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ORIGINAL PAPER

Isolation and characterization endophytic bacteria from hyperaccumulator *Sedum alfredii* Hance and their potential to promote phytoextraction of zinc polluted soil

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Abstract The aim of this study was to isolate and characterize endophytic bacteria from roots, stems and leaves of Zn/Cd hyperaccumulator *Sedum alfredii*. Endophytic bacteria were observed in roots, stems and leave of *S. alfredii*, with a significantly higher density in roots, followed by leave and stems. A total of fourteen bacterial endophytes were isolated and are closely related phylogenetically to *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Acinetobacte* by 16S rRNA sequence analysis. Most of the endophytic bacteria were found to exhibit high Zn and Cd resistance characteristics, but difference existed among this isolates. The fourteen endophytic bacteria all had the capacity to produce IAA. Moreover, strains VI_8L_1, VI_8L_2, VI_8L_4, VI_8R_2, VI_8R_3 and II_2R_3 could solubilize $Ca_3(PO_4)_2$, strains VI_8L_2, II_8L_4 and VI_8R_2 could produce siderophore, and strains VI_8L_2 and VI_8R_3 had the capacity of nitrogen fixation. Both plate and broth assay proved that strain VI_8L_1, VI_8L_2, II_8L_4 and VI_8R_2 were able to effectively solubilize $ZnCO_3$ and $Zn_3(PO_4)_2$. The filtrate liquid media after growth of strains VI_8L_1, VI_8L_2, II_8L_4 and VI_8R_2 extracted much higher Zn from artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils than those extracted by axenic SMS broth, and the filtrates of the culture media supporting growth of strains VI_8L_2, II_8L_4 and VI_8R_2 also extracted significantly greater quantities of Zn from the Dabaoshan contaminated soils. This Zn mobilizing, plant growth promoting and metal resistant endophytic bacteria may offer promise as inoculants to increase soil Zn bioavailability and improve growth and Zn accumulation by *S. alfredii*.

Keywords Endophytic bacteria · Heavy metal resistance · Plant growth promoting bacteria · Phytoextraction

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Introduction

Endophytic bacteria are defined as bacteria that colonize healthy plant tissue without causing obvious disease symptoms in host plant. Endophytic bacteria seem to be ubiquitous in plant kingdom and have been isolated from roots, leaves and stems, and a few from flowers, fruits and seeds (Lodewyckx et al. 2002). Endophytic bacteria may complement certain metabolic properties, such as promoting plant growth, controlling soil-borne pathogens, or helping host plant to overcome stress responses to environmental insults (Mastretta et al. 2006; Ryan et al. 2008). Generally, researches reported that endophytes promote plant growth by a number of similar mechanisms like soil plant growth promoting bacteria (PGPB), including phosphate solubilization activity (Verma et al. 2001; Wakeli et al. 2004), indole acetic acid (IAA) production (Lee et al. 2004) and production of siderophore (Costa and Loper 1994). Endophytic organisms can also supply essential vitamins to plants (Pirttilä et al. 2004). Moreover, a number

of other beneficial effects on plant growth have been attributed to osmotic adjustment, stomatal regulation, modification of root morphology, enhanced uptake of minerals and alteration of nitrogen accumulation and metabolism (Compant et al. 2005a, b).

Metal hyperaccumulators are plants which accumulate extreme amounts of trace metals in their aboveground biomass when growing in metal enriched habitats (mg kg^{-1}; $>$ 10,000 (Mn or Zn), $>$ 1,000 (Cu, Co,Cr, Ni, Pb) or $>$ 100 (Cd) (Baker et al. 2000). Currently, the interactions between endophytes and hyperaccumulator plants have attracted the attention of several investigators due to biotechnological applications for bioremediation and for studying the composition of bacterial communities living on a naturally contaminated environment (Lodewyckx et al. 2002; Idris et al. 2004). For example, various pink-pigmented facultative methylotrophic were obtained from the rhizosphere and endosphere of Ni hyperaccumulating plant *Thlaspi goesingense* grown in Redschlag, Austria. Methylobacteria also showed high abundance and diversity among rhizosphere and endophyte isolates, and were characterized by high Ni tolerance, siderophore production and in some strains ACC deaminase activity (Idris et al. 2004, 2006). Barzanti et al. (2007) isolated 83 endophytic bacteria from roots, stems, and leaves of Ni hyperaccumulator *Alyssum bertolonii*. They pointed out that, despite the high concentrations of heavy metals present in its tissues, *Alyssum bertolonii* harbors an endophytic bacterial flora showing a high genetic diversity as well as a high level of resistance to heavy metals, which could potentially help plant growth and Ni hyperaccumulation.

Phytoextraction, an emerging low-cost and ecologically benign technology for decontamination of soils, is the use of metal-accumulating plants to remove contaminants from soils, sediments or water into harvestable plant biomass. Research confirmed that plant biomass production, shoot metal concentration and soil metal bioavailability are the major factors determining the efficiency of the phytoextraction process (McGrath and Zhao 2003). Unfortunately, most of the natural hyperaccumulator grow slowly and have small shoot biomass, while large-biomass crop plant can not tolerate high metal stress and have low metal concentration factor. In addition, metal uptake by plants is usually limited by low metal solubility at field condition (Salt et al. 1998). Therefore, many efforts are still necessary for selection of appropriate agricultural management and rhizosphere manipulation to promote plant growth and improve metal accumulation by plants. In metal contaminated soil, plant associated bacteria, both rhizobacteria and endophyte, may play an important role in plant growth and metal accumulation (Rajkumar et al. 2009). In addition to plant growth promoting potential, certain metal resistant endophytes have been shown to be able to alter heavy metal toxicity and availability to the plant through acidification, or by producing siderophores, organic acids and/or mobilizing the metal phosphates (Saravanan et al. 2007; Sheng et al. 2008). For instance, Saravanan et al. (2007) reported the production of 5-ketogluconic acid, a major gluconic acid derivative product that aids in the solubilization of different Zn compounds by endophyte *Gluconacetobacter diazotrophicus* under in vitro conditions. Sheng et al. (2008) observed that the inoculation of *Brassica napus* with Pb resistant endophytic bacteria increased Pb uptake into the shoot from 76 to 131% (*Pseudomonas fluorescens*) and from 59 to 80% (*Microbacterium sp.*), compared to the dead bacterial-inoculation control. A possible explanation might be the production of siderophore or by solubilization of Pb. Mastretta et al. (2009) found that the inoculation of *Nicotiana tabacum* with Cd resistant endophyte *Sanguibacter* sp. *S_d* increased the concentration of Cd in shoot tissues by approximately three-fold compared with respective un-inoculated control. These studies suggest that it will be possible to improve the metal extraction potential of hyperaccumulator plants by inoculating the seeds/rhizosphere with selected metal resistant PGPB endophytes.

