Journal of Sciences, Islamic Republic of Iran 33(3): 205 - 211 (2022) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Isolation and Characterization of Heavy Metal-Resistant Plant Growth Promoting Rhizobacteria (PGPR) as Candidates for Simultaneous Enhancement of Plant Growth and Bioremediation of Agricultural Metal-Polluted Soils

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Received: 12 March 2022 / Revised: 29 July 2022 / Accepted: 17 August 2022

Abstract

Application of metal-resistant plant growth-promoting bacteria is an efficient method for enhancing crop yields by improving biomass accumulation and plant tolerance to heavy metals. The present study aimed to isolate and characterize these bacteria from rhizosphere soil and root exudates of plants grown on agricultural soils contaminated with heavy metals. Plant growth-promoting properties of isolated strains were assayed by evaluating their abilities to solubilize insoluble phosphate, produce indole-3-acetic acid (IAA), and fix nitrogen. Resistance to metals toxicity and metal removal potential of the selected strains were investigated by MIC and MBC values determination and atomic absorption spectroscopy (AAS), respectively. *Pantoea agglomerans*exhibited the maximum solubilization of insoluble phosphate and IAA production. In the case of Cd^{2+}, the highest MIC value belonged to *Enterobacter ludwigi*. *Pseudomonas taiwanensis, P. agglomerans*, and *E. ludwigi* exhibited the greatest MIC value (8mM) in the case of Pb^{2+}. *P. agglomerans* and *E. ludwigi* showed the highest MBC of Pb^{2+}(>120mM) and the greatest MBC of Cd^{2+} (30mM) belonged to *P. agglomerans*. Also, *E. ludwigi* exhibited the highest metal removal percentage for Pb^{2+} as 31.81% and Cd^{2+} as 37.58%. As results showed, these four isolated strains can be used as novel and efficient agents for improving plant growth, especially in heavy metals polluted agricultural soils.

Keywords: Heavy metals removal; Nitrogen fixation; Phosphate solubilizer; IAA production.

Introduction

Heavy metals are elements that enter into their surrounding environments at low amounts through natural sources, including the erosion of soil and the earth's crust. But, in recent decades, human activities like mining, discharge of untreated industrial effluents and urban sewage, and use of chemical pesticides and

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fertilizers have raised dramatically metals concentration to the dangerous doses for biological functions in all living organisms [1]. Heavy metals have harmful effects on the soil texture and its fertility, the number and activity of soil microbial population, and physiological and morphological features of plants that lead to crop yield reduction [2, 3]. Also, heavy metals can easily be uptake by the plant roots and subsequently arrive into the food chain of animals and humans. Metals can cause severe diseases in humans through damage to vital biological substances, such as lipids, proteins, and nucleic acids through the production of free radicals. Among the heavy metals, which can cause environmental pollutions, lead and cadmium have become an important global problem, because they have no essential role in biological reactions [4, 5].

Unlike other environmental contaminants, heavy metals are persistent and accumulative pollutants because they cannot break down to non-toxic elements gradually. So, it is necessary to use suitable techniques for cleaning up heavy metals from contaminated environments to reduce their toxicity [6]. Numerous chemo-physical methods which are used for metal remediation have various deficiencies such as high expense, loss of soil fertility, and generation of secondary pollutants [6, 7].

Bioremediation is a simple, low-cost, and eco-friendly alternative method to remove heavy metals from polluted areas [8]. Microbial remediation, using heavy metal resistant bacteria can be applied as an effective and reliable type of bioremediation methods [9]. Heavy metal-resistant plant growth-promoting bacteria (PGPB) can be used for enhancing plant biomass production as well as removing of heavy metals from the polluted agricultural soils [10]. These bacteria stimulate plant growth by different mechanisms such as increasing availability of essential nutrients through phosphorus solubilization, nitrogen fixation, and siderophore production for iron uptake. Also, they regulate many plant developmental processes by secretion of phytohormones like indole-3-acetic acid (IAA), and protect plants against deleterious effects of phytopathogens by the synthesis of antibiotics, HCN, and degradative enzymes [11-13].

