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Characterization of *Azomonas agilis* PY101, a Cadmium-Resistant Strain Isolated from Anyang Stream

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A cadmium-resistant strain isolated from Anyang stream, *Azomonas agilis* PY101 exhibited strong resistance to 1000ppm of cadmium ion(Cd^{2+}). *A. agilis* PY101 also exhibited resistance to various antibiotics, such as amoxicillin, ampicillin, bacitracin, cefazolin, erythromycin, penicillin, tetracycline, and vancomycin. In the presence of Cd^{2+}, the growth of *A. agilis* PY101 started after an extended lag phase and produced a green-fluorescent pigment induced by cadmium. The dramatic decrease(approximately 400ppm) of concentration of cd^{2+} in the culture medium during the growth phase of *A. agilis* PY101 was confirmed by the inductively coupled plasma-atomic emission spectrophotometer. Transmission electron microscopic analysis revealed that *A. agilis* PY 101 actively accumulated Cd^{2+} in the cytoplasm.

Key words: cadmium-resistant strain, *Azomonas agilis* PY101, green-fluorescent pigment.

Cadmium is a heavy metal used extensively in industry for various applications, including electroplating, protection against corrosion, and stabilizing plastic. As a result of industrial release of cadmium into the environment, it ranks as a major pollutant (1, 3). Cadmium is toxic to microbial and other life forms (15). The toxic effect of cadmium ion(Cd^{2+}) is caused by the binding of the ions to thiol groups(-SH) in essential proteins (6). In spite of this toxic effect, microorganisms show resistance and adapt to the presence of cadmium (19). As a result, new acquired phenotypes are inherited in microbial communities under the influence of cadmium (8, 18).

Bacterial resistance to toxic cadmium is known to be based on a small number of basic mechanisms (1). First, there is the possibility of sequestration and binding of toxic cadmium either in the cell wall or to highly specific intracellular components, such as metallothionein (4). Metallothioneins have been isolated from diverse organisms including mammals, yeast, algae, and fungi (10). *Pseudomonas putida* actively accumulates cadmium from the medium, and the resistance mechanism involves both polyphosphate and a series of low molecular weight cysteine-rich cadmium proteins induced during different growth phases (4). Second, there is the possibility of blocking cellular uptake by altering the uptake pathway available in sensitive cells. This system has been demonstrated in *Bacillus subtilis* 168 (12). Third, once the toxic mercury has reached the intracellular cytoplasm, it can be pumped out again rapidly by a highly specific efflux system (17). Cadmium resistance mechanisms in gram-positive bacterium are known in *Staphylococcus aureus* (11, 16). Cadmium resistance in gram-negative *Alcaligenes eutrophus* is effected by a plasmid-borne efflux system, the *czc* system. It confers resistance to cobalt, zinc, and cadmium (5).

The aim of the present study was to determine the basis of cadmium resistance mechanism in *Azomonas agilis*. In this paper, we present the characterization of cadmium resistance in *A. agilis* PY101.

Materials and Methods

Bacterial strain

Sample was taken from Anyangstream in Kyonggido and plated on LB agar containing 500 ppm of cadmium chloride($CdCl_2$). The plate was incubated at 30°C for 2 days. Six successive streaking from single colony were done to ensure the purity and to confirm the cadmium-

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resistance of the strain. The isolated strain was identified as *A. agilis* according to the VITEK system (McDonald Douglas Health System Company) and the Bergey's Manual of Systematic Bacteriology (9).

Media

The Mueller Hinton Broth(MHB) media were used also for antibiotic resistance, cadmium resistance, and bacterial growth. The Heart Infusion Broth(HIB) media were used for liquid culture of *A. agilis* PY101. $CdCl_2$ was sterilized by the membrane filteration and added to the autoclaved media. The pH value of these media was adjusted to 7.0 with 1N NaOH or 1N HCl before autoclaving. The growth was assessed by the OD_{600nm} and the viability counted on MHB agar.