S. alfredii has been studied extensively with respect to its Cd/Zn hyperaccumulation characteristics. This plant displayed high translocation of soil Cd and Zn to shoots when compared to non-accumulating plants (Long et al. 2002; Yang et al. 2004). However, no study has been reported to the relationships between *S. alfredii* and their associated endophytic bacteria. The purpose of this study is (1) to isolate and characterize endophytic bacteria from Zn/Cd hyperaccumulator *S. alfredii* collected from a phytoremediation field experiment site; (2) to select endophytic bacteria which have the ability of solubilizing insoluble Zn compound; (3) to assess isolate's plant growth promoting traits like production of IAA and siderophore, phosphate solubilization, and to grown on nitrogen-free liquid medium. The creation of such metal tolerant plant–microbe associations is aimed at improving the efficiency of phytoremediation of heavy metal polluted soils.

Materials and methods

Isolation of endophytic bacteria from *Sedum alfredii*

Healthy plants *S. alfredii* were collected from a phytoremediation field experiment, which was conducted on a paddy soil located at Fogang, in Northern Guangdong, China. The soils had been contaminated with Zn and Cd due to surface irrigation with the Pb/Zn mining wastewater since 2002 (Zhou 2009). The planting treatments included mono-planting *S. alfredii* and co-planting of *S. alfredii* with *Zea mays*. The properties and total heavy metal

concentrations of the paddy soils were: pH 4.69, organic matter 34.76 g kg^{-1}, total N 1.74 g kg^{-1}, available P 58.82 g kg^{-1}, available K 76.89 g kg^{-1}, total Zn 284 mg kg^{-1}, total Cd 1.01 mg kg^{-1}, total Pb 104 mg kg^{-1}. Plant samples were washed with tap water followed by three rinses with deionized water, and then separated into roots, stems and leaves. Healthy root, stem and leaf samples were sterilized by sequential immersion in 97% ethanol for 1 min, 30% H_2O_2 and 3% sodium hypochlorite for 30 min, 97% ethanol for 1 min, and then surface-sterilized samples were washed in sterile deionized water three times to remove surface sterilization agents. To confirm the surface disinfection process was successful, triplicate root, stem and leaf samples and water from the final rinse were separately plated out on Petri plates of Luria–Bertani's (LB) agar for 7 days. No contamination was found. Root, stem and leaf (5 g, fresh weight) were ground in a sterile mortar. Serial dilutions of this suspension were prepared (10^1–10^3) and from each dilution of the series, 0.1 ml suspension was spread on plates containing LB agar. After incubation for 7 days at 30°C, the number of aerobic heterotrophic bacteria was determined as colony-forming units (CFUs), and colony variation in morphology was picked and repeatedly re-streaked on LB medium for three times until the colony morphology of each isolate was homogenous. Then each isolate was stored on slants with fresh LB medium for further use.

Identification of the isolates

The bacteria strains were identified based on morphological and biochemical features, including gram staining, catalase, sugar fermentation, starch hydrolysis, cellulose decomposition, and motility of these isolates according to the method described by Dong and Cai (2001). For further characterization, genomic DNA of the test bacterial strains grown on LB broth was extracted with MiniBEST Bacterial Genomic DNA Extraction Kit. Full-length 16S rRNA gene was PCR amplified by using 100 ng genomic DNA as template with 20 pmol of bacteria universal primers 27f (5′-AGAGTTTGATCATGGCTCAG-3′) and 1500R (5′-AAGGAGGTGATCCAGCCGC-3′). The PCR mixture (50 µl) contained 1 µl template, 5 µl of 10 × Tap DNA polymerase buffer (Mg^{2+} plus), 4 µl dNTP at 2.5 mM, 0.25 µl of 5 unite Taq polymerase. The PCR was performed in a DNA Engine Thermal Cycler (TaKaRa TP 600, Germany) with a hot start performed at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by a final extension performed at 72°C for 5 min. The amplification products were sequenced by Shanghai Invitrogen Biotechnology Company, Limited (Shanghai, China).

Determination of metal minimal inhibitory concentration

The bacterial level of resistance to Cd and Zn was analyzed in LB medium supplemented with the appropriate amount of soluble metals, checking for growth after 7 days incubation at 30°C. The different concentration series of Cd ($CdSO_4$) ranged from 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4 and 5 mmol L^{-1}, while that of Zn ($ZnSO_4$) ranged from 0, 0.5, 1, 2, 4, 6, 8, 10, 15 and 20 mmol L^{-1}.

Evaluation of plant growth promoting activities

Phosphate solubilization

The phosphate-solubilizing activity of each strain was determined by measuring the zone size formed by solubilization of insoluble phosphate on Pikovskaya's agar plates (in g l^{-1}: $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; $MnSO_4$, 0.002; $FeSO_4{\cdot}7H_2O$, 0.002; KCl, 0.2; yeast extract, 0.5; glucose 10.0; $Ca_3(PO_4)_2$, 5.0; pH 7.0 ± 0.02 and agar 15.0). The zone of clearance around the colony was observed after 2 ~ 7 days.

