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REGULAR ARTICLE

Metal-tolerant *Enterobacter* sp. strain EG16 enhanced phytoremediation using *Hibiscus cannabinus* via siderophore-mediated plant growth promotion under metal contamination

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Abstract
Aims This study is aimed to investigate the efficiency of plant growth-promoting (PGP) strategies of *Enterobacter* sp. strain EG16 under metal stress and its potential application in phytoremediation.
Methods Production of siderophores and indole-3-acetic acid (IAA) by EG16 were assessed in a hydroponic system in which *Hibiscus cannabinus* was grown with different concentrations of Cd and Fe. A pot experiment was also carried out to evaluate the practical effect of EG16 on *H. cannabinus* growth and remediation efficiency.
Results Inoculation with EG16 significantly improved plant growth, probably as a result of increased plant uptake of Fe and immobilization of Cd^{2+}, which resulted in decreased plant accumulation of Cd. Increased production of siderophores by EG16 in response to Cd exposure appeared to be the PGP strategy functioning in the EG16–*H. cannabinus* association. The bacterial Cd response system promoted plant and bacterial uptake of Fe, alleviated Cd-induced inhibition of bacterial IAA production, and potentially assisted in metal immobilization in the rhizosphere.
Conclusions The EG16–*H. cannabinus* association may be useful for phytostabilization, as it exhibits good plant growth, low plant accumulation of metals, and reduced metal bioavailability in soil.

Keywords *Enterobacter* sp. EG16 · Plant growth-promoting strategy · Siderophore · Metal contamination · *Hibiscus cannabinus* · Phytostabilization

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Introduction

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that are able to colonize the surface of the root system (and sometimes the root inner tissues) and stimulate the growth and health of the plant through several direct and indirect mechanisms (Vacheron et al. 2013). PGPR can enhance plant nutrition and improve plant growth via N_2 fixation, production of siderophores and plant growth hormones such as indole-3-acetic acid (IAA), phosphate solubilization, and reduction of ethylene levels in plants by 1-aminocyclopropane-1-carboxylic acid deaminase (Glick 2005; Habibi et al. 2014; Richardson and Simpson 2011; Vacheron et al. 2013). Some PGPR have been reported to help plants withstand abiotic stresses, including heavy metal toxicity, as well as to promote plant growth in heavy

metal-contaminated soils (Belimov et al. 2005; Prapagdee et al. 2013; Tak et al. 2013). As a result, the combination of plants and PGPR has been considered an important component in phytoremediation of heavy metal- contaminated soil (Khonsue et al. 2013; Ma et al. 2011; Rajkumar et al. 2012; Sessitsch et al. 2013).

However, the practical effect on plant growth of PGPR is affected by environmental metal concentrations. Belimov et al. (2005) reported that stimulation of the root length of *Brassica juncea* VIR3129 seedlings by several plant-associated bacteria was more pronounced in the presence of Cd. Tripathi et al. (2005) inoculated mung bean with the Pb- and Cd-resistant siderophore-producing *Pseudomonas putida* strain KNP9 and observed stronger growth-promoting effects in the presence of Pb and Cd than in the control that lacked added metals. Furthermore, Remans et al. (2012) observed plant growth promotion by PGPR only in the case of Cd stress, and proposed that the plant-associated bacteria may possess stress-relieving characteristics and thus promote plant growth only under stress conditions. Understanding the effect of environmental factors such as metal stress on the characteristics of PGPR and the practical effect of such factors on plant growth is of great importance for a better application of PGPR in phytoremediation.

Phytoremediation using proper plant-microbe consortia to enhance extraction (phytoextraction) or stabilization (phytostabilization) of metals has been proven to be a reliable and sustainable approach for the cleanup of metals and restoration of soil quality (Hao et al. 2015). However, interactions between plants, PGPR, and heavy metals may greatly affect the efficiency of phytoremediation. The bioavailability of soil heavy metals to plants can be influenced by soil factors, including plant root exudates and microbial activities (Brown et al. 1999; Traina and Laperche 1999). For survival and adaption under metal-stressed conditions, PGPR have developed a range of mechanisms by which they can immobilize, mobilize, or transform heavy metals, thereby rendering them inactive and nontoxic for their own development (Nies 1999; Tak et al. 2013) and resulting in either enhanced or repressed metal transfer from soil to plant (Phieler et al. 2014). Hence, specific plant-PGPR associations and the mechanisms involved in their interactions need to be carefully selected and tested before they are applied in the field, in order to successfully establish different phytoremediation systems (Phieler et al. 2014). At present, research on the interactions of plants, PGPR strains, and metals is still limited.

Enterobacter sp. EG16 is a Cd-resistant strain isolated from the rhizosphere of *H. cannabinus* growing in a multi-metal polluted mine tailing by our laboratory (Guangdong Provincial Key Lab of Environmental Pollution Control and Remediation Technology, Sun Yat-sen University, Guangzhou, China). It was shown to accumulated Cd by both surface biosorption and intracellular accumulation, and was able to produce siderophore enterobactin and plant hormone IAA (Chen et al. 2016). However, its ability to promote plant growth under conditions of metal stress and its practical effect on phytoremediation has not been studied. *H. cannabinus* has been suggested to apply in phytostabilization due to its fast growth and high metal tolerance (Banuelos et al. 1997; Mun et al. 2008; Taiwo et al. 2016; Yang et al. 2013). In this work we re-inoculated the EG16 strain to the rhizosphere of *H. cannabinus* and investigated the effect of Cd on the plant growth promoting (PGP) traits of EG16 as well as the role of EG16 in phytostabilization with *H. cannabinus*. Based on the results of our previous work, we hypothesized that: (1) Cd-induced siderophore production is an effective PGP strategy used by plant–EG16 associations in a Cd-polluted environment; and (2) EG16 would be a suitable candidate to assist with *H. cannabinus* in phytostabilization of Cd-polluted soils.