This study aimed to isolate new heavy metal-resistant bacterial strains with plant growth-promoting properties for enhancing plant growth in heavy metal contaminated soils. Therefore, the main objectives of this study were (1) to isolate and characterize bacterial strains from the rhizosphere soil and plant root exudate, (2) to investigate the plant growth-promoting traits of bacterial isolates, and finally (3) to evaluate their potential to resist against metals (i.e., Pb^{2+} and Cd^{2+}) toxicity and their metal removal efficiency.

Materials and Methods

1. Collection of samples and bacterial isolation

Bacteria were isolated from the soil of the rhizosphere and root exudates of two herb plants include alfalfa (*Medicago sativa*), and wheat (*Triticum aestivum*) grown on agricultural soils contaminated with heavy metals, sited in Talkhooncheh village (32.2307° N, 51.5360° E). To obtain rhizosphere strains, the soil particles that closely adhered to the root surfaces were separated gently from the roots. One-gram soil was transferred to 99 ml of normal saline and incubated at 150 rpm for 45 min on a rotary shaker (Infors HT CH-4103 Bottmingen, Germany). To discard the sediments, the suspensions were allowed to settle for 1 hour. Subsequently, each sample was serially diluted tenfold (10^{-2}-10^{-8} dilutions) with normal saline. Also, the isolation of the microorganisms from root exudates was done as follows: plant roots were carefully washed with water, sterilized by soaking in 10% sodium hypochlorite and rinsed three times with distilled water. The roots were ground to prepare their extracts. Finally, the root extracts were streaked on dishes containing suitable culture media(such tryptic soy agar or nutrient agar) [14].

2. Evaluation of plant growth-promoting activities

2.1. Solubilization of inorganic phosphate

This ability was screened on Pikovskaya's (PVK) agar medium. Briefly, 0.1ml of each sample was spread on PVK agar medium and incubated at 30 °C for 5 days. The P-solubilization potential was indicated with the development of a clear zone around the bacterial colony. Quantitative analysis of phosphate solubilization potential of the isolated bacteria was done in PVK liquid medium. Briefly, 0.1ml of fresh bacterial inoculum was added to 10ml of PVK liquid medium and incubated at 30 °C on a rotary shaker at 180 rpm for 4 days. The supernatant was harvested at 8000 rpm for 10 min. Then, 0.5 ml of the supernatant was mixed with 2.5 ml of Barton's reagent and the final volume was adjusted to 50 ml with deionized water. After 10 min, the intensity of the yellow color (due to phosphomolybdate complex formation) was measured at 430 nm (Shimadzu UV-160 UV-Vis-NIR Spectrophotometer) [15]. The concentrations of P-liberated was estimated from a calibration curve, which prepared by using different concentrations of phosphate instead of supernatant.

2.2. Production of indole-3-acetic acid (IAA)

One milliliter from overnight bacterial culture

(OD=1.5) was inoculated to 100 ml liquid Luria-Bertani (LB) medium supplemented with 0.1% L-tryptophan and incubated at 30 °C in darkness on a rotational shaker. After 3 days, the pellet was removed by centrifugation (5000 rpm for 20 min). Then, 1ml of supernatant was added to 2ml of Salkowski reagent and the optical density was read at 530nm after 30min [14]. The IAA concentration was determined by IAA standard curve.

2.3. Nitrogen fixation ability

Individual colonies were picked up and transferred to N-free medium comprising (l^{-1}): 20 g glucose, 3 g $CaCO_3$, 0.4 g $FeSO_4$, 0.25 g KH_2PO_4, 0.75 g K_2HPO_4, 0.5 g $MgSO_4$, 0.005 g $FeSO_4$, 0.02 g Na_2MoO_4, and 15 g agar and incubated at 30 °C for 14 days. The growth of bacterial colonies on the nitrogen-free medium exhibited the bacterial nitrogen fixation ability [16].

