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Effects of inoculation of biosurfactant-producing *Bacillus* sp. J119 on plant growth and cadmium uptake in a cadmium-amended soil

Xiafang Sheng *, Linyan He, Qingya Wang, Hesong Ye, Chunyu Jiang

MOA Key Laboratory of Microbiological Engineering of Agricultural Environment, College of Life Science, Nanjing Agricultural University, Nanjing 210095, PR China

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Abstract

A biosurfactant-producing *Bacillus* sp. J119 isolated from heavy metal contaminated soils was investigated for its effects on the plant growth-promoting characteristics and heavy metal and antibiotic resistance. A pot experiment was conducted for investigating the capability of the biosurfactant-producing bacterial strain *Bacillus* sp. J119 to promote the plant growth and cadmium uptake of rape, maize, sudangrass and tomato in soil artificially contaminated with different levels of cadmium (Cd) (0 and 50 mg kg^{-1}). The strain was found to exhibit different multiple heavy metal (Pb, Cd, Cu, Ni and Zn) and antibiotic (kanamycin, streptomycin, ampicillin, tetracycline and rifampin) resistance characteristics. The strain had the capacity to produce indole acetic acid (IAA) and siderophores. Cd treatment did not significantly decreased growth of tomato, maize and rape plants, but Cd treatment significantly decreased growth of sudangrass ($p < 0.05$). In the Cd-added soil, above-ground biomass and root dry weights of tomatoes were increased by 24 and 59%, respectively, in live bacterial inoculation compared to dead bacterial inoculation control. There were no obvious differences in the above-ground tissue and root dry weight of maize and sudangrass between live bacterial inoculation and dead bacterial inoculation. In the soil treated with 50 mg Cd kg^{-1}, increase in above-ground tissue Cd content varied from 39 to 70% in live bacterium-inoculated plants compared to dead bacterium-inoculated control. In addition, among the inoculated plants, tomato was the greatest Cd accumulator. The bacterial strain was also able to colonize and develop in the rhizosphere soils after root inoculation.

Keywords: Biosurfactant-producing bacterium; Cd; Phytoremediation; Rape; Maize; Sudangrass; Tomato

1. Introduction

Cadmium (Cd) is a common heavy metal contaminant in the environment. Activities such as mining, dumping of municipal sewage, and fertilizers containing high levels of Cd are responsible for soil Cd contamination [1]. Cd is a non-essential element in metabolic processes in plants and animals, and it can accumulate to levels that are toxic to organisms. The threat of Cd pollution to public health and wildlife has led to an increased interest in developing systems that can remove or neutralize its toxic effects in soil [2]. Phytoremediation offers significantly more benefits than conventional technologies to accumulate heavy metals from the soil due to its lower cost and safety to humans and the environment [3,4]. In recent years, some plant species (identified as hyperaccumulators) growing in heavy metal contaminated sites have been found with the ability to accumulate unusually high concentrations of heavy metals without impacting on their growth and development [5]. However, most hyperaccumulators identified so far are not suitable for field phytoremediation applications due to their small biomass and slow growth [6]. In addition, low bioavailability of heavy metals in soils may also limit the efficiency of phytoremediation [7].

The efficacy of phytoremediation could be increased by using chemicals (chelators) such as EDTA and organic acids to increase heavy metals accumulation by plants [8]. Biosurfactants have shown the capability to solubilize heavy metals and remove them from soils and sediments [9,10]. EDTA-assisted phytoremediation has been used to artificially enhance heavy metal solubility in soil and thus increase heavy metal phytoavailabity [11–13]. However, some synthetic chelators are too expensive and pose a threat to soil and groundwater quality [14–16]. Microbial activity strongly influences metal speciation and transport in the environment [17]. Many soil bacteria are tolerant to heavy

* Corresponding author. Tel.: +86 25 84395125; fax: +86 25 84396326.
E-mail address: xfsheng604@sohu.com (X. Sheng).

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metals and play important roles in mobilization of heavy metals [12,18,19]. Rhizosphere bacteria were found to increase the concentration of Zn in *Thlaspi caerulescens* [20] and Ni in *Alyssum murale* [21]. However, little is known about the potential of biosurfactant-producing and heavy metal-resistant bacteria on the phytoremediation of heavy metal contaminated soils. Previous work with *Bacillus* sp. J119 has shown that the strain could produce biosurfactants and mobilize lead efficiently in the soil [22]. However, research to determine the potential effect of *Bacillus* sp. J119 on the growth of rape, maize, sudangrass and tomato in Cd-amended soils has not been performed. The major objectives of the reported research were to characterize the biosurfactant-producing *Bacillus* sp. J119 and to evaluate the enhancement of plant growth promotion and Cd uptake in rape, maize, sudangrass and tomato plants grown in Cd-amended soil for improving the efficiency of phytoremediation of Cd-polluted soils.

