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Isolation and Characterization of Pb-Solubilizing Bacteria and Their Effects on Pb Uptake by *Brassica juncea*: Implications for Microbe-Assisted Phytoremediation

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The aim of this study was to isolate and characterize lead (Pb)-solubilizing bacteria from heavy metal-contaminated mine soils and to evaluate their inoculation effects on the growth and Pb absorption of *Brassica juncea*. The isolates were also evaluated for their plant growth-promoting characteristics as well as heavy metal and salt tolerance. A total of 171 Pb-tolerant isolates were identified, of which only 15 bacterial strains were able to produce clear haloes in solid medium containing PbO or $PbCO_3$, indicating Pb solubilization. All of these 15 strains were also able to dissolve the Pb minerals in a liquid medium, which was accompanied by significant decreases in pH values of the medium. Based on 16S rRNA gene sequence analysis, the Pb-solubilizing strains belonged to genera *Bacillus*, *Paenibacillus*, *Brevibacterium*, and *Staphylococcus*. A majority of the Pb-solubilizing strains were able to produce indole acetic acid and siderophores to different extents. Two of the Pb-solubilizing isolates were able to solubilize inorganic phosphate as well. Some of the strains displayed tolerance to different heavy metals and to salt stress and were able to grow in a wide pH range. Inoculation with two selected Pb-solubilizing and plant growth-promoting strains, (*i.e.*, *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151) and their consortium enhanced the growth and Pb uptake of *B. juncea* plants grown in a metal-contaminated soil. The bacterial strains isolated in this study are promising candidates to develop novel microbe-assisted phytoremediation strategies for metal-contaminated soils.

Keywords: Metal-solubilizing bacteria, lead, PGPR, contaminated soils, phytoremediation

Introduction

The growing industrialization and urbanization worldwide have resulted in serious environmental hazards due to the wide array of pollutants, including heavy metals, released into the soil environment. Although trace concentrations of certain heavy metals such as Fe, Mn, Zn, and Cu are essential for living cells, others like Cd, Hg, and Pb have no known biological or physiological functions and are even considered to be toxic not only to humans but to plants and microorganisms as well [1]. Among these toxic metals, Pb has the greatest detrimental impact on human health. Most Pb concentrations found in the environment are connected to such human activities as metal mining and smelting, lead battery production, leaded petroleum consumption, agricultural use of sewage sludge, and uncontrolled disposal of industrial wastes [2]. Chronic exposure to Pb increases the risks of high blood pressure, diseases of the nervous system and kidneys, hyperactivity, anemia, and infertility [2].

Accumulation of heavy metals in soils enhances their risk of entering the food chain via crop uptake. Humans and animals may also be exposed to these metals through inhalation of contaminated dust and consumption of contaminated waters [3]. To avoid the harmful effects of heavy metals, it is necessary to remediate metal-contaminated sites. Although effective in remediating soils contaminated with heavy metals, particularly those contaminated with high heavy-metal concentrations, traditional cleanup technologies (*e.g.*, thermal treatment, physical separation, electrochemical processes, and acid leaching) are usually expensive and they might also disturb the structure, biogeochemistry, and productivity of the remediated soils [4]. These limitations have led to a growing interest in alternative, less expensive, environmentally friendly, and nondestructive technologies with in situ applicability and high public appeal. Phytoextraction, a common process of phytoremediation, is one such technology that has attracted a lot of attention among researchers. It exploits the potential of green plants to absorb contaminants from the soil and accumulate them in their above-ground tissues, which can then be harvested using conventional agricultural techniques [5].

Metal hyperaccumulators are mostly slow-growing plants with a small biomass, limiting the efficacy of phytoextraction in remediating metal-contaminated sites [6]. Plant growth and biomass production in contaminated soils, and thereby phytoextraction efficiency, can be improved by utilizing plant growth-promoting rhizobacteria (PGPR). PGPR have positive effects on plant performance via such varied mechanisms as phosphorus mobilization, atmospheric nitrogen fixation, iron sequestration by siderophores, and production of phytohormones including auxins like indole acetic acid (IAA) [7–9].

Phytoremediation efficiency is not only limited by the slow growth and low biomass production of hyperaccumulator plants but also by the low bioavailability of heavy metals in many contaminated soils [9, 10]. A large proportion of heavy metals present in soils is, indeed, either bound to the organic and inorganic soil constituents or precipitated as sparingly soluble minerals. Carbonates, hydroxides, and phosphates are the most important minerals that control the solubility of metals in soils within a pH range of 5.5–7.5 [11]. Adding chelating agents to soil has been suggested as a means of increasing metal availability to plants [12]. However, application of these expensive compounds has faced increasing criticism on the grounds of their phyto- and microorganism-toxicity traits [13, 14]. One promising alternative proposed to overcome this limitation is the bioaugmentation of contaminated soils with microorganisms that are able to increase metal solubility while also enhancing plant growth and biomass production, and thereby overall metal uptake by plants [8–10]. Jiang *et al*. [7] reported that an isolated heavy metal-resistant bacterium highly capable of mobilizing Pb and Cd carbonates increased not only tomato plant growth but its Cd and Pb uptake as well. Sheng *et al*. [10] showed that soil inoculation with *Pseudomonas fluorescens* and *Microbacterium* sp. strains significantly increased the water solubility of lead. *Pseudomonas* and *Bacillus* strains have also been reported for their capability to solubilize Zn from sparingly soluble salts [15, 16]. The release of protons, organic acids, siderophores, and other chelating agents, generation of special metabolites, solubilization of phosphates, and mediation of redox transformations have been suggested as important mechanisms involved in the metal mobilization processes performed by metal-solubilizing bacteria [16, 17].

