Effect of heavy-metal on synthesis of siderophores by

Pseudomonas aeruginosa ZGKD3

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Abstract. Most siderophore-producing bacteria could improve the plant growth. Here, the
effect of heavy-metal on the growth, total siderophore and pyoverdine production of the Cd
equivalent Pseudomonas aeruginosa ZGKD3 were investigated. The results showed that
ZGKD3 exhibited tolerance to heavy metals, and the metal tolerance decreased in the order
Mn²⁺ > Pb²⁺ > Ni²⁺ > Cu²⁺ > Zn²⁺ > Cd²⁺. The total siderophore and pyoverdine production of
ZGKD3 induced by metals of Cd²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Pb²⁺ and Mn²⁺ were different, the total
siderophore and pyoverdine production reduced in the order Cd²⁺ > Pb²⁺ > Mn²⁺ > Ni²⁺ > Zn²⁺>
Cu²⁺ and Zn²⁺ > Cd²⁺ > Mn²⁺ > Pb²⁺ > Ni²⁺ > Cu²⁺, respectively. These results suggested that
ZGKD3 could grow in heavy-metal contaminated soil and had the potential of improving
phytoremediation efficiency in Cd and Zn contaminated soils.

1. Introduction
Soil contamination with heavy metals has become increasingly prominent with the rapid development
of human industry and agriculture which causes great hazard to natural environment [1]. Heavy metal
ions could be absorbed by plant root and then transport from roots to shoots and cause a considerable
threat to human health through food chain due to its high toxicity [2]. Nowadays, in comparison with
conventional methods such as physical separation, washing and stabilization, more attention has been
paid to plant-microorganism combined bioremediation phytoremediation [3-4]. Various studies have
reported that heavy-metal tolerant microbes can directly improve the efficiency of phytoremediation,
the possible strategies including metal mobilization, metal chelation and oxidation/reduction reactions
[5-7]. In particular, many metal resistant bacteria are capable of synthesizie siderophores which
provides benefits to plants [8-9]. Siderophores play an important role in phytoremediation due to its
strong affinity for Fe³⁺, Cu²⁺, Zn²⁺, Ni²⁺ and Cd²⁺. Siderophore-producing Pseudomonas aeruginosa
increased the concentrations of Cr and Pb in maize by mobilizing Cd and Pb in soils [10]. Sharma
reported that siderophore-producing P. aeruginosa GRP3 alleviated the chlorotic symptoms and
significantly enhanced chlorophyll content and biomass of Vigna radiate L [11]. Ni tolerant
endophytic bacteria isolated from Alyssum bertoloni were capable of producing siderophores,
enhanced the biomass and Ni accumulation of inoculated plants [12]. Similarly, Dimkpa found that
siderophores produced by Streptomyces acidiscabies E13 alleviated oxidative stress induced by heavy
metal of cowpea [13]. A large amount of siderophore-producing bacteria were screened and applied to improve phytoremediation efficiency, and it has been evidenced that siderophores produced by bacteria can protect microbes against the toxicity of heavy metals [14-15], whereas the mechanism of heavy-metal tolerance of siderophore-producing bacteria is still unknown.

In the present study, P. aeruginosa ZGKD3 isolated from soil contaminated by gangue pile of coal area in our laboratory exhibited high tolerance to Cd²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Pb²⁺ and Mn²⁺. The objectives of this study were to investigate the effect of different concentrations of multiple heavy metals on synthesis of siderophores of P. aeruginosa ZGKD3. Furthermore, determine the potential of ZGKD3 in improving phytoremediation efficiency in heavy-metal contaminated soils and these results may provide a base for revealing the heavy metal tolerance mechanism of bacteria.

2. Materials and Methods

2.1. Bacteria and media.
The heavy-metal resistant P. aeruginosa ZGKD3 were isolated from soil contaminated by gangue pile of coal area of Shanxi province in our laboratory. ZGKD3 were cultivated in broth medium which contained 3.0 g beef extract, 10.0 g peptone, and 5.0 g sodium chloride per liter with an initial pH of 7.2 and MSA (sugar-Asp) medium which contained 20.0 g sucrose, 2.0 g aspartic acid, 1.0 g K₂HPO₄ and 0.5 g MgSO₄ per liter with an initial pH of 7.2 [16].