2. Materials and methods

2.1. Soil and plant

The soil, a yellow brown soil (Alfisol) for the pot experiment was sampled to a depth of 0–10 cm from a non-fertilized field site in Nanjing, East China. The soil was kept in a cooler (4 °C) prior to laboratory analyses. The properties of the soil used in the experiments were: pH (1:1, w/v water) 6.71; organic matter 20.18 g kg^{-1}; total N 1.58 g kg^{-1}; available P 11.7 mg kg^{-1}; available K 80.5 mg kg^{-1}; CEC 20.2 cmol kg^{-1}; organic matter content and total N, available P, available K and CEC were determined following the methods described in the *Physical Chemical Analysis of Soil* [23]; total bacterial count (dilution-plate method) 4.5×10^8 cells g^{-1}; cadmium-resistant bacteria count (dilution-plate method) 6.10×10^3 cells g^{-1}. Rape (*Brassica napus* variety Huiyou-50), maize (*Zea mays* L. variety Denhai-11), sudangrass (*Sorghum bicolor* × *Sorghum sudanense*) and tomato (*Lycopersicon esculentum* variety Shanghai-906) were used in the inoculation experiments due to their fast growth, large biomass production.

2.2. Bacterium

Bacillus sp. J119 was isolated from heavy metal contaminated soils in Nanjing, China. According to Ye et al. [22], strain J119 was able to produce a lipopeptide-biosurfactant and possess resistance to Cd (100 mg l^{-1}) and Pb (200 mg l^{-1}).

2.3. Heavy metal and antibiotic resistance of the bacterium

J119 strain was tested for heavy metal and antibiotic resistance using Luria–Bertani's (LB) agar medium with the addition of 50 mg l^{-1} Cu ($CuSO_4$), 20 mg l^{-1} Ni ($NiCl_2$), or 100 mg l^{-1} Zn ($ZnSO_4$), 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} streptomycin, 300 μg ml^{-1} ampicillin, 50 μg ml^{-1} tetracycline or 100 μg ml^{-1} rifampin, which were added aseptically to the medium after autoclaving. Cultures were incubated at 30 °C for 7 days.

2.4. Indole acetic acid (IAA), siderophore production

The *Bacillus* sp. J119 was cultured for 4 days in flasks containing 20 ml of sucrose-minimal salts (SMS) medium (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.2%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) supplemented with 0.5 mg ml^{-1} of tryptophane. After incubation, a 1 ml cell suspension was transferred into a tube and mixed vigorously with 2 ml of Salkowski's reagent (150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$) [24] and allowed to stand at room temperature for 20 min, after which a pink color is developed in the cell suspensions. The absorbance of pink color developed after 25 min incubation was read at 530 nm. The IAA concentration in culture was determined using a calibration curve of pure IAA as a standard following linear regression analysis.

Siderophore secretion by the *Bacillus* sp. J119 was detected by the "universal" method of Schwyn and Neilands [25] using blue agar plates containing the dye chrome azurol S (CAS). Orange halos around the colonies on blue agar were indicative of siderophore excretion.

2.5. Pot experiment

The outdoor pot experiment was carried out for studying the effects of bacterial strain on plant growth and Cd uptake. Each pot contained 300 g of soil with 0 and 50 mg Cd kg^{-1} soil added as $CdCO_3$. The $CdCO_3$ was mixed thoroughly with the soil in a plastic bag before use. Three replicates were used for each treatment. Five surface-sterilized seeds were placed in each pot at a 2 cm depth. After germination (14 days), plants were thinned to two plants per pot.

To determine the survival rate of the inoculated bacterium in rhizosphere soil, a mutant of the heavy metal-resistant and biosurfactant-producing bacterial strain J119 marked with antibiotic resistance was obtained after plating of the parental strain onto LB agar amended with rifampicin (100 mg l^{-1}). After incubation for 4 days at 28 °C, the rifampicin-resistant strain was selected based on similarities in colony morphology, heavy metal-resistance and biosurfactant-producing ability with the parent strain and was recultured on rifampicin-free medium to check stability of the antibiotic resistance marker.