Indole acetic acid (IAA) production

The IAA production by the isolates was quantitatively assayed by the method of Libbert et al. (2006). In brief, each strain was cultured in flasks containing 100 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 L-tryptophan with or without Zn^{2+} (1 mM, 2 mM), then cultivation was performed in the dark at 30°C on a shaker (160 rpm). 10 ml of culture was removed from each flask and filtered through a sterile filter paper (0.22 µm pore size). To one part of the supernatant one part of the Salkowski's reagent (50 ml of 35% $HClO_4$ + 1 ml of 0.5 M $FeCl_3$) was added, and allowed to stand at room temperature for 20 min. Development of pink colour indicates IAA production. The absorbance of pink color developed was read at 530 nm using pure IAA as a standard.

Nitrogen fixation

To estimate nitrogen fixation ability, a full loop of bacteria was inoculated into 50 ml of nitrogen-free liquid medium (Jiang 2005): per liter containing mannitol, 10 g; KH_2PO_4, 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.2 g; $CaSO_4{\cdot}2H_2O$, 0.1 g; $CaCO_3$, 5.0 g; pH 7.0 ~ 7.5 and was then incubated at 28°C with shaking for 2 ~ 3 days. The bacteria growing in this medium were subcultured by transfer 1 ml of the

culture into another flask with the same medium. Vigorous growth after three cycles of subculture demonstrated that the endophytic bacterium have ability of N_2 fixation.

Siderophore production

The Chromeazurol S (CAS) assay (Schwyn and Neilands 1987) was used to detect siderophores. The CAS liquid assay was performed as follows: 0.013 g CAS was dissolved in 10 ml of deionised water, and mixed with 2 ml of a Fe^{3+} solution (1 mmol l^{-1} $FeCl_3 \cdot 6H_2O$ in 10 mmol l^{-1} HCl). While stirring, this solution was slowly mixed with 0.016 g of exadecyltri-methylammonium bromide (HDTMA) previously dissolved in 8 ml water. The resulting dark-blue solution was autoclaved, cooled to 50°C ~ 60°C and mixed with 20 ml of sterile medium MM9, 6.04 g of Pipes previously dissolved in 150 ml water and 15% agar. The 50% NaOH was added until the pH of the solution was 6.8. Finally, the above solution were mixed with 0.2 ml of 1 mmol l^{-1} $CaCl_2$, 4 ml of 1 mmol l^{-1} $MgSO_4 \cdot 7H_2O$, 2 ml of 20% glucose, and 6 ml of 0.5 mg ml^{-1} L-tryptophan. This medium was allowed to gel on Petri dishes.

Strains were cultured three times in an iron deficient liquid medium (MM9). The medium was composed of 3 g l^{-1} KH_2PO_4; 60 g $l^{-1}Na_2HPO_4$; 5 g l^{-1} NaCl; 10 g l^{-1} NH_4Cl; and 30.24 g l^{-1} PIPES. This solution was autoclaved and supplemented with 1 ml of 1 mmol $CaCl_2$ and 20 ml of 1 mM $MgSO_4 \cdot 7H_2O$, 20 ml of 20% glucose, and 60 ml of 0.5 mg/ml L-tryptophan. These strains grown well in liquid medium were subsequently inoculated in Petri dishes and incubated in the dark (30°C for 10 days). Positive results were indicated by the formation of a clear halo around the colonies, showing a visual change in color form dark-blue to yellow. Each assay was performed in triplicate.

In vitro solubilization of insoluble zinc compound

The strains grown in LB broth for 24 h were spotted in 10 µl volumes to SMS plates (in g l^{-1}: sucrose, 10; $(NH_4)_2SO_4$, 1; K_2HPO_4, 2; $MgSO_4$, 0.5; NaCl, 0.1; yeast extract, 0.5; $CaCO_3$, 0.5; pH 7.2) amended with insoluble Zn compounds (ZnO, $ZnCO_3$, $Zn_3(PO_4)_2$,) at 30°C for 7 days. The diameters of the clearing zones around the colonies were measured. If the strain can produce clear zone, broth cultures were conducted to certify its solubilization potential. 100 µl log-phase cultures of the strains were inoculated to 100 ml of LB broth supplemented with insoluble Zn compounds in a gyratory shaker (160 rpm) for 7 days. Uninoculated medium served as a control. The Zn concentrations of the culture supernatants and pH were estimated at different time.

Effects of bacterial metabolite on the mobility of soil Zn

The bacterial strains were cultured in SMS medium at 28°C in shaken flasks (200 rpm) for 48 h. Bacterial cells were harvested by centrifugation (10,000 rpm) at 4°C for 5 min, the supernatant including the bacterial metabolite was used to extract Zn from two artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils and a polluted paddy soil from Dabaoshan located at South Guangdong province of China, using the sterile SMS medium and deionized water as control. Ten milliliter cell supernatant or SMS medium, or water was added to two grams soil. Soil suspension were vibrated at 25°C for 2 h, and then centrifugated at 4,000 rpm for 15 min. Zinc concentrations in the extracted solutions were determined by AAS (Z-5300). The artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils were prepared as follows: A clean soil was collected from the farm of South China Agricultural University. The basic properties of the soil samples were pH (1:2 w/v water) 5.30; organic matter, 90.69 g kg^{-1}; total Zn, 74.3 mg kg^{-1}. Fine powder of $ZnCO_3$ or $Zn_3(PO_4)_2$ was added and mixed with 10 kg soil to given the 500 mg kg^{-1} Zn, and incubated at room temperature for 60 days before use. The basic properties of the Dabaoshan polluted paddy soil samples were pH (1:2 w/v water) 4.68; total Zn, 332 mg kg^{-1}, total Cd, 0.54 mg kg^{-1}, total Cu, 407 mg kg^{-1} and total Pb 490 mg kg^{-1}.

