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Isolation and Characterization of Pigment-deficient Mutants from *Azomonas agilis* PY101

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To investigate the mechanism of cadmium tolerance in a cadmium-resistant *Azomonas agilis* PY101 that produces a specific fluorescent pigment promoted by cadmium, we carried out Tn5 mutagenesis and isolated four pigment-deficient mutants. In these mutants, Ppg1, Ppg2, and Ppg3 remarkably reduced the pigment production to 15.3%, 11.2%, and 13.9%, respectively. Especially, Ppg4 mutant did not produce the pigment at all. None of the mutants grew in the presence of 1500 ppm of $CdCl_2$ in growth medium, and they exhibited differential sensitivities to cadmium. Ppg1, Ppg2, Ppg3, and Ppg4 mutants were sensitive to 900 ppm, 1100 ppm, 1000 ppm, and 800 ppm of $CdCl_2$, respectively. These mutants also showed noticeable increase, from 8.8-fold to 13.2-fold, in the size of growth inhibition zone compared with that of the wild type after treatment with cadmium. Therefore, the pigment production of *A. agilis* PY101 was found to decrease the toxic effects of cadmium to the bacterium.

Key words: *Azomonas agilis*, cadmium toxicity, fluorescent pigment

Cadmium is a nonessential heavy metal used extensively in industry for various applications including electroplating, preventing corrosion, and stabilizing plastic. Over the last century, increased industrial use of cadmium has led to cadmium contamination of the environment, plants, animals, food product, and human and to deleterious effects on organisms. Disruption of essential protein function can occur through binding of cadmium ions to sulfhydryl groups (-SH) (2). The toxic effects of cadmium on microorganisms are well documented (6, 13). Some bacteria contain cadmium resistance determinants and are thus less susceptible to the toxic effects (3, 5, 12, 18, 19). Microbial populations exposed to toxic concentrations of cadmium have developed three main tolerance mechanisms. The first involves the alteration of membrane transport systems involved in initial accumulation, thus blocking or reducing entry of cadmium. This system has been demonstrated in *Bacillus subtilis* 168 (10). The second mechanism is intracellular or extracellular sequestration by specific binding to a biopolymer (1, 4, 7, 8). The third mechanism is energy-dependent cadmium ion efflux. Two plasmid-determined mechanisms for cadmium efflux have been intensively studied: the *cadCA* system of *Staphylococcus aureus* (3, 9, 17, 20, 21) and *czcCBAD* system of *Alcaligenes eutrophus* (5, 14, 15, 16).

Azomonas agilis PY101 was isolated from the samples collected from an Anyang stream and is able to grow on agar plate containing high levels of cadmium (22). This microorganism also produces a specific fluorescent pigment induced by cadmium ion (Cd^{2+}), and the pigment displays a significant affinity for Cd^{2+} (23). This phenomenon can affect the toxicity of Cd^{2+} to *A. agilis* PY101. Therefore, a correlation is thought to exist between the pigment production and cadmium resistance.

In this study, the relationship between the pigment production and the cadmium resistance in *A. agilis* PY101 was investigated. We carried out transposon mutagenesis with Tn5 and isolated four pigment production deficient mutants. Isolation and characterization of these mutants suggested that the pigment production is important in the cadmium resistance of *A. agilis* PY101.

Materials and Methods

Bacterial strains, plasmid, and growth conditions

Gram-negative *A. agilis* PY101 producing specific fluorescent pigment was resistant to ampicillin (60 μg/ml) and tetracycline (30 μg/ml). *Escherichia coli* C 600 (pGS9) was used as a Tn5 donor in transposon mutagenesis (5). The characteristics of strains and

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Table 1. Bacterial strains and plasmid

Strains and plasmid	Relevant characteristics
Strains	
A. agilis PY101	Wild type, spontaneously Ap^r, Tc^r strain
E. coli C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*
Plasmid	
pGS9	30.5 kb, Tra^+, p15A replicon, Cm^r, Km^r (Tn5)

Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Tra^+, self transmissible.

plasmid used in this study are listed in Table 1.

