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Cadmium-tolerant bacteria reduce the uptake of cadmium in rice: Potential for microbial bioremediation

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ABSTRACT

We selected 24 bacterial isolates that could tolerate up to 2500 μM $CdCl_2$ from the soil of rice fields downstream from a zinc-mineralized area contaminated with a high level of cadmium (Cd). In the presence of 500 μM $CdCl_2$, all isolates grew slower and with a prolonged lag-phase compared to in the absence of Cd. Cd-binding capacity was high and ranged from 6.38 to 9.38 log[Cd(atom)]/cell. The stability of Cd complexes in bacteria was affected by 1 mM EDTA. In 500 μM $CdCl_2$, all isolates produced 0.7 to 4.8-fold more inorganic sulfide and 0.6 to 2.2-fold more thio-rich compounds containing SH groups. Out of 24 Cd-tolerant bacterial isolates, KKU2500-3, -8, -9 and -20 were able to promote the growth of Thai jasmine rice (*Kao Hom Mali* 105) seedlings in the presence of 200 μM $CdCl_2$, and KKU2500-3 produced the highest numbers of fibrous root. Interestingly, these 4 isolates increased Cd tolerance and decreased the accumulation of Cd in rice by 61, 9, 6, and 17% when grown in the presence of 200 μM $CdCl_2$. Of the 4 isolates, KKU2500-3 produced more inorganic sulfide when grown in $CdCl_2$ at 500–2000 μM. XANES analyses indicated that this isolate precipitated a detectable amount of cadmium sulfide (CdS) when grown in 500 μM $CdCl_2$. Thus, the isolate KKU2500-3 could possibly transform toxic, soluble $CdCl_2$ into non-toxic, insoluble CdS. These 4Cd-tolerant bacterial isolates were identified via 16S rDNA sequencing and classified as *Cupriavidus taiwanensis* KKU2500-3 and *Pseudomonas aeruginosa* KKU2500-8, -9, and -20.

1. Introduction

Heavy metal pollution is one of the most widespread and serious environmental problems facing the biosphere. In 2002, the International Water Management Institute (IWMI), a research agency focusing on the wise use of water and land resources, found samples of soil, rice, garlic, and soya beans containing harmful levels of bone-damaging cadmium (Cd) in Thailand. Soil samples collected in the riverside villages of Mae Tao Mai and Pha Dei in the Mae Sot district, downstream from a zinc (Zn)-mineralized area, showed that 84.59% of the fields exceeded European Union (EU) standards for the Cd content in soils (Simmons et al., 2005a, 2005b). The levels were up to 1893-times greater than the standard set in Thailand. Almost 70% of the fields produce rice grains containing Cd levels above the international standard of 0.2 mg Cd/ kg of rice. In fact, 0.7 to >2 mg of Cd per kg of rice was detected in more than 30 rice samples collected by officials from the Pollution Control Department. In 2004, the Cd contamination area extended for approximately 21 km^2, affecting the health and environmental biosphere of more than 12,000 people in12 villages (Pollution Control Department, 2004; National Research for Environmental and Hazardous Waste Management, 2005; Tak Provincial Office, 2005; Honda et al., 2010).

Agricultural soils are primarily contaminated with ionic Cd^{2+} because of the excessive use of phosphate fertilizers, dispersal of sewage sludge and atmospheric deposition. Currently, the metal processing industries also result in a widespread Cd contamination of soil. Cd^{2+} is readily absorbed by numerous types of crops, including cereals, potatoes, rice, and fruits (Ingwersen and Streck, 2005). Consumption of rice grown in paddy soils contaminated with Cd^{2+} may pose a serious health risk to humans, as 22–24% of the total metal content in the rice biomass is concentrated in the rice grains (Wang et al., 2003). Thus, contamination by Cd^{2+} is increasing in human food and the agricultural environment. Plants readily absorb Cd^{2+} from the soil, and exposure to high levels of Cd^{2+} that exceed the limit of plant tolerance could have severe effects on their growth and could ultimately lead to death.