For inoculation, the strain marked with antibiotic resistance was grown in LB medium. Cells in the exponential phase were collected by centrifugation at 925 × *g* for 15 min at 6 °C, washed with sterile distilled water, and recentrifuged. Bacterial inoculum was prepared by resuspending pelleted cells in sterile distilled water to obtain an inoculum density of ca. 10^8 colony forming units (cfus) ml^{-1}. Bacterial suspension (10 ml pot^{-1}) was sprayed on the soil surface 4 weeks after seedling emergence. A dead bacterial (autoclaved at 121 °C for 40 min) inoculated sample was prepared as control. Bacterial inoculated and dead bacterial inoculated plants were grown in pots. Pots were placed outdoors and were covered or moved to indoors in order to protect them from the rainfall. During cultivation, minimal and maximal temperature ranged from 17 to 22 and

26 to 32 °C, respectively. The soil was moistened with water and maintained at 60% water holding capacity. The plants were harvested 4 weeks after inoculation. Above-ground tissue and roots were separated and washed, first in several changes of 0.01 M EDTA and then in distilled water to remove any non-specifically bound Cd and dried at 105 °C before determining the above-ground and root tissue dry weight. The oven-dried samples were ground using a stainless steel mill (FZ102, Tianjing, China) to 0.5 mm for analysis. Subsamples of above-ground samples (200 mg) and root samples (30 mg) were then digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, v/v) [26]. The volume of each sample was adjusted to 10 ml using double deionized water. The concentrations of Cd in the samples were determined using an atomic absorption spectrometer (TAS-986, Beijing, China). Reagent blank and analytical duplicates were used where appropriate to ensure accuracy and precision in the analysis.

Survival of antibiotic-resistant bacteria after inoculation was conducted after harvesting. The adhering soil was removed from plant roots. For determination of rhizosphere soil colonization, 1 g soil removed from the roots was shaken with 10 ml sterile water and 1% fungicidin (USP, Amresco, USA) solution for 30 min. The resulting suspensions were evaluated for cfu according to the dilution-plate method on LB agar with addition of 100 mg l^{-1} rifampicin. By adding fungicidin and rifampicin, the native fungal and bacterial flora were mostly excluded from the plates. After incubation for 4 days at 28 °C, the reisolated, rifampicin-resistant strain was identified for colony characteristics, heavy metal resistance and hemolytic activity (detected as the presence of a clear zone around a colony on blood agar plates according to Carrillo et al. [27] and Plaza et al. [28] against the parent strain).

2.6. Statistical analysis

Analysis of variance and the Student–Newman–Keuls test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 10.0.

3. Results and discussion

3.1. Characteristics of the strain

The bacterial strain *Bacillus* sp. J119 was found to exhibit different multiple heavy metal and antibiotic resistance characteristics. *Bacillus* sp. J119 was resistant not only to Pb (200 mg l^{-1}) and Cd (20 mg l^{-1}), but also to Cu (50 mg l^{-1}), Ni (20 mg l^{-1}) and Zn (100 mg l^{-1}). The *Bacillus* sp. J119 exhibited antibiotic resistance to kanamycin (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}), ampicillin (300 μg ml^{-1}), tetracycline (50 μg ml^{-1}) or rifampin (100 μg ml^{-1}).

After incubation at 28 °C for 48 h, orange halos around the colonies of *Bacillus* sp. J119 on blue agar were formed, indicating siderophore excretion of the strain. The *Bacillus* sp. J119 also had the capacity to produce IAA (15.8 μg ml^{-1}) in culture when the medium was supplemented with L-tryptophane.

Table 1
The influence of strain J119 on the dry weight (mg) of test plants on a yellow brown soil treated with 0 and 50 mg kg^{-1} of Cd (pot experiment, mean and standard deviation of three replicas per treatment)

Plant	Above-ground tissue		Root	
	0	50	0	50
Rape				
+Strain J119	975 ± 63	862 ± 73	289 ± 16	42 ± 11
+Dead Strain J119	958 ± 56	900 ± 43	264 ± 13	209 ± 14
Maize				
+Strain J119	2416 ± 104	2255 ± 92	1024 ± 68	939 ± 21
+Dead Strain J119	2282 ± 87	2173 ± 26	988 ± 32	956 ± 23
Sudangrass				
+Strain J119	514 ± 52	350 ± 66	1524 ± 85	1320 ± 74
+Dead Strain J119	510 ± 44	434 ± 15	1496 ± 78	928 ± 109
Tomato				
+Strain J119	2514 ± 116	2373 ± 82	405 ± 42	352 ± 61
+Dead Strain J119	2058 ± 104	1909 ± 91	296 ± 56	222 ± 21