Results

Isolation and identification of endophytic bacteria isolates

Cadmium concentrations in root and shoot tissues of *S. alfredii* ranged from 71.2 to 100.3 mg kg^{-1} (average 86.9 ± 9.7 mg kg^{-1}, $n = 8$) and from 42.0 to 93.0 mg/kg (average 68.8 ± 17.2 mg kg^{-1}, $n = 8$), respectively. Zn concentrations in root and shoot tissues of *S. alfredii* were in the range of 6,298 ~ 13,366 mg kg^{-1} (average 10,642 ± 2,520 mg kg^{-1}, $n = 8$) and 6,993 ~ 12,668 mg kg^{-1} (average 10,972 ± 1,988 mg kg^{-1}, $n = 8$), respectively. Cadmium and zinc concentrations of roots and shoots were all significantly higher than those of soil, while no significant differences of Zn and Cd concentrations were found between roots and shoots.

The surface sterilization protocol was a critical prerequisite for isolating plant endophytic bacteria. This study proved that the surface sterilization protocol was effective in removing epiphytic microorganism, and that the bacterial isolates can be considered to be true endophytic bacteria. Though with high Zn and Cd concentration in plant tissues, *S. alfredii* hosted an abundance of endophytic bacteria. The

total culturable bacterial densities were significantly different among root, stem and leaf tissues, endophytic bacteria count ranged from 2,000 to 816,000 CFU g^{-1} for roots, 3,000 to 3,300 CFU g^{-1} for stems, and 199 to 18,8000 CFU g^{-1} for leaves, respectively. Based on colony morphology, a total of fourteen endophytic bacteria isolates were randomly picked up, with five from leaves, four from stems and five from roots. There was a large variation in colonial morphology—color, shape and size among these isolates. Both Gram positive and Gram negative bacteria were found, but the number varied in different tissues. All the isolated strains had flagellar motility, three strains had the ability of starch hydrolysis, and four strains had the ability of cellulose decomposition (Table 1). The 16S rDNA sequences indicated that the majority of these isolates are closely related phylogenetically to *Pseudomonas fluorescens*, *Bacillus cereus*, *Bacillus subtilis*, *Stenotrophomonas maltophilia*, *Acinetobacter calcoaceticu*, *Pseudomonas synxanthas* (Table 1).

The isolated strains were found to exhibit different multiple heavy metal resistance characteristics (Table 1). Extremely high Zn resistance (up to the concentration of 20 mmol L^{-1}) was observed for the strains VI_8R_3 and II_2L_1, followed by stains VI_8R_2 and VI_8L_2 (15 mmol L^{-1} Zn), strain II_2R_3 and VI_8L_1 (10 mmol L^{-1} Zn), whereas other strains showed relatively low tolerance to Zn (equal to or lower than 6 mmol L^{-1} Zn). Furthermore, strain VI_8R_3 was also resistant to 5 mmol L^{-1} Cd, strain II_2L_1 tolerated 3 mmol L^{-1} Cd, and strains VI_8R_2, II_2R_1, VI_8L_2, VI_8L_1 and VI_8L_4 tolerated 2 mmol L^{-1} Cd. Generally, endophyte bacteria isolated from the leaves and roots showed higher resistance to Zn and Cd than those isolated from the stems (Table 1).

In vitro screening of zinc solubilizing endophyte isolates

Firstly, plate assays were conducted to assess the Zn solubilization potential of the 14 endophyte strains supplementing with various Zn compounds ($ZnCO_3$, $Zn_3(PO_4)_2$ and ZnO) at one gram per liter in SMS medium containing either glucose or sucrose as carbon sources. When glucose was used as carbon sources, eight strains (VI_8L_1, VI_8L_2, II_8L_4, II_8L_5, II_2S_1, $II_2^SS_2$, VI_8R_2, and II_2R_3) could effectively solubilize $ZnCO_3$, seven strains (II_2L_1, VI_8L_2, II_8L_4, VI_8L_5, II_2S_1, VI_8R_2, and II_2R_3) could effectively solubilize $Zn_3(PO_4)_2$ (Fig. 1). Strains II_8L_4, II_2S_1 and II_2R_3 were also able to produce clear halo on ZnO supplemented medium (data not shown). However, when sucrose was used as carbon sources, only four isolates (II_8L_4, II_8L_5, II_2S_1 and $II_2^SS_2$) were able to produce clear halo on SMS medium supplemented with $ZnCO_3$, and strain II_2S_1 could solubilized $Zn_3(PO_4)_2$ (data not shown).

The diameter of solubilization halo produced by five most efficient strains (VI_8L_1, VI_8L_2, IIL_4, VI_8R_2, and II_2R_3) grown on SMS medium added with $ZnCO_3$ and $Zn_3(PO_4)_2$ is presented in Table 2. Strains II_8L_4 and VI_8R_2 showed greater diameter of solubilization with $ZnCO_3$, while VI_8L_2 and II_8L_4 showed greater diameter of solubilization with $Zn_3(PO_4)_2$. Generally, the same strain produced larger clear halo on $ZnCO_3$ supplemented medium than on $Zn_3(PO_4)_2$ supplemented medium (Table 2).

Table 1 Morphological and physiological characterization of 14 endophytic bacteria isolated from *Sedum alfredii*

Isolates	Origin	Colony color	Gram staining	Starch hydrolysis	Cellulose decomposition	MIC of Zn	MIC of Cd	Closest relative
II_2L_1	Leave	Orange	−	+	+	20 mM	3 mM	*Pseudomonas fluorescens*(99%)
VI_8L_1	Leave	Orange	−	+	+	10 mM	2 mM	*Pseudomonas fluorescens* (99%)
VI_8L_2	Leave	Beige	−	−	+	15 mM	2 mM	*Bacillus pumilus*(100%)
II_8L_4	Leave	Orange	−	−	−	8 mM	2 mM	*Pseudomonas fluorescens* (99%)
II_8L_5	Leave	Orange	+	−	−	0 mM	0.6 mM	*Bacillus subtilis* (99%)
II_2S_2	Stem	Beige	−	−	−	6 mM	0.2 mM	*Stenotrophomonas maltophilia* (99%)
II_2S_1	Stem	Pale orange	+	−	−	6 mM	0.6 mM	*Bacillus cereus*
$II_2^SS_2$	Stem	Orange	−	−	−	6 mM	0.2 mM	*Pseudomonas synxantha* (99%)
$II_2^SS_1$	Stem	Pale orange	+	−	−	4 mM	0 mM	*Bacillus cereus*(99%)
VI_8R_2	Root	Yellow-green	−	−	+	15 mM	2 mM	*Pseudomonas fluorescens* (99%)
VI_8R_3	Root	Beige	+	−	−	20 mM	5 mM	*Bacillus pumilus*(98%)
II_2R_3	Root	Pale orange	−	−	−	8 mM	1 mM	*Pseudomonas fluorescens* (99%)
II_2R_1	Root	Orange	+	+	−	8 mM	1 mM	*Bacillus pumilus*(98%)
II_2R_3	Root	Beige	−	−	−	10 mM	2 mM	*Acinetobacter calcoaceticus* (99%)