A. agilis PY101 cells were cultured in Mueller Hinton Broth (MHB, Difco laboratories) medium at 30°C, whereas *E. coli* (pGS9) cells were grown in Luria-Bertani (LB) medium at 37°C.

Tn5 mutagenesis

Cells of donor *E. coli* C600 harboring pGS9 (5) and recipient *A. agilis* PY101 were grown to late-log phase (OD_{600}=0.8). They were washed twice with saline (0.9% NaCl) and resuspended in 200 μl of saline. Diparental mating was carried out by placing 20 μl of each strain with micropipette onto pre-warmed LB agar plates. The plates were dried and incubated at 30°C. After 8 h of incubation, cells were collected by scraping, and transconjugants were selected on agar plates containing ampicillin (60 μl/ml), tetracycline (30 μl/ml), and kanamycin (80 μl/ml). Colonies were picked on replica MHB agar plates containing 500 ppm of $CdCl_2$. After that pigment-deficient mutants were isolated. It was confirmed, through Southern hybridization with Tn5 DNA fragments as probes, that these pigment-deficient cells were derived from *A. agilis* PY101 by single Tn5 insertion.

Pigment measurement

The Heart Infusion Broth (HIB, Difco) was also used for measuring the amount of pigment produced by the mutants and *A. agilis* PY101. The cells cultured in liquid HIB supplemented with 500 ppm of $CdCl_2$ were sampled at the mid-log phase (OD_{600}= 0.6) of growth and then centrifuged at 15,000 rpm for 20 min. The growth was assessed by spectrophotometer (Milton Roy Company, Spectronic 20). The amount of pigments in these supernatant was measured with the UV-VIS spectrophotometer (Beckman DU650).

Biochemical characteristics of mutants

The biochemical characteristics of pigment-deficient mutants were charcterized by the GNI (gram-negative strain identification) card with the VITEK system (bioMérieux Vitek, Inc.).

Minimum inhibitory concentration

The lowest $CdCl_2$ concentration that completely inhibited cell growth was determined on MHB agar plate. Fresh culture inoculated with overnight stationary-phase culture was grown for 3 h in LB broth and diluted 1,000-fold in LB broth. Drops (3 μl) of each culture were placed on the series of plates which contained dilutions of $CdCl_2$ (0, 100 to 2000 ppm). After 24 h incubation at 30°C, the lowest concentration at which there was no growth was recorded as the minimum inhibitory concentration (MIC).

Growth inhibition zone

Overnight culture of *A. agilis* PY101 and the mutants (200 μl) were spread on MHB agar plates, and 6 mm diameter paper disks (BBL Microbiology Systems, Cockeysville, Md.), preloaded with 10 μM of $CdCl_2$, were placed on the plates. The plates were incubated at 30°C for 24 h, and the diameters of growth inhibition zones surrounding the disk were measured.

Results and Discussion

Isolation of pigment-deficient mutants

To investigate the molecular mechanism of cadmium resistance in *A. agilis* PY101, we tried to isolate pigment-deficient mutants generated by transposon insertion. We attempted to mate cadmium-resistant strain and *E. coli* C600 carrying pGS9::Tn5.