3.2. Plant growth promotion

Cd treatment significantly decreased sudangrass growth. Root growth of rape was also significantly ($p < 0.05$) inhibited. Cd treatment did not significantly decrease the growth of maize or tomato. However, significant increases of above-ground tissue dry weight of tomato and root dry weight of tomato and sudangrass were observed when the soil was inoculated with strain J119, compared to the dead bacterium-inoculated soil (Table 1). Above-ground tissue and root dry weights of tomato increased by 24 and 59%, respectively, in the inoculated soil compared to the dead bacterium-inoculated soil (Table 1). The study demonstrated that the strain J119 could facilitate tomato, maize and sudangrass growth. Bacteria that could produce IAA and siderophores are capable of stimulating plant growth and helping plants acquire sufficient iron for optimal growth [29,30]. An increase in plant growth, generally promoted by IAA and siderophore-producing rhizobacteria [30,31], was also observed under toxic Cd concentrations in tomato inoculation experiments using *Bacillus* sp. J119. Rhizobacteria belonging to different genera such as *Pseudomonas*, *Bacillus*, *Mycobacterium*, *Agrobacterium* and *Arthrobacter* were found to have plant growth-promoting characteristics that can potentially support heavy metal uptake and reduce stress symptoms in plants [32].

3.3. Effect of bacterium on Cd uptake by plants

Bacillus sp. J119 was tested for its effect on Cd uptake by plants. There was no significant ($p < 0.05$) difference in above-ground tissue Cd concentrations of maize and sudangrass between bacterial inoculation and dead bacterial inoculation (Fig. 1). Significant increases ($p < 0.05$) of above-ground tissue Cd concentrations of rape and tomato and root Cd concentrations of rape, maize and tomato were observed when the soil was inoculated with *Bacillus* sp. J119, compared to the dead bacterium-inoculated soil. For the rape and

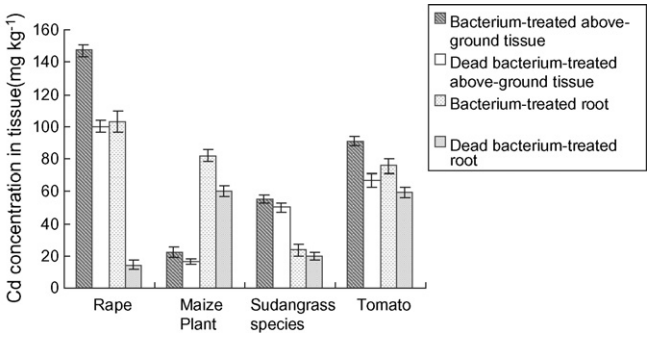

Fig. 1. Influence of bacterial inoculation on above-ground tissue and root Cd concentration (mg kg^{-1}) of the plants on a yellow brown soil treated with 50 mg kg^{-1} of Cd. Error bars are ±standard deviation ($n = 3$).

tomato plants grown in soil inoculated with J119, above-ground tissue and root Cd concentrations increased from 36 to 47% ($p < 0.05$) and from 28 to 622% ($p < 0.05$) compared to the dead bacterial inoculation control, respectively (Fig. 1). Although the rape accumulated much more Cd in the above-ground biomass (147.3 ± 3.5 mg kg^{-1}) than the tomato (91.0 ± 3.0 mg kg^{-1}), the total Cd uptake in the above-ground tissue (215.9 ± 12.5 μg plant^{-1}) of the tomato was much greater than the rape (127.0 ± 3.6 μg plant^{-1}) (Fig. 2) due to the greater biomass of the tomato (Table 1). In the inoculated soils, 1.6-, 2.6- and 6.6-fold increases in Cd accumulation in the above-ground tissues of the rape were obtained compared to the tomato, sudangrass and maize, respectively. Total Cd uptake in the above-ground tissues of the tomato was increased by 1.7-, 4.2- and 11.1-fold compared to the rape, maize and sudangrass, respectively.

Low bioavailability of heavy metals in soils and low biomass production of the plants may limit the efficiency of phytoremediation. Although the accumulation of metals by plants can be enhanced by addition of chemical chelates [33], these expensive compounds can increase the metal leaching risk and impart negative effects on soil fertility or soil structure [8,14,16,34]. Column experiments showed that the rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* BS2 could solubilize the heavy metals in the soil and increase the removal of the heavy metals [10] indicating that biosurfactant technology can be an effective and non-destructive method for bioremediation of Cd- and Pb-contaminated soil [10]. Our strain J119 produced lipopeptide-biosurfactant and effectively solubilized Pb in the soil [22]. In the solution culture, the strain J119 solubilized $CdCO_3$, but no obvious change in pH was observed during the experiment indicating the solubilization of the $CdCO_3$ was not related to the production of H^+ produced by the strain J119 (data not shown). Studies have demonstrated that heavy metal-resistant bacteria can enhance metal uptake by hyperaccumulator plants [20,35]. The same results were reached in our experiment, that Cd uptake was significantly enhanced by the heavy metal-resistant and biosurfactant-producing *Bacillus* sp. J119. Pot experiments demonstrated that *Bacillus* sp. J119 could effectively promote the growth of tomato, consequently increasing total Cd accumulation of the plants even under non-sterile conditions. Plant type could influence bacterial effects on plant growth and Cd uptake. Further understanding of the basic mechanisms of plant–microbe interactions is essential for enhancing Cd-contaminated soil phytoremediation.

