

ADAPTIVE AND CROSS-PROTECTIVE RESPONSES AGAINST CADMIUM AND ZINC TOXICITY IN CADMIUM-RESISTANT BACTERIUM ISOLATED FROM A ZINC MINE

Benjaphorn Prapagdee*, Anchulee Watcharamusik

Laboratory of Environmental Biotechnology, Faculty of Environment and Resource Studies, Mahidol University, Salaya,
Nakhonpathom 73170, Thailand.

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ABSTRACT

Cadmium (Cd) is a major environmental hazard, which usually is detected in its ionic form of Cd^{2+} . It also causes adverse toxic effects on human health and other living organisms. Cd-resistant bacteria were isolated from Cd-contaminated soils. One isolate, TAK1, was highly resistance level to Cd toxicity. TAK1 was isolated from soil contaminated with a high Cd concentration (204.1 mg.kg^{-1}). The result of 16S rDNA sequence analysis found that the TAK1 showed the similarity to *Ralstonia* sp. Physiological adaptive and cross-protective responses to Cd and Zn killing were investigated in *Ralstonia* sp.TAK1. Exposure to a low concentration of Cd induced adaptive resistance to higher concentrations of Cd. In addition, pretreatment of *Ralstonia* sp.TAK1 with an inducing concentration of Cd conferred cross-protective response against subsequent exposure to the lethal concentrations of Zn. The induced adaptive and cross-protective response *Ralstonia* sp.TAK1 required newly synthesized protein(s). Cd-induced adaptive and cross-protective responses against Cd and Zn toxicity are the important mechanisms used by *Ralstonia* sp.TAK1 to survive in the heavy metal contaminated environments. These findings might lead to the use of *Ralstonia* sp.TAK1 for microbial based remediation in Cd and Zn-contaminated soils.

Key words: Bacterial responses; Cadmium; Zinc; Metal Resistance; *Ralstonia* sp.

INTRODUCTION

Cadmium (Cd), classified as a heavy metal, is widely distributed in the earth's crust. It is commonly regarded as a pollutant of worldwide concern (1, 16). High Cd concentration in soil is more commonly found in areas containing deposits of zinc, lead and copper ores. Weathering results in the riverine transport of Cd into the oceans and represents a major flux of the global Cd cycle. Cd has been

intentionally and accidentally released into the environment by industrial and agricultural activities, causing serious environmental problems. The activities involved in the mining, production and consumption of Cd and other non-ferrous metals results in the release of significant quantities of Cd into the environment (15). In addition, the intensive application of phosphate fertilizers in the agricultural areas increased the level of Cd accumulation in soils (22, 34, 35).

Discharge of Cd into the environment causes direct and

*Corresponding Author. Mailing address: Faculty of Environment and Resource Studies, Mahidol University, Salaya, Nakhonpathom 73170, Thailand.; Tel: 662 441 5000 ext.1319 Fax: 662 441 9509 to 10.; E-mail: enbrp@mahidol.ac.th

indirect effects on microorganisms. Cd is well-known for being highly toxic to soil microorganisms even at very low concentrations. Cd can enter the cell through divalent cation uptake systems (Zn^{2+} , Ca^{2+} , Mn^{2+}) (25). The effects of Cd exposure have been investigated in many microorganisms (4, 31). Cd has been reported in field studies to be responsible for changes in species composition in microbial populations (16). However, trace amounts of some metals such as Zn, Co and Ni are essential for microbial metabolic pathways. When the concentration of these essential metals exceeds the required amount, they become highly toxic to microorganisms (23). The exposure of microorganisms to excessive concentrations of metals adversely affects their growth, morphology and biochemical activity (8, 14, 18, 30).

Microorganisms have several resistant mechanisms that can prevent heavy metal toxicity either by inducing development of tolerance or resistance. The general heavy metal resistance mechanisms are an active metal efflux, synthesis of metal-binding peptides, proteins or polysaccharides such as metallothioneins, extracellular polymeric substance (EPS) and the increasing of detoxification enzymes expression (13). Heavy metal resistance is known to occur in many bacterial genera. Bacteria use various types of resistance mechanisms in response to heavy metal toxicity.

In heavy metal contaminated sites, soil bacteria are usually exposed to heavy metals resulting in the establishment of heavy metal-resistant bacterial populations (11, 27). Heavy metals are able to induce increased resistance levels in soil bacteria and modify bacterial responses to environmental conditions either by inducing mutations or by altering physiological responses (38). Generally, exposure of bacteria to a low dose of one stress can induce a subsequent increase in resistance to the same (adaptive) or unrelated (cross protection) stress (20).