Materials and methods

Bacterial strain and growth conditions

The Cd-resistant bacterium *Enterobacter* sp. EG16 was isolated from the rhizosphere of *Hibiscus cannabinus* growing in multi-metal polluted tailings in Dabao Mountain, Guangdong, China (Chen et al. 2016). It has been deposited in the Guangdong Culture Collection Centre of Microbiology with the strain number GIMCC1.808, and a partial sequence of its 16S rRNA gene (1377 bp) was deposited in the GenBank database (accession no. KP406619). EG16 was maintained at 30 °C on solid nutrient broth (peptone, 1 %; beef extract, 0.5 %; sodium chloride, 0.5 %; agar, 20 %). For root inoculation, cells were grown in fresh liquid nutrient broth (peptone, 1 %; beef extract, 0.5 %; sodium chloride, 0.5 %) for 12 h at 30 °C, collected by centrifugation at 8000 rpm and 4 °C for 10 min, washed twice with the hydroponic nutrient solution (Guoqing et al. 2006), and resuspended in the same solution to get an OD_{600} of 1.0,

corresponding to a viable cell number of approximately 3.8×10^8 CFU mL^{-1}.

Hydroponic experiment

The hydroponic nutrient solution was prepared by the method of Guoqing et al. (2006) with modifications. Four stock solutions were prepared as following: solution A contained 0.15 *M* $Ca(NO_3)_2 \cdot 4H_2O$ and 0.5 *M* KNO_3, solution B contained 0.1 *M* $(NH_4)H_2PO_4$ and 0.1 *M* $MgSO_4$, solution C contained 0.1 *M* NaCl, 0.02 *M* H_3BO_3, 5 m*M* $MnSO_4 \cdot H_2O$, 0.4 m*M* $ZnSO_4 \cdot 7H_2O$, 0.2 m*M* $CuSO_4 \cdot 5H_2O$ and 0.05 m*M* $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$], and solution D contained 0.05 *M* EDTA-FeNa. For the seedling cultivation stage, the hydroponic nutrient solution 1 (NS1) consisted of solutions A, B, C, and D diluted 200, 200, 1000, and 1000 times, respectively. For the experiment with different Fe and Cd concentrations and bacterial inoculation, the hydroponic nutrient solution 2 (NS2) consisted of solutions A, B, and C diluted 100, 100, and 500 times, respectively. Both solutions (NS1 and NS2) were adjusted to a pH of 4.5 and filter sterilized (0.2μm cellulose acetate filtration membrane, Sartorius Stedim Biotech, Germany) before use.

H. cannabinus seeds were surface sterilized using 10 % H_2O_2 for 30 min and then soaked in sterile deionized water for 2 h. Sterilized seeds were incubated on the same solid nutrient broth medium mentioned above for 24 h to verify sterilization efficiency. Seeds were then placed on filter paper in sterile glass Petri dishes containing 6 mL sterile deionized water and germinated at 25 °C in the dark for 2 days. Germinated seedlings were moved to sterile trays containing a mixture of perlite and vermiculite, provided with the hydroponic nutrient solution 1 (NS1), and incubated at 25 °C and 70 % relative humidity with 16 h photoperiod and 140 μmol m^{-2} s^{-1} light intensity. The hydroponic solution was continuously aerated and renewed every 4 days.

Seven days later, perlite and vermiculite were washed from the roots, and four uniformly grown seedlings were transferred to each sterilized vessel containing 500 mL NS2 with varying concentrations of Fe and Cd. Fe was supplied as $FeCl_3$ at final concentrations in the hydroponic system of 0, 50, 100, and 150 μ*M*, and Cd was added as $CdCl_2$ at final concentrations of 0 and 100 μ*M*. Metal solutions were filter sterilized (0.22-μm filter, Micro PES, MEMBRANA, Germany) before addition. Eight metal treatments were set up as follows: No metal, Cd100, Fe50, Cd100 + Fe50, Fe100, Cd100 + Fe100, Fe150, and Cd100 + Fe150. All metal treatment solutions received an addition of 5 mL of bacterial suspension (OD_{600} = 1.0) or 5 mL sterile deionized water (uninoculated control, CK). Each treatment was replicated 4 times. The hydroponic experiment was conducted in a greenhouse at 25 °C and 70 % relative humidity with 16 h photoperiod and 140 μmol m^{-2} s^{-1} light intensity. The hydroponic solution was renewed every 3 days. Plants were harvested after 30 days of growth under the experimental conditions.

Production of siderophores and IAA in the hydroponic experiment

For quantification of siderophores in the hydroponic systems, 5 mL of nutrient solution was collected at 24, 48, and 72 h, and the OD_{600} was measured. After centrifugation at 10,000 rpm for 10 min (4 °C), the supernatant was collected by filtration using a 0.22-μm filter (Micro PES, MEMBRANA, Germany). For detection of siderophore (Dimkpa et al. 2008a), the filtered solution was mixed with chrome azurol S (CAS) indicator solution (1:1, *v*/v) prepared by the method of Alexander and Zuberer (1991). After a 60-min reaction period, absorbance was measured at 630 nm using the UV-visible spectrophotometer (UV2450, Shimadzu, Japan). Treatments without bacterial inoculation were used as controls. Deferoxamine mesylate (DFOM, Sigma, US) was used to prepare a standard curve (Alexander and Zuberer 1991). Each treatment was conducted in 4 replicates.

For estimation of IAA production, 1 mL of supernatant collected in the previous section was mixed with 4 mL Sackowski's reagent (Patten and Glick 2002) and placed at room temperature (25 °C) for 20 min before absorbance at 535 nm was measured. The concentration of IAA was determined by comparison with a standard curve made using an IAA standard (Sigma, US). Each treatment was conducted in 4 replicates.

