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Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth

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ABSTRACT

Naturally occurring bacteria play an important role in bioremediation of heavy metal pollutants in soil and wastewater. This study identified high levels of resistance to zinc, cesium, lead, arsenate and mercury in eight copper resistant *Pseudomonas* strains previously isolated from Torch Lake sediment. These strains showed variable susceptibility to different antibiotics. Furthermore, these metal resistant strains were capable of bioaccumulation of multiple metals and solubilization of copper. Bacterial strains TLC 3-3.5-1 and TLC 6-6.5-1 showed high bioaccumulation ability of Zn (up to 15.9 mg/g dry cell) and Pb (80.7 mg/g dry cell), respectively. All the strains produced plant growth promoting indole-3-acetic acid (IAA), iron chelating siderophore and solubilized mineral phosphate and metals. The effect of bacterial inoculation on plant growth and copper uptake by maize (*Zea mays*) and sunflower (*Helianthus annuus*) was investigated using one of the isolates (*Pseudomonas* sp. TLC 6-6.5-4) with higher IAA production and phosphate and metal soubilization, which resulted in a significant increase in copper accumulation in maize and sunflower, and an increase in the total biomass of maize. The multiple metal-resistant bacterial isolates characterized in our study have potential applications for remediation of metal contaminated soils in combination with plants and metal contaminated water.

1. Introduction

Worldwide contamination of soils and water with heavy metals pose a serious threat to both the ecosystem and human health. Removal of metals from contaminated soil, wastewater and industrial waste is particularly challenging because heavy metals are non-biodegradable [1]. Conventional physicochemical techniques for metal remediation such as filtration, acid leaching, electrochemical processes or ion exchange are expensive and may not be very effective [2]. Bioremediation based on microorganisms, plants or other biological systems offers a cost-effective and environment-friendly method for metal clean-up [2–5].

The most common bioremediation method for soil remediation is phytoremediation, which utilizes plants to remove toxic metals from soils. Currently, various plant species such as alpine pennycress (*Thlaspi caerulescens*), sunflower (*Helianthus annuus*), Indian mustard (*Brassica juncea*), and willow (*Salix* spp.), are being used to absorb and accumulate heavy metals [4–6]. However, the metal accumulation efficiency of plants is limited because of the low bioavailability of metals in soils that exist in insoluble-bound forms in various soil components. Application of synthetic chemical chelators such as EDTA to enhance the efficiency of phytoremediation has potential environmental risks [7].

Recently, an alternative strategy has been developed that provides plants with certain beneficial soil microorganisms [8]. This remediation process known as rhizoremediation utilizes plant-microbe interactions to improve the efficiency of phytoremediation. The inoculation of genetically modified, cadmium-resistant bacteria (*Pseudomonas putida*) enhanced 40% more cadmium accumulation in sunflower plants (*H. annuus*) than the control group [9]. Additionally, a few naturally occurring microorganisms with high metal resistance in plant rhizosphere significantly increased metal uptake in plants and reduced metal toxicity [10–13]. Some metal-resistant microorganisms, such as plant-growth promoting bacteria (PGPB), are able to produce plant hormones and siderophores in addition to solubilizing phosphate, which reduces metal stress and promotes plant growth resulting in improved total metal uptake by plants [14–19]. Siderophores are small molecular weight, high-affinity Fe(III)-complexing compounds that increase availability of soluble iron in soil for uptake by plants [20]. However, all these characteristics are not shown by a single PGPB strain. Furthermore, some microbes have been reported to solubilize metals, which enhance metal bioavailability [21,22]. Most of this research was performed using one specific plant [14,16,23,24]. There is lit-

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tle information on whether microbe-assisted phytoremediation is strain and plant species specific.

Metal contaminated water clean-up using bacteria with high metal resistance is regarded as a cheap and effective method [25]. Various bacterial strains of *Bacillus* and *Pseudomonas* (living and dead biomass) have been successfully used as metal adsorbing agents because of their high metal binding capability [26–28]. It was reported that high metal-binding capability of bacteria is due to the charge of cell wall, high surface-volume ratio, S-layer protein, and metal-binding proteins [29,30].

In our previous work, we isolated eight copper-resistant bacteria from Torch Lake sediments which were contaminated by copper mine tailings [31]. All these strains belong to various *Pseudomonas* species based on sequencing of DNA gyrase B subunit (*gyrB*) and sigma 70 factor (*rpoD*) genes and biochemical tests. In the present study, we screened copper-resistant bacterial isolates for resistance to multiple metals, elucidated the effect of these bacteria on plant growth and copper uptake by maize (*Zea mays*) and sunflower (*H. annuus*), and evaluated the potential use of these bacterial isolates for bioaccumulation of heavy metals in an aqueous solution.

2. Materials and methods

2.1. Screen of multiple heavy metal resistance

Eight copper-resistant bacterial strains isolated from Torch Lake sediment in our previous work were identified as *Pseudomonas* species based on sequencing of *gyrB* and *rpoD* genes [31]. GenBank accession numbers of these sequences are FJ212441–FJ212449 and FJ151403–FJ151409. These bacterial strains were further tested for their resistance to zinc, lead, cesium, arsenic, and mercury. The minimal inhibitory concentration (MIC) was determined on Basal Salt Medium (BSM) supplemented with a range of concentrations of Zn^{2+}, Pb^{2+}, Cs^{+}, AsO_4^{3-} and Hg^{2+}, separately. Glycerol (1%, v/v) was used as the carbon source in BSM medium and the pH of the medium was adjusted to 7. The plates with bacteria were incubated for 3 days at 30 °C.