2.2. The growth of ZGKD3 under heavy-metal stress.
The growth of P. aeruginosa ZGKD3 under different concentrations of heavy-metal stress was assayed in MSA. ZGKD3 were grown on nutrient broth medium for 16 h, 2 ml cells of ZGKD3 were inoculated into 100 ml Erlenmeyer flasks containing 50 mL of sterile MSA medium with CdCl₂, CuCl₂, ZnCl₂, NiCl₂, Pb(NO₃)₂ and MnCl₂ (0, 200, 400 and 1000 µM), respectively, and incubated in an rotary shaker (150 rpm) at 37 °C for 24 h, the effect of heavy-metal on growth and the ability of alkaline production of ZGKD3 were investigated at 16 h. The biomass of bacterial cell was determined by a UV-Visible spectrophotometer, the absorbance was measured at 600 nm (OD₆₀₀). All of the chemical reagent were analytical reagent.

2.3. Quantitative analysis siderophore synthesis by ZGKD3 under heavy-metal stress.
The effects of different concentrations of heavy-metal on siderophore synthesis of ZGKD3 were test. Bacterial samples were obtained from MSA medium contained CdCl₂ (0, 200, 400, 1000 and 3000 µM), CuCl₂, ZnCl₂, NiCl₂, Pb(NO₃)₂ and MnCl₂ (0, 200, 400 and 1000 µM), respectively. Detection of total siderophores and pyoverdine production by ZGKD3 were carried out, the total siderophore production was assayed by chromo azurol S (CAS) plate assay [17]. For quantification of siderophore and pyoverdine production were investigated at 12, 24 and 48 h by a UV-Visible spectrophotometer at the absorbance of 630 and 400 nm, respectively.

2.4. Statistical analysis.
All data were analyzed by SPSS 16.0 for significant differences (P<0.05). Statistical analyses were performed by one-way ANOVA.

3. Results

3.1. The growth of ZGKD3 in MSA medium under heavy metal stress
The effect of six heavy metals on growth of ZGKD3 was assayed. As shown in Figure 1, the concentrations of Cd²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Pb²⁺ and Mn²⁺ (200, 400, 1000 µM) inhibited the growth of ZGKD3 and as a consequence of the increasing dose of heavy meal, the stronger inhibitive effects on growth of bacterial were observed, suggested that there is a significant negative correlation between the heavy-metal concentration and the growth of ZGKD3. Moreover, ZGKD3 could tolerate Ni²⁺, Pb²⁺
and Mn$^{2+}$ at the concentrations of 200, 400 and 1000 μM, and the growth of ZGKD3 was significantly inhibited by Cd$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ at the concentration of 200 μM. The strongest inhibitory effect on the growth of ZGKD3 was observed at the high concentrations of Cd$^{2+}$, the biomass (OD$_{600}$) of ZGKD3 decreased from 0.792 to 0.098 with the increasing dose of Cd$^{2+}$. Thus, the effects of various heavy metals on growth of ZGKD3 were different.

Figure 1. Effect of heavy metals on the growth of strain ZGKD3 in MSA medium contained heavy metals at the concentration of 0, 200, 400 and 1000 μM at 16 h

3.2. The total siderophore and pyoverdine production of ZGKD3 in MSA medium under heavy metal stress
Siderophore production of ZGKD3 was confirmed by adding the culture supernatant into the holes on CAS agar plate and orange haloes were observed. Cd$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$ and Mn$^{2+}$ exhibited different effects on siderophore production of bacteria (Figure 2). The largest orange haloes was found in the Zn$^{2+}$ group, next is Cd$^{2+}$. Significant increase in the total siderophore and poverdine production of strain ZGKD3 was found, with the increase of 8-90% for 200-1000 μM of Cd$^{2+}$ and 35-242% for 200-1000 μM Zn$^{2+}$. However, Cu$^{2+}$ significantly inhibited the total siderophore and poverdine production of strain ZGKD3, and the reduction was range from 26-88%. In comparison with control, Ni$^{2+}$, Pb$^{2+}$ and Mn$^{2+}$ had no markedly difference in total siderophore and poverdine production (Figure 3 and 4), the total siderophore and pyoverdine production reduced in the order Cd$^{2+}$ > Pb$^{2+}$ > Mn$^{2+}$ > Ni$^{2+}$ > Zn$^{2+}$ > Cu$^{2+}$ and Zn$^{2+}$ > Cd$^{2+}$ > Mn$^{2+}$ > Pb$^{2+}$ > Ni$^{2+}$ > Cu$^{2+}$, respectively.

