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The Role of Exopolymers in Protection of *Ralstonia* sp., a Cadmium-resistant Bacterium, from Cadmium Toxicity

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Abstract

Production of exopolymers is one of heavy metal resistance mechanisms in bacteria. *Ralstonia* sp. TAK1, a cadmium-resistant bacterium, was isolated from a high cadmium (Cd) contaminated soil at the zinc mine, Tak province, Thailand. The bacterium was cultivated in LB broth and its growth was monitored. The yields of exopolymers were measured by the phenol-sulfuric method at different growth phases. The levels of Cd resistance were quantitatively determined by survival cell assay. The highest amount of exopolymers (0.69 mg glucose equivalent/ mg dry weight) was found at the stationary phase and sharply decreased at the late-stationary phase. In addition to high production of exopolymers at the stationary phase, *Ralstonia* sp. TAK1 was more resistant to Cd than that of exponential phase cells. These results suggested that the resistance to Cd toxicity in *Ralstonia* sp. TAK1 at the stationary phase is mediated by exopolymer production. Contradictorily, there was no correlation between Cd resistance level and exopolymer production of cells at exponential phase indicating that other mechanism(s) is responsible for Cd resistance of exponential phase cells. In addition, 0.4 mM $CdCl_2$ was able to induce the increasing of exopolymers at the mid-exponential phases compared to uninduced cells. Exopolymer production of Cd-induced cells was constant from the mid-stationary to late-stationary phase. However, the highest exopolymers was found in uninduced cells at the stationary phase.

Keywords: cadmium-resistance mechanism; exopolymers; *Ralstonia* sp.

1. Introduction

Cadmium (Cd) is commonly regarded as an environmental pollutant of worldwide concern (IPCS, 1992). Cd is highly toxic to living organisms and ecosystems even at low concentrations but effects of Cd on biological function are unknown. The toxicity effects of Cd exposure have been investigated in many microorganisms (Roane and Pepper, 2000; Banjerdkij *et al*., 2003). The exposure of microorganisms to excessive concentrations of metals adversely affects their growth, morphology and biochemical activity (Roane and Kellogg, 1996). However, microbes use various types of resistance mechanisms in response to heavy metal toxicity, either by inducing development of tolerance or resistance. The production of exopolymers or extracellular polymeric substances is one of the defensive mechanisms of bacteria that use to keep them alive when exposed to stressful environments or toxic conditions (Gadd, 2004).

Microbial exopolymers are mostly polysaccharides and are derived from algae, fungi or bacteria. Exopolymers tend to bind metal ions into complex forms resulting in metal stabilization or immobilization, thereby reducing metal toxicity and also preventing metal entry into the cells (Chen *et al*., 1995b; Maier *et al*., 2000). As stated in several previous investigations, exopolymers are effective metal-binding or metal-cheating agents (Chen *et al*., 1995b; Jensen-Spaulding *et al*., 2004; Iyer *et al*., 2005). Exopolymers have the potential of binding to metal ions in aqueous solution. It has been reported that exopolymers produced by *Bacillus firmus* were able to remove metal ions, *e.g.*, lead, copper and zinc from aqueous solutions (Salehizadeh and Shojaosadati, 2003). In addition, exopolymers are soluble in water and do not adsorb strongly to the soil matrix. Exopolymers could promote metal mobilization in the soils as carriers facilitating transport of Cd in the soils (Chen *et al*., 1995a). Exopolymers are also thought to be important in controlling metal distribution in the environment (Gadd, 2004). Due to the properties of exopolymers, the need for cost-effective and environmental friendly techniques for removal of heavy metals from wastewater and contaminated soils has directed attention to exopolymer production in microorganisms.

Ralstonia sp. TAK1, which was isolated from Cd-contaminated soil in a zinc mine at Tak Province is highly resistant to Cd toxicity (Prapagdee *et al*., 2006). The colonies characteristic of this bacterial strain on agar plate are smooth-surfaced, slimy or mucoid. It was reported that typical mucoid colonies of bacteria indicated characteristics of exopolymer producing-bacteria (Whitfield, 1998). The resistance mechanisms

that *Ralstonia* sp. uses as defense against metal toxicity are not clearly understood. Therefore, exopolymer production reflecting resistance mechanisms at different growth phase in *Ralstonia* sp. TAK1 were investigated. The correlation between exopolymer production and Cd resistance level in Cd resistant bacteria has not previously been studied. In the present study, the toxic effects of Cd on the survival of *Ralstonia* sp. TAK1 were determined, as well as the effects of Cd on the induction of exopolymer production. The findings could provide a potential application of either the bacterial cells or its exopolymers for bioremediation in metal-contaminated soils, sediments and waters.