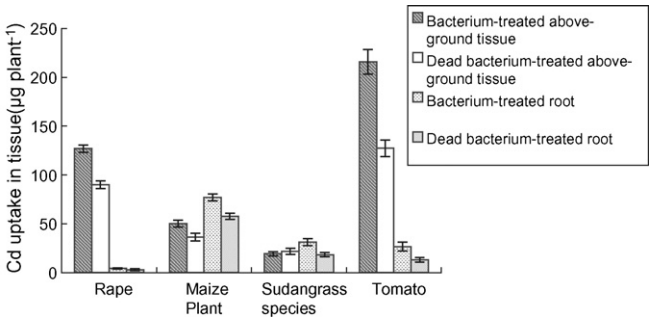

Fig. 2. Influence of bacterial inoculation on above-ground tissue and root Cd uptake (μg plant^{-1}) of the plants on a yellow brown soil treated with 50 mg kg^{-1} of Cd. Error bars are ±standard deviation ($n = 3$).

Table 2
Colonization of strain J119 in rhizosphere soil of test plants after inoculation in a yellow brown soil treated with 0 and 50 mg kg^{-1} of Cd (pot experiment, mean and standard deviation of three replicas per treatment, values in 10^3 cfu 1 g^{-1} of fresh soil)

Concentration of Cd (mg kg^{-1})	Rape	Maize	Sudangrass	Tomato
0	1135 ± 75	784 ± 46	515 ± 28	1217 ± 98
50	910 ± 49	550 ± 35	462 ± 14	1130 ± 64

3.4. Survival and establishment of antibiotic-resistant bacteria in rhizosphere soil

Bacillus sp. J119 was tested for its ability to colonize rhizosphere soils of rape, maize, sudangrass and tomato plants. Cd treatment significantly decreased the growth of strain J119 in rhizophere soils of maize and sudangrass plants, but did not noticeably affect *Bacillus* growth in rhizophere soils of rape and tomato (Table 2). In the Cd-amended soil, the inoculated bacterial strain was detected in the rhizophere soils for 4 weeks after inoculation (from 4.62×10^5 to 1.13×10^6 cfu 1 g^{-1} of fresh soil). However, bacterial survival was better in tomato rhizophere soil than rape, maize or sudangrass rhizophere soil (Table 2). As successful inoculants, bacteria must be able to rapidly colonize the root system during the growing season [36]. The results obtained showed that in the Cd-contaminated soils, *Bacillus* sp. J119 was able to colonize rhizosphere soils of the test plants and enhance plant uptake of Cd from the soil. However, the extent of stimulation of plants by the tested bacterial strain and the persistence of plant growth-promoting activity under actual Cd-contaminated field conditions remains unclear. Thus, experiments concerning stimulation of the plants and Cd accumulation must be followed by investigations under Cd-contaminated field conditions.

4. Summary and conclusion

Phytoremediation is an environment-friendly, cost-effective and plant-based solution for the remediation of heavy metal

contaminated soils. Low biomass production and slow growth of the plants and the low availability of heavy metals in soil limited effective remediation [37,38]. Effective phytoremediation could be accomplished by bacteria having the potential of solubilizing heavy metals and promoting plant growth in contaminated soils. Our study demonstrated that the biosurfactant-producing and heavy metal-resistant *Bacillus* strain J119 could colonize the rhizosphere soils of selected plants. Pot experiments demonstrated that the application of the biosurfactant-producing and heavy metal-resistant bacterial strain J119 significantly enhanced biomass of tomato plants and Cd uptake in plant tissue so that Cd accumulation increased in above-ground parts of plants. In our experiment, plant type influenced root colonization activity of the introduced strain and bacterial effects on plant growth and Cd accumulation. A further understanding of the relationship between the plant and the biosurfactant-producing bacterial strain J119 is a critical prerequisite for the development of effective phytoremediation of heavy metal contaminated soils. This may therefore provide a new microbe assisted-phytoremediation strategy for metal-polluted soils.

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