; Grichko et al., 2000; Dell'Amico et al., 2005; Khan, 2005).

3.7. Mobilization of lead to plants

Based on the bacterial Pb mobilization and root length promotion, two Pb-resistant strains *P. fluorescens* G10 and *Microbacterium* sp. G16 were selected for the mobilization of Pb in rape plants. There was no significant difference in root Pb concentrations of rape plants between bacterial inoculation and dead bacterial inoculation (Fig. 2). However, significant increase in shoot Pb concentrations of rape and water-soluble Pb concentrations in rhizosphere soil was obtained in the bacterial-inoculation treatments compared to the dead bacterial-inoculation control (Fig. 2, Table 5). No significant difference in shoot Pb concentrations was observed between the two dead bacterial-inoculation treatments. The two Pb-resistant strains *P. fluorescens* G10 and *Microbacterium* sp. G16 significantly increase the total Pb uptake in shoots (μg $plant^{-1}$) of rape plants when the Pb was added at rate of 400 and 800 mg kg^{-1} soil. No significant increases of root total Pb uptake (μg $plant^{-1}$) were observed when the soil was inoculated with strains G10 and G16, compared to the two dead bacteria-inoculated soil when the Pb was added at rate of 800 mg kg^{-1} soil. For the rape plants grown in soil inoculated with the strains G10 and G16, shoot total Pb uptake (μg $plant^{-1}$) was increased from 76 to 131% ($p < 0.05$) for strain G10 and from 59 to 80% ($p < 0.05$) for strain G16, root total Pb uptake (μg $plant^{-1}$) was increased from 29 to 33% for strain G10 and from 20 to 25% for strain G16, compared to the dead bacterial-inoculation control, respectively. In addition, in the bacteria-inoculated rape plants, the total Pb uptake in shoots was from 4- to 5-fold increase for strain G10 and from 3- to 5-fold increase for strain G16, compared to the Pb uptake in roots, respectively (Table 5).

Studies have evidenced that heavy metal-resistant bacteria can enhance metal uptake by hyperaccumulator plants (de Souza et al., 1999; Whiting et al., 2001). The same results were obtained in our experiment that the total Pb uptake by rape plants was significantly enhanced by the heavy metal-resistant endophytic bacterial strain G10 and G16. Although a number of studies have demonstrated the importance of bacterial inoculation for plant growth and heavy metal accumulation in heavy metal-polluted environments (Abou-Shanab et al., 2003; Idris et al., 2004; Khan, 2005; Sheng and Xia, 2006), to the best of our knowledge, this is the first research report elucidating the role of heavy metal-resistant endophytic *P. fluorescens* G10 and *Microbacterium* sp. G16 in Pb solubilization in solution and soil and Pb accumulation by rape with concurrent promotion of plant growth in a pot experiment. These increases in Pb uptake may translate into higher water-soluble Pb concentrations in rhizosphere soils of the inoculated plants (Table 5). In this study, we found that the application of the heavy metal-resistant endophytic bacterial strains effectively increased the bioavailability of Pb in the rhizosphere soils and promoted the growth of rape plants, consequently increasing the total Pb uptake of the rape plants even under nonsterile conditions.

3.8. Survival and establishment of antibiotic resistant bacteria in rape

Mutants of the antibiotic resistant endophytic bacterial strains were obtained to determine the survival rate of the inoculated bacteria in rhizosphere soil and tissue interior. Two rifampicin and

Table 5
The influence of the endophytic isolates on the total Pb uptake (μg $plant^{-1}$) of rape and the water-soluble Pb (mg kg^{-1} soil) in rhizosphere soil on a yellow brown soil added with 400 and 800 mg kg^{-1} of Pb

Treatment	Shoot		Root		Water-soluble Pb	
	400	800	400	800	400	800
+Strain G10	40.9 ± 4.4*	58.7 ± 7.0*	8.9 ± 0.7*	13.4 ± 2.1	2.75 ± 0.60*	6.58 ± 1.55*
+Dead strain G10	23.2 ± 2.3	25.4 ± 2.7	6.7 ± 0.6	10.4 ± 1.0	1.80 ± 0.15	4.00 ± 0.84
+Strain G16	42.2 ± 4.5*	40.3 ± 3.3*	8.0 ± 0.5*	13.0 ± 1.6	2.40 ± 0.44*	5.76 ± 1.20*
+Dead strain G16	23.5 ± 2.6	25.3 ± 2.9	6.4 ± 0.7	10.8 ± 0.9	1.65 ± 0.20	3.50 ± 0.95

An asterisk (*) denotes a value significantly greater than the corresponding control value ($p < 0.05$).

