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Toxicity Decrease of Cadmium by the Pigment Produced by *Azomonas agilis* PY101 in the Culture of Bacterial Cells and Vero Cells

Kyung Man You, Soo-Youn Lee[1] and Yong Keun Park[1,*]

Department of Entomology, Cornell University, NYSAES, Geneva, NY 14456, USA
[1]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Abstract - The morphological patterns and the cytopathogenicity time of the Vero cells induced by free Cd^{2+} and pigment-bound Cd^{2+} were observed by inverted microscope in order to investigate the difference of cadmium toxicity. The Vero cells induced by free Cd^{2+} of 0.1 mM were shown to have a fatal toxic effect and the cytopathogenicity could be seen early after 6±2 hours of incubation. Partially affected cells induced by pigment-bound Cd^{2+} of 0.1 mM were shown and the cytopathogenicity could be seen after 20 hours of incubation. The Vero cells grown with free 0.001 mM Cd^{2+} were also affected and the cytopathogenicity could be seen after 17 hours of incubation, whereas the Vero cells grown with 0.001 mM pigment-bound Cd^{2+} were unaffected. The sensitivity of *Escherichia coli* DH5α bacterial cells was also examined after a short treatment with free Cd^{2+} or pigment-bound Cd^{2+}. About 5% of cells survived after 0.01 mM of free Cd^{2+} treatment, while about 68% of cells survived after 0.01 mM of pigment-bound Cd^{2+}.

Key words : Vero cells, *Azomonas agilis*, pigment, cadmium, toxicity

Introduction

Cadmium is a nonessential heavy metal used extensively in industry for a variety of applications, including electroplating, protection against corrosion, and stabilizing plastic. Over the last century, its increased industrial use has led to cadmium contamination in the environment, plants, animals, food product, and humans, and exhibits deleterious influences on organisms.

The toxic effects of cadmium on microorganisms are well documented (Babich and Stotzky 1977; Duxbury 1981; Mitra and Bernstein 1978) and derive from several mechanisms (Beveridge and Doyle 1989). Disruption of essential protein function can occur through binding of cadmium to sulfhydryl groups (-SH) (Aiking *et al.* 1982). This potent toxic effects can result in prolonged lag phase, decreased growth rate, lower cell density, or death for bacteria at levels below 1 ppm of cadmium (112.4 ppm = 1 mM) (Aiking *et al.* 1982; Mitra and Bernstein 1978; Shapiro and Keasling 1996). Some bacteria contain cadmium resistance determinants and are thus less susceptible to the toxic effects (Trevors *et al.* 1986). A common plasmid-encoded mechanism employs cadmium efflux pumps, which specifically capture and eject undesirable cadmium ion (Cd^{2+}) through the cell membrane (Chopra 1975). Alternatively, cadmium can be sequestered by binding to detoxifying proteins (Denise and Peter 1984), insoluble sulfides (Aiking *et al.* 1982; Daryl and Lundie 1993), or insoluble phosphate (Aiking *et al.* 1984).

Azomonas agilis PY101 was isolated from samples collected in 1994 from a Anyang stream of Gyeonggi-Do, Korea and able to grow in toxic concentration of

* Corresponding author: Yong Keun Park, Tel: +82(2) 3290-3422, Fax: +82(2) 927-9028, E-mail. ykpark@korea.ac.kr

cadmium (You *et al.* 1996). This microorganism produces specific pigment induced by Cd^{2+} and converts free Cd^{2+} into pigment-bound Cd^{2+} (You and Park 1998).

It is described here that the pigment of *A. agilis* PY101 that is able to detoxify cadmium in microplate culture of Vero cells and in culture of bacterial cells.

Materlals and Methods

1. Vero cell culture

Cell line maintained by serial passages in Eagle's Minimal Essential Medium (MEM) with 10% (v/v) fetal bovine serum. Single cell suspensions were obtained from confluent monolayers of Vero cells by treatment with a solution of 0.1% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate-buffered saline, pH 7.2. The released cells were adjusted to a final concentration of 1.0×10^4 cells per well in 1% Eagle's MEM.

2. Free Cd^{2+} and pigment-bound Cd^{2+} preparation

The cadmium chloride ($CdCl_2$) solution was used as a free Cd^{2+} source. Purified pigment-bound Cd^{2+} was obtained by FPLC using a column HR10/30 of Superdex 75 as previously described (You and Park 1998). The concentration of free Cd^{2+} and Cd^{2+} bound to this pigment was measured by atomic absorption spectrophotometry (Varian AA-1495) with a cadmium lamp. Dilutions of $CdCl_2$ prepared with sterilized distilled water and treated like the experimental sample were used as standards.

3. Procedure for free Cd^{2+} and pigment-bound Cd^{2+} titration

Free Cd^{2+} and pigment-bound Cd^{2+} could be added to the Vero cells at the time of seeding. In order to standardize the number of cells at the time of addition of free Cd^{2+}/pigment-bound Cd^{2+}, the addition of freshly trypsinized cells to free Cd^{2+} and pigment-bound Cd^{2+} diluted directly in the culture microplate was chosen. Use of 1.0×10^4 cells per well gave consistent and easily observable end-point titres after 24 hours of incubation and was therefore chosen for free Cd^{2+} and pigment-bound Cd^{2+} titrations.