Lots of works have been carried out using PGPR to improve metal phytoextraction, but studies on the application of those PGPR strains capable of metal solubilization to overcome low metal availability in soils, as a bottleneck in phytoremediation efficiency, are rare. Microbial strains with both metal-solubilizing and plant growth-promoting abilities may effectively be used for bioaugmentation-phytoremediation of heavy metal-contaminated soils. This study was designed and implemented in pursuit of the following objectives: (i) to isolate and characterize Pb-resistant bacteria from metal contaminated mine soils that are capable of both solubilizing $PbCO_3$ and PbO as sparingly soluble Pb compounds, (ii) to examine the plant growth-promoting characteristics of the isolated Pb-solubilizing bacteria, and finally (iii) to evaluate the inoculation effects of the isolated bacteria on the growth and Pb uptake of Indian mustard (*Brassica juncea*) grown in a contaminated soil.

Materials and Methods

Site Description and Soil Sampling

Two study sites located inside metal mining areas in Isfahan Province, Iran, were selected for soil sampling. Three soil samples were collected from the Bama Pb/Zn Mine (51° 32′ E and 32° 28′ N) and two from the Nakhlak Pb Mine (53° 50′ E and 33° 34′ N). A portion of the soil samples was kept in sterile zip-lock plastic bags and stored at 4°C for bacterial isolation and characterization procedures. Another portion was air-dried, passed through a sieve (2 mm), and analyzed for important soil properties. The soil pH and electrical conductivity (EC) were determined in 1:2

Table 1. Some characteristics of the soils that the Pb-solubilizing bacteria were isolated from.

Soil[a]	pH[b]	EC[b] (dS/m)	OC (%)	DTPA-extractable metals (mg/kg)				Total concentration of metals (mg/kg)	
				Cu	Cd	Pb	Zn	Pb	Zn
B_1	7.4	0.86	1.27	2.70	0.72	696	225	5,489	4,200
B_2	7.8	0.47	1.10	3.80	0.99	727	352	7,909	4,387
B_3	7.6	1.68	2.85	1.00	0.45	174	79.80	1,343	2,325
N_1	8.5	11.70	0.22	0.92	0.32	294	645	5,128	3,850
N_2	7.6	17.90	0.08	0.75	0.17	688	11.10	1,866	887

[a]B and N represent the Bama and Nakhlak mine soils, respectively.

[b]The pH and EC were measured in a 1:2 soil:water suspension.

EC, electrical conductivity; OC, organic carbon; DTPA, diethylenetriaminepentaacetic acid.

(soil:water) extracts with a glass electrode (Cyberscan 2100) and a conductivity meter (Elmetron CC-501), respectively. The soil organic carbon content was determined using the potassium dichromate-sulfuric acid method [18], total Pb and Zn concentrations were measured via digesting soil samples in 6N HNO_3 [19], and bioavailable Cd, Cu, Pb, and Zn concentrations were estimated using the diethylenetriaminepentaacetic acid (DTPA)-$CaCl_2$-triethanolamine extraction method [20]. Metal concentrations in the extracts were determined with a flame atomic absorption spectrophotometer (FAAS, Z-5300). Some of the properties and metal concentrations of the soils are listed in Table 1.

Isolation and Purification of Pb-Tolerant Bacteria

The Pb-resistant bacteria were isolated using the Tris-buffered low-phosphate agar (TLP) medium containing sucrose as the carbon source to minimize Pb complexation to phosphate [21]. The medium was sterilized by autoclaving at 120°C for 20 min and subsequently supplemented with a filter-sterilized $Pb(NO_3)_2$ solution containing 50 mg-Pb/l. To prevent soil fungi growth, the media were supplemented with 10 mg/l of cycloheximide after autoclaving. The soil samples were then serially diluted with a sterile saline solution (0.85% NaCl) before 0.1 ml of each dilution was plated on the surface of the TLP medium. The bacterially inoculated plates were incubated at 30°C for up to 4 days, and individual colonies of different morphological characteristics, including color, size, form, opacity, and elevation, were selected and streaked on the same medium 3 to 4 times until pure cultures were obtained. The bacterial colonies were then regrown on Luria-Bertani (LB) medium and stored for further studies. A total of 171 bacterial isolates showing different morphological appearances on the agar medium were selected for studying the solubilization of Pb carbonate and oxide minerals.

Isolation of Pb-Solubilizing Bacteria

In the initial screening test for Pb tolerance, 110 Pb-resistant strains from the Bama Mine soils and 61 Pb-resistant strains from the Nakhlak Mine soils were isolated using a spread plate procedure and the pH-neutral TLP medium. These strains were subsequently screened to assess their potential to solubilize sparingly soluble Pb compounds (PbO and $PbCO_3$). This ability was revealed by the appearance of clear haloes of solubilization around the colonies in the medium containing sucrose as the carbon source. For this purpose, inocula of the Pb-resistant bacterial isolates were prepared by centrifugation of 2 ml of logarithmic phase grown bacteria at 10,062 ×*g* for 5 min at 4°C, washed twice with saline solution (0.85% NaCl), and adjusted to about 10^8 CFU/ml by suspending in sterile saline solution. Forty microliters of the inocula was spotted onto plates containing a sucrose-minimal salt low-phosphate (SLP) medium (1% sucrose, 0.1% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.01% NaCl, and 0.05% yeast extract, pH 7.2) supplemented with the sparingly soluble Pb compounds PbO or $PbCO_3$. The concentration of the metal oxide added to the medium was 1,115 mg/l and that of the metal carbonate was 500 mg/l. Centrally inoculated plates in triplicate were incubated at 30°C for 10 days in the dark. A clear halo against an opaque background was interpreted as the sign of metal solubilization.