3. Assessment of bacterial reactions to heavy metals

3.1. Resistance of bacterial strain to heavy metals

The resistance patterns of bacterial strains to cadmium and lead were identified by measuring MIC and MBC values in a 96-well microtitre plate. Each well contained 100 µl of TSB medium, 50 µl of different concentrations of each metal (cadmium chloride and lead acetate), and 50 µl of bacterial suspension (O.D. ~ 0.1). After 24 h incubation at 30 °C, bacterial growth was evaluated at 630 nm. Control wells were contained TSB and bacterial suspension without metals. The experiment was carried out in triplicate [6].

3.2. Heavy metal removal experiments

Collecting of bacterial cells was carried out in the late-exponential growth phase by centrifugation at 3000rpm. 20mg of bacterial biomass was added to 20 ml of metal solutions (2mM of $CdCl_2.2H_2O$ and 5mM of $Pb.3H_2O(CH3COO)_2$ and incubated 2 h at 150 rpm. In the next step, the supernatant was harvested by centrifugation at 5000 rpm for 15 min. Finally, the residual metal ions were measured by atomic absorption spectrophotometer. Samples without bacterial inoculum served as controls [17].

4. Identification and characterization of bacterial isolates

Based on Bergey's Manual of Systematic Bacteriology, the morphological and biochemical characteristics of each isolate were determined. Also, molecular identification was conducted by sequencing of 16S-rRNA gene by using universal primers16SP0F (5'-AAGAGTTTGATCCTGGCTCAG-3') and 16SP6R (5'-CTACGGCTACCTTGTTACGA-3'). PCR reaction mixture composition and thermocycling conditions were as follows: The PCR mixture (25 µl) contained 0.5 µl of dNTPs, 1 µl of each primer, 2 µl of DNA template, 0.75 µl of $MgCl_2$, 2.5 µl of PCR buffer, and 0.25 µl of Taq DNA polymerase. The PCR reaction was started by heating the mixture for 5 min at 95 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C, 45 s at 72 °C, and final extension for 7 min at 72 °C. The products of PCR were electrophoresed in agarose gel and stained with ethidium bromide to reveal DNA bonds. In the final step, the sequences of desired genes were analyzed using the NCBI BLAST platform.

Results and Discussion

1. Assessment of plant growth-promoting activities

PGPRs can increase the production of plant biomass via different processes such as phosphate solubilization, fixation of atmospheric nitrogen, and secretion of phytohormones like IAA [18-20]. Therefore, in this study, we tried to isolate new bacterial strains which can increase plant growth as well as remove heavy metals from polluted soils. At first, we screened phosphate solubilizing bacteria on PVK agar. A total of 23 bacterial colonies with a clear zone were isolated. Among them, 10 isolates which formed the largest transparent zone on PVK agar, were selected for quantitative estimation of phosphate solubilization. These strains include: RA and SV isolated from alfalfa rhizoids, ET isolated from wheat rhizoids, RO, HO, MR, EO, EME1,2, and 3 isolated from rhizosphere. Among these potent isolates, two strains RA and ET exhibited the maximum phosphate solubilization capability (Figure 1). Also, IAA production was evaluated and strains RA and RO showed the highest and the lowest IAA production ability, respectively (Figure 2). Then, nitrogen fixation ability was detected in N-free medium. After 14 days of incubation at 30 °C, growth of strains HO, SV, RA, ET and MR on this medium indicated the ability of these five strains to fix nitrogen. According to these results, strains HO, SV, RA, ET, and MR showed more ability to enhance plant growth than other isolates. So, they were selected for further analysis in the next steps of this study.