Free Cd^{2+} and pigment-bound Cd^{2+} preparations were diluted in 1% Eagle's MEM in the 96-well culture microplate (NUNC 16 7008, Roskilde, Denmark). Ten thousand freshly trypsinized Vero cells in 0.1 ml of culture medium were then added to each well. After mixing, the plate was incubated at 37°C for 24 hours in an incubator (CF Autoflow CO_2 incubator, NU-1700) with an atmosphere of 5% (v/v) CO_2 in air. The morphological patterns were observed directly using an inverted microscope (ZEISS Germany, Axiovert 135) without staining of the cells.

4. Cadmium sensitivity of bacteria cells

10 ml of Luria-Bertani (LB) medium was inoculated into 50 ml culture bottle. By overnight culture at 37°C, *Escherichia coli* DH5α bacterial cells were grown until an OD_{600} of 0.6 was reached. The culture was taken and plated on LB agar with serial dilution. To the diluted culture, free Cd^{2+} and pigment-bound Cd^{2+} toxin (0.1 mM or 0.001 mM) were added and the mixture was incubated with agitation for 15 min. Cells were plated on LB agar, and colonies were counted after 24 hours of incubation. Survival rates were calculated from the number of colonies present before and after free Cd^{2+} or pigment-bound Cd^{2+} toxin treatment.

Results and Discussion

1. Effects of free Cd^{2+} and pigment-bound Cd^{2+} toxin on the morphology of Vero cells

The morphological patterns and the cytopathogenicity time observed in microplate with free Cd^{2+} and pigment-bound Cd^{2+} are shown in Fig. 1 and Table 1.

Plate A and B are control of Vero cells that resulted in confluent monolayer. Fatal toxic effect of Vero cells resulted by 0.1 mM free Cd^{2+} are shown in plate C. The cytopathogenicity in plate C could be seen after 6 ± 2 hours of incubation. However, a partially affected cells induced by 0.1 mM pigment-bound Cd^{2+} are shown in plate D. The cytopathogenicity in plate D could be seen lately after 20 hours of incubation. The Vero cells grown