Figure 2. Pyoverdine production by ZGKD3 exposed to heavy metals at the concentrations of 200, 400, 1000 and 3000 μM. The orange haloes on each plate are: A and B in No.1 row indicate negative control and positive control, respectively. The numbers from 1 to 6 indicate Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, Pb$^{2+}$ and Mn$^{2+}$, respectively.
Discussion

4.1. The heavy metals inhibited the growth of ZGKD3
Various studies have evidenced that heavy metals could induce inhibitory effects on growth of bacteria. For instance, Zhang found that the biomass of Bacillus subtilis decreased by 96.1% at 0.2 mM of Cd [18]. Jiang reported that the growth of Bacillus subtilis was inhibited and decreased by 95% at the concentration of 0.25 mM Cd compared to control [19]. In this paper, an experiment was used to determine the growth of ZGKD3 in response to different concentrations of heavy metals. According to the growth curves, ZGKD3 present a great variance in tolerance towards different heavy metals. In this study, an increase in bacteria biomass of ZGKD3 was observed under Ni$^{2+}$ stress of 200 and 400 μM, the result was similar with the reports previously [20]. Thus, low concentration of heavy metal might promote the growth of bacteria, in the contrary, high concentrations of Ni inhibited the growth of ZGKD3. For the Cd$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Pb$^{2+}$ and Mn$^{2+}$, all the concentrations of heavy metals exhibited inhibitory effect on bacteria. Therefore, the role of heavy metals on bacteria was dependent on their respective concentration and the kind of heavy metals, suggested that ZGKD3 had different tolerance to multiple heavy metals, ZGKD3 is more sensitive to Cd$^{2+}$ than other metals and the metal tolerance of ZGKD3 decreased in the order Mn$^{2+}$ > Pb$^{2+}$ > Ni$^{2+}$ > Cu$^{2+}$ > Zn$^{2+}$ > Cd$^{2+}$. 
4.2. The heavy metals induced the siderophore production of ZGKD3
In the present study, Cd\(^{2+}\) and Zn\(^{2+}\) were the heavy metal that showed the greater toxicity than that of other heavy metals. In this sense, most of bacteria couldn’t grow at high concentrations of heavy metals. Furthermore, bacteria could remove heavy metals from their growth environment with intracellular and surface accumulation of Cu, Zn, Pb and Cd [21]. A large number of bacteria can produce diffusible light-green pigment on a CAS agar plate, and the light-green pigment has been characterized previously to be siderophores [22]. Most of *P. aeruginosa* could produce siderophores, including pyoverdine and pyochelin. It has been suggested that Al, Cu, Mn, Ga and Ni could induce synthesis of siderophores [10]. Similarly, Sinha demonstrated that Cd-resistant strain KUCd1 induced siderophore production maximally at 1.75 mM of Cd concentration [23]. Dao found that the presence of 0.125-1 mM of Cd could stimulate pyoverdine production of *P. aeruginosa* strain PAO1 [24]. Dimlpa observed that Al, Cd, Cu and Ni induced three hydroxamate siderophores by *Streptomyces* sp. Strains [25]. Furthermore, it has been confirmed that siderophores produced by bacteria could chelate many heavy metals, such as Al, Cd, Zn, Cu and Pb, and it was different in chelate ability of bacteria for every heavy metal [26]. Some studies demonstrated that siderophore could increase or decrease the toxicity of heavy metals in bacteria. Pyochelin produced by *P. aeruginosa* increased the toxicity of vanadium to bacteria [27]. However, Braud found synthesis of siderophores decreased the toxicity of multiple heavy metals to *P. aeruginosa* [28]. According to our results, the effects of heavy metals on growth of ZGKD3 were different, the ability of producing siderophore of ZGKD3 varied with different heavy metals might be one of the reasons. The toxicity of heavy metals on bacteria dependent on the amount of heavy metal accumulation in bacteria cells. Hence, the more siderophore produced, the more heavy metals were absorbed by ZGKD3 through the chelation of siderophores for heavy metals, which found to be high toxic to bacteria. In the present study, Cd\(^{2+}\) and Zn\(^{2+}\) significantly induced the synthesis of pyoverdine, and remarkably inhibited the growth of ZGKD3, indicated that the absorption of ZGKD3 for Cd\(^{2+}\) and Zn\(^{2+}\) was more than that for other metals. Therefore, *P. aeruginosa* ZGKD3 might have the potential of improving the phytoextraction efficiency in Cd and Zn contaminated soils.