2.2. Sensitivity to antibiotics

The antibiotic sensitivity of the bacterial isolates was determined by disk diffusion method using antibiotic susceptibility test discs (BBL, MD, USA). The bacterial strains were grown overnight in LB liquid medium and spread on Mueller Hinton agar using sterile swabs. Antibiotic discs were placed on the agar plates and incubated at 30 °C. The diameter of the inhibition zones was measured after 24 h. The bacterium was classified as resistant (R), intermediate (I) or susceptible (S), according to the information supplied by the manufacturer (BBL, MD, USA). The following antibiotic discs were used: ampicillin (10 μg), amikacin (20 μg), carbenicillin (100 μg), chloramphenicol (30 μg), kanamycin (30 μg), neomycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg) and trimethoprim (5 μg).

2.3. IAA-producing capability and mineral phosphate solubilization activity

IAA production by bacterial isolates was quantitatively assayed by the Gordon and Weber method [32]. The bacteria were grown in LB broth with L-tryptophan (0.5 mg/L) for 96 h in the presence and absence (control) of heavy metals (Cu, Zn, Pb, As, Hg). After centrifugation (6000 rpm, 10 min), the supernatant was mixed with Salkowski's reagent and the absorbance was measured at 530 nm. The phosphate solubilization ability of the bacterial isolates was screened using NBRIP broth [33] with $Ca_3(PO_4)_2$ (5 g/L) as the only P source and in the presence or absence of metals (Cu, Zn, Pb, As, Hg). The solubilized phosphate in the culture was quantified by ammonium molybdate spectrophotometric method as described by Dhar et al. [34]. All tests were done in triplicate.

2.4. Production of siderophores

Overnight cultures of bacterial isolates grown in LB broth were centrifuged and the pellet was redissolved in sterile water. 100 μl of cell suspension was cultured in 30 ml of Basal Salt Medium without ferric ions (BSM-Fe) in the presence or absence of heavy metals (Cu, Zn, Pb, As, and Hg). After 72 h of incubation, the bacterial culture was harvested by centrifugation at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 400 nm to detect siderophore production in iron-limiting conditions [35]. BSM medium with $FeCl_3$ was used as the control. These experiments were performed in triplicate.

2.5. Metal solubilization by the bacterial isolates

Bacterial isolates grown in LB broth overnight were spotted in 10 μl volume onto Bushnell Haas (BH) agar medium containing $CuCO_3$ (5 g/L) as the sole copper source and 10 g/ml of glucose as the carbon source. The solubilization halo was observed after plate incubation at 30 °C for 3 days. For broth assay, bacterial stains were cultured in 20 ml BH medium supplemented with 5 g/L $CuCO_3$ and 10 g/ml of glucose in a controlled environment shaker at 30 °C. After 3 days, the culture broth was centrifuged at 8000 rpm for 10 min. The supernatant was passed through 0.45 μm filter and the concentration of soluble Cu in the supernatant was determined by AAS in flame mode (air-acetylene) (AAS-3100, PerkinElmer, USA). The pH of the broth was also recorded. Three triplicates were used for each experiment.

2.6. Bioaccumulation of heavy metals by bacterial isolates

For bioaccumulation studies, 0.5 ml of overnight-grown bacterial culture was inoculated into 50 ml each of LB with 3 mM copper, zinc, lead, 300 mM arsenate, and 0.45 mM of mercury. Three replicates were made. These cultures were incubated for 48 h at 30 °C. Cells were harvested by centrifugation and the cell pellets were washed three times with deioned water and dried at 60 °C. The bacterial pellet was digested with nitric acid for metal analysis. The concentration of copper, zinc and lead in the pellet was measured as described earlier. The arsenic concentration was measured by AAS in graphite furnace mode with HGA 600 autosampler (AS-72, PerkinElmer, USA). Mercury concentration in the pellet was measured by Thio-Michler's Ketone spectrophotometric method as described by Niazi et al. [36]. Bacterial isolates grown without metals were used as blank for spectrophotometric analysis.

2.7. Effect of bacterial strains on copper uptake by maize and sunflower

Copper contaminated soil was prepared by autoclaving soil and mixing with aqueous solution of $CuCl_2$ to achieve the final concentration of 500 mg/kg soil. Soil was left in greenhouse for 2 weeks and mixed occasionally so that copper was evenly dispersed in the soil. The *Z. mays* (maize) and *H. annuus* (sunflower) seeds were germinated in non-contaminated soil for 15 days. Seedlings were then divided into four groups: (1) plants + non-contaminated soil (2) plants + copper contaminated soil (3) plants + non-contaminated soil + bacteria and (4) plants + copper contaminated soil + bacteria.

Table 1
Minimum inhibitory concentration (MIC) of different metals for bacterial isolates.

Sample ID	MIC of heavy metals (mM)					
	Copper	Zinc	Cesium	Lead	Arsenate	Mercury
TLC3-3.5-1	4	6	10	5	400	0.4
TLC3-3.5-2	5	6	10	5	400	0.4
TLC3-3.5-3	5	6	10	5	400	0.2
TLC3-3.5-4	5	6	10	5	400	0.2
TLC6-6.5-1	4	6	11	5	400	0.3
TLC6-6.5-2	4	6	10	5	400	0.3
TLC6-6.5-3	5	6	10	5	400	0.3
TLC6-6.5-4	5	6	10	5	400	0.3

Table 3
IAA production and phosphate solubilization by bacterial isolates without metal stress.