Traditional, non-selective methods of sequestering trace elements have involved chemical engineering approaches in which the elements are precipitated, reduced and/or absorbed from contaminated media. More selective approaches, such as the use

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of chelating resins that concentrate trace elements, have been considered, but large-scale application of ion exchange and/or ion-selective resins is expensive (Logan and Traina, 1993). Therefore, there is a need for effective and renewable systems to selectively sequester and concentrate toxic trace metals without interference from other metals. Biological trace metal recovery systems meet many of the objectives for safe, effective, and renewable recovery of trace metals from industrial waste and contaminated sites (Tebo, 1995; Siripornadulsil et al., 2002).

Many microorganisms in the soil are able to solubilize unavailable forms of heavy metal-bearing minerals by excreting organic acids. In addition, many soil bacteria are tolerant of heavy metals and play important roles in the mobilization of these metals (Gadd, 1990; Nies, 1999). Several tolerance mechanisms have been proposed for the bacteria that live under heavy metal stress (Lima et al., 2006). Nevertheless, unlike other toxic but degradable organic compounds, only three basic mechanisms underlying the heavy metal resistance are possible: (i) an active efflux system as observed in Gram-positive bacteria, including *Listeria monocytogenes* and *Staphylococcus aureus* (Lebrun et al., 1994; Bruins et al., 2000), and Gram-negative bacteria, including *Cupriavidus necator*, *Cupriavidus metallidurans* CH34 and *Pseudomonas* spp. (Nies et al., 1989; Perron et al., 2004; Zoropogui et al., 2008; Gibbons et al., 2011); (ii) complex formation with thiol-containing molecules such as glutathione-mediated Cd sequestration, as observed in *Rhizobium leguminosarum* (Lima et al., 2006); and (iii) transformation into less toxic forms as observed in *Klebsiella aerogenes* (Aiking et al., 1985). However, for many toxic heavy metals, the detoxification tends to involve more than one mechanism.

Rice is an economically important crop in Thailand and several countries. Rice fields in the Cd-contaminated Mae Sort District have been previously known as one of the best agricultural areas for the production of Thai jasmine rice (*Kao Hom Mali* 105) in Thailand. Hence, the toxic level of accumulated Cd in rice due to its transport from contaminated soil into the rice plants is a major problem that needs an urgent solution. Rice can absorb Cd^{2+} ions from the soil, and studies have indicated that there are 4 major transport processes: root uptake, root to shoot through xylem, redirection at nodes and remobilization from leaves to grains (Uraguchi and Fujiwara, 2012). Therefore, perspectives for reducing the Cd content in rice and other cereals are desired to improve human health (Reeves and Chaney, 2008).

Soil microorganisms are essential components of most terrestrial ecosystems due to their role in mineral cycles and their living habitat around the plant roots, also known as the rhizosphere. Thus, toxic heavy metal stress can tremendously affect their survival, population and diversity and can subsequently result in the unsuitable growth of living plants. The mechanisms by which rhizosphere microorganisms reduce heavy metal toxicity in plants have been investigated. A nickel-resistant and plant growth-promoting bacterium, *Kluyvera ascorbata* SUD165, seems to lower the level of stress ethylene induced by nickel in canola and tomato seedlings (Burd et al., 1998). Metal-tolerating methylotrophic bacteria isolated from rice reduced nickel and Cd uptake and promoted the growth of tomato plants (Madhaiyan et al., 2007). *Pseudomonas* sp. PsA4 and *Bacillus* sp. isolated from heavy metal-contaminated soils protected Indian mustard plants against the inhibitory effects of chromium, likely due to the production of IAA, siderophores and the solubilization of phosphate (Rajkumar et al., 2006).

Here, we characterize Cd-tolerant bacteria that were isolated from the soils of Cd-contaminated rice fields. We demonstrate that the Cd-tolerant bacteria were likely able to precipitate toxic, soluble $CdCl_2$ as nontoxic, insoluble CdS. Furthermore, they increase the $CdCl_2$ tolerance of Thai jasmine rice (*Kao Hom Mali* 105) and decrease the $CdCl_2$ accumulation in rice plants when grown in the presence of a toxic $CdCl_2$ concentration of 200 μM. Thus, it may be possible to treat rice plants with the Cd-tolerant bacteria to decrease the Cd content of rice grains and to remediate Cd-contaminated soils in rice fields in the Mae Sot District where the problem remains.