Resistance to antibiotics

The antibiotic patterns of a cadmium-resistant strain were measured by 19 antibiotic discs(BBL Microbiology systems, Cockeysville, Md.). *A. agilis* PY101 was tested for resistance to amikacin(30 μg/ml), amoxicillin(30 μg/ml), ampicillin(30 μg/ml), bacitracin(10 μg/ml), cefazolin(30 μg/ml), cefoperazone(75 μg/ml), chroramphenicol(30 μg/ml), colistin(10 μg/ml), doxycycline(30 μg/ml), erythromycin(15 μg/ml), gentamycin(10 μg/ml), imipenem(10 μg/ml), kanamycin(30 μg/ml), neomycin(30 μg/ml), penicillin(10 μg/ml), tetracycline(30 μg/ml), ticarcillin(75 μg/ml), tobramycin(10 μg/ml), and vancomycin(30 μg/ml).

Cadmium analysis

The cadmium-resistant strain cultured in HIB supplemented with 1000 ppm of $CdCl_2$ at 30°C was sampled according to optical density(0, 0.3, 0.6, 0.9, and 1.2 at OD_{600nm}) and then centrifuged at 15,000 rpm for 10min. The concentration of $CdCl_2$ in supernatants was measured with the inductively coupled plasma-atomic emission spectrophotometer(ICP-AES, Perkin Elmer Plasma 40) at wavelength scan.

Growth of the cadmium-resistant strain

The cadmium-resistant strain was grown in 100 ml of HIB containing 1000 ppm of $CdCl_2$ in 250 ml shake flask. This flask was incubated in a rotary shaker(VS-8480SR, VISION SCIENTIFIC CO., LTD.) at 30°C and 200 rpm. Growth was assessed by the OD_{600nm}. Cells described as control were in HIB without $CdCl_2$, whereas other cells were cultured in HIB supplemented with $CdCl_2$(0.1~1.0 mg/ml).

Electron microscopy

Electron microscopy was performed by the Electron Microscope Laboratory at Korea University. Samples were examined in an JEM-100C(X) transmission electron microscope. The analyzed cultures were control culture without cadmium and induced culture with cadmium.

Results and Discussion

Resistance to antibiotics

The genus *Azomonas* can be seperated from *Azotobacter* by lack of cyst formation. They occur in soil and water. *A agilis* PY101, isolated from factory wastewater of Anyang stream, was able to survive in the presence of cadmium. A cadmium-resistant strain, *A. agilis* PY101 exhibited resistance to 1000 ppm of Cd^{2+}. The antibiotic patterns of cadmium-resistant *A. agilis* PY101 were measured b ml of amoxicillin, 30 μg/ml of ampicillin, 10 μg/ml of bacitracin, 30 μg/ml of cefazolin 15 μg/ml of erythromycin, 10 μg/ml of penicillin, 30 μg/ml of tetracycline, and 30 μg/ml of vancomycin. *A. agilis* PY101 were sensitive to 30 μg/ml of amikacin, 75 μg/ml of cefoperazone, 30 μg/ml of chroramphenicol, 10 μg/ml of colistin, 10 μg/ml of gentamycin, 10 μg/ml of imipenem, 30 μg/ml of kanamycin, 30 μg/ml of neomycin, 75 μg/ml of ticarcillin, and 10 μg/ml of tobramycin. *A. agilis* PY101 also had a slight sensitivity to 30 μg/ml of doxycycline(Table 1). Cadmium resistance determinants have been found both on plamids (11, 21) and chromosomally (4, 17). The lack of plasmid in *A. agilis* PY