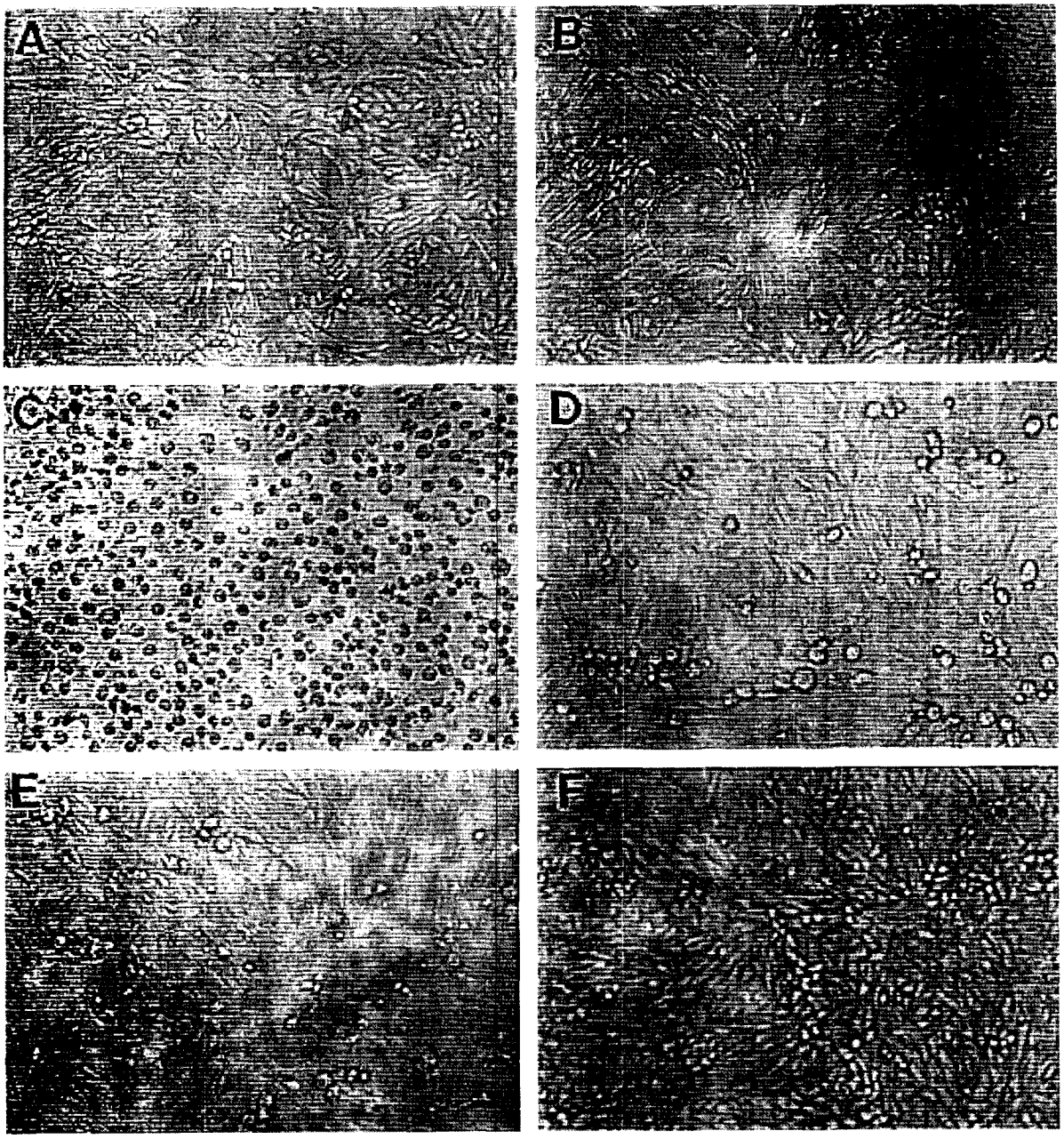

Fig. 1. Morphological patterns of Vero cells in microplate titrated with free Cd^{2+} and pigment-bound Cd^{2+} after 24 hours incubation. A and B, Control Vero cells. C, free Cd^{2+} (0.1 mM). D, pigment-bound Cd^{2+} (0.1 mM). E, free Cd^{2+} (0.001 mM). F, pigment-bound Cd^{2+} (0.001 mM). Magnification, ×250.

with 0.001 mM free Cd^{2+} were also affected and the cytopathogenicity could be seen in several parts of plate E after 17 hours of incubation. Further incubation of affected cells by 0.001 mM free Cd^{2+} resulted in cell death. The Vero cells grown with 0.001 mM pigment-bound Cd^{2+} were unaffected and normal cells could be seen in all parts of plate F. It is likely that the pigment of *A. agilis* PY101 converted Cd^{2+} from more toxic into less toxic states.

2. Effects of free Cd^{2+} and pigment-bound Cd^{2+} toxin on cadmium-sensitive bacteria cells

The sensitivity of *E. coli* DH5α bacterial cells was also

Table 1. Cytopathogenicity time of Vero cells induced by free Cd^{2+} or pigment-bound Cd^{2+} toxin

Plate	Condition	Cytopathogenicity time (h)[a]
A and B	Control	–
C	Free Cd^{2+} 0.1 mM	6±2
D	Pigment-bound Cd^{2+} 0.1 mM	20
E	Free Cd^{2+} 0.001 mM	17
F	Pigment-bound Cd^{2+} 0.001 mM	–

[a] The cytopathogenicity was not seen in A and B, F plate and cytopathogenicity time were counted as described in Materials and Methods.

Table 2. Effects of free Cd^{2+} and pigment-bound Cd^{2+} toxin on cadmium-sensitive bacterial cells

Strain [a]	Condition	Colony count[b]
E. coli DH5α	Control	19,750
	Free Cd^{2+} 0.01 mM	993
	Pigment-bound Cd^{2+} 0.01 mM	13, 429

[a] *E. coli* DH5α, a cadmium-sensitive control bacteria cells.
[b] Cells were treated with free Cd^{2+} or pigment-bound Cd^{2+} toxin (0.1 mM or 0.001 mM) for 15 min, and surviving cells were counted as described in Materials and Methods. Numbers are CFU normalized to 10^6 cadmium-treated cells. Values are the averages of results from three independent experiments.

examined after a short treatment with free Cd^{2+} or pigment-bound Cd^{2+}. The bacterial cells cultivated in LB broth to log phase were treated with 0.01 mM of free Cd^{2+} or pigment-bound Cd^{2+}, respectively. The treated cells were serially diluted and plated on LB agar and the colonies that appeared were counted. For *E. coli* DH5α, about 5% of cells survived after 0.01 mM of free Cd^{2+} treatment, while about 68% of cells survived after 0.01 mM of pigment-bound Cd^{2+} (Table 2).

These results seemed to suggest a specific role of the pigment as a defense reserve against toxic cadmium in bacterial cells as well as Vero cells.

It is supposed that formation of pigment-bound Cd^{2+} is probably the most important mechanism of detoxification in this strain. Cadmium detoxification in *P. putida* was accomplished by production of three cadmium-binding proteins induced during different growth phase (Denise and Peter 1984). *K. aerogenesi* was achieved by insoluble cadmium sulfide formation at the cell surface (Aiking *et al.* 1982) or by insoluble phosphate within the cell (Aiking *et al.* 1984). The bacterial cadmium-binding pigment added important information to our knowledge of cadmium detoxification in bacteria.

We therefore purpose that the pigment produced by *A. agilis* PY101 promotes the decrease of Cd^{2+}-induced toxicity from Cd^{2+}-rich environment. Further study of this bacterial system may provide a useful insight into the relationship between toxic cadmium and metabolism of the pigment.

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