Bacterial growth in the hydroponic systems

Root colonization by the isolate was determined by the method of Hossain et al. (2008) and Islam et al. (2016) with some modifications. After bacterial inoculation for 72 h, roots were harvested randomly from plants of each hydroponic system, washed with running tap water,

rinsed three times in sterilized distilled water, and blotted dry. Then 0.1 g of dried root was cut into pieces and homogenized in 1 mL of the nutrient solution of each hydroponic system. Serial dilutions were prepared on nutrient broth plates and incubated at 30 °C. The number of colony-forming units (cfu) per milliliter root suspension was determined after 48 h of incubation. Roots in treatments without bacterial inoculation were harvested and treated similarly as the controls. Each treatment was replicated 4 times.

EG16-associated phytoremediation pot experiment

Soil used in the pot experiment was collected from the South China Agricultural University (unpolluted soil) and the multi-metal polluted mine tailings in Dabao Mountain, Guangdong, China (metal polluted soil). The soil was air dried and sieved (2 mm) to remove stones and plant materials. Soil physicochemical properties and total heavy metal concentrations are listed in Table S1. *H. cannabinus* seeds were pretreated as in the hydroponic experiment and sown in plastic pots containing 1 kg of polluted or unpolluted soil. Two-week-old *H. cannabinus* seedlings were treated by soaking with 10 mL bacterial suspension (OD_{600} = 1.0) or sterile deionized water (bacterial control) and then grown for 30 days before harvest. Plants were grown in a glasshouse at 25 °C with a photoperiod of 16 h. Pots containing soil with and without bacterial inoculation but without plants served as plant controls. The soil was moistened with sterile deionized water and maintained at a moisture content or 60 %. Each pot containing 7 of *H. cannabinus* seedlings was defined as one replicate, and each treatment was performed in 7 replicates.

Growth and Cd/Fe uptake of *H. cannabinus*

Plants in both the hydroponic and pot experiments were carefully harvested and rinsed thoroughly with sterile deionized water. For the pot experiment, total number of leaves and total shoot height in each pot were recorded, and several parameters of the root were measured using a WinRHIZO (Epson Perfection V700 Photo Scanner, Epson, Japan). After the fresh weight of both roots and shoots was obtained, plants were heated at 105 °C for 2 h and then dried at 60 °C for 48 h before dry weight was determined. Heavy metals accumulated in the plants were extracted by digestion (4:1, $HNO_3/HClO_4$,), and concentration of Fe and Cd were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 5300 DV, Perkin-Elmer Instruments, USA). Each treatment in the hydroponic and pot experiments was conducted in 4 and 7 replicates, respectively.

Bioavailability of heavy metals in soil

The bioavailability of heavy metals in the pot experiment was assessed by a one-step soil extraction procedure using NH_4NO_3 (Gupta and Sinha 2007; Symeonides and McRae 1977). In detail, 10 g of soil was added to 50 mL of 1 *M* NH_4NO_3 and shaken for 2 h at room temperature. The mixture was filtered, and metal concentrations in the filtrate were estimated using ICP-OES (Optima 5300 DV, Perkin-Elmer Instruments, USA) and Polarized Zeeman Atomic Absorption Spectrophotometer (AAS, Z-5000, HITACHI, Japan). Each treatment was performed in 7 replicates.

Evaluation of phytostabilization potential

The phytostabilization potential was evaluated by the bioconcentration factor (BF) and the translocation factor (TF). According to Mendez and Maier (2008), BF is defined as the ratio of the metal concentration in shoot tissue to that in the soil, whereas TF is the ratio of metal concentration in the shoot to that in the root.

Results

Bacterial growth in the hydroponic systems

Growth of EG16 in the 8 metal treatments was determined 72 h after inoculation. As shown in Table 1, bacterial growth was enhanced with increased Fe supply. Addition of Cd significantly inhibited bacterial growth in the treatment without Fe supply (independent-samples *t*-test, $P < 0.05$, Table 1). However, in treatments supplied with Fe, there was no significant effect of 100 μ*M* Cd on the growth of the strain EG16 (Table 1).

Effect of Cd on bacterial siderophore and IAA production

As shown in Table 1, the unit output of siderophore by EG16 at 72 h was decreased with increasing Fe, while was induced by Cd addition in treatments containing no

Table 1 Growth, siderophore production, and IAA production of EG16 in a hydroponic system under the interplay of Cd and Fe after inoculation for 72 h

Treatments	Bacterial growth (10^5 cfu mL^{-1})	Total amount of Siderophore production (μM DFOM equivalent)				Total amount of IAA production (μM)			
		24 h	48 h	72 h	Unit output of at 72 h (10^{-8} μmol DFOM (cfu^{-1}))	24 h	48 h	72 h	Unit output of at 72 h (10^{-9} μmol (cfu^{-1}))
No metal	2.30 ± 0.42	42.46 ± 3.41	55.53 ± 1.28	55.18 ± 0.43	24.60 ± 4.65	0.67 ± 0.26	1.63 ± 0.52	3.14 ± 0.21	14.10 ± 3.33
Cd100	1.25 ± 0.35	51.81 ± 1.24	57.21 ± 1.07	59.10 ± 0.96	50.12 ± 13.53	Nd	1.17 ± 0.38	2.19 ± 0.22	18.80 ± 6.14
Significance level	*	**		**	*			**	
Fe50	7.35 ± 0.42	47.34 ± 2.22	49.05 ± 5.17	51.79 ± 3.92	7.06 ± 0.63	1.14 ± 0.10	2.68 ± 0.23	4.32 ± 0.33	5.89 ± 0.48
Cd100 + Fe50	6.98 ± 0.39	49.08 ± 1.47	53.58 ± 2.11	59.44 ± 2.16	8.54 ± 0.64	1.00 ± 0.17	1.18 ± 0.08	2.01 ± 0.37	2.88 ± 0.52
Significance level				*	*		**	**	**
Fe100	48.50 ± 5.69	44.26 ± 0.88	44.58 ± 1.74	45.24 ± 2.40	0.94 ± 0.09	1.20 ± 0.20	1.83 ± 0.24	2.32 ± 0.13	0.48 ± 0.05
Cd100 + Fe100	47.50 ± 5.45	44.97 ± 1.36	46.99 ± 0.99	49.55 ± 2.46	1.03 ± 0.15	Nd	0.76 ± 0.19	0.88 ± 0.14	0.19 ± 0.05
Significance level				*			**	**	***
Fe150	46.30 ± 3.40	26.69 ± 0.77	26.60 ± 0.77	26.11 ± 1.00	0.57 ± 0.03	1.34 ± 0.58	1.69 ± 0.59	2.06 ± 0.27	0.45 ± 0.08
Cd100 + Fe150	41.50 ± 7.19	27.30 ± 0.08	27.22 ± 0.08	27.26 ± 0.21	0.67 ± 0.10	Nd	0.70 ± 0.19	0.73 ± 0.26	0.18 ± 0.08
Significance level							*	**	**