Presently, little is known regarding the protective responses of soil bacteria to Cd toxicity. These responses are an important strategy for bacterial survival in Cd-polluted environment. A better understanding of the bacterial

responses to heavy metals toxicity could be helpful for bioremediation of heavy metal contaminated sites. For this study, Cd-resistant bacteria were isolated from Cd-contaminated soils at a zinc mine and examined for Cd resistance. Consequently, we investigated the ability of Cd to induce adaptive and cross-protective responses to Cd and Zn killing in Cd-resistant bacteria.

MATERIALS AND METHODS

Soil sampling and analysis of Cd concentration in contaminated soil

Five composite soil samples were collected from the top 10 cm of soil surface horizon at zinc mine located near Maetao Creek in Tak province, Thailand. High concentrations of Cd in soil, water and sediment in this area have been previously reported by Department of Primary Industries and Mines, Ministry of Industry, Thailand in year 2005. Collected soil samples were analyzed for Cd concentrations by atomic adsorption spectrometry (AAS) as described previously (26).

Isolation and identification of Cd-resistant bacteria

Cd-resistant bacteria were counted and isolated by using a modification of the plate sensitivity assay described previously by Delaunay *et al.* (9). Briefly, serial 10-fold dilutions of soil suspensions were plated onto minimal salt medium (MSM) (31) amended with 4mM $CdCl_2$ (Fluka, Switzerland). The plates were incubated at 28°C and inspected regularly for bacterial colonies. Only colonies from the highest concentration of Cd were selected for further study based on differing colony morphology. Bacterial colonies were streaked on fresh Cd-amended MSM plate. Bacterial numbers in soil samples were enumerated using R2A agar plates (Difco, USA).

Pure culture of Cd-resistant bacterial isolate was identified by the analysis of 16S rDNA sequencing (28) using universal primers of 27f (5'-AGA GTT TGA TCC TGG CTC AG -3') and 1525r (5'-AAG GAG GTG ATC CAG CC-3').

The fragment of 16S rDNA was amplified in a Thermocycler (PE Applied Biosystems, USA). The nucleotide sequence of 16S rDNA was determined by ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with sequencing enzyme AmpliTaq DNA polymerase from Applied Biosystems (ABI, USA). The 16S rDNA sequence of selected bacterial isolate was analyzed by using BLAST program (2) from NCBI database.

Bacterial growth condition

All bacterial strains were maintained on tryptic soy agar (TSA) (Difco, USA) plates. For the experiment, all bacteria strains were aerobically cultivated in TSB at 28°C with continuous shaking at 120 rpm. Overnight cultures were inoculated into fresh TSB to give an OD₆₀₀ of about 0.1. Bacteria in the exponential phase (OD₆₀₀ about 0.5, after 4 h of growth) were used in all experiments, as indicated.

Determination of Cd-resistance levels in Cd-resistant bacteria

Analysis of the killing effects of Cd on Cd-resistant bacteria was performed by the use of inhibition zone assay (19). Briefly, exponential phase bacteria (OD₆₀₀ ~ 0.5 after growth 4 h) were mixed with 10 ml of pre-warmed (50°C) top agar (MSM containing 0.7% agar) and overlaid on the top of MSM agar plates (14 cm diameter petri-dishes and poured with 50 ml of MSM agar). The agar plates were left at room temperature for 15 min to let the top agar solidify. Five µl of 3M CdCl₂ was applied to 5-mm diameter paper discs made from Whatman filter paper and subsequently placed on the lawn of bacteria. The diameters of growth inhibition zones were measured after an overnight incubation at 28°C.

Determination of Cd-inducible responses against Cd and Zn toxicity

Cd-induced adaptive and cross-protective experiments in Cd-resistant bacterium were performed by survival curve

determination according to some modification method described by Vattanaviboon *et al.* (36) and Vattanaviboon and Mongkolsuk (37). Bacterial culture was grown in TSB at 28°C with shaking for 3 h (exponential-phase culture) and subsequently induced with 400 µM CdCl₂. This culture was grown for an additional 30 min before aliquots of cells were removed and treated with lethal concentrations of CdCl₂ (50, 100, 150 and 200 mM) and ZnCl₂ (100, 200, 300 and 400 mM) for 30 min. After treatment, the cells were removed and washed once with fresh TSB medium before determination of cell survival by plating appropriate dilutions on TSA plates. Colonies were counted after 48 h of incubation at 28°C. The percent survival was defined as the number of CFU recovered after treatment divided by the number of colony forming unit (CFU) prior to treatment multiplied by 100.