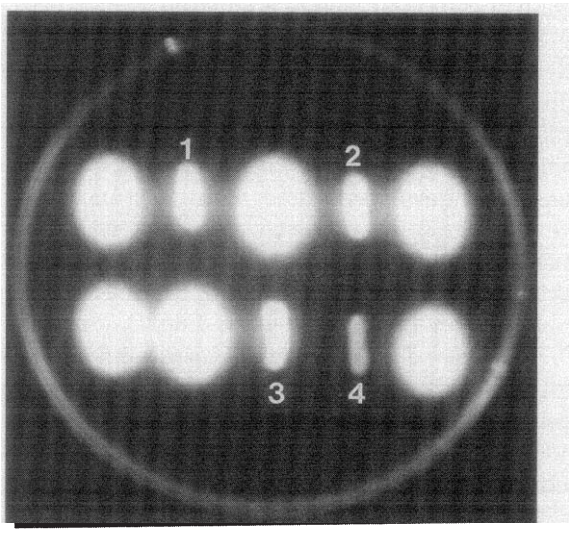

Fig. 1. Photograph of pigment-deficient mutants isolated from *A. agilis* PY101 by Tn5 mutagenesis on MHB agar plate containing 500 ppm of $CdCl_2$ under ultraviolet. Number 1, Ppg1; Number 2, Ppg2; Number 3, Ppg3; Number 4, Ppg4.

Table 2. Pigment production levels of pigment-deficient mutants

Strains	The amount of pigment	
	†$A_{400\,nm}$	%
PY101	1.20827	100.0
Ppg1	0.18479	15.3
Ppg2	0.13545	11.2
Ppg3	0.16832	13.9
Ppg4	0.00035	0.0

† Samples were analyzed three times, and obtained values were averaged.

Transposon mutagenesis was carried out by conjugative transfer of Tn5 from *E. coli* C600 (pGS9::Tn 5) to *A. agilis* PY101, and pigment-deficient mutants were screened by replica plating on the plates overlaid with cadmium. From the screening of about 5,000 colonies, we isolated four mutants which were defective in pigment production in the cadmium-overlaid plates (Fig. 1). *A. agilis* PY101 produces the fluorescent pigment (absorption peak at 400 nm) promoted by 500 ppm of $CdCl_2$. But, in pigment-deficient mutants, Ppg1, Ppg2, and Ppg3 remarkably reduced the pigment production to 15.3%, 11.2%, and 13.9%, respectively. Futhermore, Ppg4 mutant did not produce the pigment at all (Table 2). These mutants are probably defective in pigment production gene(s) and thus were called *ppg* mutants. Southern hybridization with Tn5 fragments as probe showed a single hybridized band in all mutants, revealing that they were single transposon insertion mutants.

Characterization of pigment-deficient mutants

In the biochemical characteristics analysis with VITEK system, the characteristics of these pigment-deficient mutants were similar to those of the wild type (Table 3). Especially, the characteristics of Ppg4 mutant were the same as those of the wild type. However, unlike the parental strain or Ppg4 mutant, strains Ppg1, Ppg2, and Ppg3 lacked xylose oxidation ability. It is reasonable to think that the lack of xylose oxidation-related gene(s) would lower the extracellular level of the pigment production. Therefore, we suggest that *A. agilis* PY101 increasingly produces fluorescent pigment when the strain can use xylose.

All *ppg* mutants, Ppg1, Ppg2, Ppg3, and Ppg4, grew more slowly than the parent in MHB medium containing cadmium. Especially, the pigment-deficient mutants isolated from *A. agilis* PY101 were no longer resistant to 1500 ppm of $CdCl_2$ at which the wild type was permitted to grow actively (Fig. 2).

For determining the sensitivities of these mutants to cadmium, the MICs and the growth inhibition zones were examined after treatment with various concentration of $CdCl_2$ (Table 4). In result, mutants exhibited differential sensitivities to cad-

Table 3. Biochemical characteristics of pigment-deficient mutants

Substrates	PY101	Ppg1	Ppg2	Ppg3	Ppg4
DP300	−	−	−	−	−
Glucose (Oxidative)	+	+	+	+	+
Growth Control	+	+	+	+	+
Acetamide	+	+	+	+	+
Esulin	−	−	−	−	−
Plant Indican	−	−	−	−	−
Urea	−	−	−	−	−
Citrate	+	+	+	+	+
Malonate	+	+	+	+	+
Tryptophan	−	−	−	−	−
Polymyxin B	−	−	−	−	−
Lactose	−	−	−	−	−
Maltose	−	−	−	−	−
Mannitol	+	+	+	+	+
Xylose	+	−	−	−	+
Raffinose	−	−	−	−	−
Sorbitol	−	−	−	−	−
Sucrose	−	−	−	−	−
Inositol	−	−	−	−	−
Adonitol	−	−	−	−	−
p-Coumaric	−	−	−	−	−
H_2S	−	−	−	−	−
ONPG	−	−	−	−	−
Rhamnose	−	−	−	−	−
Arabinose	−	−	−	−	−
Glucose (Fermentative)	−	−	−	−	−
Arginine	+	+	+	+	+
Lysine	−	−	−	−	−
Base Control	−	−	−	−	−
Ornithine	−	−	−	−	−
Code for Oxidase Reaction	+	+	+	+	+