[a] IAA production: strains were cultured in SMS medium with 0.5 mg ml^{-1} of L-tryptophan for 96 h, +, moderate; ++, high; +++, very high

[b] P-solibilization: Result were based on solid culture, +, positive; −, negative

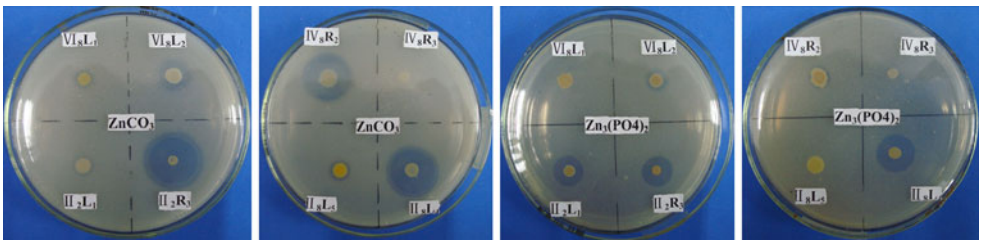

Fig. 1 The growth of endophytic bacteria on SMS medium containing 0.21% $ZnCO_3$ or 0.42% $Zn_3(PO_4)_2$

Table 2 The ability of solubilization of $ZnCO_3$ and $Zn_3(PO_4)_2$ by the five endophytic bacteria strains in the plate assay

Strain	$ZnCO_3$			$Zn_3(PO_4)_2$		
	Colony diameter (mm)	Clearing zone (mm)	Zn solubilization efficiency (%)*	Colony diameter (mm)	Clearing zone (mm)	Zn solubilization efficiency (%)
VI_8L_1	8	2	25	8	0	0
VI_8L_2	9	7	78	8	8	100
II_8L_4	8	18	225	6	11	182
VI_8R_2	9	17	189	6	3	50
II_2R_3	5	13	260	3	9	300

* Zn solubilization efficiency (%) was calculated based on the clear zone diameter/colony diameter × 100

Zinc solubilization in liquid cultures

Based on the first plate assay results, five strains (VI_8L_1, VI_8L_2, II_8L_4, VI_8R_2 and II_2R_3) were further selected for broth assay to confirm their ability to sulubilize $ZnCO_3$ and $Zn_3(PO_4)_2$. For the $ZnCO_3$ amendment (Fig. 2), the Zn concentrations in the supernatants medium inoculated with strains VI_8L_1, VI_8L_2 and VI_8R_2 were significant higher than that of the uninoculated control, and increased with incubation time, especially for strain VI_8L_2. The results confirmed that strains VI_8L_1, VI_8L_2 and VI_8R_2 were able to effectively solubilize the insoluble $ZnCO_3$ compound, which was consistent with the results of plate assay. Though strains II_8L_4 and II_2R_3 produced clear solubilization halo on solid medium with $ZnCO_3$, the Zn concentrations in the supernatants medium inoculated with strains II_8L_4 and II_2R_3 decreased with incubation time, even lower than that of the uninoculated control after incubation of 6 days. Among the test isolates, strain VI_8L_2 solubilized maximum amounts of $ZnCO_3$ (42.4 mg L^{-1} at the 10th day), followed by the strain VI_8L_1 (23.8 mg L^{-1} at the 10th day), and strain VI_8R_2 (16.0 mg L^{-1} at the 10th day) (Fig. 2).

For the $Zn_3(PO_4)_2$ amendment, incubation of strain VI_8L_1, VI_8L_2, VI_8L_4 and VI_8R_2 all resulted in increase of Zn concentrations in the culture supernatants, and also linearly increased with incubation time, while there was no significant change of Zn concentration for the uninoculated control (Fig. 2). During the first 8 days of incubation, Zn concentration in the culture supernatants incubated with strain II_2R_3 increased slowly, but it sharply increased after incubation of 10 days, which was about 100 times of the control (Fig. 2). Among the test isolates, strain VI_8L_1 solubilized maximum amounts of $Zn_3(PO_4)_2$ (145.5 mg L^{-1} at the 8th day), followed by strain II_8L_4 (77.6 mg L^{-1} at the 10th day), strain VI_8L_2 and VI_8R_2 (64.0 and 61.4 mg L^{-1} at the 10th day, respectively), and strain II_2R_3 (31.8 mg L^{-1} at the 10th day).

For the $ZnCO_3$ amendment, incubation of strain VI_8L_1 induced pH drop in the culture medium compared with the unincubated control, but pH in the culture medium incubated with the other four strains (VI_8L_2, VI_8L_4, VI_8R_2 and II_2R_3) were even higher than the control during the first 6 days of incubation; however, after growth of 8 or 10 days, the five strains all resulted in a drop of pH in the culture medium compared with the control (Fig. 2). For the $Zn_3(PO_4)_2$ treatment, pH in the culture medium incubated with strain VI_8L_1, VI_8L_2, II_8L_4 and VI_8R_2 were significantly lower than the control, and the pH changed with growth times; however, no significant difference were found between the incubation of strain II_2R_3 and the control (Fig. 2).

