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Isolation of Cadmium Ion-resistant Bacteria and Resitance to Various Heavy Metals

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카드뮴 내성균의 분리와 각종 중금속에 대한 저항성의 연구

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ABSTRACT

Cadmium ion-resistant microorganism was isolated from the sludge of wastewater. The physiological, morphological and other cultural data showed that this strain belonged to *Citrobacter freundii.* A clearcut distinction of growth among nutrient broth, tryptic soy broth and synthetic medium was demonstrated. The resistant cells showed only slight mutagenic action. During the growth of bacterial population in resting state, the organisms reduced the initial level of resistance to cadmium ions when they were not kept in contact with cadmium ions in bacterial multiplication. And cadmium ion-resistant and cadmium ion-sensitive strain were found to show equal, lower or higher sensitivity to other heavy metals.

INTRODUCTION

Cadmium compounds are generally toxic for microorganisms, but some of bacteria are able to grow in the medium or wastewater containing certain concentration of heavy metal ions. Penicillinase plasmid, and some related extrachromosomal elements in *Staphylococcus aureus*, could specify resistance to inorganic ions including cadmium and mercuric ions. At the sametime, there were some tentative evidences that resistance to mercuric ions was due to the impermeability of the cells to the ions and not to higher concentration of free-SH group in resistant cells(6). The deposition of cadmium into a soil or sediment containing montmorillonite(2, 3) and incorporation of cysteine, zinc, magnesium and chelating agents(1) could lessen the toxidity. Mercury taken up by the genus *Pseudomonas* on the basis of taxonomic observation was mainly located on the cell surface and vaporization of a mercury compound by multiple drug-resistant strains of *Escherichia coli* was demonstrated(16, 17, 30, 31). There were also studies on the removal of inorganic mercury compounds in waste by the cell-reused method of mercury-resistant bacterium named *Pse-*

udomonas K62(27). Doyle *et al.* (9) found at 40 and 80 μg of cadmium per m*l*, *Escherichia coli* and *Bacillus cereus* grew well and some species including *Lactobacillus acidophilus* were repressed. The freqency of metal resistance was higher than that of antibiotic resistance(21) and *Micrococcus luteus* cells in medium containing lead salts exhibited a sequence of changes in the quanity of total cellular lipid(24). Biochemical experiments of resistance to cadmium ions showed that there was a marked decrease of cadmium uptake by resistant cells, which were identified as *Staphylococcus aureus* strain 8325 when compared with strains that lack the *cad*^{r} gene and cadmium uptake was estimated(17). In recent years, Horitsu *et al.* (15) reported that heavy metal-resistant strain of *Pseudomonas aeruginosa* was not resistant to streptomycin. They also studied on the distribution of cadmium ions in the cell. There was also a report on the isolation and identification of cadmium ion-resistant bacteria named *Klebsiella rhinoscleromatis* and on the accumulation of cadmium ions(22).

This experiment was designed to investigate the effect of growth in the media containing various concentrations of cadmium ions and to study the sensitivity to various heavy metals.

MATERIALS AND METHODS

Organisms: Cadmium ion-resistant or sensitive bacterium was isolated from the sludge of wastewater in the manufacturing district adjacent to Busan and those were used throughout this study.

Media and determination of resistance: Resistant or sensitive strain was determined by means of the gradient platetechniqe. To make the determination more accurate confirm-test was employed, Nutrient broth plus 10 per cent glucose and cadmium ions in 50, 100, 200~3,000 ppm was prepared. Resistance to cadmium ions was detected from the gas production appeared as the result of cell growth. By these ways resistance of bacteria was determined and they were identified through the various biochemical tests. Standard media employed were nutrient broth and nutrient agar. For the growth of cadmium ion-resistant or sensitive bacteria in the various media, 200ml of nutrient broth, tryptic soy broth and synthetic medium(glucose 2.5 g, potassium phosphate monobasic 0.01 g, magnesium sulfate 0.2 g, calcium chloride dihydrate 0.02 g, sodium chloride 0.1 g, ferrous sulfate 0.2 g) were prepared. Nutrient broth containing various concentrations of cadmium ions was used for the growth of cadmium ion-restant or sensitive bacteria.