+, positive response; −, negative response.

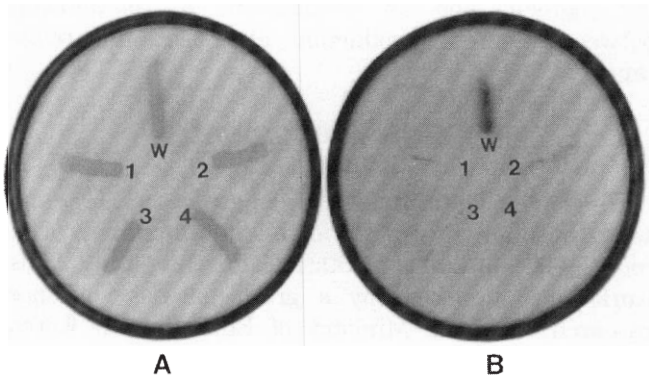

Fig. 2. Photographs of pigment-deficient mutants and *A. agilis* PY101 grown in MHB plate without or with 1500 ppm of $CdCl_2$. A, MHB without $CdCl_2$ (control); B, MHB with $CdCl_2$. Number 1, Ppg1; Number 2, Ppg2; Number 3, Ppg3; Number 4, Ppg4; W, PY101.

Table 4. Cadmium tolerance levels of pigment-deficient mutants

Strains	MIC on plate (×100 ppm $CdCl_2$)	Zone of inhibition (mm)[a]
PY101	20	1.2
Ppg1	9	10.5
Ppg2	11	11.3
Ppg3	10	10.7
Ppg4	8	15.8

[a] 10 μM amount of $CdCl_2$ per disk of cadmium for *A. agilis* PY101 and four mutants were used. After 24 h of incubation at 30°C, the diameters of the inhibition zones surrounding the disks (minus the 6 mm diameter of the paper disks) were measured.

mium in growth. Ppg1, Ppg2, Ppg3, and Ppg4 mutants were sensitive to 900 ppm, 1100 ppm, 1000 ppm, and 800 ppm of $CdCl_2$, respectively.

These mutants also showed noticeable increase (from 8.8-fold to 13.2-fold) in the sizes of growth inhibition zone compared with that of the wild type after treatment with cadmium. Especially, Ppg4 mutant showed higher sensitivity to cadmium and formed bigger growth inhibition zone by cadmium stress than the others. In contrast, the wild type showed no noticeable increase in the size of growth inhibition zone. The fluorescent pigment was known to possess higher binding constant for cadmium (23). Concluding from the results obtained in this study, the pigment production is likely to affect the resistance of *A. agilis* PY101 against cadmium. The pigment was found to decrease the toxic effects of cadmium to the strain, as revealed by significant decrease of the growth of pigment-deficient mutants in the presence of cadmium. Thus, we conclude that the pigment production was one of the cadmium-resistance determinants in *A. agilis* PY101. Further researches should be addressed for purification of the pigment and for elucidations of relationship between pigment production and cadmium resistance.