2. Assessment of bacterial reactions to heavy metals

Microbes can usually tolerate a narrow range of heavy metals because these elements will cause adverse effects on several physiological and biochemical mechanisms, including enzymatic functions, ion regulation, and the formation of DNA and protein [21]. These adverse changes will have negative effects on

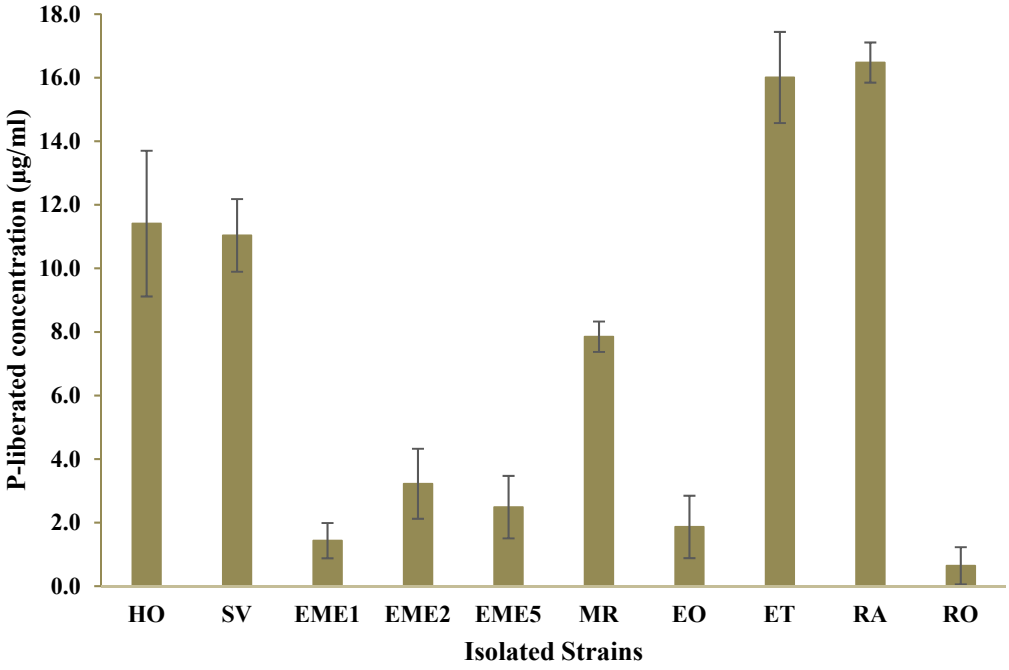

Figure 1 Comparison of p-liberated concentrations by the bacterial isolates

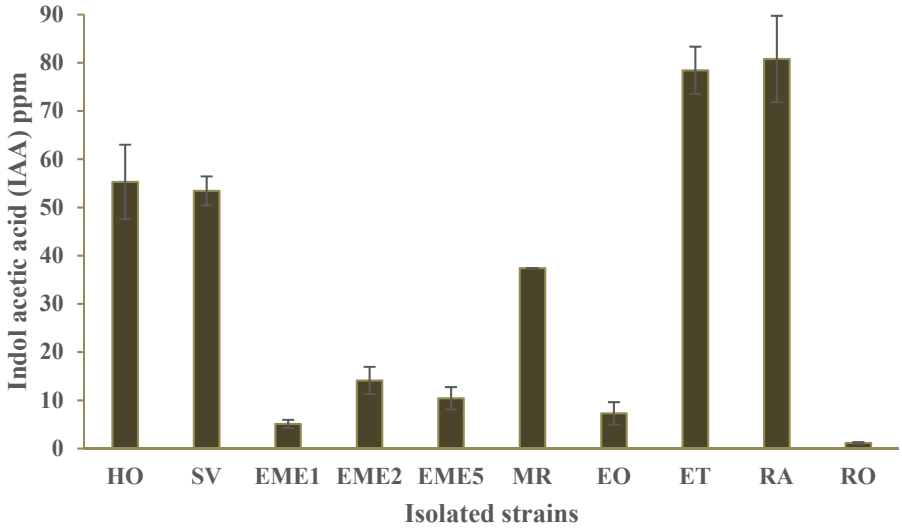

Figure 2 Efficiency of isolated bacteria to produce IAA (ppm)