Effect of bacterial filtrate on the mobility of Zn in soil

The Zn concentrations extracted from the artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils by filtrate liquid media after 48 h growth of the strain VI_8L_1, VI_8L_2, II_8L_4 and VI_8R_2 were all significantly higher than those extracted by water and axenic SMS broth, while the culture medium supporting strain II_2R_3 growth decreased Zn solubilization in all the three tested soils (Fig. 3). It indicated that

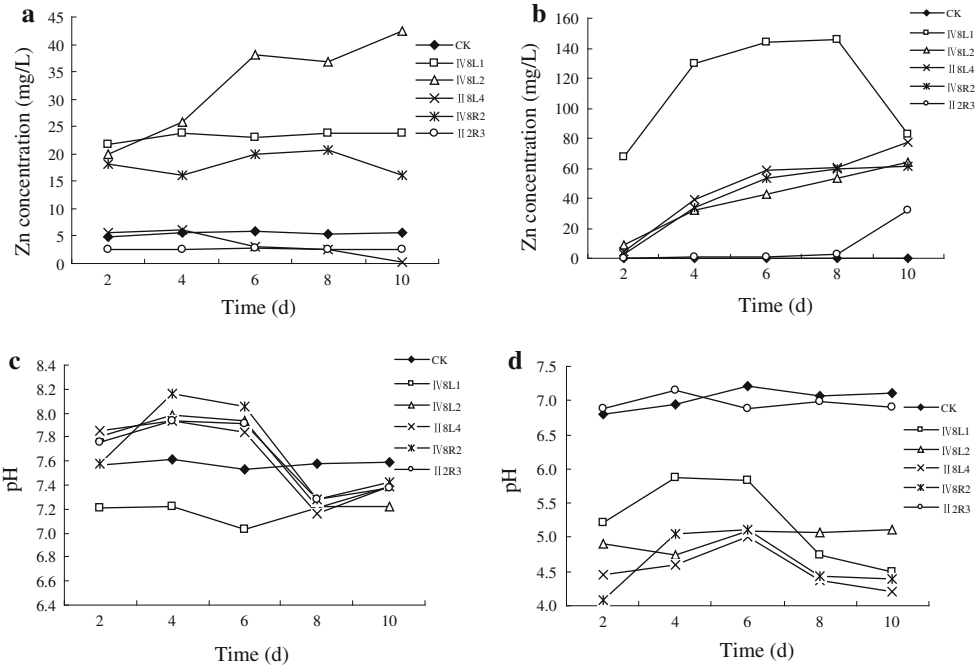

Fig. 2 The change of Zn concentration and pH in the SMS liquid medium supplemented with (**a**, **c**) 0.21% $ZnCO_3$ and (**b**, **d**) 0.42% $Zn_3(PO4)_2$. Each value is the mean of triplicates

the products of bacterial strains VI_8L_1, VI_8L_2, VI_8L_4 and VI_8R_2 growth could effectively mobilize Zn from $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils. Compared with water and axenic SMS broth, the filtrates of the culture media supporting growth of strain VI_8L_2, II_8L_4 and VI_8R_2 also extracted significantly greater quantities of Zn from the Dabaoshan contaminated soils, but the amount of Zn extracted by culture medium supporting strains VI_8L_1 and II_2R_3 growth were even lower than water and axenic SMS medium, expecially for strain II_2R_3 (only about 66.2% of Zn extracted by axenic SMS medium) (Fig. 3).

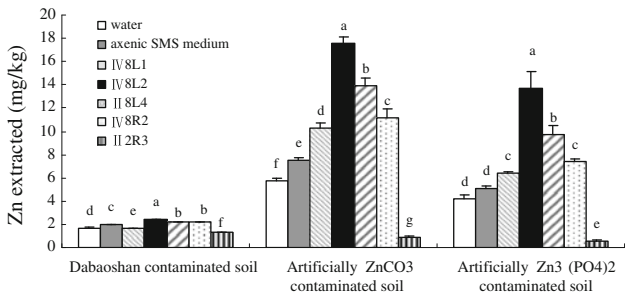

Fig. 3 The ability of bacterial metabolite to extract Zn from artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils and Dabaoshan contaminated soils. Each value is the mean of triplicates. *Error bars* represent standard deviation. For the same soil, significant differences according to (*least significant difference*) LSD at $P = 0.05$ levels are indicated by *different letters*

Evaluation of plant growth promoting activities

Five isolates were screened in vitro for their plant growth promoting traits like production of IAA, siderophore, phosphate solubilization, and abilities to grow on nitrogen-free liquid medium. The five tested strains were all capable of producing IAA and solubilizing calcium phosphate, and significant differences among strains were observed in the amount of IAA produced and P solubilized (Table 3). Among the five strains, strain VI_8R_2 produced highest amount of IAA, followed by VI_8L_2, II_8L_4, VI_8L_1 and II_2R_3. On CAS agar plates, strains VI_8L_2, II_8L_4 and VI_8R_2 showed the siderophore activity, which produced a 8.3, 3.3, and 5.6 mm colored zone on CAS plates, respectively (Table 3). After three cycles of subculturing in nitrogen-free liquid medium, only strains VI_8L_2 had vigorous growth, while the other four isolates could not grow.