Sample ID	IAA production (μg/ml)	Soluble phosphate (μg/ml)
TLC 3-3.5-1	5.78 ± 0.25bc	116.61 ± 2.90b
TLC 3-3.5-2	5.65 ± 0.78cd	60.61 ± 4.53f
TLC 3-3.5-3	1.42 ± 0.11g	106.17 ± 6.12c
TLC 3-3.5-4	1.82 ± 0.17f	73.34 ± 3.01e
TLC 6-6.5-1	2.27 ± 0.11e	102.42 ± 1.29c
TLC 6-6.5-2	7.16 ± 0.36a	79.15 ± 3.71d
TLC 6-6.5-3	6.03 ± 0.28b	126.32 ± 2.50a
TLC 6-6.5-4	5.35 ± 0.15d	129.19 ± 5.17a

All the values are mean of three replicates ± standard deviation (SD). Values in rows in each column indexed by different letters are significantly different ($p < 0.05$) according to Tukey's test.

For inoculation, 50 ml of bacterial culture (TLC 6-6.5-4) grown for 16 h in LB medium with 3 mM copper was centrifuged at 6000 rpm for 10 min. The bacterial pellet was washed twice with sterile water, recentrifuged, and resuspended in 0.85% NaCl solution. The bacterial suspension in 0.85% NaCl solution was adjusted to an absorbance of 0.5 at 600 nm (equivalent to approximately 7.5×10^8 cfu ml^{-1}). The 15-day-old plant roots were soaked in the bacterial culture 2 h for bacterial colonization and transferred to pots with 200 g of copper contaminated soil in each pot. The bacterial culture was also added into rhizosphere during transplantation. Pots were sealed to prevent any metal leaching. After 30 days, plants were harvested. For determination of bacterial root and soil colonization, plant roots with some attached soil were soaked in PBS (pH 7) and left on shaker for 1 h to recover the bacteria. The number of bacteria surviving in plant root rhizosphere in LB agar with 3 mM of copper and 20 μg/ml of streptomycin was calculated by plate dilution technique [37]. Plants were washed with deioned water and dried at 60 °C. Plant roots and shoots were separated and biomass (dry weight) was recorded. Plants were digested with nitric acid and copper concentration in plants was measured using AAS in flame mode (air-acetylene) (AAS-3100, PerkinElmer, USA) according to the protocol of Andra et al. [38]. Five replicates were used for each test.

2.8. Statistical analysis

Values of the mean and standard deviations (mean ± standard deviation) were calculated and *t*-test was used to identify significant differences ($p < 0.05$) between treated and control groups. For multiple comparisons, one-way analysis of variance (ANOVA) was performed followed by Tukey's test using Minitab 15. The significance analyses of different treatments were conducted with a 5% least significant difference test ($p < 0.05$).

3. Results

3.1. Identification of multiple heavy metal resistant bacterial strains

Bacteria growing in 1 mM copper, zinc and lead, 125 μM mercury, and 25 mM arsenate are considered to be metal resistant [39–41]. The minimal inhibitory concentration (MIC) of six heavy-metal ions for bacterial isolates is listed in Table 1. All the eight copper-resistant bacterial strains isolated from Torch Lake sediment showed high resistance to zinc, lead, cesium, arsenic, and mercury. These bacterial isolates have very similar MIC values for these heavy metals. The order of toxicity of heavy metals on the bacterial isolates was Hg > Pb = Cu > Zn > Cs > AsO_4. For subsequent experiments, a working concentration below MIC was chosen for each metal. These concentrations challenged the bacteria but did not inhibit their growth.

3.2. Antibiotic susceptibility of multiple metal resistant bacteria

To further characterize the bacterial isolates, susceptibility to different antibiotics was tested. The results indicate that these bacterial isolates were either resistant or intermediate resistant to carbenicillin, penicillin, tetracycline and trimethoprim but susceptible to amikacin and kanamycin (Table 2). In addition, all the strains were sensitive to neomycin except TLC 3-3.5-4 which showed intermediate resistance. The resistance and sensitivity to three other antibiotics – chloramphenicol, streptomycin, and tobramycin – varied among the eight bacterial isolates. TLC 6-6.5-2 was the only isolate susceptible to chloramphenicol. Bacterial isolates TLC 3-3.5-2, TLC 6-6.5-1 and TLC 6-6.5-3 were susceptible to streptomycin whereas TLC 3-3.5-3 and TLC 6-6.5-4 showed intermediate resistance and resistance, respectively.

Table 2
Antibiotic susceptibility of bacterial isolates.

Antibiotics (μg/disc)	Diameter of inhibition zone (mm)							
	TLC 3-3.5-1	3-3.5-2	3-3.5-3	3-3.5-4	6-6.5-1	6-6.5-2	6-6.5-3	6-6.5-4
Carbenicillin (100)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)
Chloramphenicol (30)	8(R)	12(R)	9(R)	14(1)	11(R)	20(S)	7(R)	7(R)
Ampicillin (10)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)
Tetracycline (30)	15(1)	16(1)	11(R)	11(R)	15(1)	10(R)	16(1)	15(1)
Trimethoprim (5)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)	7(R)
Amikacin (20)	19(S)	20(S)	20(S)	22(S)	21(S)	20(S)	20(S)	23(S)
Kanamycin (30)	22(S)	20(S)	23(S)	22(S)	21(S)	23(S)	22(S)	26(S)
Neomycin (30)	19(S)	19(S)	13(1)	17(S)	24(S)	19(S)	20(S)	19(S)
Streptomycin (10)	13(1)	19(S)	10(R)	11(R)	20(S)	13(1)	18(S)	9(R)
Tobramycin (10)	20(S)	20(S)	17(1)	30(S)	21(S)	20(S)	20(S)	12(R)

S: means susceptible; R means resistance; I means intermediate resistance.