5. Conclusions
*P. aeruginosa* ZGKD3 exhibited different tolerance to multiple heavy metals. In comparison with Cd\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\) and Mn\(^{2+}\), and the metal tolerance decreased in the order Mn\(^{2+}\) > Pb\(^{2+}\) > Ni\(^{2+}\) > Cu\(^{2+}\) > Zn\(^{2+}\) > Cd\(^{2+}\). Moreover, Cd\(^{2+}\) and Zn\(^{2+}\) significantly stimulated the total siderophore and pyoverdine production of strain ZGKD3. Therefore, *P. aeruginosa* ZGKD3 can act as siderophore-producing bacteria and applied to microbe and plants combined remediation in Cd and Zn contaminated soils.

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References
[1] Kermani J N, Ghasemi F, Khosravan, A. Farahmand, et al. 2010 *Iran. J. Environ. Health. Sci. Eng.* 7 279-286
[2] Nawrot T S, Staessen J A, Roels H A, Munters E, Cuypers A, Richart T, Ruttens A, Smeets K, Clijsters H, Vangronsveld J 2010 *Biometals* 23 769-782
[3] Chehregani A, Noori M, Yazdi H L 2009 *Ecotoxicol Environ Saf* 72 1349–53
[4] Haque N, Peralta-Videa J R, Jones G L, Gill T E, Gardea-Torresdey J L 2008 *Environ Pollut* 153 362–368
[5] Ma Y, Prasad M N V, Rajkumar M, Freitas H 2011 *Biotechnol Adv* 29 248–258
[6] Rajkumar M, Ae N, Prasad MNV, Freitas H 2010 *Trends Biotechnol* 28 142–149
[7] Kidd P, Barcelo J, Bernal M P, Navari-Izzo F, Poschenrieder C, Shilev S, Clemente R, Monterroso C 2009 *Environ Exp Bot* 67 243–59
[8] Kuffner M, Puschenreiter M, Wieshammer G, Gorfer M, Sessitsch A 2008 Plant Soil 304 35-44
[9] Rajkumar M, Ae N, Prasad M N V, Freitas H 2010 Trends Biotechnol 28 142-149
[10] Braud A, Jézéquel K, Bazot S, Lebeau T 2009 Chemosphere 74 280–286
[11] Sharma A, Johri B N, Sharma A K, Glick B R 2003 Soil Biol. Biochem 35 887–894
[12] Barzanti R, Ozino F, Bazzicalupo M, Gabrielli R, Galardi F, Gonnelli C, Mengoni A 2007 Microb. Ecol 53 306-316
[13] Dimkpa C O, Merten D, Svatoš A, Büchel G, Kothe E 2009 Soil Biol Biochem 41 154–162
[14] Cortese M S, Paszczynski A, Lewis T A, Sebat J L, Borek V, Crawford I 2002 Biometals 15 103-120
[15] Fiedler H P, Krastel P, Müller J, Gebhardt K, Zeeck A 2001 FEMS Microbiol. Lett 196 147-151
[16] Shin S H, Lim Y, Lee S E, Yang N W, Rhee J H. 2001 Journal of Microbiological Methods 44 89-95
[17] Schwyn B, Neiland J B 1987 Anal Biochem 160 47–56
[18] Zhang W, Chen G H, Guo J, Zhao Y J, Gao Y C, Wang J N 2013 Industrial safety and environmental protection 39 42-45
[19] Ma G F, Wang J, Zhang C B 2009 Microbiology 35 882-887
[20] Cabrero A, Fernandez S, Fernando M, Julian G 1998 Water research 32 1355-62
[21] Maria V C, Antonio C H, Antonio F V, Maria V M T 2014 Advances in Microbiology 4 644-655
[22] Meyer J M, Abdallah M A 1978 J Gen Microbiol 107 319–328
[23] Sinha S, Mukherjee S K 2008 Curr Microbiol 56 55-60
[24] Dao K H T, Hamer K E, Clark C L, Harshman L G 2001 Ecol Appl 9 441–448
[25] Dimkpa C O, Svatos A, Dabrowska P, Schmidt A, Boland W, Kothe E 2008 Chemosphere 74 19-25
[26] Schalk I J, Hannauer M, Braud A 2011 Microbial 13 2844-54
[27] Baysse C, Vos D D, Naudet Y, Vandermonde A, Ochsner U, Meyer J M 2000 Microbiol-SGM 146 2425-34
[28] Braud A, Geoffroy V, Hoegy F, Mislin G L A, Schalk I J 2010 Environ Microbiol Report 2 419-425