*, **, and *** indicate significant differences between the zero Cd and the 100 μM Cd treatments at the 0.05, 0.01, and 0.001 levels, respectively, by independent-samples *t*-test ($n = 4$); Cd100 indicates treatments containing 100 μM Cd; Fe50, Fe100, and Fe150 indicate treatments containing 50, 100, and 150 μM Fe, respectively. Data were displayed as mean ± SD. (Siderophore and IAA production of EG16 were calculated from the same raw data of 72 h shown in Figs. 1 and 2)

Fe and low Fe (50 μ*M*) supply (independent-samples *t*-test, $P < 0.05$). For the total amount of siderophore in the hydroponic systems (Table 1), treatments with lower Fe supply similarly showed a higher amount of siderophore after bacterial inoculation for 72 h. Notably, the inducing effect of Cd on siderophore production was also more pronounced in the treatment with lower Fe supply (0 μM, $P < 0.01$; 50 and 100 μ*M*, $P < 0.05$).

The unit output of IAA by EG16 similarly decreased with increasing Fe (Table 1). Exposure to Cd conversely led to a significant decrease in IAA production by EG16 except in the treatment without Fe supply (Table 1). After inoculation for 72 h, the total amount of IAA in the hydroponic systems was reduced by 30, 54, 62 and 65 % under Cd exposure in the treatments containing 0, 50, 100, and 150 μ*M* Fe, respectively (independent-samples *t*-test, $P < 0.01$, Table 1).

Effects of bacterial inoculation on plant growth

In the hydroponic experiment, inoculation with EG16 severally promoted significantly plant growth in 0, 50 and 100 μ*M* Fe treatments (independent-samples *t*-test, $P < 0.05$ or $P < 0.01$), except the shoot biomass of the Cd-free treatment with 100 μ*M* Fe ($P > 0.05$ Fig. 1a–c). When Fe concentration increased to 150 μ*M*, the EG16 strain showed no PGP effect ($P > 0.05$, Fig. 1d). In Cd-free treatments, bacterial inoculation increased shoot biomass by 9 (0 μ*M* Fe) and 15 % (50 μ*M* Fe), and root biomass by 8, 16, and 11 % in the 0, 50 and 100 μ*M* Fe treatments, respectively ($P < 0.05$ or $P < 0.01$). Interestingly, the PGP effect of EG16 was even stronger in the Cd-added treatments, with increases in shoot biomass of 22, 38, and 11 % and in root biomass of 16, 43, and 13 % in the 0, 50, and 100 μM Fe treatments,

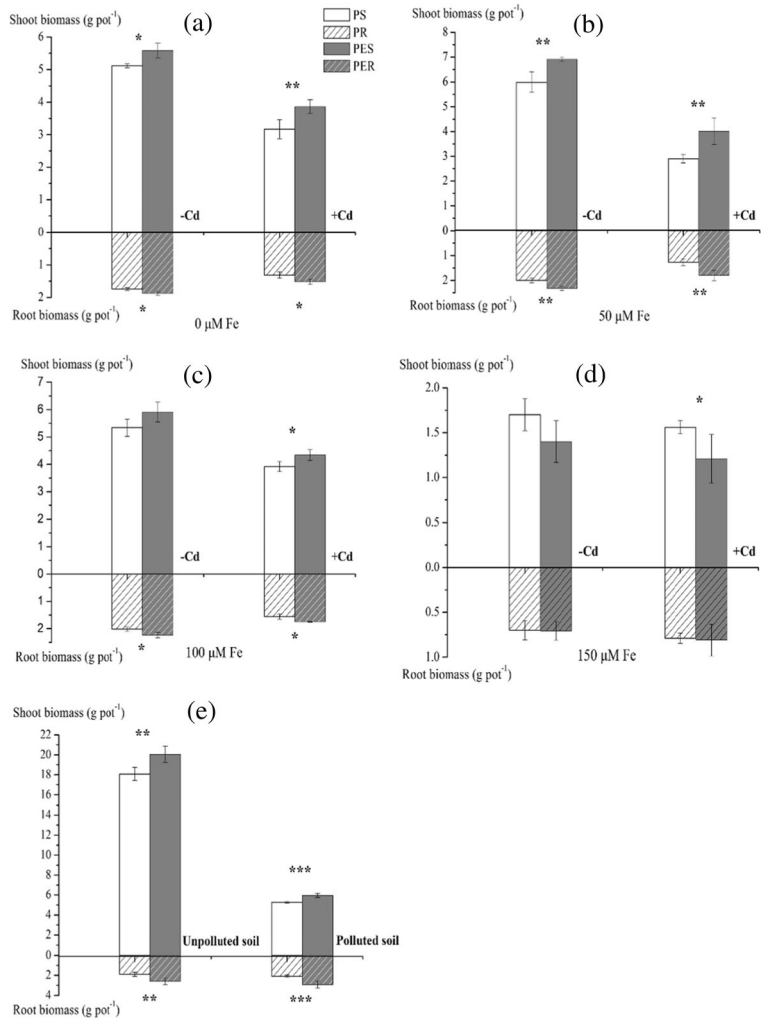

Fig. 1 Effects of inoculation with EG16 on the growth of *H. cannabinus*. (**a-d**) plant growth in treatments containing 0, 50, 100, and 150 μ*M* Fe, respectively, in the hydroponic experiment; (**e**) plant growth in unpolluted and polluted soils in the pot experiment; P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with the EG16 (plant + EG16); *, **, and *** indicate a significant difference between the P and PE treatments at the 0.05, 0.01, and 0.001 levels, respectively, by independent-samples *t*-test (hydroponic experiment, n = 4; pot experiment, n = 7). *Error bars* indicate the standard deviation (created by Origin 8.0)