Table 4
Accumulation of heavy metals in eight bacterial isolates.

Sample ID	Accumulated metals in bacterial cells (μg/g dry bacterial pellet)				
	Cu	Zn	Pb	As	Hg
TLC 3-3.5-1	556 ± 23c	15877 ± 1526a	44709 ± 1994c	12.55 ± 0.6b	0.39 ± 0.22a
TLC 3-3.5-2	678 ± 23b	3598 ± 764b	26741 ± 3666c	11.81 ± 0.7b	0.08 ± 0.03d
TLC 3-3.5-3	120 ± 6d	4465 ± 683c	188 ± 210d	16.21 ± 0.8a	0.40 ± 0.024a
TLC 3-3.5-4	718 ± 9b	3577 ± 392b	43957 ± 6673bc	15.47 ± 2.4a	0.19 ± 0.05cd
TLC 6-6.5-1	158 ± 20d	5874 ± 1084b	80666 ± 7856a	14.94 ± 0.3a	0.25 ± 0.02bc
TLC 6-6.5-2	509 ± 47c	2971 ± 319c	30618 ± 925c	14.79 ± 0.2a	0.14 ± 0.03d
TLC 6-6.5-3	543 ± 26c	2963 ± 155c	35825 ± 1389c	9.21 ± 0.2c	0.31 ± 0.16a
TLC 6-6.5-4	838 ± 60a	2477 ± 208c	75075 ± 8824ab	6.18 ± 0.1d	0.34 ± 0.21ab

All the values are mean of three replicates ± SD. Values in rows in each column indexed by different letters are significantly different ($p < 0.05$) according to Tukey's test.

3.3. Indole-3-acetic acid (IAA) production and phosphate solubilization

The multiple heavy metal resistant bacterial isolates were screened for IAA-production and phosphate solubilization. The results showed that all the strains were able to utilize L-tryptophan as precursor to produce IAA (Table 3). Strain TLC 6-6.5-2 produced the highest (7.16 μg/ml) whereas TLC 3-3.5-3 produced the lowest (1.42 μg/ml) IAA among the eight isolates tested. In addition to IAA production, all eight strains demonstrated the potential for phosphate solubilization (Table 3). The maximum phosphate solubilization was achieved by the strain TLC 6-6.5-4 (129 μg/ml). Furthermore, the effect of heavy metals on IAA production and phosphate solubilization by the bacterial isolates was investigated using TLC 6-6.5-4 (Fig. 1). Results from these experiments indicate that the presence of 3 mM copper, 3 mM zinc, and 100 mM arsenate did not suppress the production of IAA ($p > 0.05$) (Fig. 1a). However, IAA production was suppressed by lead and mercury ($p < 0.05$). Phosphate solubilization ability was not inhibited by lead (Fig. 1b). Copper, zinc, and mercury in the medium reduced phosphate solubilization by 43%, 15%, and 69%, respectively, compared to the control. Only arsenate increased phosphate solubilization by 108%.

3.4. Production of siderophores

The production of low molecular weight, iron-chelating siderophores by the eight multiple-metal resistant bacterial strains was detected by the absorbance at 400 nm, as described in materials and methods. 2.2-fold variation in siderophore production was observed among the eight isolates with TLC 3-3.5-1 and TLC 3-3.5-4 showing highest and lowest siderophore levels released in the supernatant (Fig. 2a). In addition, 3 (TLC 3-3.5-4) to 9 (TLC 6-6.5-3) fold difference in absorbance at 400 nm was observed between bacterial cultures grown in the absence versus presence of iron. Furthermore, the effect of heavy metals on siderophore production by the bacterial isolates was investigated using TLC 6-6.5-4 (Fig. 2b). Results from these experiments indicate that the presence of 3 mM copper or 100 mM arsenate in the medium improved the production of siderophore by 114% and 84%, respectively, compared to the control ($p < 0.05$). However, siderophore production was suppressed by zinc and lead, while 0.2 mM mercury did not show a significant change in siderophore production.

3.5. Bioaccumulation of heavy metals by bacterial isolates

Most of the bacterial isolates exhibited high bioaccumulation of copper, zinc, and lead, while the accumulation of arsenate and mercury was relatively low (Table 4). The maximum copper accumulation observed was 838 μg/g in the dry pellet of TLC 6-6.5-4, while TLC 6-6.5-1 was lead hyperaccumulator, which accumulated 80666 μg/g of dry bacterial pellet. The bacterial strain TLC 3-3.5-1 showed the highest amount of zinc uptake (15877 μg/g dry pellet). Interestingly, TLC 3-3.5-3 was the lowest copper and lead accumulator but accumulated the highest arsenate and mercury among the eight isolates tested. These results indicate that bacterial isolates TLC 6-6.5-4, 6-6.5-1, and 3-3.5-1 are strong candidates for bioremediation of copper, lead, and zinc, respectively.