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References

1. **Aiking, H., A. Stijnman, C. van Garderen, H. van Heerikhuizen, and J. van't Riet.** 1984. Inorganic phosphate accumulation and cadmium detoxification in *Klebsiella aerogenes* NCTC418 growing in continuous culture. *Appl. Environ. Microbiol.* **47**, 374-377.
2. **Aiking, H., K. Kok, H. van Heerikhuizen, and J. van't Riet.** 1982. Adaptation to cadmium by *Klebsiella aerogenes* growing in continuous culture proceeds mainly via formation of cadmium sulfide. *Appl. Environ. Microbiol.* **44**, 938-944.
3. **Chopra, I.** 1975. Mechanism of plasmid-mediated resistance to cadmium in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **7**, 8-14.
4. **Denise, P.H. and J.S. Peter.** 1984. Cadmium-resistant *Pseudomonas putida* synthesizes novel cadmium proteins. *Science* **225**, 1043-1046.
5. **Dietrich, H.N. and S. Silver.** 1989. Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. *J. Bacteriol.* **171**, 896-900.
6. **Duxbury, T.** 1981. Toxicity of heavy metals to soil bacteria. FEMS Microbiology Let. **11**, 217-220.
7. **Khazaeli, M.B. and R.S. Mitra.** 1981. Cadmium-binding component in *Escherichia coli* during accommodation to low levels of this ion. *Appl. Environ. Microbiol.* **41**, 46-50.
8. **Kojima, Y. and J.H.R. Kagi.** 1978. Metallothionein. *Trends Biochem. Sci.* **3**, 90-93.
9. **Kondo, I. T. Ishikawa, and H. Nakahara.** 1974. Mercury and cadmium resistances mediated by the penicillinase plasmid in *Staphylococcus aureus*. *J. Bacteriol.* **117**, 1-7.
10. **Laddaga, R.A., R. Bessen, and S. Silver.** 1985. Cadmium-resistant mutant of *Bacillus subtilis* 168 with reduced cadmium transport. *J. Bacteriol.* **162**, 1106-1110.
11. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
12. **Mitra, R.S., R.H. Gray, B. Chin, and I.A. Bernstein.** 1975. Molecular mechanisms of accommodation in *Escherichia coli* to toxic levels of Cd^{2+}. *J. Bacteriol.* **121**, 180-1188.
13. **Mitra, R.S. and I.A. Bernstein.** 1978. Single-strand breakage in DNA of *Escherichia coli* exposed to Cd^{2+}. *J. Bacteriol.* **133**, 75-80.
14. **Nies, D.H.** 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (*czc* system) in *Alcaligenes eutrophus*. *J. Bacteriol.* **174**, 8102-8110.
15. **Nies, D.H.** 1995. The cobalt, zinc, and cadmium efflux system *czc*ABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. *J. Bacteriol.* **177**, 2707-2712.
16. **Nies, D.H., A. Nies, L. Chu, and S. Silver.** 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proc. Natl. Acad. Sci. USA* **86**, 7351-7355.
17. **Novick, R.P. and C. Roth.** 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* **95**, 1335-1342.
18. **Silver, S. and M. Walderhaug.** 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**, 195-228.
19. **Trevors, J.T., K.M. Oddie, and B.H. Belliveau.** 1985.

Metal resistance in bacteria. *FEMS Microbiol. Rev.* **32**, 39-54.

20. **Tynecka, Z., A. Malm, and T. Skwarek.** 1989. Effect of Cd^{2+} on growth of the cadmium-resistant and -sensitive *Staphylococcus aureus*. *Acta Microbiol. Polon.* **38**, 117-129.
21. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Reduced cadmium transport determined by a resistant plasmid in *Staphylococcus aureus*. *J. Bacteriol.* **147**, 305-312.
22. **You, K.M., J.H. Lee, J.K. Kim, N.J. Hah, Y.N. Lee, and Y.K. Park.** 1996. Characterization of *Azomonas agilis* PY101, a cadmium-resistant strain isolated from Anyang stream. *J. Microbiol.* **34**, 289-293.
23. **You, K.M. and Y.K. Park.** 1998. Adaptation of *Azomonas agilis* PY101 exposed to cadmium via production cadmium-binding pigment promoted by Cd^{2+}. *J. Microbiol.* **36**, 159-163.