respectively ($P < 0.05$ or $P < 0.01$). However, addition of 150 μ*M* Fe inhibited plant growth, and bacterial inoculation did not improve it in this case.

In the pot experiment as well, the PGP effects of EG16 were stronger under conditions of high metal exposure. In the metal polluted soil, inoculation increased shoot and root biomass by 13 and 40 %, respectively (independent-samples *t*-test, $P < 0.001$), while the corresponding increases in the unpolluted soil were 10 and 37 % ($P < 0.01$, Fig. 1e). Results for some other parameters of plant growth also reflected a stronger PGP effect of EG16 under heavy metal stress conditions (Table S2).

Cd and Fe accumulation in *H. cannabinus*

Results from the hydroponic experiment showed that *H. cannabinus* with lower Fe supply has an increased Fe concentration when inoculated with the EG16 strain, no matter whether there was Cd addition (one-way ANOVA, $P < 0.05$, Fig. 2a, b). As for total amount of Fe in the plant, Cd addition significantly inhibited plant Fe uptake in treatment with 0, 50 and 100 μ*M* Fe supply (one-way ANOVA, $P < 0.05$, Fig. 2c). Bacterial inoculation contributed to improve plant Fe uptake in all the Cd-added treatments, with a 72 and 106 % increase in treatment with 0 and 50 μ*M* Fe supply, respectively, and a reduced effect (only 40 and 53 % increase of Fe uptake) on higher Fe supply treatments (100 and 150 μ*M* Fe) (one-way ANOVA, $P < 0.05$, Fig. 2c). By contrast, both Cd concentration and total Cd amount in *H. cannabinus* were significantly reduced in Cd-added treatments containing 50 and 100 μ*M* Fe when inoculated with EG16 (independent-samples *t*-test, $P < 0.05$ or $P < 0.01$, Fig. 2d–f). In the treatments with EG16 inoculation, the total

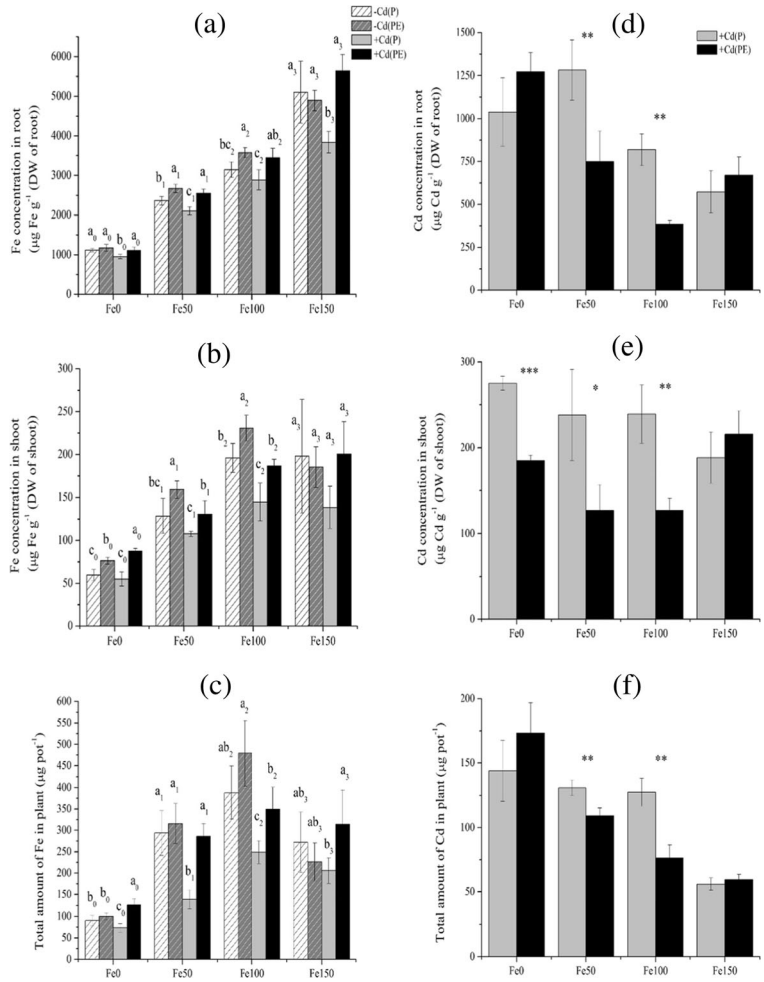

Fig. 2 Effect of inoculation with EG16 on uptake of Fe and Cd by *H. cannabinus* in the hydroponic experiment. Fe concentration (**a, b**) and Cd concentration (**d, e**) of root and shoot, respectively. Total amount of Fe (**c**) and Cd (**f**) in plant of each pot. P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with the EG16 (plant + EG16). *, **, and *** indicate a significant difference between the P and PE treatments at the 0.05, 0.01, and 0.001 levels, respectively, by independent-samples *t*-test. Means not sharing the same letter are different at the 0.05 level by one-way ANOVA with Duncan's correction ($n = 4$). *Error bars* indicated the standard deviation (created by Origin 8.0)

Cd amount in plant decreased with increasing Fe supply (Fig. 2f).