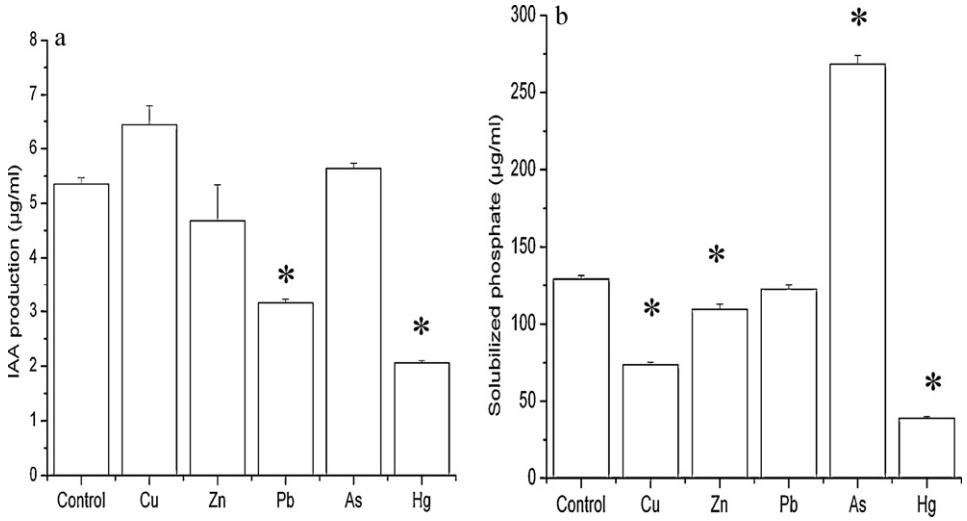

Fig. 1. (a) IAA production and (b) phosphate solubilization by TLC 6-6.5-4 in the presence of heavy metals. Control: absence of heavy metals. The concentration of copper, zinc and lead was 3 mM in the broth, arsenate was 100 mM and mercury was 0.2 mM. Error bars represent standard deviation of the mean ($n = 3$). Values differing significantly from the control ($p < 0.05$) using t-test are labeled with asterisks (*).

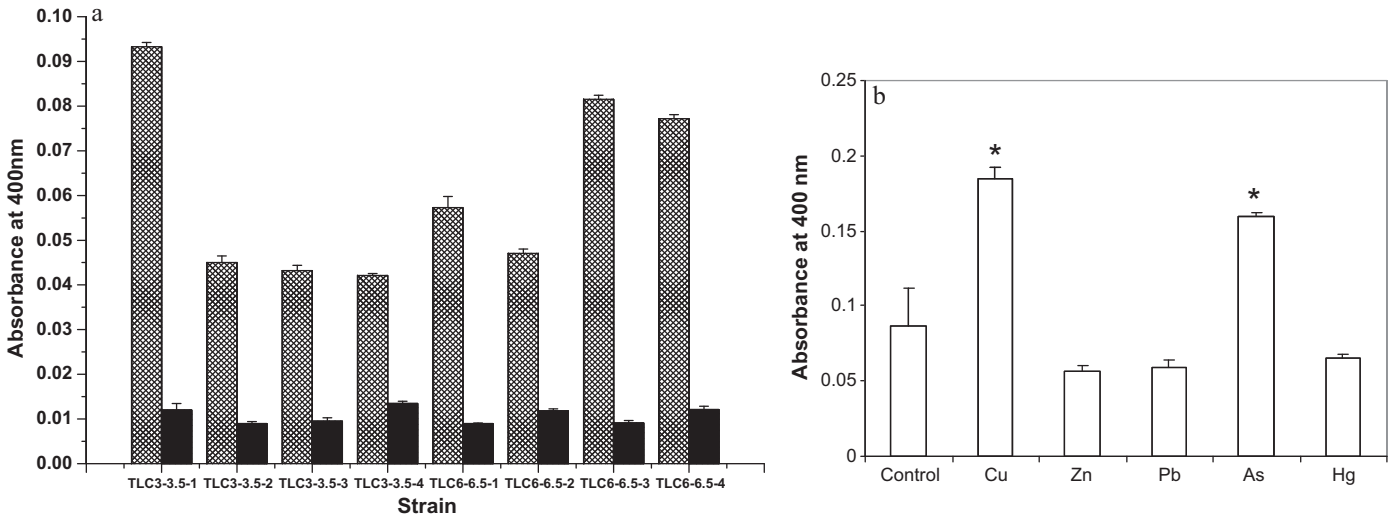

Fig. 2. (a) Siderophore production by the bacterial isolates. Hatched and solid bars represent cultures grown in the absence and presence of $FeCl_3$, respectively. (b) Siderophore production by TLC 6-6.5-4 in the presence of heavy metals without $FeCl_3$. Control: absence of heavy metals. The concentration of copper, zinc and lead was 3 mM, arsenate was 100 mM and mercury was 0.2 mM. Error bars are mean ± SD ($n = 3$). Values differing significantly from the control ($p < 0.05$) using t-test are labeled with asterisks (*).

3.6. Solubilization of copper

The ability of the bacterial isolates to solubilize copper was first tested in BH agar medium supplemented with $CuCO_3$. The solubilization halo was observed after 3 days (Fig. 3). The metal solubilization potential was further measured in a broth assay. The ability to solubilize copper ranged from 9.9 to 41.8 μg/ml with concomitant change in pH ranging from 4.9 to 6.1 for different isolates (Table 5). TLC 6-6.5-4 showed the highest ability for solubilizing copper. For this isolate, the soluble copper concentration in the medium was 41.8 μg/ml on the 3rd day after bacterial inoculation, accompanied by a decrease in pH from 7 to 4.9.