The five isolates were also tested for the quantitative estimation of IAA in the presence of different concentrations of Zn in the growth medium. The result revealed that these five endophytic bacteria all had the capacity to produce IAA with (1 and 2 mM Zn) or without Zn in the growth medium. When no Zn was supplied in the growth medium, IAA production of the five strains showed a non-linear and time-dependent change (Fig. 4). The result shows that the effect Zn on IAA production varied significantly from inhibition to stimulation of IAA production depending on the strains. Zinc added to the growth medium stimulated IAA production for strain VI_8R_2, decreased IAA production for strain II_8L_4, but no significant difference was noticed between 1 and 2 mM Zn treatments for both

Table 3 Plant growth promoting activities of the five endophytic bacteria (each value is a mean ± **standard deviation of three replicates)**

Strain	IAA (μg ml^{-1})[a]	*P* solubilization in solid medium		*P* solubilized in liqiud medium (μg ml^{-1})[b]	Siderophores on CAS agar[c]		Nitrogen fixation[d]
		Colony diameter (mm)	Clearing zone (mm)		Colony diameter (mm)	Clearing zone (mm)	
VI_8L_1	10.9 ± 1.5	14.5 ± 1.47	6.8 ± 2.1	1.85 ± 0.53	0	0	−
VI_8L_2	30.2 ± 1.9	10.6 ± 1.23	3.8 ± 0.7	1.24 ± 0.25	9.7 ± 0.6	8.3 ± 2.1	+
II_8L_4	32.2 ± 6.1	12.2 ± 0.59	11.8 ± 1.0	1.81 ± 0.42	14.7 ± 0.6	3.3 ± 1.5	−
VI_8R_2	45.6 ± 5.6	10.3 ± 0.58	3.9 ± 0.1	1.76 ± 0.40	15.0 ± 0.0	5.6 ± 1.1	−
II_2R_3	4.2 ± 0.6	6.23 ± 0.06	6.8 ± 1.0	1.17 ± 0.20	0	0	−

[a] Indole acetic acid concentration in the liquid growth medium after bacteria growth of 2 days

[b] Soluble *P* in the liquid growth medium after bacteria growth of 7 days

[c] Chrome Azurol S agar

[d] + Indicates a vigor growth, −indicates no growth

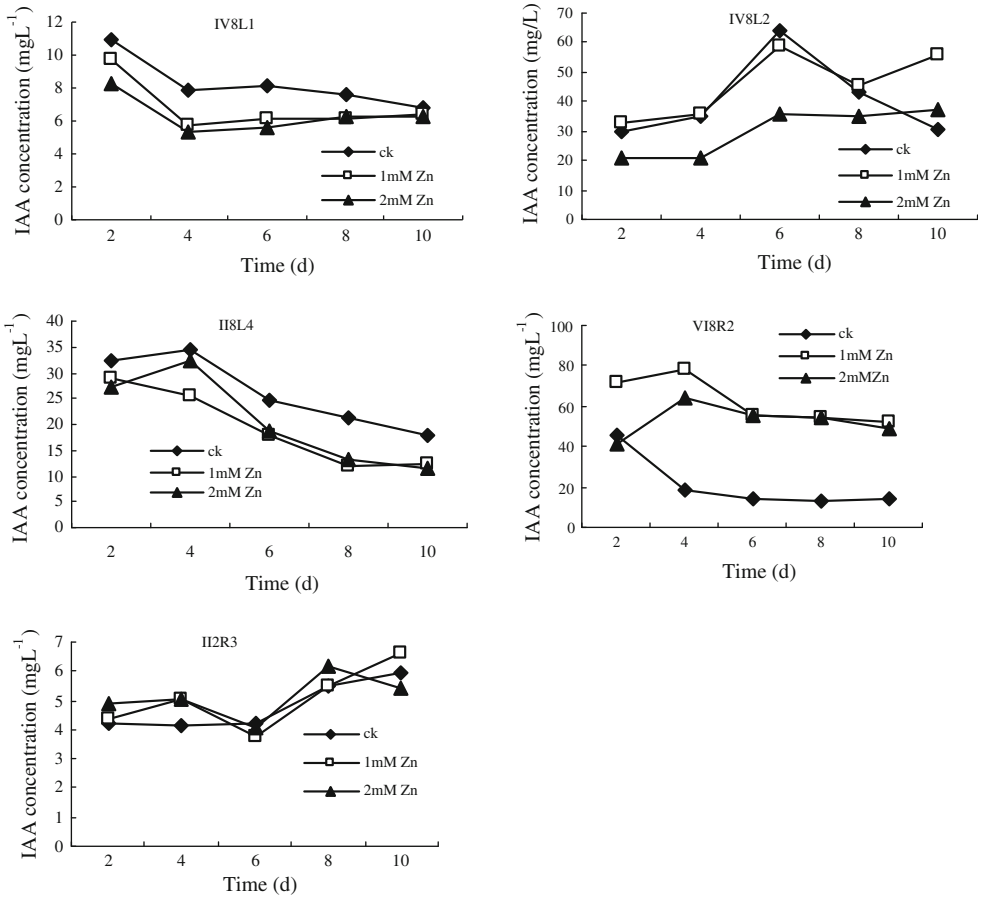

Fig. 4 Effect of Zn in growth medium on IAA production by the five endophytic bacteria. Each value is the mean of triplicates

strains. However, Zn addition had no significant effect on IAA production for the other three strains (Fig. 4).

Discussion

Bacterial endophytes have been defined as "bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease" (Lodewyckx et al. 2002). Endophytic bacteria have been found in virtually every plant studied, but most of these studies have focused on bacteria from the crop plants. Currently, endophytic bacteria associated with hyperaccumulator plants have attracted the attention of several investigators. For example, research found that Zn

hyperaccumulator *Thlaspi caerulescens* (Lodewyckx et al. 2002), Ni hyperaccumulator *Thlaspi goesingense* and *Alyssum bertolonii* (Idris et al. 2004; Barzanti et al. 2007), and Cu hyperaccumulator *Elsholtzia splendens* (Sun et al. 2009) were all colonized simultaneously by a high number of different divisions, genera, and species of metal resistant endophytic bacteria. In this study, we found Zn/Cd hyperaccumulator *S. alfredii* also harbored an abundance of culturable endophytic bacteria, and varied between roots, stems and leaves (Table 1). Fourteen strains were isolated and analyzed by sequencing of 16S rDNA for the taxonomic interpretation. Most isolates belonged to *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Acinetobacter*, which are common soil and endophytic bacteria. These results were in agreement with Idris et al. (2004) and Sheng et al. (2008). However, an abundance of highly Zn or Ni resistant pink pigmented *Methylobacterium* spp were found in the stems of Zn hyperaccumulator *T. caerulescens* and Ni hyperaccumulator *T. goesingense* (Lodewyckx et al. 2002; Idris et al. 2004). In this study, no *Methylobacterium* spp were isolated from Zn/Cd hyperaccumulator *S. alfredii*.