Investigation of the nascent polypeptide synthesis involved in the adaptive and cross-protective responses to Cd and Zn toxicity

To investigate Cd-induced adaptive and cross protective resistance required newly synthesized proteins, the induction experiments were repeated. However, 100 µg/ml of chloramphenicol (Cm) (20), a protein synthesis inhibitor, was added to *Ralstonia* sp. TAK1 culture prior to pretreatment with 400 µM CdCl₂. The induced and uninduced cultures were then treated with lethal concentrations of CdCl₂ or ZnCl₂ to investigate the adaptive and cross-protective resistance as mentioned above.

Statistical analysis

All experiments were independently repeated at least three times. The significance of the differences observed when comparing two strains was determined statistically using Student's t-test, one-way analysis of variance (ANOVA). The posthoc pairwise comparison with a least significant difference test at $p \leq 0.05$ was used when more than two strains were compared.

RESULTS

Cd-resistant bacteria isolated from Cd-contaminated soils

Five samples of metal-polluted soils were collected from the different locations of the zinc mine and in the vicinity of a paddy field. Cd concentrations in soil samples ranged from 36.2 to 204.1 mg.kg⁻¹ of soil (Table 1). The levels of Cd in the most of soil samples, but not in the soil sample collected from sampling site 4, were found to be above the permissible limits of soil quality standard for agricultural use in Thailand (not to exceed 37 mg.kg⁻¹ of soil) (21). The highest Cd concentration in the soil was found at sampling site 1. Cd contamination in soil even at low concentration causes a detrimental effect on the survival of soil microorganisms. The effects of Cd on microbial population in the environment have been so far lacking study. The culturable bacterial population sizes in the soil samples were determined by plating appropriate dilutions onto R2A medium. The result showed that culturable bacterial number in soil sample collected from sampling site 1 was lower than that of other soil samples (Table 1) due to the high concentration of Cd stress imposed in the soil. This result indicated that Cd toxicity adversely affected the quantity of viable soil bacteria.

Cd-resistant bacteria in Cd-contaminated soils were counted and isolated by plating soil suspensions on MSM amended with 4 mM CdCl₂. High numbers of Cd-resistant bacteria (3.5x10⁴ CFU.g⁻¹ of dry soil) were found in the soil sample collected from the sampling site 1 (Table 1) which was associated with the effect of higher concentration of Cd in soil. Significant numbers of Cd-resistant bacteria were not found in other soil samples (Sampling sites 2 to 5). This evidence suggested that differences in the number of Cd-resistant bacteria among the soils were only observed at higher levels of Cd concentration. In addition, the six isolates of Cd-resistant bacteria from culturable bacteria on Cd-amended MSM plates were chosen for further study based on their colony morphology and their potential to grow on MSM plate supplemented with 4 mM CdCl₂.

Table 1. Effects on cadmium contaminated in soils on the number of viable cells and Cd-resistant bacteria

Soil samples	Cd Conc. (mg.kg ⁻¹)	Total viable bacteria ^a (CFU.g ⁻¹ dry soil)	Cd-resistant bacteria ^b (CFU.g ⁻¹ dry soil)
1	204.1	5.2x10 ⁵	3.5x10 ⁴
2	38.6	4.3x10 ⁷	2.2x10 ³
3	48.3	2.2x10 ⁷	1.9x10 ³
4	36.2	3.9x10 ⁷	2.9x10 ³
5	78.2	1.3x10 ⁷	4.1x10 ³

^aSoil samples were plated onto R2A medium

^bSoil samples were plated onto MSM amended with 4mM CdCl₂

The levels of Cd resistance in soil bacteria

The resistance levels against Cd toxicity were determined in the exponential-phase cultures of the six bacterial isolates using the growth inhibition zone assay. The result clearly revealed that the TAK1 isolated from soil sample at sampling site 1 was highly resistant to 3M CdCl₂ toxicity (clear zone diameter 13.8 mm) as compared to other isolates. This result implied that bacteria isolated from highly Cd-contaminated soil exhibited higher Cd resistance levels than bacteria isolated from uncontaminated soil. Some Cd-resistant bacterial strains increase resistance under higher levels of Cd toxicity because Cd could induce the alteration in the bacterial response or resistance mechanisms.

From the morphological study, the TAK1 is Gram-negative and short-rod shape. Its colony on TSA plate was cream-colored, smooth, rounded-margin and butyrous or viscid. The colony diameter was 4-5 mm after incubation for 24 h. Using 16S rDNA sequencing, the Cd-resistant bacterium, the TAK1, showed 96% similarity to *Ralstonia* sp. from GenBank database accession number AB167178. Thus, the isolate TAK1 could be identified as *Ralstonia* sp.