Table 6
Colonization of the effective isolates in the rhizosphere soil and root interior of rape after inoculation in a yellow brown soil added with 0, 400 and 800 mg kg^{-1} of Pb (pot experiment, mean and standard deviation of three replicas per treatment, values in 10^3 cfu g^{-1} of fresh soil or root)

Concentration of Pb (mg kg^{-1})	Strain G10		Strain G16	
	Rhizosphere soil	Root interior	Rhizosphere soil	Root interior
0	1685 ± 176	1.24 ± 0.08	1328 ± 141	2.05 ± 0.48
400	950 ± 85	0.45 ± 0.06	1020 ± 110	1.60 ± 0.35
800	1220 ± 130	0.36 ± 0.05	970 ± 80	1.36 ± 0.28

Pb-resistant endophytic bacterial strains of *P. fluorescens* G10 and *Microbacterium* sp. G16 were tested for their ability to colonize rape rhizosphere soil and tissue interior. Low and high Pb treatments significantly decreased the bacterial rhizosphere soil colonization. The bacteria were detectable in the bacteria-inoculated rape rhizophere soil and root interior for 3 weeks after the inoculation treatments (Table 6). The bacteria were no detectable in the dead bacteria-inoculated rape rhizophere soil and root interior. However, the survival of the inoculated bacterial strain G16 was better on rape root interior (1.36–1.60 × 10^3 cfu g^{-1} of fresh root) than that of the inoculated bacterial strain G10 (0.36–0.45 × 10^3 cfu g^{-1} of fresh root).

As successful plant growth-promoting inoculants, bacteria must be able to rapidly colonize the root system during the growing season (Defreitas and Germida, 1992). Although the colonization of the endophytic *P. fluorescens* G10 and *Microbacterium* sp. G16 in the shoots or foliages could not be detected (data not shown), the tested strains were able to colonize the rhizosphere soils or root interiors (i.e. a facultative endophyte) of rape (Table 6). According to Paau (1989), competitive and effective bacterial strains must be screened and isolated from the pool of indigenous bacteria, which supposedly are adapted to the particular conditions of the site.

4. Conclusion

Phytoremediation is an environmental friendly, cost-effective and plant-based solution for the remediation of heavy metal-contaminated soils. Low biomass production and slow growth of the plants and the low availability of heavy metals in soil limited effective remediation (Kayser et al., 2000; Quartacci et al., 2006). Effective phytoremediation could be accomplished by bacteria having the potential of solubilizing heavy metals and promoting plant growth in contaminated soils. Our researches demonstrated that heavy metal-resistant, Pb-solublizing and plant growth-promoting endophytic bacteria could be isolated from rape plants grown in heavy metal-contaminated site and chosen as the bioinoculant for the effective phytoremediation of Pb-contaminated soil. The *P. fluorescens* G10 and *Microbacterium* sp. G16 were facultative endophyte which could colonize the rhizosphere soils and plant tissue interiors. The endophytic bacteria colonizing in the plant tissue interiors might protect plants against the inhibitory effects of high concentrations of Pb and promote the plant growth by production of IAA or ACC deaminase, and the endophytic bacteria colonizing in the rhizosphere soils of rape might promote plant growth and Pb uptake by production of IAA, siderophore or ACC deaminase or by solubilization of Pb in soils as shown in our research. A further understanding of the relationship between the plant and the plant growth-promoting endophytic bacteria is essential for the development of effective phytoremediation of Pb-contaminated soils. This may therefore provide a new endophytic bacteria-assisted phytoremediation of Pb-contaminated soil.

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