Quantification of Pb Solubilization

Into 250-ml Erlenmeyer flasks containing 100 ml of the sterile liquid SLP, 500 mg/l of Pb as $PbCO_3$ or 1,115 mg/l of Pb as PbO was added, and the medium was then inoculated with 1 ml of the logarithmic phase inoculum at approximately 10^8 CFU/ml. Media without metal addition and without bacterial inoculation were used as controls. The test flasks were incubated in a rotary shaker in the dark at 180 rpm and 30°C. After 10 days of incubation, 25 ml of the culture was centrifuged at 8,000 ×*g* for 20 min and filtered through a 0.22 μm Millipore filter. The Pb concentration in the supernatants was determined by FAAS. The pH levels of the supernatants were measured with a pH meter [10].

Plant Growth-Promoting Properties of the Isolates

IAA production. The Pb-solubilizing bacterial strains were cultured in flasks containing 25 ml of LB medium supplemented with 0.5 mg/ml of L-tryptophan (filter-sterilized) at 30°C on an orbiter shaker at 150 rpm. After incubation for 24, 48, and 96 h, 1 ml of the cell-free suspension was mixed vigorously with 2 ml of Salkowski's reagent [22] and allowed to stand at room temperature

for 20 min. The absorbance of the pink color developed after 25 min of incubation was read at 530 nm. The IAA concentration in the culture was determined using a calibration curve of pure IAA.

Siderophore production. Siderophore produced by the isolated strains was detected according to the chrome azurol S (CAS) agar method described in Alexander and Zuberer [23]. The bacterial strains were grown twice in the Fe-free SLP medium before the inocula were prepared and adjusted to about 10^8 CFU/ml by suspending in a sterile saline solution. Subsequently, 40 µl of the inocula was spotted on CAS-agar plates and incubated in the dark for 10 days. Orange-yellow haloes around the colonies on the blue agar were interpreted as siderophore excretion.

Phosphate-solubilizing ability. The phosphate solubilization ability of the strains was determined in Pikovskaya's Agar medium containing 0.5% tricalcium phosphate [24]. Forty microliters of each bacterial inoculum (about 10^8 CFU/ml) was spotted in Pikovskays's Agar medium and incubated in the dark for 10 days. A clear halo around the colony was interpreted as the bacteria's phosphate solubilization ability.

Genetic Identification

Characterization of each selected metal-solubilizing bacterium at the genus level was accomplished by partial sequencing of the 16S ribosomal DNA gene. Genomic DNA was extracted with the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA) and amplified by the polymerase chain reaction (PCR) using the universal bacterial primers fD1 (5′-AGAGTTTGATCC TGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) [25]. Each PCR contained 1 µl of gDNA and 0.4 µM (final concentration) of each primer in a final volume of 50 µl. The following PCR conditions were used: initial denaturing time of 5 min at 95°C, followed by 35 PCR cycles of 95°C for 1 min; 55°C, 1 min; and 72°C, 2 min; and a final extension step at 72°C for 10 min. Five microliters of the PCR products was subjected to electrophoretic investigation on a 1% TAE agarose gel and purified with the Ultra Clean PCR Clean-up Kit (MO BIO Laboratories, Inc.,) according to the manufacturer's instructions. The purified PCR products were sequenced by Secugen (Madrid, Spain). Pairwise alignments were conducted using DNASTAR Lasergene (ver. 7.00) and the 16S rRNA gene sequences thus obtained were compared with those in the GenBank Database using the BLAST program from the NCBI website to determine the percent similarity.

Maximum Tolerance Concentration (MTC) of the Isolates

The maximum tolerance of the selected isolates against increasing concentrations of heavy metals (Zn, Pb, Cd, and Cu) was evaluated on SLP medium at pH 6.8 until the strains were unable to grow colonies on the agar plates. The maximum concentration of heavy metals at which the bacteria still presented growth was designated as the MTC. For this purpose, 10 µl of the logarithmic phase bacterial culture at approximately 10^8 CFU/ml was drop-inoculated onto Petri dishes in three replicates. A 100 mM stock solution of each metal was prepared using $CdCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, and $Pb(NO_3)_2$ and filtered through a 0.22 µm Millipore filter. The plates were incubated at 28°C for up to 10 days. This experiment was repeated twice in three replicates [26].

Isolate Tolerance to Acid and Alkaline pH and Sodium Chloride

Experiments were performed to monitor the ranges of pH and NaCl concentration at which the isolated bacterial strains maintained their ability to grow on the SLP agar medium [27]. For this purpose, the medium was buffered with 0.1 M sodium acetate/acetic acid at pH 4.5, 25 mM MES at pH 5.5 and 6.5, 25 mM HEPES at pH 7.5, 25 mM Tris-HCl at pH 8.5, and 25 mM sodium carbonate at pH 9.5 and 10.5. Subsequently, 10 µl of the exponential phase growing bacteria in the LB medium, at approximately 10^8 CFU/ml, was drop-inoculated on the medium and incubated at 28°C for up to 10 days. In the case of increasing salt concentration, NaCl from 3% up to 10% was added to the medium before autoclaving and the same amount of bacterial inoculum was used each time. These assays were repeated twice in three plates for each treatment.

Influence of Selected Isolates on the Growth and Pb Uptake of *B. juncea*

Preparation of bacterial inocula. Two isolated strains, *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151, both with Pb-solubilizing and plant growth-promoting properties, were selected for inoculation of *B. juncea* in a greenhouse study. Inocula of the bacterial isolates were prepared by centrifugation of 2 ml of logarithmic phase grown bacteria at 10,062 ×*g* for 5 min at 4°C, washed twice with 0.85% NaCl solution, and adjusted to about 10^8 CFU/ml by suspending in the same sterile saline solution. A consortium of these two strains was also prepared by mixing equal amounts of the bacterial suspensions.