Table 1. Antibiotic resistance patterns of *A. agilis* PY101

Antibiotics	Conc. of antibiotic[a](μg/ml)	Diameter of clear zone[b]	Resistance patterns
Amikacin	30	23.0	S
Amoxicillin	30	0	R
Ampicillin	30	0	R
Bacitracin	10	0	R
Cefazolin	30	0	R
Cefoperazone	75	21.0	S
Chroramphenicol	30	20.5	S
Colistin	10	12.5	S
Doxycycline	30	8.0	±
Erythromycin	15	0	R
Gentamycin	10	18.0	S
Imipenem	10	23.0	S
Kanamycin	30	15.5	S
Neomycin	30	18.5	S
Penicillin	10	0	R
Tetracycline	30	0	R
Ticarcillin	75	24.5	S
Tobramycin	10	23.5	S
Vancomycin	30	0	R

[a]Sensi-Discs were employed to determine resistance to the antibiotics.

[b]Clear zone size(mm).

S, sensitive; R, resistant; ±, very slightly sensitive.

101 suggested that cadmium resistance in the strain was chromosomally encoded.

Growth of *A. agilis* PY101 in the presence of cadmium

The cadmium-resistant *A. agilis* PY101 was incubated at 30°C with HIB supplemented with various concentrations of $CdCl_2$. In higher cadmium concentration, the growth of *A. agilis* PY101 was slower and the lag phase of this organism increased significantly(Table 2). As a result, *A agilis* PY101 in the presence of 1000 ppm of Cd^{2+} grew after an extended lag phase (18h). Mitra and Bernstein (14) observed that cadmium produced considerable single-strand breaks in the DNA of *Escherichia coli* and suggested that the extended lag phase was due to the time required for the induction of DNA repair mechanisms. We assume it's due to the inducible production of essential protein for DNA repair or cadmium-binding materials such as metallothionein in *A. agilis* PY 101 which prevent cadmium toxicity by sequestration of the cadmium once it enters the cell.

Table 2. Growth of *A. agilis* PY101 in the presence of cadmium

Cadmium[a] (mg/ml)	Length of lag phase(h)	Relative growth[b] (% of control)
0(control)	1	100
0.1	4	57
0.2	7	38
0.3	8	34
0.4	9	31
0.5	9	24
0.6	10	23
0.7	11	21
0.8	13	19
0.9	15	17
1.0	18	14

[a]Cells were incubated at 30°C with HIB supplemented various concentration of $CdCl_2$.

[b]Relative growth rate were determined by measuring the optical density (OD_{600nm}; SPECTRONIC 20, Milton Roy Company) of culture medium.

Green-fluorescent pigment induced by cadmium in *A. agilis* PY101

Pigments are produced by a wide range of microorganisms. *A. agilis* PY101 produce a green-fluorescent pigment, when grown under high concentration of Cd^{2+} condition(Fig. 1). This pigment was water-soluble and

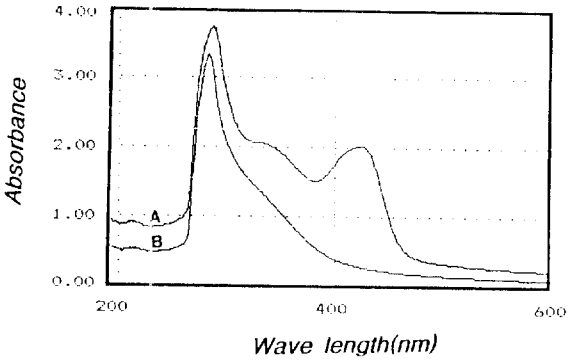


Fig. 2. UV and Visible spectra of green-fluorescent pigment. The absorbance of the fluorescence was measured from 380 nm to 450 nm. Distilled water was used as blank and the absorbance of component of MHB was measured from 260 nm to 370 nm. A, MHB containing pigment with 1000 ppm of $CdCl_2$; B, MHB(control) with 1000 ppm of $CdCl_2$.