bacterial viability and subsequently, they will lead to the reduction of bioremediation efficiency. To overcome this limitation, there is a need to screen and identify heavy metal-resistant bacteria [22]. Isolated bacteria from metal-polluted areas are usually more resistant to high concentrations of heavy metals than those isolated from intact sites [19]. The resistance of each isolated strain to heavy metal toxicity was evaluated by determining the MIC and MBC values. In the case of Cd^{2+}, the highest MIC value belonged to strain HO, followed by strains EO, SV, and RA. Strains MR, EO, SV, and RA showed the greatest MIC value (8mM) in the case of Pb^{2+}. Strains RA and MR showed the highest MBC of Pb^{2} (>120mM) and the greatest MBC of Cd^{2+} (30mM) belonged to strains EO and RA (Table 1). According to these results, strains HO, MR, SV, and

Table 1 MIC and MBC values of lead and cadmium for isolated strains

Isolated Strains	MIC of Cd^{2+}(mM)	MBC of Cd^{2+}(mM)	MIC of Pb^{2+}(mM)	MBC of Pb^{2+}(mM)
HO	10	20	5	20
SV	5	15	8	30
EME1	2.5	15	0.2	30
EME2	2	20	2	20
EME5	2	15	2.5	30
EO	5	30	8	>30
MR	2.5	15	8	>120
ET	2.5	15	7	30
RA	5	30	8	>120
RO	2	10	7	15

Bold names are selected strains in this paper.

RA, which were more resistant to metal toxicity than other isolates, were selected for metal removal experiments. Strain MR showed the highest metal removal percentage for Pb^{2+} as 31.81% and Cd^{2+} as 37.58%. Strains HO and RA removed the lowest quantities of Cd^{2+} and Pb^{2+}, respectively (Figure 3). So, based on PGP characteristics and metal resistance, strains HO, RA, SV, and MR were suggested as new enhancers of plant growth agents in contaminated soils with heavy metals in this work.

3. Identification and characterization of bacterial isolates

The phenotypic properties of selected strains were summarized in Table 2. Molecular characterization of bacteria was carried out by amplification and sequencing of the 16s rRNA gene (PCR amplicons with the expected size (~1500 bp)) (Figure 4). The isolates HO, SV, and RA BLAST results indicated 99% similarity to *Enterobacter cloacae*, *Pantoea*

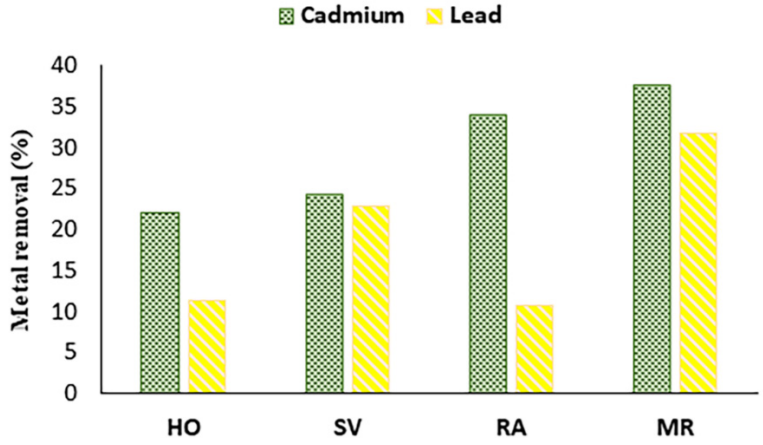

Figure 3. Metal removal (%) by isolated strains with initial metal concentration 5mM of Pb^{2+} and 2mM of Cd^{2+}