Soil. Soil collected from a metal-contaminated site around the Bama Pb/Zn Mine (51° 33′ 47/69′′E and 32° 30′ 45/79′′N) was air-dried and sieved to finer than 4 mm. It was characterized as a calcareous silty soil (15.6% sand, 83.5% silt, 0.9% clay) with pH 7.4 and an EC equal to 0.72 dS/m. The total Pb, Zn, and Cd concentrations of the soil were 446, 413, and 6.2 mg/kg, respectively, as determined by FAAS after soil digestion in 6N HNO_3 [19].

Pot experiment. *Brassica juncea* was used for the pot trial because of its fast growth as well as its potential to survive and absorb heavy metals in contaminated soils [5]. Seeds of *B. juncea* were surface sterilized by soaking in 96% ethanol for 30 sec and in 5% sodium hypochlorite solution for 2 min. The seeds were then thoroughly rinsed at least 7 to 8 times with sterile distilled water. Seed inoculations were made by soaking the sterilized seeds in the bacterial suspension for 1 h. Seeds soaked in a sterile saline solution (0.85% NaCl) were used as controls. Ten seeds of *B. juncea* were sown, in triplicates, in a plastic pot containing 2.3 kg of the metal-contaminated soil. One week after the seedlings had

emerged, the plants were thinned to six plants per pot and grown under greenhouse conditions at a daytime temperature of 28–34°C and a night-time temperature of 17–23°C. The soil moisture was maintained at 60% of water holding capacity by adding water on a weight basis throughout the growth period. Four weeks after germination, the bacterial suspensions (20 ml/pot) were sprayed on the soil surface. The control plants received 20 ml of sterile saline solution (0.85% NaCl).

Plant analysis. Plant growth parameters including shoot length as well as shoot and root dry weights were measured. The maximum shoot height was measured before harvesting on day 55 of the growth period. Subsequently, roots were separated and washed extensively with 1 mM $Ca(NO_3)_2 \cdot 4H_2O$ and then with distilled water in order to remove surface-adsorbed Pb ions. Shoot and root dry matters were recorded after oven-drying at 75°C for 72 h.

The oven-dried plant samples were digested in a mixture of concentrated HNO_3-HCl (70%) and H_2O_2 (30%) [28], and the Pb concentration in the extracts was determined using FAAS. Total Pb uptake by the plant shoots was calculated as the product of the shoot dry weight and Pb concentration.

Statistical Analysis

One-way analysis of variance followed by the least significant difference (LSD) test ($p < 0.05$) using STATISTICA 8 (StatSoft, USA) was applied to compare the potential significant differences between treatments.

Results and Discussion

General Characteristics of the Metal-Contaminated Soils

The properties of the soils from which the bacteria were isolated are reported in Table 1. Soil pH values ranged from 7.4 to 8.5. The soils collected from the Bama Mine had EC values in the range of 0.47–1.68 dS/m whereas those of the Nakhlak Mine soil ranged from 11.7 to 17.9 dS/m. The total Pb concentration in the soils ranged from 1,343 to 7,909 mg/kg and the available (DTPA-$CaCl_2$ extractable) fraction of Pb in the soils varied from 174 to 727 mg/kg. As judged from these values, all the soils were contaminated with Pb [29]. The values for total and available metals indicate that the soils were also contaminated with Zn and Cd [29].

Plant Growth-Promoting Characteristics of the Pb-Solubilizing Strains

IAA production. Plant growth enhancement by IAA is mostly due to its stimulating effect on root development, plant cell elongation, and cell division [30–32]. The root system enhancement, in turn, leads to a higher uptake of ions as a result of the larger soil volume made available to the roots.

All the Pb-solubilizing isolates, except YSP66 and YSP40n,

Table 2. Indole acetic acid (IAA) production by the Pb-solubilizing isolates after 24, 48, and 96 h of incubation.[a]

Isolate	IAA (mg/l)					
	LB medium			LB medium + L-tryptophan (500 mg/l)		
	24 h	48 h	96 h	24 h	48 h	96h
YSP15	ND	ND	ND	0.67 ± 0.09 pq	0.53 ± 0.14 q	0.49 ± 0.12 q
YSP17	0.46 ± 0.18 hj	0.39 hk	0.43 ± 0.17 hk	4.69 ± 0.43 hi	4.2 ± 0.17 hk	4.25 ± 0.29 hk
YSP18	1.14 ± 0.11 df	1.19 ± 0.11 d	1.16 ± 0.22 de	1.10 ± 0.14 nq	1.61 ± 0.13 lq	1.05 ± 0.14 nq
YSP24	ND [b]	1.03 ± 0.11 dg	2.33 ± 0.30 b	0.58 ± 0.09 pq	72.72 ± 2 b	58.88 ± 2.78 d
YSP36	1.24 ± 0.33 d	0.88 ± 0.10 g	0.88 ± 0.09 g	0.92 ± 0.30 pq	1.08 ± 0.30 nq	1.03 ± 0.32 nq
YSP40	2.58 ± 0.54 a	2.85 ± 0.35 mn	1.51 ± 0.15 n	6.64 ± 0.68 g	77.71 ± 3.33 a	61.65 ± 5.49 c
YSP40n	ND	0.07 ± 0.05 ln	ND	ND	0.12 ± 0.03 q	ND
YSP66	ND	ND	0.49 ± 0.13 hi	ND	ND	0.97 ± 0.24 oq
YSP69	ND	0.19 ± 0.06 kn	ND	ND	3.94 ± 0.11 hk	1.14 ± 0.18 nq
YSP95	0.55 ± 0.04 h	0.48 ± 0.10 hi	0.41 ± 0.08 hk	0.55 ± 0.07 q	1.60 ± 0.16 lq	1.51 ± 0.17 mq
YSP104	0.37 ± 0.18 hk	ND	ND	3.41 ± 0.42 hl	3.94 ± 0.16 hk	3.89 ± 0.37 hk
YSP110	0.51 ± 0.15 hi	0.47 ± 0.15 hi	0.46 ± 0.10 hj	1.39 ± 0.20 nq	5.23 ± 0.23 gh	3.78 ± 0.12 hk
YSP143	0.24 ± 0.06 jm	0.21 ± 0.06 kn	0.31 ± .03 il	2.41 ± 0.11 kp	3.24 ± 0.30 im	4.04 ± 0.29 hk
YSP149	0.45 ± 0.13 hj	0.42 ± 0.08 hk	0.43 ± 0.12 hk	2.85 ± 0.35 in	3.25 ± 0.37 im	2.80 ± 0.25 jo
YSP151	0.93 ± 0.20 eg	0.92 ± 0.24 fg	1.62 ± 0.14 c	4.45 ± 0.41 hj	9.21 ± 0.78 f	11.41 ± 0.94 e

