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Characterization of an Hg(II)-volatilizing *Pseudomonas* sp. strain, DC-B1, and its potential for soil remediation when combined with biochar amendment

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ABSTRACT

Hg contamination is a critical environmental problem, and its remediation using cost-effective and environmentally friendly methods is highly desirable. In this study, a multi-metal-resistant bacterium showing strong Hg(II) volatilization ability, *Pseudomonas* sp. DC-B1, was isolated from heavy metal-contaminated soils. DC-B1 volatilized 81.1%, 79.2% and 74.3% of the initial Hg^{2+} from culture solutions with initial Hg^{2+} concentrations of 5.1, 10.4, and 15.7 mg/L, respectively, within 24 h. Microcosm experiments were performed to investigate the remediation of Hg(II)-spiked soils inoculated with DC-B1 coupled with sawdust biochar amendment. The efficiency of Hg removal from two types of soil samples with different properties and an initial Hg(II) content of approximately 100 mg/kg was enhanced 5.7–13.1% by bio-augmentation with inoculation of the bacterial strain DC-B1, 5.4–10.7% by amendment of 4% (w/w) biochar, and 10.7–23.2% by the combination of DC-B1 and biochar amendments over an incubation period of 24 d over the efficiency in the control treatment under flooded conditions. Longer root lengths were observed in lettuce grown in the treated soils than in lettuce from the control soil, confirming the bioremediation efficacy of the two bioagents for soil Hg contamination.

1. Introduction

Mercury (Hg) is a significant global contaminant, is ranked sixth among the ten most hazardous elements, has high toxicity and mobility and persists for long periods in the environment (Nascimento and Chartone-Souza, 2003). Hg is usually released into the environment from both natural (such as forest fires, volcanic and geothermal activities, and re-emission from soil and ocean) and anthropogenic sources (such as metal mining and refining, fossil fuel burning, waste incineration, and other industrial activities) (Li et al., 2009; Pirrone et al., 2010; Driscoll et al., 2013). Although the majority of globally presented Hg originates from natural processes, anthropogenic sources are responsible chiefly for the increasing content of Hg in the biosphere (Driscoll et al., 2013).

Hg tends to accumulate in the ecosystem through the food chain, eventually posing a severe toxic risk to animal and human health and resulting in brain and liver damage, disorder in the central nervous system, and cardiac failure, even at a low lethal dose (Kim et al., 2004; Guzzi and La Porta, 2008; Dash and Das, 2012). Hg pollution in the soil system through food chain circulation has become one of the most critical environmental concerns worldwide. In China, the Hg content in 1.6% of the farmland soils exceeds the safety limit according to a recent national-scale survey by the Ministry of Environmental Protection and the Ministry of Land and Resources of China (MEP, 2014). Although the natural attenuation of Hg in contaminated soils occurs through various pathways, including biodegradation, dispersion, dilution, sorption, volatilization, reduction and chemical or biological stabilization, high Hg content beyond the attenuation capacity could lead to soil Hg pollution (Li et al., 2009; Xu et al., 2015). Therefore, in addition to reducing the use and emission of Hg, the remediation of Hg-contaminated soils, either by removing Hg from soil or transforming toxic forms to less toxic or immobile forms, is urgently required for the protection of environmental and human health (Wang et al., 2012).

Several physicochemical techniques such as thermal treatment, precipitation and washing with chemical reagents, solidification and stabilization have conventionally been applied for the remediation of environments polluted by Hg (Wang et al., 2012; Xu et al., 2015). However, these technologies are usually expensive, of low specificity,

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and destructive to ecosystems and can produce substantial amounts of secondary pollutants (Mahbub et al., 2017a). So far, almost no viable physicochemical method that is both cheap and environmentally friendly has been developed.

Recently, biotechnological strategies, which offer approaches that are more cost-effective and eco-friendly, cast a light on the remediation of Hg-polluted systems, especially those with low Hg concentrations (Velasquez-Riano and Benavides-Otaya, 2016). Some microorganisms gradually develop resistance to Hg pollution after exposure, most commonly through the *mer* operon, which allows the transformation of transform both organic and inorganic forms of Hg to less toxic Hg° that can escape from water and soil. These microorganisms are considered promising candidates for the bioremediation of Hg-contaminated environments (Yu et al., 2014; Mahbub et al., 2016a; Giovanella et al., 2017). In addition to volatilization, microorganism-mediated adsorption, accumulation and immobilization can also contribute to the remediation of Hg pollutants (Ahluwalia and Goyal, 2007; Lefebvre et al., 2007; Francois et al., 2012). Various bacteria isolated from Hg-polluted locations such as a *Psychrobacter* sp. (Pepi et al., 2011), *Pseudomonas putida* (Cabral et al., 2013; Giovanella et al., 2017), an *Enterobacter* sp. (Sinha et al., 2013), *Bacillus thuringiensis* (Giri et al., 2014), *Staphylococcus epidermidis* (Yu et al., 2014), a *Sphingopyxis* sp. (Mahbub et al., 2017c), a *Sphingobium* sp. (Mahbub et al., 2016a), and a *Pseudoxanthomonas* sp. (Mahbub et al., 2016b) have been reported to exhibit potential for Hg removal from wastewater by biovolatilization, biosorption or bioaccumulation. Nevertheless, the remediation of Hg-contaminated soil by Hg-resistant bacteria has been poorly investigated. Recently, Mahbub et al. (2017) and McCarthy et al. (2017) reported that the efficiency of Hg removal from soils can be enhanced by bio-stimulation and/or bio-augmentation using Hg-resistant bacteria, indicating that the microbial remediation of Hg pollution in soil is likely feasible. However, the efficacy of Hg-resistant microorganisms for the remediation of Hg-contaminated soil still needs to be explored.