Since the contaminated soil used in the pot experiment was multi-metal polluted and contained excessive levels of Pb, Zn, Cu, and Cd (Table S1), plant accumulation of Pb, Zn, and Cu was measured in addition to that of Cd and Fe. As shown in Table 2 and Table 3, EG16 inoculation significantly reduced the concentration and the total amount of Pb, Zn, Cd in *H. cannabinus* cultivated in the metal polluted soil (independent-samples *t*-test, $P < 0.05$, 0.01 or 0.001), whereas plant Cu accumulation was unaffected by bacterial inoculation ($P > 0.05$). As for Fe, bacterial inoculation promoted Fe uptake in plants growing in the unpolluted soil ($P < 0.05$) while having no significant effect on those in the metal polluted soil ($P > 0.05$).

Effect of strain EG16 on Cd bioconcentration and translocation factors

The bioconcentration factor (BF) and translocation factor (TF), indices for identifying suitable phytostabilization plant candidates and restricting metal accumulation in the aerial parts of plants and safeguard their economic use (Mendez and Maier 2008), were calculated for the metal polluted soil in pot experiment. Results showed rather low BF and TF values (0.02 to 0.44, Table 4) in *H. cannabinus* cultivated in the metal polluted soil. Inoculation with EG16 resulted in a further decrease in the BF values of Pb, Zn and Cd, and TF values of Zn and Cd (independent-samples *t*-test, $P < 0.05$, 0.01 or 0.001, Table 4).

Bioavailability of heavy metals in soil

The mobility of metals and their bioavailability affect their eco-toxicity to plants and depend strongly on their specific chemical forms. Thus, it is important to predict bioavailable metals rather than the total metals in contaminated soil in order to assess their toxic effects and the environmental quality of contaminated soil. (Gupta and Sinha 2007). In the pot experiment of this study, the bioavailability of metals was defined through a one-step soil extraction procedure using NH_4NO_3 solution (Gupta and Sinha 2006; Gupta and Sinha 2007). Both cultivation of *H. cannabinus* and inoculation with EG16 resulted in significant reductions in the bioavailability of Pb, Zn, and Cd, especially in the metal polluted soil (one-way ANOVA, $P < 0.05$, Table 5). *H. cannabinus* cultivation also was effective at immobilizing Cu in the metal polluted soil ($P < 0.05$, Table 5).

Discussion

PGPR colonizing the rhizosphere of plants play important beneficial roles that directly or indirectly influence plant growth and development (Gerhardt et al. 2009; Glick et al. 1999; Islam et al. 2016). In the current study, a PGPR strain, *Enterobacter* sp. EG16, was re-inoculated to the rhizosphere of *H. cannabinus* and was observed to significantly improved the growth of *H. cannabinus*. Interestingly, its PGP effect was more pronounced in metal-polluted conditions (Fig. 1 and Table S2). Some other PGPR strains were found similarly to present a better PGP performance under metal stress (Belimov et al. 2005; Tripathi et al. 2005). Other researchers have described bacterial strains that show PGP effects only in the presence of metals (Remans et al. 2012; Xu et al. 2015). Finally, there have been reports of bacterial strains that exhibit PGP traits in metal-free physiological and biochemical tests while failing to promote plant growth in a metal contaminated environment (Becerra-Castro et al. 2012; Belimov et al. 2005; Guo and Chi 2014). In addition, some metals were observed to have active or negative effects on different PGP traits of PGPR strains (Gaonkar and Bhosle 2013; Kamnev et al. 2005; Schalk et al. 2011). These results indicated that the effective PGP strategies and the actual PGP effect might be quite different in the interactions between different plants, bacteria, and metals, which would greatly influence the final remediation efficiency of PGPR-associated phytoremediation. However, studies on the interactions and mechanisms behind such an associated system are limited. Accordingly, this study devoted to understanding the functioning PGP strategies as well as the actual effect of the EG16 strain in the metal-exposed EG16-*H. cannabinus* association in order to optimize this PGPR-associated phytoremediation.

Production of IAA and the siderophore enterobactin were the only two potential PGP strategies that EG16 possessed (Chen et al. 2016). Production of siderophore is reported as one of the most common bacterial strategies for acquisition of iron under Fe-limited conditions (Rajkumar et al. 2010). Actually we did observe increased siderophore production by EG16 with the reducing Fe supply (Table 1 and Fig. 1). Beyond the effect

Table 2 Bacterial effect on metal concentrations in *H. cannabinus* in the pot experiment

Treatments	Metal concentrations in *H. cannabinus* (10^1 μg g^{-1} (DW of plant))									
	shoot					root				
	Pb	Zn	Cu	Cd	Fe	Pb	Zn	Cu	Cd	Fe (10^3 μg g^{-1})
Unpolluted soil_P	Nd	3.60 ± 0.73	0.93 ± 0.15	0.09 ± 0.02	11.50 ± 0.74	0.82 ± 0.10	4.18 ± 0.67	1.85 ± 0.26	0.21 ± 0.04	5.10 ± 0.33
Unpolluted soil_PE	Nd	3.26 ± 0.36	0.96 ± 0.12	0.08 ± 0.01	15.78 ± 1.67	0.76 ± 0.10	4.02 ± 0.85	1.49 ± 0.17	0.13 ± 0.04	5.93 ± 0.39
Significance level					**			*	*	*
Bacterial effect (%)		-9.34 %	3.61 %	-13.64 %	+37.26 %	-8.27 %	-3.86 %	-19.32 %	-37.37 %	+16.17 %
Polluted soil _P	4.97 ± 0.64	17.19 ± 0.75	1.07 ± 0.18	0.15 ± 0.01	15.21 ± 2.92	59.59 ± 1.60	62.45 ± 3.92	23.60 ± 4.00	0.50 ± 0.07	18.83 ± 1.29
Polluted soil_PE	3.77 ± 0.36	13.88 ± 1.37	1.00 ± 0.07	0.11 ± 0.01	18.62 ± 2.96	54.65 ± 1.56	50.76 ± 3.56	22.53 ± 1.48	0.37 ± 0.05	17.13 ± 1.50
Significance level	**	**		***		**	***		**	
Bacterial effect (%)	-24.31 %	-19.23 %	-7.16 %	-22.35 %	+22.38 %	-8.30 %	-18.73 %	-4.55 %	-25.78 %	-9.04 %