[a]Data represent the average of three replicates ± standard deviation. Means in each medium followed by the same letter are not significantly different according to LSD ($p < 0.05$). ND: not detected.

were found capable of producing IAA in the LB medium supplemented with L-tryptophan (Table 2). The strains YSP40 and YSP24 recorded the highest IAA production rates (Table 2). Moreover, the strains YSP18, YSP24, YSP36, YSP40, and YSP151 produced IAA in the culture medium lacking L-tryptophan (Table 2). Since there are several tryptophan-independent pathways for IAA synthesis [33], it is possible that the latter strains were able to produce IAA without its major precursor, tryptophan. The strains YSP24, YSP40, YSP69, and YSP110 recorded reductions in their IAA content in the medium supplemented with tryptophan after 48–96 h (Table 2). This might have been due to the degradation of IAA by IAA oxidase secreted by the bacteria or to the storage of IAA in the bacterial cells as previously suggested [34]. The production of IAA has been reported to be common in *Bacillus* and *Paenibacillus* species, such as *B. subtilis*, *B. pumilus*, and *B. cereus* [10, 16, 24].

Siderophore production. Bioaugmentation with siderophore-producing bacteria has been proposed as an efficient strategy to enhance plant growth and metal uptake in phytoremediation practices [35]. In addition, chelation of heavy metals by siderophores has been found to promote bacterial IAA synthesis through reduced oxidative degradation of IAA by free heavy metals [36].

Ten out of the 15 Pb-solubilizing strains were able to produce siderophores (Table 3). Siderophore production levels by the different bacterial strains were compared on the basis of the ratio of colony + haloe diameter to colony diameter. The strains YSP151 and YSP149 exhibited the highest capability for siderophore production (Table 3).

Production of metal-chelating substances, such as organic acids and siderophores, plays a special role in metal detoxification, and thereby in increasing metal tolerance in bacteria. These chelating agents may form complexes with heavy metals that are considered to be less toxic so as to inactivate and minimize the cytological impacts of free metal ions [37]. Abou-Shanab *et al*. [38] reported that 61.6% and 42.9% of the Pb-resistant bacterial strains isolated from the rhizosphere of *Diplachne fusca*, grown in industrial sites, were siderophore producers and acid producers, respectively.

Phosphate solubilization. Only two Pb-solubilizing strains (YSP24 and YSP95) were able to produce clear haloes on Pikovskaya's medium containing sparingly soluble $Ca_3(PO_4)_2$ as the sole source of phosphate (Table 3). Low P availability may retard plant growth and metal uptake in phytoremediation practices of contaminated soils [24]. Therefore, selection of bacterial strains capable of increasing P solubility in these soils would be favorable for improving plant growth and, in turn, enhancing metal uptake from these soils [7].

Lead Solubilization Ability of the Isolates

Only 15 (8.8%) out of the 171 Pb-resistant isolates showed clear halos in agar plates amended with PbO or $PbCO_3$. Hence, this ability was not a common feature among the isolated bacteria. Colonies that formed zones of clearance around the colony were selected for further studies.

The isolated strains that were able to form zones of clearance around the colony on the solid medium containing PbO or $PbCO_3$ were also able to dissolve these minerals in the liquid medium. The results of Pb solubilization by the isolates and the associated pH changes in the liquid (SLP) medium are reported in Table 4. Clearly, maximum values of $PbCO_3$ solubilization in the SLP medium were recorded in the presence of strain YSP17 (4.28 mg-Pb/l), followed by YSP104 (2.64 mg-Pb/l) and YSP69 (2.16 mg-Pb/l), which were 26.7, 16.5, and 13.5 times higher than that of the control, respectively (Table 4). In the PbO-containing SLP medium, the maximum value of Pb concentration was recorded with inoculation of the strains YSP69, YSP40n, and YSP110 (8.99, 8.73, 8.42 mg-Pb/l, respectively), which were more than 30 times higher than that of the control. The bacterial isolates might have increased dissolution of the Pb-bearing minerals through such mechanisms as ligand-promoted dissolution induced by the bacterial metabolites and proton-promoted dissolution triggered by a pH decrease [39, 40]. Nevertheless, the magnitude of metal solubilization by a bacterial isolate varies depending

Table 3. Siderophore production and P solubilization abilities of the Pb-solubilizing isolates.