2. Materials and methods

2.1. Bacterial growth and maintenance

Ralstonia sp. TAK1 was cultured in Luria-Bertani (LB) broth (Criterion, USA) with continuous shaking (150 rpm) at 28 ^{o}C. This bacterium was maintained on LB agar plate and kept at 4 ^{o}C for short-term storage. For long term storage of bacterial strain, fresh colonies were inoculated into LB broth with agitation until exponential growth phase. Subsequently, 800 µl of the culture was mixed with 400 µl of 45% sterile glycerol (15% glycerol of final concentration) and kept in -70 ^{o}C deep freezer.

2.2. Quantitative determination of exopolymer production

For exopolymers production, *Ralstonia* sp. TAK1 was cultivated in LB broth at 28°C with continuous shaking at 150 rpm for 72 hr. Samples of the fermentation broth were collected at 4, 8, 12, 24, 48 and 72 hr, respectively. Bacterial exopolymers were extracted by heating in a water bath at 100 °C for 15-20 minutes. Cells were completely separated from medium by centrifugation at 9000 rpm, 4 °C for 30 min. Cell pellets were dried and weighed. Subsequently, an equal volume of ice-cold ethanol was added to the supernatant for exopolymer precipitation (Kunito *et al*., 2001). Extracted bacterial exopolymers were analyzed by the phenol-sulfuric method (Dubois *et al*., 1956). The absorbance of each sample was measured in a spectrophotometer wavelength 490 nm. The amounts of exopolymers were calculated in glucose equivalents from a glucose standard curve.

2.3. Determination of cadmium resistance level

Growth phase resistance to Cd in *Ralstonia* sp. TAK1 was quantitatively determined by survival cell assay using a modification of the method described by Prapagdee *et al*. (2004). *Ralstonia* sp. TAK1 was grown aerobically in LB broth at 28 ^{o}C. Mid-exponential phase (OD_{600} of 0.5, after 4 hr of growth), late-exponential phase (OD_{600} of 1.5, after 8 hr of growth), mid-stationary phase (OD_{600} of 5, after 24 hr of growth) and late-stationary phase (OD_{600} of 5, after 32 and 48 hr of growth) cells were used in Cd treatments. Aliquots of cells from the cultures were treated with 10 mM of $CdCl_2$ for 30 min at 28 ^{o}C. Untreated cells added to an equal volume of sterile LB broth instead of $CdCl_2$ served as controls. The cells were collected by centrifugation at 6,000 rpm for 5 min at room temperature. Cell pellets were washed once with fresh LB broth and then diluted in a 10-fold series of dilutions in LB broth. Appropriate dilutions were plated on LB agar plates and incubated at 28 ^{o}C for 24-48 hr. Colonies on agar plates were counted and surviving cells were calculated. The percentage of surviving cells is defined as the number of colony forming units (CFU) recovered after treatment divided by the number of CFU prior to treatment and multiplied by 100. The survival curves were determined by plotting the percentages of survival cells versus the time of cultivation

2.4. Evaluation of the induction of exopolymer production by cadmium

Ralstonia sp. TAK1 was cultivated in LB broth containing with various concentrations of $CdCl_2$ to give a final concentration of 0.2, 0.4 and 1.0 mM, respectively. Cells were aerobically grown at 28 ^{o}C in an incubator shaker (150 rpm) for 2 days. Exopolymer production at different growth phases was determined according to the method as mentioned above.

2.5. Statistical analysis

The means and standard deviations of the exopolymer production and survival cells of bacteria were calculated. Data were statistically analyzed by using one-way analysis of variance (ANOVA). The post hoc pairwise comparison with a Least Significant Difference (LSD) test was used when more than two treatments were compared. In either case, a value of $P<0.05$ was considered significant. All experiments were independently repeated at least three times with standard errors represented as bars.

3. Results and Discussion

3.1. Production of exopolymers at different growth phases

Exopolymers are mostly anionic charged, thus they can bind cationic metal ions outside the cells (Bruin *et*

Figure 1. Slimy conoly of *Ralstonia* sp. TAK1 on LB agar plate after 24 h of cultivation at 28 °C.

al., 2000). Exopolymers act as an efficient barrier preventing metal ions from entering the cells and protecting the cells from metal toxicity (Maier *et al*., 2000). Several metal resistant bacteria have been reported to be the potent exopolymer producers (Gadd, 2004). A typical colony of *Ralstonia* sp. TAK1 on agar plate is smooth surfaced and slimy (Fig. 1). Exopolymers production was low during the exponential phase (0.12 mg glucose equivalent/ mg dry weight) and sharply increased when cells entered the stationary phase of growth (Fig. 2). The amount of exopolymers peaked at the mid-stationary phase (24 hr) (0.69 mg glucose equivalent/ mg dry weight) and then declined when cells entered the late-stationary phase (48 hr) (0.24 mg glucose equivalent/ mg dry weight).