3.7. Effect of bacterial inoculation on maize and sunflower biomass and copper uptake

TLC 6-6.5-4 was chosen for testing its rhizoremediation potential because of its relatively higher IAA production and mineral phosphate and copper solubilization ability among the eight multiple metal-resistant strains. TLC 6-6.5-4 significantly improved the accumulation of copper in both maize and sunflower plants by 2.8-fold and 1.7-fold, respectively ($p < 0.05$) (Fig. 4). Copper uptake in copper-contaminated soil-with or without bacterial inoculation by sunflower-was higher compared to maize. Presence of copper in the soil reduced the dry weight of maize roots by 58% and shoots by 65%, while the total biomass of maize plant was reduced by 63% (Table 6). Inoculation with TLC 6-6.5-4 reduced the copper toxicity and significantly increased the root biomass and plant height of maize compared to the uninoculated control (Fig. 5 and Table 6). However, bacterial inoculation, in presence of copper in the soil, resulted in an increase in sunflower root biomass, while no significant changes in shoot biomass and plant height were observed. The presence of the inoculated strain in the rhizosphere was determined using its properties of metal (copper) and antibiotic (streptomycin) resistance as described in materials and methods. The population of TLC 6-6.5-4 was 6.72×10^7 CFU/g in maize rhizosphere and 4.91×10^6 CFU/g in sunflower rhizosphere under copper stress, which reflects better growth characteristics in maize compared to sunflower.

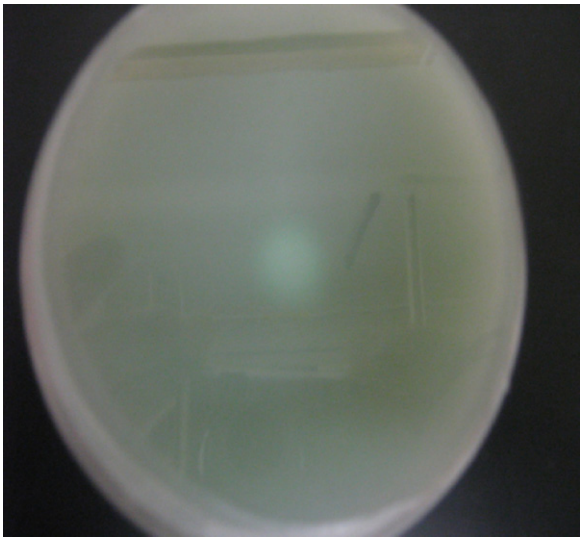

Fig. 3. Solubilization zone produced by TLC 6-6.5-4 in BH medium with $CuCO_3$.

4. Discussion

For the development of an effective bioremediation system using microbes or plant-microbe interactions, one of the important steps is to identify microorganisms which can survive in high levels of heavy metal-contaminated sites. Multiple metal-resistant microorganisms are promising candidates for rhizoremediation because many sites are co-contaminated with a myriad of heavy metals. Recently, a few gram-negative and gram-positive bacte-

Table 5
Copper solubilization by different bacterial isolates.

Sample ID	Soluble copper (μg/ml)	Final pH
TLC3-3.5-1	16.1 ± lcd	5.2
TLC3-3.5-2	19.6 ± 6.8c	6.1
TLC3-3.5-3	31 ± 3.3b	5.5
TLC3-3.5-4	26.5 ± 3.7b	5.2
TLC6-6.5-1	14.6 ± 2.8cd	5.6
TLC6-6.5-2	19.7 ± 1.8c	5.1
TLC6-6.5-3	9.9 ± 0.1d	5.7
TLC6-6.5-4	41.8 ± 0.7a	4.9

The initial pH of the medium was 7. All the values are mean of three replicates ± SD. Soluble copper values indexed by different letters are significantly different ($p < 0.05$) according to Tukey's test.

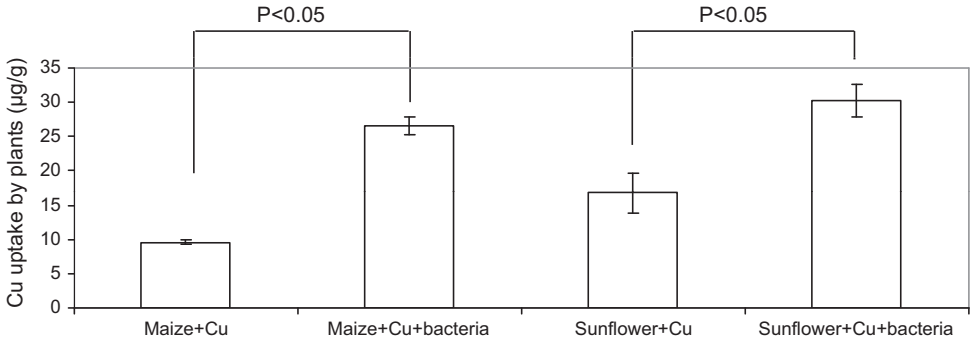

Fig. 4. Copper uptake by maize and sunflower with or without bacterial inoculation. Error bars represent standard deviation of the mean of five replicates ($n = 5$). The differences in copper uptake by maize and sunflower with or without TLC 6-6.5-4 were significant ($p < 0.05$) according to student's *t*-test.