	Isolate														
	YSP15	YSP17	YSP18	YSP24	YSP36	YSP40	YSP40n	YSP66	YSP69	YSP95	YSP104	YSP110	YSP143	YSP149	YSP151
Siderophore production[a]	0	1.12	0	0	1.92	1.55	0	0.8	1.67	1.00	0.33	1.41	0	2.25	3.83
P solubilization[b]	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–

[a]Data represent the colony + haloes diameter/colony diameter ratio on CAS-agar medium.

[b]+, Clear halo produced on Pikovskaya's medium; –, No clear halo on Pikovskaya's medium.

Table 4. Total soluble Pb and final pH measured in $PbCO_3$- and PbO-containing SLP broth medium after inoculation with Pb-solubilizing bacterial isolates.

Bacterial isolate	Pb liberated[a] (mg/l)		Final pH of the medium		
	$PbCO_3$	PbO	$PbCO_3$	PbO	Control[b]
YSP15	1.48 ± 0.26 e	0.91 ± 0.08 g	5.95 ± 0.08 b	6.98 ± 0.07 b	6.76 ± 0.18 ab
YSP17	4.28 ± 0.15 a	2.13 ± 0.44 ef	4.04 ± 0.12 g	6.60 ± 0.26 c	4.54 ± 0.06 h
YSP18	0.36 ± 0.02 i	1.81 ± 0.15 ef	5.71 ± 0.12 b	5.60 ± 0.17 e	6.85 ± 0.09 a
YSP24	1.98 ± 0.18 cd	1.74 ± 0.10 f	4.27 ± 0.50 efg	4.01 ± 0.16 h	4.81 ± 0.07 g
YSP36	1.65 ± 0.38 de	6.73 ± 0.47 b	4.46 ± 0.14 de	4.21 ± 0.03 h	5.72 ± 0.17 c
YSP40	1.61 ± 0.20 de	2.35 ± 0.25 de	4.94 ± 0.14 c	6.26 ± 0.13 d	6.53 ± 0.08 b
YSP40n	1.02 ± 0.02 fg	8.73 ± 0.38 a	4.38 ± 0.11 def	4.27 ± 0.05 h	5.45 ± 0.06 de
YSP66	1.45 ± 0.39 e	0.84 ± 0.08 gh	4.07 ± 0.08 fg	6.01 ± 0.13 d	5.26 ± 0.14 ef
YSP69	2.16 ± 0.02 c	8.99 ± 0.26 a	4.43 ± 0.19 de	4.27 ± 0.14 h	4.49 ± 0.13 h
YSP95	0.21 ± 0.11 i	2.18 ± 0.72 ef	5.93 ± 0.18 b	5.12 ± 0.16 g	5.1 ± 0.16 f
YSP104	2.64 ± 0.41 b	2.86 ± 0.20 d	4.63 ± 0.37 cd	5.54 ± 0.11 ef	5.73 ± 0.15 c
YSP110	0.81 ± 0.02 gh	8.42 ± 0.76 a	4.15 ± 0.13 efg	4.20 ± 0.19 h	5.26 ± 0.09 ef
YSP143	0.47 ± 0.03 hi	0.67 ± 0.08 gh	4.46 ± 0.17 de	5.65 ± 0.30 e	5.58 ± 0.05 cd
YSP149	1.98 ± 0.06 cd	0.81 ± 0.21 gh	4.60 ± 0.15 d	6.59 ± 0.05 c	4.19 ± 0.18 i
YSP151	1.36 ± 0.42 ef	5.82 ± 0.52 c	4.43 ± 0.11 de	5.29 ± 0.17 fg	5.18 ± 0.20 f
Control[c]	0.16 ± 0.10 i	0.27 ± 0.05 h	7.56 ± 0.06 a	7.36 ± 0.18 a	6.90 ± 0.10 a

[a]SLP medium supplemented with 1,115 mg/l PbO or 500 mg/l $PbCO_3$ and inoculated with different bacterial strains.

[b]SLP medium inoculated with different bacterial strains without Pb salts.

[c]SLP medium supplemented with 1,115 mg/l PbO or 500 mg/l $PbCO_3$ without bacterial inoculation.

Means in each column followed by the same letter are not significantly different according to LSD ($p < 0.05$).

on the mineral composition and solubilization mechanism(s) exploited by the isolate. Li *et al*. [39], for example, showed that *B. cepacia* possessed different mobilization abilities on metal compounds with the descending order of $ZnCO_3$ > $CdCO_3$ > ZnO > > $PbCO_3$.

Bacterial-induced metal solubilization of minerals has frequently been attributed to ligand-promoted dissolution of minerals caused by bacterial metabolites such as siderophores, carboxylic organic acid anions, and biosurfactants [41]. Abou-Shanab *et al*. [42], for instance, demonstrated that growth products of *B. subtilis* and *B. pumilus* increased Zn, Cu, and Pb solubilization in soils. They suggested that siderophore production by these bacterial strains had a major effect on metal solubilization. Siderophores can form stable soluble complexes with heavy metals such as Pb or Fe [43, 44] and increase the solubility of metal-bearing minerals. *Pseudomonas aeruginosa* was reportedly able to produce siderophores when inoculated in Cr- and Pb-polluted soils, leading not only to increased concentrations of bioavailable Cr and Pb in the rhizosphere but to significantly enhanced Cr and Pb accumulation in maize shoots as well [43]. In addition, bacterially mediated scavenging of metals from insoluble mineral sources has been attributed to the production and secretion of such organic acids as gluconic, oxalic, tartaric, formic, and lactic acids [7, 9, 15, 39]. It has been suggested that the presence of sparingly soluble metal compounds in the growth medium might induce the production of carboxylic acid anions, leading to the enhanced dissolution of metal ions from the solid phase [39].