Cd-induced adaptive and cross-protective responses to Cd and Zn toxicity and required nascent synthesized polypeptide(s)

Inducible protective responses to Cd toxicity are an important response for soil bacteria. The effect of Cd pretreatment on *Ralstonia* sp. TAK1 physiological responses to Cd killing treatment was investigated. The exponential phase cells were challenged with an inducing concentration of CdCl₂ at 400 μM for 30 min before being treatments with various concentrations of CdCl₂ at 0, 50, 100, 150 and 200 mM for 30 min. The results clearly showed that exposure to sub-lethal concentrations of Cd induced adaptive protection to a subsequent treatment with higher, normally lethal concentrations of Cd (Fig.1A). Cd-induced cells were 10-fold more resistant to 200 mM CdCl₂ than the un-induced control cells. The results indicated that challenging of *Ralstonia* sp.TAK1 cells with a sub-lethal concentration of Cd was able to induce adaptive protection to subsequent killing treatments with Cd.

Experiments were then performed to find out whether

Cd-induced cells had resistance to other metals, particularly Zn. The Cd-induced cells were treated with various concentrations of ZnCl₂ at 100, 200, 300 and 400 mM for 30 min. Interestingly, Cd-induced cells were more than 250-fold more resistant to subsequent killing treatments with 400 mM ZnCl₂ (Fig.1B). These data indicated that Cd could induce the cross-protective response to Zn toxicity. In addition, the results found that the adaptive and cross-protective responses to Cd and Zn in *Ralstonia* sp.TAK1 were eliminated by the presence of Cm in culture broth. The percentages of survival cells after treatment with high concentrations of CdCl₂ (Fig.1A) and ZnCl₂ (Fig.1B) were significantly decreased when Cm was added prior to an induction treatment. These results suggested that Cd and Zn-induced adaptive and cross-protection responses against Cd and Zn toxicity required newly synthesized protein(s).

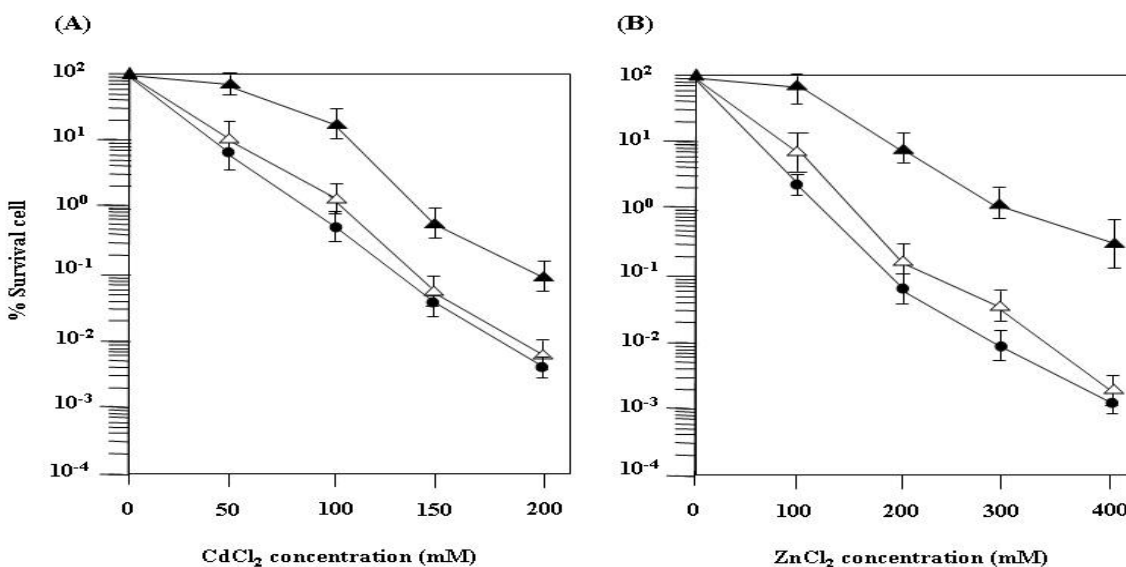


Figure 1. Cd-induced adaptive and cross-protective responses to Cd and Zn killing in *Ralstonia* sp. TAK1 and the involvement in the nascent protein(s) synthesis *Ralstonia* sp.TAK1 was grown aerobically in TSB broth. Cd induction and killing treatment with Cd and Zn were performed as described in materials and methods. The survival curves of exponential phase cultures of *Ralstonia* sp.TAK1 were pretreated with CdCl₂ as Cd-induced cells (▲), uninduced cells (△) and Cd-induced in the presence of chloramphenicol (●) and then exposed to the high concentrations of CdCl₂ (A) and ZnCl₂ (B) at indicated concentrations. The values presented are mean and standard deviation of three replicates.