Exopolymer production in *Pseudomonas atlantica* peaked during the stationary phase (Uhlinger and White, 1983). In contrast to the study of Mian *et al*. (1978), they found that maximum rate of exopolymer production of *Pseudomonas aeruginosa* in batch culture occurred during exponential growth. Moreover, Sheng and Yu (2006) found that total exopolymer was increased rapidly in the exponential phase, but then stayed almost unchanged during the stationary phase. During the stationary phase, *R. acidophila* produced less exopolymer than that during the exponential phase, indicating that exopolymer production was closely associated with the cell growth. However, exopolymer production of microorganisms is under nutritional control and depends on the C:N ratio during cultural conditions (Singh *et al*., 1999). Exopolymers were produced at a lower rate under carbon limitation conditions (Mian *et al*., 1978). Moreover, the types of carbon source affected the yield of exopolymers. *Azotobacter* sp. AC2 produced the highest proportion of exopolymers when it was cultured in medium containing sucrose (Emtiazi *et al*., 2004). The highest yield of exopolymers in *P. atlantica* was obtained when used galactose as the major carbon source (Uhlinger and White, 1983).

3.2. Correlation between exopolymer production and cadmium resistance level

The finding that *Ralstonia* sp. TAK1 produced a high proportion of exopolymers prompted us to investigate the involvement of exopolymer production on the protection of bacterial cells from Cd toxicity. To test this, *Ralstonia* sp. TAK1 was cultured in LB broth and collected at 4, 8, 24, 32 and 48 hr, respectively. Exopolymer production and resistance to 10 mM $CdCl_2$ during different growth phases were determined. The results demonstrated that *Ralstonia* sp. TAK1 produced a high exopolymers during the stationary phase (Fig. 3). During the mid-stationary phase, the cells became more resistant to 10 mM $CdCl_2$ than during other phases of growth. These data indicate that exopolymer production in *Ralstonia* sp. TAK1 was responsible for protection cells from Cd toxicity. As stated by Guibaud *et al*. (2003), the

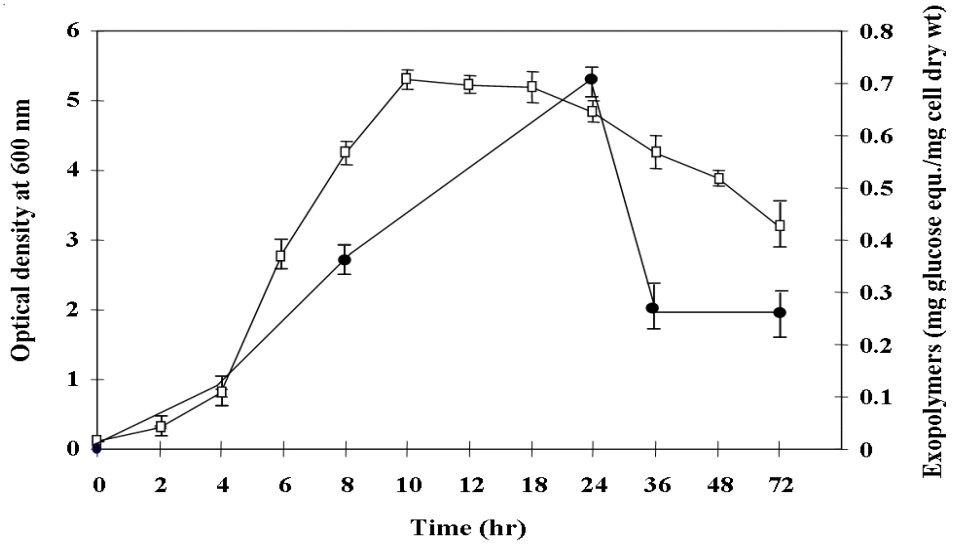

Figure 2. Exopolymer production (●) of *Ralstonia* sp. TAK1 at various growth phases (□) cultivated in LB broth at 28 °C. (means ± SEM; n=3).