Biochar, made from the pyrolysis of various organic materials, is another attractive agent for the remediation of heavy metal-contaminated soil. Bioavailability of heavy metals and their uptake by plants in agricultural fields can be reduced by amendments with biochar, which supplies abundant functional groups for metal binding and subsequent stabilization (Ahmad et al., 2014). In addition, other environmental benefits, including the improvement of fertility and soil structure, carbon sequestration and agricultural by-product/waste recycling, are provided by the application of biochar (Ahmad et al., 2014). A few recent studies have investigated the impact of biochar amendment on Hg mobility and transfer in plant-soil systems (Shu et al., 2016; O'Connor et al., 2018). However, there is no report on the effect of biochar amendment or the synergistic effects between bacterial inoculation and biochar amendment on Hg removal from Hg-contaminated soil.

Accordingly, in this study, an Hg(II)-resistant bacterial strain was isolated from a heavy metal-contaminated site, and its potential for the remediation of Hg-polluted soil when combined with pine sawdust biochar amendment under flooded conditions was evaluated through microcosm experiments. Flooding frequently occurs in some field soils, which may affect Hg behaviors in the soil; however, there are no reports on the bioremediation of Hg-contaminated soils under flooded conditions. The main purposes were (1) to isolate a Hg(II)-resistant bacterium with high potential for remediation of Hg pollution and determine its Hg(II) volatilization ability from solution and (2) to investigate and evaluate the Hg bioremediation potential of the isolate coupled with biochar amendment for two different Hg-contaminated soils under flooded conditions (105% water holding capacity).

2. Materials and methods

2.1. Isolation of Hg-resistant bacteria

Soil samples (0–5 cm) polluted by multiple heavy metals were collected at Dongchuan District, Yunnan Province, China (26°3'56"N, 103°9'43"E). The average contents of Hg, As, Cr, Pb, Cu and Zn in the soil samples were determined to be 2.50, 281.0, 30.0, 291.0, 206.0 and 584.0 mg/kg (dry weight), respectively. The isolation of Hg-resistant bacteria was conducted using a serial dilution method. Mixed soil (1 g) was suspended in 10 mL sterile deionized water and then serially diluted. Dilutions of 10^{-3}-10^{-5} were spread onto Luria broth (LB) agar medium (10 g peptone, 5 g yeast extract, 10.0 g NaCl, 15 g agar, 1 L deionized water, pH 7.5) containing 20.0 mg/L Hg^{2+} and then incubated at 30 °C for 48 h. Then, several visually distinct single colonies were streaked repeatedly on the same type of agar plates to obtain pure bacterial cultures. The purified cultures were preserved in 15% glycerol broth at −80 °C. A resistant bacterial strain named DC-B1 exhibited high Hg removal from culture solution (Section 2.4) and was selected for the present study.

Throughout the experiment, $HgCl_2$ was used, and a stock solution of 1000 mg Hg(II)/L was prepared after sterilization at 121 °C.

2.2. Molecular identification of the isolate DC-B1

Phylogenetic analysis by 16 S ribosomal RNA (rRNA) gene sequencing was performed to identify the Hg(II)-resistant bacterial strain DC-B1. Genomic DNA was extracted from pure culture of DC-B1 by using a bacterial genomic DNA extraction kit (Sangon Inc., Shanghai, China) according to the manufacturer's instructions. The 16 S rRNA genes of the strain were amplified using the universal forward primer 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492 R (5'-ACGG CTACCTTGTTACGA-3') in a T3 thermocycler (Biometra, Germany). All reactions were carried out in 50 μL mixtures containing approximately 10–100 ng of bacterial DNA, 1 × reaction buffer, 200 μM dNTP, each primer at 10 pmol, 1.5 mM $MgCl_2$ and 5 U Taq polymerase. Negative controls were performed using sterile ultrapure water instead of the template. The PCR program was an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified products were purified using a DNA Gel Extraction kit and then sent for sequencing (Sangon Inc., Shanghai, China).

The 16 S rRNA gene sequence of the strain was then submitted to the NCBI GenBank Database to determine its homology with the archived 16 S rRNA sequences by using the BLASTN program. A phylogenetic tree was inferred, and bootstrap analysis (1000 replicates) was performed by MEGA 5.0 software using the neighbor-joining DNA distance algorithm for elucidating the phylogenetic position of the isolate.

2.3. Trace metal resistance of isolate DC-B1

The resistance of the strain DC-B1 to inorganic Hg, Cr, Cu, Zn, Cd, Pb and As were determined through serial dilution methods. Bacterial growth in LB liquid medium containing gradient concentrations of metals was monitored. Sterilized liquid medium (10 mL) in test tubes was amended with 0–30 mg/L Hg (as $HgCl_2$), 0–500 mg/L Cr (as $K_2Cr_2O_7$), 0–300 mg/L Cu (as $CuCl_2$), 0–150 mg/L Zn (as $ZnCl_2$), 0–500 mg/L Cd (as $CdCl_2$), 0–500 mg/L Pb (as $Pb(NO_3)_2$) and 0–150 mg/L As (as Na_3AsO_3), and 0.25 mL of an overnight-grown culture of DC-B1 with an adjusted optical density at 600 nm (OD_{600}) of 0.6 was added to each tube. The negative control was LB medium containing metals and lacking bacterial inoculation, and the positive control was inoculated LB medium without added metals. Then, all the tubes were incubated at 30 °C in a shaker at 150 rpm. After an incubation period of 48 h and a stable bacterial growth phase was