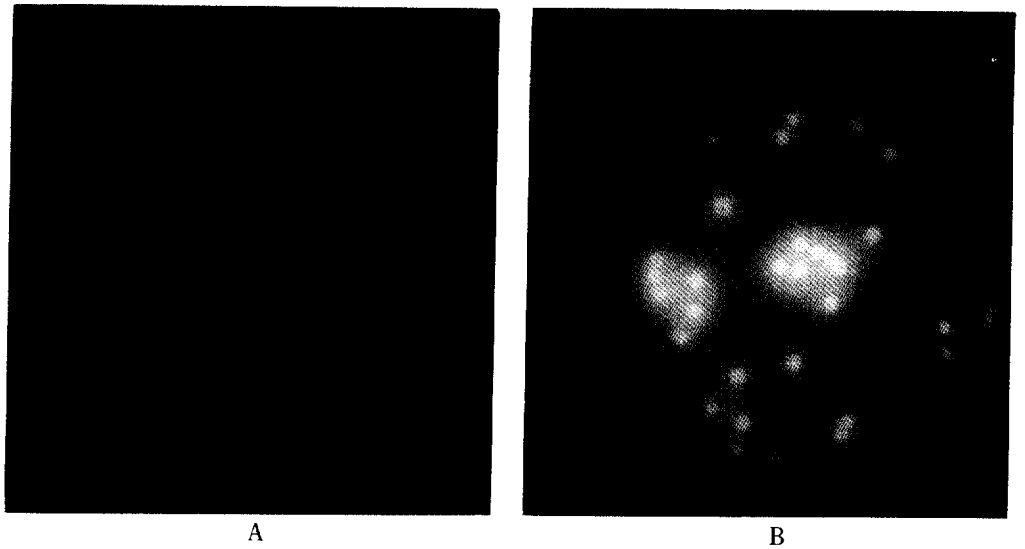


Fig. 1. Photographs of *A. agilis* PY101 grown in MHB plate without and with 1000 ppm of $CdCl_2$. A, MHB without $CdCl_2$(control); B, MHB with $CdCl_2$.

diffused into the medium and was fluorescent under ultraviolet light. Its synthesis was specifically stimulated by cadmium. The absorbance of fluorescence of the green-fluorescent pigment was measured from 380 nm to 450 nm by UV-vis spectrophotometer(Fig. 2). In the analytical result of element analysis and atomic absorption spectrometry, we measured crudely that this extracellular pigment contained an amount of sulfur atom and cadmium(data not shown). This revealed that the green-fluorescent pigment of *A. agilis* PY101 possess a high-affinity cadmium binding property. As a result, this pigment may act as an impermeable barrier for toxic cadmium. We suggested that at least one of the detoxification mechanisms of *A. agilis* PY101 involved the formation cadmium-binding pigment.