Table 6
Dry weights of maize and sunflower with or without inoculation of the bacterial isolate TLC 6-6.5-4.

Treatment	Dry weight (g)/plant					
	Maize			Sunflower		
	Roots	Shoots	Total	Roots	Shoots	Total
Untreated control	0.31 ± 0.017b	0.85 ± 0.031a	1.16 ± 0.019b	0.19 ± 0.023b	1.54 ± 0.059a	1.73 ± 0.054a
$CuCl_2$ (500 mg/kg)	0.13 ± 0.028c	0.3 ± 0.046b	0.43 ± 0.027c	0.15 ± 0.02b	1.48 ± 0.154a	1.64 ± 0.148a
Bacterial isolate	0.37 ± 0.023a	l ± 0.094a	1.37 ± 0.115a	0.18 ± 0.02b	1.69 ± 0.142a	1.86 ± 0.122a
Bacterial isolate + $CuCl_2$	0.29 ± 0.033b	0.89 ± 0.154a	1.18 ± 0.132b	0.24 ± 0.013a	1.56 ± 0.243a	1.8 ± 0.258a

Data is mean ± SD of five replicates ($n = 5$). Data of rows indexed by different letters in each column are significantly different ($p < 0.05$) according to Tukey's test.

ria with multiple metal resistance have been identified [18,42,43]. However, our work here is the first report on naturally occurring bacterial isolates (*Pseudomonas* sp.) with high resistance to six common heavy metal pollutants-copper, cesium, zinc, lead, arsenic, and mercury. High bacterial metal resistance is an important factor to be considered for remediation of heavy metals because it is directly related to the survival and growth of bacteria in metal contaminated soil or water. In comparison to other reported metal-resistant strains, our bacterial isolates exhibited relatively higher metal resistance [18,43,44].

The presence of high levels of heavy metals appears to co-select antibiotic resistance along with metal tolerance in bacteria [45,46]. It is likely that both heavy metal and antibiotic resistance in bacteria isolated from metal contaminated sites are mediated by efflux pumps in their membranes [47]. The biochemical and molecular mechanism used by the bacterial isolates described in this study, for multiple metal and antibiotic resistance needs to be investigated.

Effective rhizoremediation depends on the specific plant species, the bioavailability of heavy metals in soil, and the interaction between plants and metal resistant microorganisms in plant root rhizosphere [11]. In this study, crop species *Z. mays* and *H. annuus* were used to demonstrate rhizoremediation because these plants have commercially available seeds, good cultivation techniques, fast growth rate as well as high biomass. Recently, Vamerali et al. [48] reviewed the application of field crops for phytoremediation in the last fourteen years and ranked *H. annuus* and *Z. mays*

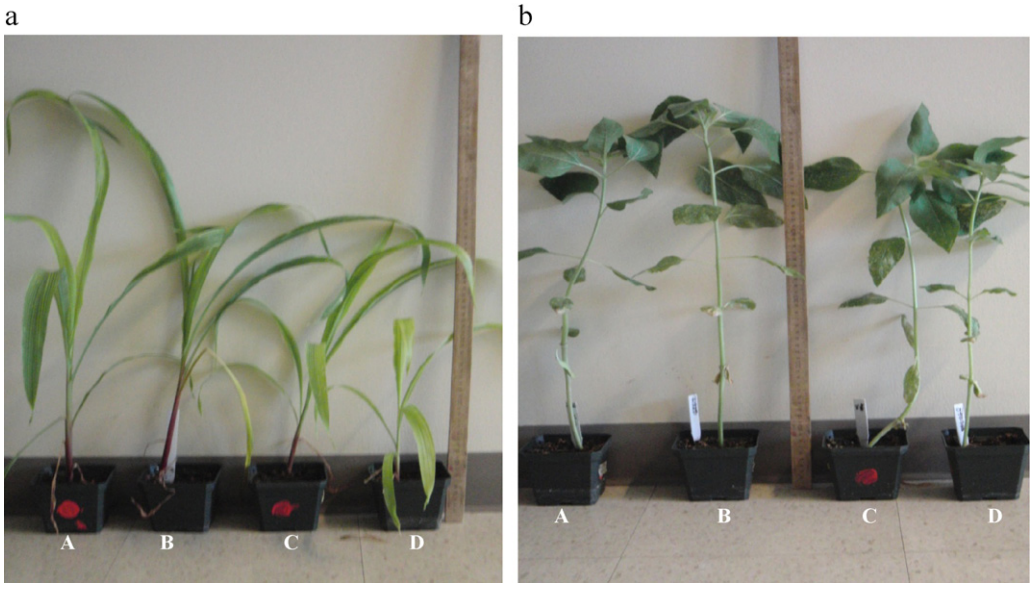

Fig. 5. Effect of TLC 6-6.5-4 on the growth of (a) maize and (b) sunflower: (A) control (no bacteria and copper), (B) soil + bacteria, (C) soil + bacteria + copper, and (D) soil + copper.

Table 7
Bioaccumulation and biosorption of zinc and lead by different bacteria.