2. Materials and methods

2.1. Isolation of Cd-tolerant bacteria

Cd-contaminated soils were collected from rice fields in Mae Tao Mai and Pha Dei. Each 2.5 g sample was added to 25 mL nutrient broth (NB, 0.5% peptone, 0.3% meat extract, pH 7.0) and shaken at 30 °C, 200 rpm for 4 h. A 1-mL aliquot of the suspension was serially diluted (from 10^{-1} to 10^{-8}), and each dilution solution was spread on Nutrient Agar plates (NA, nutrient broth and 1.5% agar) in the presence of 500–2500 μM $CdCl_2$. The plates were incubated at 30 °C for 24–48 h. The growing colonies were repeatedly inoculated onto a new NA plate containing the same and increased level of Cd concentration. Finally, large colonies of 24 isolates of Cd-tolerant bacteria grown on the NA agar plates in the presence of 2500 μM $CdCl_2$ were selected for further study.

To determine the bacterial cell concentration, Cd-tolerant bacteria were grown in NB and incubated at 30 °C, 150 rpm for 18 h. The culture was diluted in PBS buffer, and the optical density at 600 nm (OD_{600}) of each dilution was measured in a range of 0.1–1.0. Each solution was diluted (10^{-1}–10^{-8}) and spread on an NA plate. Plates were incubated at 30 °C. After 24 h, colonies of Cd-tolerant bacteria were counted and calculated as colony forming units per mL (CFU/mL).The values of the OD_{600} and CFU/mL were plotted as a standard growth equation and used in all of the remaining experiments.

2.2. Cell growth in the presence of Cd

One loop full of 24 isolates of Cd-tolerant bacterial colonies grown on NA plates were inoculated into NB and incubated at 30 °C, 150 rpm to late log phase (approximately 18 h). The bacterial culture, which had been adjusted to an optical density at 600 nm to 1, was referred to as an inoculum. A 1% (v/v) bacterial inoculum was inoculated into NB in the absence or presence of $CdCl_2$ at 500 μM and incubated at 30 °C, 150 rpm for 36 h. Cell growth was determined by measuring the optical density at 600 nm every 3 h.

2.3. Cd-binding capacity of Cd-tolerant isolates

The Cd-binding capacity was measured according to the methods of Ihnat (2000). The 1% (v/v) bacterial inoculum was inoculated into NB medium containing 500 μM $CdCl_2$ and incubated at 30 °C with shaking at 150 rpm to late log phase (approximately 18 h), and the cell density was measured at 600 nm. The cells were then harvested via centrifugation (4000 × *g* for 10 min at 4 °C). The pellets were washed twice with sterile deionized water (dH_2O) and resuspended in 10 mL of sterile dH_2O. To determine the effect of EDTA on the Cd-binding capacity, the pellets were washed twice with sterile dH_2O, followed by 2 washes with 10 mL of 1 mM EDTA in 50 mM HEPES and 10 mM NaCl, and then rewashed twice with 10 mL sterile dH_2O. The pellets were subsequently digested with 10 mL 70% (v/v) HNO_3. Cd concentrations were measured with a Perkin-Elmer model AAnalyst 100 flame atomic absorption spectrophotometer.

2.4. Determination of thiol-rich compounds

Thiol-rich compounds were detected using the method of Ellman (1959). A 1% (v/v) bacterial inoculum was inoculated into NB in the absence and presence of 500 μM $CdCl_2$ and incubated at 30 °C, 150 rpm for 18 h. The bacterial cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C, washed with PBS buffer and suspended in PBS buffer. The cell pellets were lysed by the freeze & thaw method (freeze at −70 °C for 30 min and thaw at 40–60 °C for 5–10 min, 3–4 times). After cell lysis, the total protein concentrations were determined by the Bradford Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and calculated using a BSA protein standard curve. The protein samples were diluted with sterile dH_2O to mg/mL for SH-group analysis. A 100-μL aliquot of the solution to be tested was diluted in 900 μL of sterile dH_2O, and 20 μL Ellman reagent (4% (w/v) of 5′-dithionitrobenzoic acid (DTNB) in absolute ethanol) was then added. The samples were allowed to develop at room temperature for 20 min, and the yellow color formation of 2-nitro-5-mercaptobenzoic acid (TNB) was measured at 412 nm. The content of thiol-rich compounds was then calculated via the equation $A = \xi bc$, where A = Absorbance at 412 nm, ξ = 13600 M absorbability (L/mol/cm), b = path length of the sample, and c = concentration of the compound in solution (mol/L).