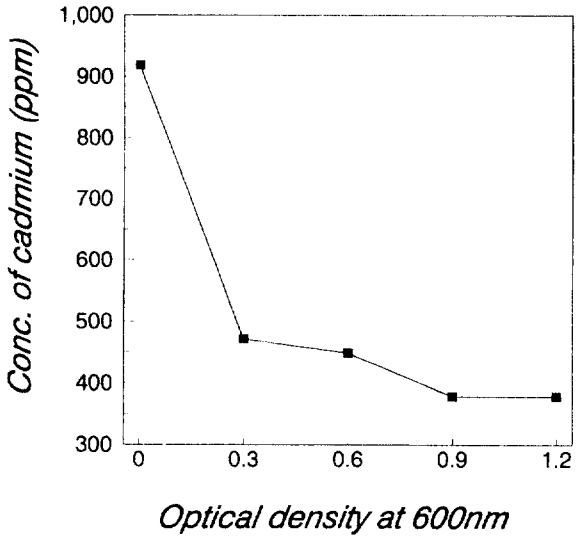
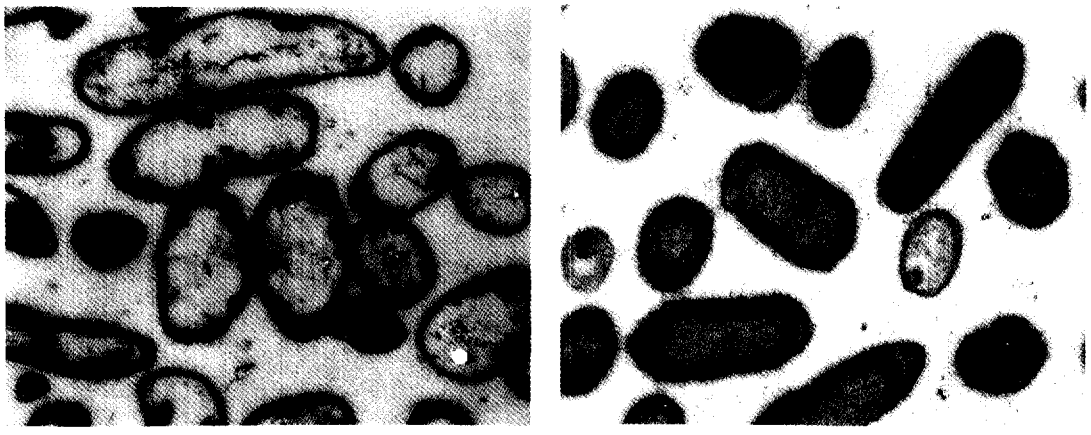


Fig. 3. Analysis for the changes of Cd^{2+} concentration in the culture medium by ICP-AES according to the optical density. *A. agilis* PY101 was cultured in HIB supplemented with 1000 ppm of $CdCl_2$. Samples were analyzed three times and averaged.

Accumulation of cadmium in *A. agilis* PY101

The quantity of $CdCl_2$ in the course of OD for culture of *A. agilis* PY101 was measured by the ICP-AES. Significant changes of the concentration of Cd^{2+} were detected in the culture medium before and after incubation with *A. agilis* PY101. The concentration of Cd^{2+} in the culture medium during growth of *A. agilis* PY101 gradually decreased to below 400 ppm of initial value(Fig. 3). In the transmission electromicrograph of bacteria grown with 1000 ppm of cadmium, electron-dense materials can be seen, which are not present in bacteria grown without cadmium. These materials were extensively located in the cytoplasm of the bacteria. That is to say, *A. agilis* PY101 actively accumulated Cd^{2+} in the cytoplasm (Fig. 4). A common property of many organisms capable of growth in the presence of cadmium is their ability to prevent accumulation of free intracellular cadmium (20, 21). In *S. aureus*, the presence of penicillinase plasmids altered the permeability properties of the cell and conferred resistance to low concentrations of cadmium(0.001 to 0.1 mM) (2). However, cadmium-resistant cells became permeable to cadmium at higher concentration(1 mM and over) of cadmium. In yeasts, cadmium resistance has been linked with hydrogen sulfide production, detoxification occurring by the formation of insoluble cadmium sulfide (7). In *Citrobacter* sp. cadmium resistance has been described by detoxification by the formation of insoluble phosphate (13). *A. agilis* PY101 was most sensitive to cadmium under condition of sulfate limitation, more so than when limited for any other nutrient, such as phosphate, potassium, or magnesium. Cadmium tolerance due to sulfate is not an uncommon feature in the microbial would. We suggested that another detoxification mechanism of cadmium by *A. agilis* PY101 involved the formation cadmium sulfate or cadmium sulfide. We therefore assume that the cadmium resistance



A B

Fig. 4. Transmission electromicrographs of *A. agilis* PY101, A, normal cells(×26400); B, cells exposed to 1000 ppm of $CdCl_2$(×26400).

of *A. agilis* PY101 in high concentration(1000 ppm) of cadmium can be achieved by precipitation or microbially binding which formed both intracellular cadmium sulfate or cadmium sulfide and extracellular cadmium-binding pigment.

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