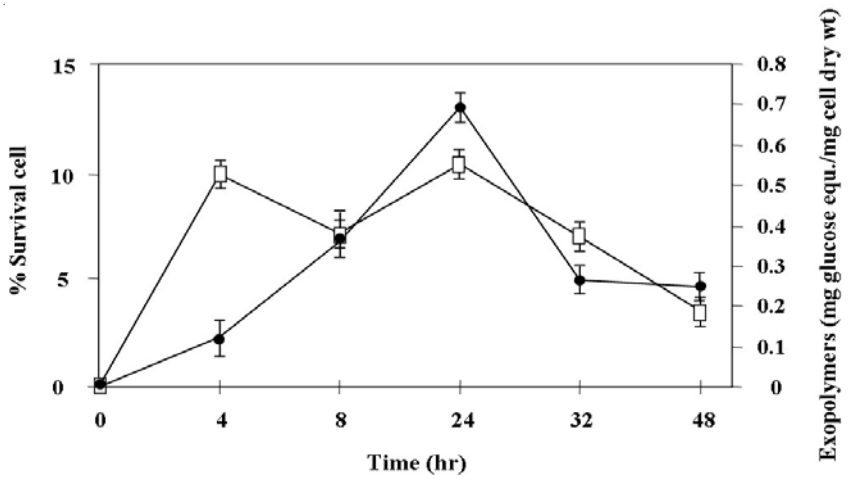

Figure 3. Relationship between exopolymer production (●) and the survival of in *Ralstonia* sp. TAK1 after exposure to 10 mM of $CdCl_2$ (□). (means ± SEM; n=3).

potential of exopolymers to detoxify copper depended on amount and the chemical composition of exopolymers. Type of exopolymers measured in *Ralstonia* sp. TAK1 was monosaccharide only in the form of glucose equivalence. The monosaccharides mostly found in extracellular polysaccharides were glucose and galactose. Extracellular polysaccharides produced by *Lactobacillus sake* 0-1 contained glucose and rhamnose (Van Den Berg *et al*., 1995).

In contrast, there was no correlation between Cd resistance level and exopolymer production during the exponential phase cells of *Ralstonia* sp. TAK1 (Fig.3). Although *Ralstonia* sp. TAK1 produced low amounts of exopolymers during the mid-exponential phase, the mid-exponential phase cells were more resistant to 10 mM $CdCl_2$ than the late-exponential phase cells. These results indicated that other resistance mechanisms play a protective role against Cd toxicity at mid-exponential phase. Cd is able to induce expression of genes and many regulons including genes directly responsible to metal transporters by efflux systems (Nies, 1995; Anton *et al*., 1999; Binet and Poole, 2000). The function of most resistance systems is based on the energy-dependent efflux of toxic ions. Thus, Cd resistance mechanisms in *Ralstonia* sp.TAK1 during the exponential phase might involve ion efflux pumps. Resistance to Cd toxicity of *Ralstonia metallidurans* CH34, a metal resistant bacterium, was mediated by CzcABC protein complex (Nies, 1995), which is the efflux pump system for divalent cations including Cd^{2+}, Zn^{2+} and Co^{2+} (Nies, 1992). Nevertheless, the exact resistance mechanisms of exponential phase cells of *Ralstonia* sp. TAK1 responsible for the Cd detoxification process remain to be investigated.

3.3. Cadmium-inducible for exopolymer production

The results of our analysis of *Ralstonia* sp. TAK1 clearly indicated that exopolymers were involved in the protection of the mid-stationary phase cells of *Ralstonia* sp. TAK1 from Cd toxicity. It has been reported that mixed-species of sulfate-reducing bacteria produced a high amount of extracellular protein and carbohydrate after exposure to Cd (White and Gadd, 1998). Therefore, the effect of Cd concentrations on the induction of exopolymer production in *Ralstonia* sp. TAK1 was evaluated. *Ralstonia* sp. TAK1 was cultivated in LB broth amended with 0.2, 0.4, or 1.0 mM $CdCl_2$. Fig. 4 illustrates the influence of different Cd concentrations on exopolymer production. The results showed that cells during the mid-exponential phase (4 hr) in LB broth, supplemented with 0.2 or 0.4 mM $CdCl_2$, produced more exopolymers than the mid-exponential phase cells, when cultured in normal LB broth. The production of exopolymers in cells induced with 0.2 and 0.4 mM of $CdCl_2$ were 0.20 and 0.28 mg glucose equivalent/mg cell dry weight, respectively. Meanwhile, exopolymer production by uninduced cells (0 mM $CdCl_2$) during the exponential phase was 0.12 mg glucose equivalent/mg cell dry weight. Optimal concentration of $CdCl_2$ for induction of exopolymer production was 0.4 mM, while the lower concentration of $CdCl_2$ (0.2 mM) slightly induced exopolymer production during the mid-exponential phase. Singh *et al*. (1999) reported that exopolymer production of *Nostoc spongiaeforme* was induced by low concentrations of nickel. Chao and Cheng (1991) found that amount of exopolymers in copper resistant bacteria, *Escherichia coli*, was significantly increased when it was grown in media amended with copper after incubation for 5.5 hr compared to control. Similar to the study of Kunito *et al*. (2001), they suggested that the addition of 0.5 mM copper to the nutrient broth could induce exopolymer production in copper resistant bacteria.