DISCUSSION

High Cd contamination in soil is a characteristic feature around non-ferrous metal mines and smelters, particularly in the case of those handling zinc ores. The mining of zinc ore has further increased the extent of Cd contamination (15). In unpolluted areas, Cd concentration in surface soil has been reported to be in the range between 0.2 and 0.4 mg.kg⁻¹ of soil. However, higher Cd in soils, up to 160 mg.kg⁻¹ of soil, was occasionally found (16). Cd shows toxic effects on a wide range of microorganisms and the resulting chronic metal stress decreases the bacterial number, diversity and activity (12, 15, 31).

Cd, a non-redox-reactive heavy metal, was able to displace Zn and Fe ions in proteins resulting in inactivation and release of free ferrous ions (Fe²⁺) that can catalyze the generation of reactive oxygen species via Fenton reaction (33). Thus, Cd toxicity is also involved in oxidative damage in many microorganisms (6). However, Cd is able to induce the expression of genes and many regulons including genes directly responsible to metal transporters (3, 5, 24). The metal transporter in bacteria involved in Cd resistance mechanisms as well as Cd toxicity is also sequestered by EPS. Several investigators have reported that bacterial communities in highly metal-contaminated soil are more resistant than uncontaminated soil communities (10, 27, 31). Culturable numbers of Cd-resistant bacteria in Cd contaminated soil were higher than that in uncontaminated soil (31). The development of metal resistant populations and metal resistance levels are directly proportional to the concentrations of metal exposure (29, 30).

As stated by Castro-Silva *et al.* (7), bacteria isolated from the coal mining environment exhibited the highly resistant to Ni and Zn but they were not resist to Cd. Normally, resistance to Cd is more prevalent in Gram-negative bacteria than Gram-positive bacteria. Thus, most bacteria isolated from metal-contaminated arable soils are Gram-negative (27). *Ralstonia* sp. TAK1, a Gram-negative bacterium, showed high resistance against Cd toxicity. It was found that the

highest amount of exopolymer or EPS production was found at the stationary phase of growth (data not shown). Thus, resistance mechanism to Cd in the isolate TAK1 probably involves an EPS production. EPS is able to bind potentially toxic metals as metal-EPS complexes and is thought to be important in controlling metals distribution in the environment (13). Iyer *et al.* (17) reported that EPS produced by *Enterobacter cloacae* could adsorb heavy metals due to its metal chelating property. High resistance to various heavy metals and high metal chelating ability of bacteria could provide a potential application of either the bacterial cells or microbial components for bioremediation of metal-contaminated soils, sediments and waters.

On the other hand, the Cd-resistance mechanism in *Ralstonia* sp.TAK1 might include ion efflux pumps. In Gram-negative bacteria, CzcABC protein complex is the efflux pump system for divalent cations namely, Cd²⁺, Zn²⁺, Co²⁺ (23). CzcABC protein complex is three polypeptides membrane-bound protein complex that functions as a chemiosmotic divalent cation-proton antiporter, not an ATPase (32). In addition to CzcABC, this efflux system contains CzcD and CzcR which are required for the regulation of *czcABC* expression (23). This system mediates resistance to Cd in the metal resistant bacterium *Ralstonia metallidurans* CH34 (previously known as *Alcaligenes eutrophus*) (24).

Inducing *Ralstonia* sp.TAK1 with a sub-lethal concentration of Cd exhibited the adaptive and cross-protective responses to subsequent killing treatments with Cd and Zn, respectively. These protective responses to Cd and Zn killings in *Ralstonia* sp.TAK1 probably involve ion efflux pumps. Cd was able to stimulate the expression of *czcABC* operon that is involved in the metal efflux pump system (24). In *Xanthomonas campestris*, Cd-induced cells showed the adaptive and cross-protective responses against subsequent exposure to higher concentrations of Cd and Zn, respectively (4). In addition, it has been reported that the induced adaptive and cross-protective resistance against stresses in microbes required nascent protein synthesis (20). Cd and Zn-induced

adaptive and cross-protection responses against Cd and Zn toxicity in *Ralstonia* sp. TAK1 involved in the synthesis of newly protein(s). The requirement for nascent polypeptide synthesis implies that Cd is able to induce expression of genes involved in metal toxicity protective pathways. The synthesis of new protein(s) by Cd induction was also reported in *X. campestris* (4). The adaptive and cross-protective responses against metal toxicity are important mechanisms for bacterial survival in metal polluted environments.

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