The hyperaccumulator accumulate huge amounts of heavy metals and can therefore provide a specific environment for bacterial endophytes that could be adapted to survive in high metal concentrations. For instance, endophytic bacteria isolated from various hyperaccumulating plants such as *Thlaspi caerulescens*, *Thlaspi goesingense*, *Alyssum bertolonii*, and *Nicotiana tabacum* were resistant to more than one heavy metals. Further, co-resistance to Ni, Cr, Zn, and Cu was the most frequent, whereas co-resistance to Ni and Co was less frequent (Lodewyckx et al. 2002; Idris et al. 2004; Barzanti et al. 2007; Mastretta et al. 2009). This study showed that most of endophytic bacteria isolated from *S. alfredii* were found to exhibit high Zn and Cd resistance characteristic. For example, most of bacterial isolates from leaves and roots were resistant to above 8 mM Zn and 2 mM Cd (Table 1). Interestingly, one isolate from leaves could even tolerate 20 mM Zn and 3 mM Cd, and one isolate from roots could tolerate 20 mM Zn and 5 mM Cd. It indicated that these endophytic bacteria populations had a marked adaptation to heavy metals under constant metal stress for a long time.

The mobility and bioavailability of heavy metal in soils is clearly a critical factor affecting the success of phytoextraction. In general, heavy metals in soils are bound to organic and inorganic soil constituents, or alternatively, present as insoluble precipitates, which are unavailable for root uptake by field grown plants, even for hyperaccumulator (Adriano 2001). Some metal resistant bacteria can produce iron chelators and siderophores that ensure iron availability, reduce soil pH, and/or solubilize metal-phosphates (Abou-Shanab et al. 2003a, b). In this study, both plate and broth assay proved that endophytic bacteria isolates VI_8L_1, VI_8L_2, II_8L_4, and VI_8R_2 were able to effectively solubilize insoluble $ZnCO_3$ and $Zn_3(PO_4)_2$ compounds, when glucose was provided as the carbon source (Figs. 1 and 2, Table 3). Solubilization of $Zn_3(PO_4)_2$ occurred by an increase in the H^+ concentration of the medium, which might be related to the consequence of ammonia assimilation and the production of organic acid (Fig. 2). However, no decrease in pH was observed in $ZnCO_3$ amended medium during the first 6 days. This might be due to the intrinsic buffering potential of the Zn compounds as demonstrated by Franz et al. (1991) with *Penicillium simplicissimum* and Saravanan et al. (2007) with *Gluconacetobacter diazotrophicus*. Interestingly, the filtrate liquid media after growth of the strains VI_8L_1, VI_8L_2, II_8L_4 and VI_8R_2 extracted much higher Zn from artificially $ZnCO_3$ and $Zn_3(PO_4)_2$ contaminated soils than those extracted by axenic SMS broth, and the filtrates of the culture media supporting growth of strains VI_8L_2, II_8L_4 and VI_8R_2 also extracted significantly greater quantities of Zn from the Dabaoshan contaminated soils. These isolates may have considerable biotechnological potential to improve the applicability and efficiency of phytoextraction. For instance, bacteria such as *Azotobacter chroococcum* (N_2-fixer), *Bacillus megaterium* (P-solubiliser) and *Bacillus mucilaginosus* (K-solubiliser) and *Bacillus* sp. RJ16 can decrease soil pH and enhance the bioavailability of Cd and Zn, probably by excreting low weight molecular acids (Wu et al. 2006; Sheng and Xia 2006). *Lupinus luteus* L, when grown on a Ni enriched substrate and inoculated with the engineered Ni-resistant endophytic bacterium *Burkholderia cepacia* L. S.2.4::*ncc-nre*, showed a significant increase (30%) of Ni concentration in the roots (Lodewyckx et al. 2001).

Endophyte associations are important in natural and managed ecosystems due to their nutritional and non-nutritional benefits to their host plants. The beneficial effects of endophytes on their hyperaccumulator host appear to occur through similar mechanisms described for PGPB, including nitrogen fixation, phosphate solubilization, IAA production and the production of a siderophore (Glick et al. 1999; Rajkumar et al. 2009). This makes sense because most of the bacterial endophytes isolated from various plants can be considered to be facultatively endophytic and are capable of living outside plant tissues as rhizospheric bacteria (Di Fiori and Del Gallo 1995). In the present study, the estimation of IAA in culture filtrate showed that all the 14 endophytic bacteria isolates had the capacity to produce IAA when the culture medium was supplemented with L-tryptophan, strains VI_8L_2, II_8L_4, VI_8R_2 and VI_8R_3 showed a high production of IAA (data not shown). Moreover, the four potential Zn solubilizing strains VI_8L_1, VI_8L_2, II_8L_4, and VI_8R_2 had the capacity to produce IAA in culture medium supplied with 1 or 2 mM Zn (Fig. 4), and could solubilize $Ca_3(PO_4)_2$, with strain

VI_8L_1 having the highest of P solubilization efficiency. In addition, three isolates (strains VI_8L_2, II_8L_4 and VI_8R_2) could produce siderophore and two isolates (strains VI_8L_2 and VI_8R_3) had the capacity of nitrogen fixation. Similarly, Idris et al. (2004) reported the siderophore production in Ni-resistant bacteria isolated from *T. goesingense*. In contrast, Lodewyckx et al. (2002) reported that the endophytes recovered from stem and root tissues of *T. caerulescen* did not produce siderophores under iron deficient condition. It suggested that such metal resistant PGPB endophytes may increase plant biomass and metal accumulation when reinoculated plants, thereby increasing the efficiency of phytoremediating heavy metal polluted soils. For example, Barzanti et al. (2007) reported 83% of bacterial isolates recovered from within *A. bertolonii* were shown to produce siderophores and promote the plant growth under Ni stress.

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