Metal	Bacterial strain	Metal uptake (mg/g dry cell weight)	Living or dead biomass	Reference
Zn	*Pseudomonas putida* CZ21	18.1	Living	[64]
	TLC3-3.5-1	15.9	Living	This study
	Bacillus weihenstephanensis	3.38	Living	[24]
	Bacillus jeotgali	128	Dead	[65]
	Geobacillus thermodenitrificans	48.26	Dead	[27]
Pb				
	P. aeruginosa PU21	110	Living	[66]
	TLC 6-6.5-1	80.7	Living	This study
	P. aeruginosa	45	Living	[67]
	Corynebacterium glutamicum	567.7	Dead	[68]
	P. stutzeri	153.3	Dead	[69]
	Geobacillus thermodenitrificans	32.26	Dead	[29]

as the second and third most frequently used crop species. Maize possesses some degree of copper tolerance and has been used for phytoremediation of copper contaminated soil by other researchers [49,50]. Sunflower is able to accumulate substantial amount of heavy metals such as copper, cadmium, chromium, and nickel in a short time [51,52].

In the present study, we have characterized eight bacterial isolates with high resistance to multiple heavy metals. Based on the relatively higher IAA production, phosphate solubilization, and copper solubilization, bacterial strain TLC 6-6.5-4 was selected for rhizoremediation experiments using copper-contaminated soil. Rhizosphere colonization is one of the first steps in plant-microbe interaction. *Pseudomonas* species are common root colonizing bacteria [53]. In our study, *Pseudomonas* sp. TLC 6-6.5-4 survived in maize and sunflower rhizosphere under copper stress. However, our study does not provide conclusive evidence for colonization of the bacteria with plant roots.

Heavy metal-bacteria interaction in plant rhizosphere is an important factor for rhizoremediation [54]. Plant-growth-promoting traits of the bacteria may be affected in presence of heavy metals. Our results showed that both IAA production and phosphate solubilization of TLC 6-6.5-4 was inhibited by mercury. Another study found that mercury has higher inhibitory effect on IAA production by *Rhizobium* compared to lead, cadmium, and barium which might be related to mercury toxicity resulting in reduced cell growth [55]. Furthermore, phosphate solubilization by *Bacillus* sp. decreased with an increase in chromium concentration in the medium [56]. The repression of phosphate solubilization in the presence of zinc might be due to increased toxicity as a result of interaction between zinc and inorganic phosphate [57]. In our experiment, zinc and lead suppressed the production of siderophores by TLC 6-6.5-4. Similar findings were reported in case of two plant-growth-promoting rhizobacterial strains under nickel stress [58]. However, the exact role of zinc and lead in siderophore production is not clear. Some researchers proposed that lead affected bacterial iron metabolism when it was chelated by siderophore and transferred into the cell through siderophore-mediated ion uptake [59].

Plant hormone synthesis and phosphate solubilization by bacteria promote plant growth and therefore increase the total metal uptake [60]. Application of TLC 6-6.5-4 stimulated maize growth compared to no significant increase in sunflower biomass. Similar results were reported by Kuffner et al. [61] where IAA producing bacterial strains did not affect willow growth. Grandlic et al. [23] showed that the effect of PGPB to influence plant growth in combination with compost was different in quailbush and buffalo grass. All these results suggest that plant growth promoted by some bacterial strains are plant specific.

Inoculation of TLC 6-6.5-4 significantly enhanced copper accumulation in maize and sunflower. In addition, we found that TLC 6-6.5-4 can increase copper uptake by the lead hyperaccumulator plant, Vetiver grass (*Chrysopogon zizanioides*) (Data not shown). This effect may be associated with copper solubilization by TLC 6-6.5-4, which improved the bioavailability of copper in plant rhizosphere. The decrease in pH suggested the production of organic acids by the bacterial isolates which act as chelators for heavy metals [62]. Solubilization of metals by bacteria would be a more efficient and environment-friendly approach than the application of synthetic chelators such as EDTA with slow degradation rate and long persistence in soil.

The bioaccumulation of copper, zinc, lead, arsenate, and mercury by the bacterial isolates were characterized to evaluate their applicability for heavy metal removal from industrial wastewaters. Differences in metal uptake rates suggest different metal transport systems and detoxification mechanisms [63]. The bioaccumulation values for lead and zinc by bacterial isolates TLC 6-6.5-1 and 3-3.5-1 are comparable to the highest amount of lead and zinc accumulation reported in living biomass of bacteria to date, but was lower than the biosorption by certain microbial dead biomass (Table 7). However, biosorption by dead biomass of bacteria is a passive process, mainly based on the physico-chemical structures of bacterial cell walls [70]. This process is nonspecific and the immobilization of metals on cell surface may not be stable. In contrast, bioaccumulation of lead and zinc by the high metal-resistant bacterial cells that we identified may take advantage of their metal detoxification mechanisms and cellular metabolism to take up the metals with an increase in biomass.

The present study elucidated the potential applicability of bacterial strain TLC 6-6.5-4 in rhizoremediation of copper. TLC 6-6.5-4 increased the bioavailability of copper in soil by solubilizing copper and thereby enhanced copper uptake by maize and sunflower. In addition, TLC6-6.5-4 has common plant growth promoting characteristics such as IAA production and phosphate solubilization. Our results suggest that plant–growth-promoting characteristics of bacteria may be plant-specific. Further investigations to understand molecular mechanisms of plant-microbe interactions for plant growth and metal uptake would lead to more efficient rhizoremediation approaches. Application of bacterial strain TLC 6-6.5-4 associated with red fescue (*Festuca rubra*) on the remediation of copper-contaminated soil is still in progress. These plants are recommended by US Environmental Protection Agency (US EPA) because they adapted to the cold climate of Lake Superior Area and are native to North America.

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