However, all tested concentrations of Cd were not able to induce the increasing of exopolymer production in *Ralstonia* sp. TAK1 during the late-

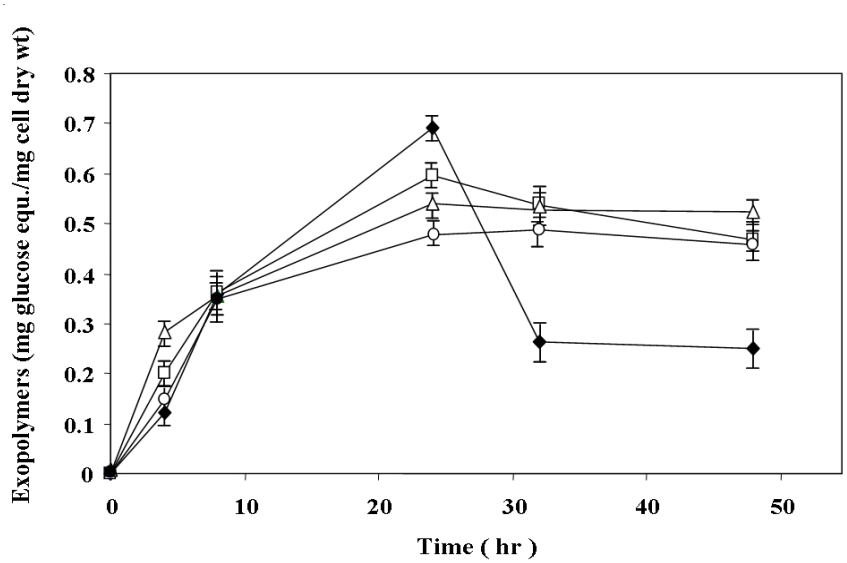

Figure 4. Effect of Cd concentrations on the induction of exopolymer production in *Ralstonia* sp. TAK1; 0 mM (◆), 0.2 mM (□), 0.4 mM (△) and 1.0 mM (○). (means ± SEM; n=3).

exponential (8 hr) and mid-stationary (24 hr) phases (Fig. 4). The amount of exopolymers in Cd- induced cells during the late-exponential and mid-stationary phases was lower than that of uninduced cells but was constant from the mid-stationary to late-stationary phase. Meanwhile, the highest exopolymer production was found during the mid-stationary phase of uninduced cells. Moreover, there was no increase in exopolymer production during the exponential phase of cells, induced with 1 mM $CdCl_2$. Additionally, exopolymer production decreased when cells were grown in LB broth amended with 2 mM $CdCl_2$ because of high toxicity of $CdCl_2$ (Data not shown). Failure of *Nostoc spongiaeforme* to produce exopolymers was previously observed when cells were induced by 20 mM nickel due to its toxic concentration (Singh *et al*. 1999).

4. Conclusion

Exopolymers are able to bind potentially toxic metals thereby reducing their toxicity and preventing metal entry into the cells. The highest exopolymer production in *Ralstonia* sp. TAK1 was observed when cells entered the stationary phase. In contrast, the mid-exponential phase cells were more resistant to Cd than cells during the late-exponential phase, although cells during the mid-exponential phase produced less exopolymers than the mid-stationary phase cells. It is concluded that exopolymers play a role in the protection of stationary phase cells of *Ralstonia* sp. TAK1 from Cd toxicity. Exopolymers were not responsible for the increased Cd resistance of exponential phase cells of *Ralstonia* sp. TAK1. Low concentrations of Cd were able to induce the increase of exopolymers production during the mid-exponential phase. However, exopolymer production in Cd-induced cells during the late-exponential and mid-stationary phase was lower than that of uninduced cells. Cd-induced exopolymer production in *Ralstonia* sp. TAK1 is 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-contaminated soils.

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