The pH of the SLP medium inoculated with the Pb-solubilizing bacterial strains decreased both in the presence and absence of Pb minerals (Table 4), suggesting the role of the bacterial strains in producing H^+ probably through organic acid secretion [7, 15]. Bacteria may also acidify their environment by the export of protons for the maintenance of charge balance [42]. The greatest drops in pH level (about 3 units) in the control SLP medium were induced by the strains YSP149 and YSP69. In the PbO-containing medium, the largest decrease in pH was measured in the presence of YSP24, whereas YSP17 and YSP66 gave rise to the largest pH decreases in the $PbCO_3$-containing medium. These results are in agreement with those reported by Ji *et al*. [34], who found that solubilization

of Cd, Pb, and Zn carbonates by *Serratia marcescens* M6 and *Rhodotorula mucilaginosa* K1 was accompanied by significant pH decreases in the medium. Long *et al*. [16] also showed that inoculation of several endophytic bacterial strains induced both a pH drop and Zn solubilization in culture media containing $ZnCO_3$ and $Zn_3(PO_4)_2$. However, they found that $Zn_3(PO_4)_2$ solubilization was 100 times greater, whereas the pH did not change, in a medium inoculated with *Pseudomonas fluorescens* (strain II2R3).

Molecular Identification of the Pb-Solubilizing Bacteria

Table 5 presents the isolated Pb-solubilizing strains identified on the basis of 16S rRNA gene sequences. The size of the PCR products generated with the selected bacterial isolates ranged from approximately 784 to 1,351 bp. Based on a sequence identity of 97% or greater, the closest matches of the isolates were determined using BLAST. The results show a preponderance of the *Bacillus* species (Table 5). This is also confirmed by other studies that revealed the presence or dominance of *Bacillus* species in mine soils or those contaminated with heavy metals [45–47].

Tolerance to Heavy Metals, NaCl, and pH

Among the 15 Pb-solubilizing isolates, 10 strains were selected for further experiments according to their best performance in Pb solubilization and PGPR traits. Mine soils most often contain more than one metal, and tolerance to several heavy metals is a desirable trait for bacteria with potential use in phytoremediation. All the isolates tested were found to exhibit multiple tolerance to high

Table 5. Identification of isolated Pb-solubilizing bacteria.

Isolates	Source soil	Length of 16S rRNA gene sequenced (bp)	GenBank Accession No.	Most closely related organism		
				Species (strain)	Accession No.	% Gene identity
YSP17	Nakhlak mine	1,173	KY887777	*Bacillus mojavensis* (strain ifo 15718)	NR_118290.1	97
YSP24	Nakhlak mine	1,346	KY887778	*Paenibacillus illinoisensis* (strain NBRC 15959)	NR_113828.1	99
YSP36	Nakhlak mine	1,065	KY887779	*Bacillus simplex* (strain RD36)	KJ_534463.1	97
YSP40	Nakhlak mine	784	KY887780	*Brevibacterium frigoritolerans* (strain DSM 8801)	NR_117474.1	99
YSP40n	Nakhlak mine	1,351	KY887781	*Staphylococcus pasteuri* (strain ATCC51129)	NR_024669.1	100
YSP69	Bama mine	1,313	KY887782	*Bacillus cereus* (strain CCM2010)	NR_115714.1	99
YSP104	Bama mine	1,158	KY887783	*Bacillus altitudinis*(strain 41KF2b)	NR_042337.1	100
YSP110	Bama mine	1,156	KY887784	*Bacillus filamentosus* (strain SGD-14)	NR_134701.1	99
YSP149	Bama mine	1,219	KY887785	*Bacillus licheniformis*(strain ATCC14580)	NR_074923.1	99
YSP151	Bama mine	1,012	KY887786	*Bacillus paralicheniformis* (strain kj-16)	NR_137421.1	99

Table 6. Maximum tolerance concentration of the Pb-solubilizing isolates against heavy metals and NaCl and ability of the isolates to grow in different pH ranges.

Isolate	Pb (mg/l)	Zn (mg/l)	Cu (mg/l)	Cd (mg/l)	NaCl (%)	pH range
YSP17	207.20	65.38	–	22.48	9	5.5–10.5
YSP24	207.20	65.38	–	11.24	5	5.5–10.5
YSP36	207.20	13.07	–	–	3	5.5–10.5
YSP40	207.20	65.38	6.35	–	3	5.5–10.5
YSP40n	207.20	163.45	–	11.24	8	5.5–9.5
YSP69	414.40	130.76	12.71	224.82	8	5.5–10.5
YSP104	414.40	196.14	6.35	112.41	8	5.5–10.5
YSP110	414.40	130.76	6.35	112.41	10	5.5–10.5
YSP149	207.20	65.38	6.35	112.41	10	5.5–10.5
YSP151	207.20	68.38	6.35	11.24	9	5.5–10.5

–, No bacterial growth observed.

concentrations of metals, especially to Pb and Zn (Table 6). The highest MTC values were detected for Pb (≥207.2 mg/l), indicating that the isolates were highly resistant to Pb. YSP69, YSP104, and YSP110 were the most tolerant to Pb, but also to Zn, Cu, and Cd. Sheng *et al*. [48] also reported that a *Bacillus* sp. strain, isolated from metal-contaminated soils, was not only resistant to Pb (200 mg/l) and Cd (20 mg/l), but also to Cu (50 mg/l), Ni (20 mg/l), and Zn (100 mg/l).