Table 2. Morphological, physiological and biochemical characteristics of selected bacterialisolates

Selected Strains	Shape	Gram reaction	Spore formation	TSI	Motility	Catalase	Oxidase	Urease	OF	Citrate utilization	Nitrate reduction	H_2S	MR	VP
HO	Rod	-	-	A/A	+	+	-	+	+/+	+	+	-	+	-
SV	Rod	-	-	A/A	+	+	-	+	+/+	+	+	-	+	-
RA	Rod	-	-	A/A	+	+	-	+	+/+	+	+	-	+	-
MR	Rod	-	-	K/K	+	+	+	+	+/-	+	+	-	-	+

A: Acid; K: Alkaline.

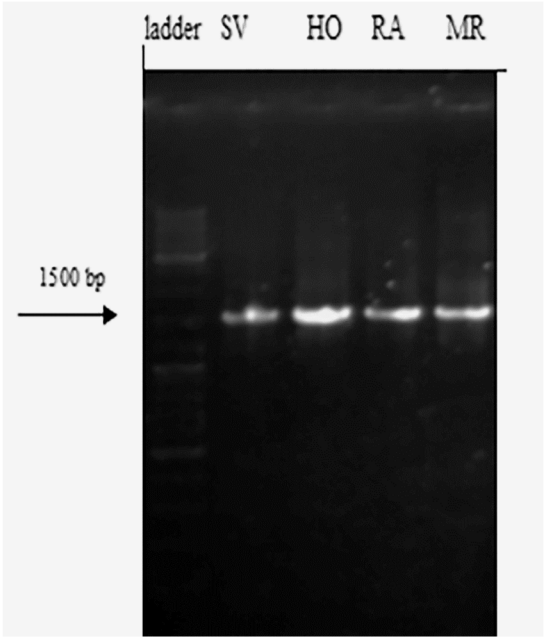

Figure 4. Agarose gel electrophoresis of PCR-products of selected strains (SV, HO, RA, MR) with universal primers 16SP0F and 16SP6R

agglomerans, and *Enterobacter ludwigi,* respectively. Also, the alignment result of strain MR showed 97% similar to the 16s rRNA region of *Pseudomonas taiwanensis*. The sequences were submitted to GenBank and the accession numbers KX865087 (HO), KX963370 (SV), KX963369 (RA), and KX963367 (MR) were achieved. In the previous study, *E. cloacae,* isolated from a heavy metal-defiled rice field, withstand up to 4000 µg/ml Cd^{2+} and 3312 µg/ml Pb^{2+} [23]. Also, *P. taiwanensis*, was reported as a heavy metal tolerant bacterial strain that could tolerate (40 ppm) Cd^{2+} and (120 ppm) Pb^{2+} [24]. In comparison with previous reports, the isolates identified in the current study were resistant to higher levels of Pb^{2+} (approximately 8mM) and Cd^{2+} (approximately 8mM). This may be attributed to the site where the soil samples were taken being contaminated with high levels of heavy metals. High-level resistance to metal toxicity is consider a significant factor in bioremediation because this factor is directly related to the survival and growth of bacteria in metal-polluted areas. So, the isolated bacterial strains in this study can be considered as new potent heavy metal tolerant agents with plant growth promoting abilities.

Conclusion

The present study focused on the isolation of multi-metal tolerant rhizobacteria. Thus, in the first step, we endeavored to isolate PGPRs from the rhizosphere and plant root exudate in metal-contaminated zones. Our findings demonstrated that *P. taiwanensis, E. cloacae*, *P. agglomerans*, and *E. ludwigi* have the potential to promote plant growth by preparing essential nutrients through the production of IAA, fixation of nitrogen, and solubilization of insoluble phosphate. Also, they could remove metals from the environment. Therefore, these newly isolated bacterial strains can be appropriate candidates for application in the bioremediation approach for eliminating contamination from multi-metals polluted soils.

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