Data were displayed as mean ± SD

P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with EG16 (plant + EG16)

*, **, and *** indicate a significant difference between the P and PE treatments at the 0.05, 0.01, and 0.001 levels, respectively, by independent-samples *t*- test ($n = 7$)

Nd not detected, *DW* dry weight

Table 3 Total amount of metals in *H. cannabinus* in the pot experiment

Treatments	Total amount of Heavy metals in plant (μg pot^{-1})				
	Pb	Zn	Cu	Cd	Fe (mg pot^{-1})
Unpolluted soil_P	1.29 ± 0.30	75.71 ± 16.16	18.98 ± 2.21	1.70 ± 0.33	0.99 ± 0.17
Unpolluted soil_PE	1.43 ± 0.19	77.86 ± 10.71	23.57 ± 2.23	1.87 ± 0.31	1.28 ± 0.30
Significance level			**		*
Bacterial effect (%)	+11.33 %	+2.83 %	+24.22 %	+10.14 %	+29.04 %
	Total amount of Heavy metals in plant (mg pot^{-1})				
	Pb	Zn	Cu	Cd (μg pot^{-1})	Fe
Polluted soil_P	0.17 ± 0.01	0.24 ± 0.01	0.06 ± 0.01	1.92 ± 0.09	4.15 ± 0.73
Polluted soil_PE	0.15 ± 0.01	0.21 ± 0.01	0.06 ± 0.01	1.68 ± 0.10	3.51 ± 0.60
Significance level	*	**		**	
Bacterial effect (%)	-10.40 %	-14.09 %	+3.29 %	-12.52 %	-15.28 %

Data were displayed as mean ± SD

P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with EG16 (plant + EG16)

* and ** indicate a significant difference between the P and PE treatments at the 0.05 and 0.01 levels, respectively, by independent-samples *t*-test (n = 7)

of Fe, other metals were found to stimulate bacterial siderophore production (Braud et al. 2010; Sinha and Mukherjee 2008). In contrast, negative effects of metals, including Fe and Cd, on bacterial IAA secretion have been observed (Dimkpa et al. 2008a; Kamnev et al. 2005). However, these results were obtained in the absence of plants, leaving confusion about the actual effect of these so-called plant-associated bacteria on plant development. In the EG16-*H. cannabinus* association of the current study, exposure to Cd significantly increased siderophore production while inhibiting the secretion of IAA, and treatments with lower Fe supply exhibited greater Cd-induced siderophore production (Table 1). As reported (Baysse et al. 2000; Dimkpa et al. 2009), some metals, including Cd, would compete with Fe for siderophore binding and thus cause Fe deficiency in microbes. Such Cd-induced Fe deficiency was also observed in the previous transcriptomic analyses of EG16 (Chen et al. 2016). In response to Cd stress, significant expression differences in genes, contributing to increasing siderophore production, was observed in EG16, whereas biosynthesis of IAA was inhibited, both

Table 4 Bioaccumulation factor (BF) and translocation factor (TF) in the metal polluted soil

Treatments	BF				TF			
	Pb	Zn	Cu	Cd	Pb	Zn	Cu	Cd
Polluted soil_P	0.03	0.44	0.02	0.08	0.08	0.27	0.04	0.36
st. dev	*0.005*	*0.015*	*0.002*	*0.008*	*0.011*	*0.054*	*0.009*	*0.043*
Polluted soil _PE	0.02	0.33	0.02	0.07	0.06	0.24	0.04	0.27
st. dev	*0.002*	*0.031*	*0.002*	*0.003*	*0.003*	*0.017*	*0.005*	*0.027*
Significance level	*	***		*		*		**
Bacterial effect (%)	-26.23 %	-24.95 %	-10.67 %	-14.93 %	-16.63 %	-8.77 %	-1.87 %	-25.27 %

Data were displayed as mean ± SD

P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with EG16 (plant + EG16); standard deviation (st. dev) is reported in italics

*, **, and *** indicate a significant difference between the P and PE treatments at the 0.05, 0.01, and 0.001 levels, respectively, by independent-samples *t*-test (n = 7)

Table 5 Bacterial effect on soil pH and metal bioavailability in polluted and unpolluted soil

Treatments	pH of soil	Bioavailability of heavy metals (μg g^{-1} (DW of soil))			
		Pb	Zn	Cu	Cd (μg kg^{-1})
Unpolluted soil	6.29 ± 0.12 b	Nd	0.26 ± 0.02 a	Nd	1.44 ± 0.06 a
Unpolluted soil _E	6.32 ± 0.06 b	Nd	0.23 ± 0.01 bc	Nd	1.09 ± 0.13 b
Unpolluted soil _P	6.44 ± 0.03 a	Nd	0.24 ± 0.02 b	Nd	1.22 ± 0.15 b
Unpolluted soil _PE	6.37 ± 0.04 ab	Nd	0.21 ± 0.02 c	Nd	1.16 ± 0.05 b
Polluted soil	3.86 ± 0.03 c	34.74 ± 1.39 a	4.28 ± 0.11 a	7.19 ± 0.06 a	45.36 ± 8.27 a
Polluted soil _E	3.90 ± 0.01 b	30.38 ± 0.82 b	3.90 ± 0.27 b	6.69 ± 0.33 ab	24.92 ± 3.86 c
Polluted soil _P	3.93 ± 0.02 a	31.21 ± 0.96 b	3.74 ± 0.24 b	6.50 ± 0.58 b	34.46 ± 2.85 b
Polluted soil _PE	3.95 ± 0.02 a	28.15 ± 0.96 c	3.25 ± 0.09 c	6.74 ± 0.17 ab	26.84 ± 1.60 c