The Pb-solubilizing bacterial strains tested displayed a notable tolerance to salt stress while they were also able to grow well in a pH range of 5.5–10.5, except for YSP40n that did not grow at pH levels higher than 9.5 (Table 6). Bioaugmentation by salt-tolerant bacteria can both promote plant growth and help extract heavy metals in phytoremediation practices of salt-affected, metal-contaminated soils of arid regions [49]. The pH and salt tolerance of the Pb-solubilizing strains tested points to the feasibility of using them in contaminated soils also affected by moderately acid or alkaline pH levels and/or elevated salinity.

Effects of Pb-Solubilizing Strains on the Growth and Pb Uptake of *B. juncea*

Effects of *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151 strains and their consortium (YSP40+YSP151) on the growth parameters of *B. juncea* are presented in Table 7. Inoculation with YSP151 led to significant ($p < 0.05$) increases in plant shoot length and dry weight by 2.9 and 2.4 times, respectively, compared with those of the non-inoculated plant. The corresponding plant shoot growth parameters were also increased by 2.8 and 2.2 times, respectively, as a result of plant inoculation with YSP40 (Table 7). Effects of the bacterial consortium were significant on the shoot length (2.6-fold) but not on the shoot dry weight of the plant (Table 7). No significant differences were observed in root dry weight between Indian mustard plants grown in inoculated and non-inoculated soils (Table 7). Growth retardation of plants due to the presence of excess heavy metals in soil is a common feature observed in many plant species [10, 50, 51]. However, plant growth-promoting bacteria can facilitate plant growth in harsh environments like metal-contaminated soils by their activities, such as IAA, siderophore, and 1-amino-cyclopropane-1-carboxylate deaminase (ACCD) production, and phosphate solubilization [32, 37, 52]. Burd *et al*. [53] reported that the plant growth-promoting bacterium *Kluyvera ascorbata* SUD165 contributed to the resistance of *Brassica napus* against nickel toxicity. Sheng *et al*. [8] also reported that inoculation of soil with *Bacillus edaphicus* NBT having IAA, siderophore, and ACCD production capabilities resulted in increases of 22% and 30%, respectively, in root and shoot dry matter values of *B. juncea* grown in the presence of 800 mg-Pb/kg. Kumar *et al*. [50] inoculated *B. juncea* with *Enterobacter* sp. to observe enhanced plant growth parameters.

Table 7 reports Pb accumulation levels in the roots and shoots of *B. juncea* plants. Clearly, inoculation with YSP151 and YSP40+YSP150 significantly increased Pb concentrations in the shoots when compared with those of the non-inoculated plants. The root Pb concentration was not, however, significantly affected by the bacterial inoculation treatments (Table 7). The increased Pb absorption by the plants might have been due to improved Pb solubilization and pH reduction by bacterial activities in the soil.

The total Pb uptake by plant shoots was 3.2 and 4.6 times higher than that of the control when the plants were inoculated with *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151 strains, respectively (Table 7). The increased total Pb uptake by plant shoots might be ascribed to enhancements in both plant shoot biomass and Pb accumulation in the shoot as a result of bacterial inoculation. Sheng *et al*. [8] reported that the shoot Pb content of *B. juncea* inoculated with *Bacillus edaphicus* NBT increased by 46%, compared with the non–inoculated plants, in a soil polluted with 800 mg-Pb/kg. Inoculation

Table 7. Inoculation effects of *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151 strains and their consortium on the growth and Pb absorption of *Brassica juncea*.

Inoculated strain	Shoot length (cm)	Shoot dry weight (g/pot)	Root dry weight (g/pot)	Shoot Pb concentration (mg/kg)	Root Pb concentration (mg/kg)	Pb uptake by shoot (μg/pot)
Non (control)	12.30 ± 2.08 b	1.84 ± 0.79 c	0.16 ± 0.19 a	38.23 ± 2.9 c	96.93 ± 6 b	69.01 ± 24.73 c
YSP40	34.83 ± 7.09 a	3.99 ± 0.59 ab	0.39 ± 0.05 a	55.31 ± 10.1 b	79.55 ± 6.5 b	224.1 ± 67.36 b
YSP151	35.68 ± 2.17 a	4.38 ± 0.55 a	0.82 ± 0.82 a	73.6 ± 13.6 a	141.7 ± 35.2 a	319.7 ± 48.62 a
YSP40+YSP151	32.30 ± 1.88 a	2.89 ± 0.36 bc	0.38 ± 0.06 a	67.16 ± 5.3 ab	80.94 ± 5.7 b	193.1 ± 14.82 b

Values are the mean ± standard deviation. Means in each column followed by the same letter are not significantly different according to LSD ($p < 0.05$).

with the YSP40+YSP110 consortium also significantly increased the Pb content of Indian mustard compared with that of the un-inoculated control; however, its beneficial effect was generally lower compared with that of YSP151 single inoculation (Table 7). Abou-Shanab *et al*. [42] also showed that a mixture of *B. subtilis*, *B. pumilus*, *Pseudomonas pseudoalcaligenes*, and *Brevibacterium halotolerans* increased the Cu concentration in shoots of *Zea mays* growing on Cu-rich soils, but this increase was less pronounced than that obtained after inoculation of *Br. halotolerans* alone.

The present study highlighted the role of bacterial strains isolated from mine soils in increasing soil metal availability and uptake by plants, for remediating metal-contaminated sites more efficiently. Since the potential of a plant for use as a phytoremediator in a metal-contaminated soil relies on the total metal quantity taken up by the harvestable part of the plant biomass, inoculation of the metal-solubilizing and plant growth-promoting strains, such as *Brevibacterium frigoritolerans* YSP40 and *Bacillus paralicheniformis* YSP151, could be recommended as a good way of increasing the phytoremediation rate and efficiency of Pb-polluted soils.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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