For each soil, means not followed by the same letter within columns are different at the 0.05 level by one-way ANOVA with Duncan's correction (n = 7)

E indicates treatments without plant cultivation but with bacterial inoculation, P indicates treatments cultivated *H. cannabinus* without bacterial inoculation, PE indicates treatments cultivated *H. cannabinus* inoculated with EG16 (plant + EG16)

Nd not detected, *DW* dry weight

of which were in line with the results of the present study. Such metal-induced siderophore production seemed to be available only in metal-resistant but not metal-sensitive bacteria (Gaonkar and Bhosle 2013). As a result, the enhanced siderophore production was suggested to be involved in the Cd response of the Cd-resistant strain EG16, which is triggered by Fe deficiency and may function better in improving plant growth under Cd exposure.

Cd-inhibited IAA production was alleviated in lower Fe supply (Table 1). Inhibition of metals on bacterial IAA production was thought to cause by either lower synthesis caused by metal stress or degradation by IAA peroxidases induced by metal-catalyzed free radical formation (Dimkpa et al. 2008b; Potters et al. 2007). Notably, the competitive siderophore binding by other metals might help to protect bacteria against metal toxicity by sequestering and/or immobilizing metals in the siderophore-chelated form (Braud et al. 2010; Ferret et al. 2015; Schalk et al. 2011). Thus it might be the decreased free Cd ions resulted from increased siderophore binding that contributing to alleviate the inhibitory effect of Cd on IAA synthesis, just as Dimkpa et al. (2008b) suggested in a plant-free system. This helped to further prove the leading role of siderophore production as a PGP strategy in the Cd-exposed EG16-*H. cannabinus* association. Consistent with this, a stronger PGP effect was observed to associate with the enhanced siderophore production under Cd exposure (Fig. 1a–c). We also detected increased Fe uptake in *H. cannabinus* with bacterial inoculation under Cd exposure, which was more evident in treatments with higher siderophore production (0 and 50 μ*M* Fe, Fig. 2c). Therefore, we suggested the Cd-induced siderophore production as the functioning PGP strategy in the Cd-exposed EG16–*H. cannabinus* association, due to its improvement in plant growth and Fe uptake, as well as its alleviation of Cd-induced inhibition of IAA production which might also help to promote plant growth.

In addition to PGP strategies, plant-associated microbes are known to affect metal mobility and bioavailability to plants through the release of chelators, acidification and/or induction of redox changes (Becerra-Castro et al. 2012; Langella et al. 2014). In phytostabilization, which primarily focus on sequestration of metals within the root and/or the rhizosphere (Mendez and Maier 2008), a microbial-mediated reduction in metal bioavailability would be greatly interesting (Langella et al. 2014). In our pot experiment, both planting with *H. cannabinus* and inoculation with EG16 led to a significant reduction in the bioavailability of Pb, Zn, and Cd in the polluted soil (Table 5). Correspondingly, we observed significant decreases in both concentration and total uptake of these metals in *H. cannabinus* inoculated with EG16 (Tables 2 and 3). In hydroponic experiment, EG16 inoculation had a similar effect on plant Cd accumulation in the Fe50 and Fe100 treatments (Fig. 2f). EG16 was proved to

accumulate and immobilize free Cd ions by both surface absorption and intracellular accumulation in response to Cd stress (Chen et al. 2016), which may contribute to decreasing free Cd ions in the system and thus reducing plant Cd accumulation. Immobilization of mobile Cd ions by EG16 is likely to get greater with the increased number of bacterial cells from treatment with 0 to 150 μ*M* Fe, in line with the phenomenon that the total Cd amount in plant decreased with increasing Fe supply in the inoculated treatments (Fig. 2f). In addition, siderophores are thought to play an important role in the biochemical cycling of metals due to their competitive chelation of metals other than Fe (Ferret et al. 2015). It was observed that the Cd content in EG16 cells was significantly higher in media with siderophore than that without siderophore (Chen et al. 2016), probably presenting a contribution of siderophore to intracellular Cd immobilization by EG16. However, it can not make certain the effect of siderophore-Cd complex on Cd availability to *H. cannabinus* in this study, since the result was acquired from interactions among the plant, the bacterium and the metal. As reported (Mun et al. 2008), *H. cannabinus* roots have shown the ability to take up large amounts of Pb and greatly restrict its transfer to aerial parts at the same time. In this study, similar results were found in Zn, Cu, Cd besides Pb (both BF and TF values <<1), indicating a wide suitability of *H. cannabinus* to apply in phytostabilization with different metal or multi-metal contamination (Mendez and Maier 2008). Moreover, EG16 inoculation led to a further decrease in the BF and TF values in some cases (Table 4), which might suggest its well cooperation with *H. cannabinus* in limiting metal translocation within the plant tissues.

Conclusion

This study focused on the functioning PGP strategies and the actual effect of a PGPR strain EG16 when it is used in microbially assisted phytostabilization. Inoculation with EG16 not only strongly improved plant growth but also helped reduce metal accumulation in *H. cannabinus* in metal polluted soil. Increased siderophore production appears to be the main and effective PGP strategy of EG16 in the EG16-*H. cannabinus* association, as it was activated by the bacterial Cd response system, promoted plant Fe uptake, and alleviated Cd-induced inhibition of bacterial IAA production. All of these were beneficial for improving plant growth and remediation efficiency. Furthermore, the combination of *H. cannabinus* and EG16 significantly reduced metal bioavailability in metal polluted soil and maintained plant growth as well as low metal translocation to the above-ground parts of the plant, suggesting its potential application in phytostabilization.

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