Cultivation: After the resistance was determined, the organisms were usually grown on a nutrient agar slant. This stock culture was suspended with sterile physiological saline, centrifuged and resuspended. One m*l* of inoculum being a bacterial suspension of 0.1 in optical absorbance was inoculated into 200 m*l* of each medium of nutrient broth, tryptic soy broth or synthetic medium. To examine growth effect of cadmium ion-resistant strain or sensitive one in the media containing various concentrations of cadmium ions, a bacterial resuspension was made to adjust optical absorbance of 0.2 on colorimeter. One m*l* of the suspension as above was inoculated into 200 m*l* of each medium. The broth media without cadmium ions as control was used for the cultivation of the resistant and sensitive strains. All cultures were cultivated at 37°C with vigorous shaking and samples were withdrawn at an interval of 4 hours.

Growth: Turbidity readings of cells were made with a Spectronic 20 colorimeter at a wave length 660 nm.

Sensitivity test: treatments of cadmium ion-resistant and sensitive strains with other heavy metals were employed. After washed cells were prepared, 1 m*l* of the resuspension (cell count: 7.8 x 10^8/ml) was added to the sterile 0.01 M phosphate buffer, *p*H 7.0, containing various concentrations of other heavy metals. These mixtures were incubated aerobically in 10 ml test tubes at their exponential growth phase at 37°C for one and a half hours. They were appropriately diluted with sterile physiological saline and plated on the E. M. B. agar media prior to their incubation at 37 C° for 48 hours.

RESULTS

Using the gradient plate technique, cadmium ion-resistant and sensitive strains were isolated on nutrient medium containing 50 to 3,000 ppm of cadmium ions and the confirm-test was also conducted. Resistance to cadmium ions was determined by both methods. Cadmium ion-resistant and sensitive bacterium possessing resistance to 300 ppm and 2,000 ppm were respectively selected.

The taxonomic morphological and cultural characteristics of the isolated organisms were listed in Table 1. Table 2 showed acid production from carbohydrates used phenol red as the pH indicator.

Table 1. Morphological and cultural characteristics of the isolated microorganism

a. Morphological characteristics
  1. Gram reaction: negative
  2. Shape and size: short rod. 0.6 by 2 ~ 5 μm
  3. Motility: positive
  4. Flagella: peritrichous
  5. Endospore: not produced
  6. Growth: aerobic

b. Cultural characteristics
  1. Surface colonies on solid media
    a. Shape: circular
    b. Chromogenesis: none
    c. Opacity: translucent
    d. Elevation: flat
    e. Surface: concentric on E. M. B. agar
    f. Margin: entire
  2. Slant culture
    a. Growth (surface): filiform
  3. Broth culture
    a. Growth: membranous
  4. Stab culture
    a. Growth: filiform

Table 2. Acid production of the isolated microorganism from carbohydrates

Substrate	Acid production
1. *D*-glucose	+
2. *D*-fructose	+
3. *D*-xylose	+
4. *L*-arabinose	+
5. Sucrose	+
6. Maltose	+
7. Lactose	+
8. Sorbitol	+
9. Mannitol	+
10. Inositol	−
11. Inulin	−

The results of the physiological characteristics of the isolated organism were recorded in Table 3. By biochemical tests above, the isolated microorganism was identified as *Citrobacter freundii*.

Table 3. Physiological characteristics of the isolated microorganism

1. Gelatin: not liquefid
2. Indole: not produced
3. Hydrogen sulfite: produced
4. Ammonia: not produced
5. Voges-proskauer reaction: negative
6. Methyl red test: positive
7. Urease: weak positive
8. Malonate: not utilized
9. Simmons citrate: used as carbon source
10. KCN broth-growth: positive
11. Gluconate: negative
12. Tyrosine nutrient agar slant: brown pigment produced
13. Lactose fermentation: delayed
14. Growth pH: from 4 to 12
15. Starch: not hydrolyzed
16. Catalase: positive
17. Growth temperature: 5 C, (−); optimum temperature, 37 C

Figure 1, 2, 3 denoted that growth curves of cadmium ion-sensitive and resistant strain cultivated on nutrient broth and tyrptic soy broth were very nearly the same, but on the synthetic medium those of both strains had differences.

The results of growth of cadmium ion-resistant and sensitive strain with time in the media containing cadmium ion in 100, 400 and 700 ppm were shown in Figures 4, 5. The maximum stationary phases of cell in various concentrations of cadmium ions were lower than that of control. After this phase, reduction in the number of cell occurred rapidly.

In Table 4, cadmium ion-resistant strain showed equal, lower or higher sensitivity to other heavy metals compared to cadmium ion-sensitive one. Cadmium ion-resistant strain of *Citrobactern freundii* was found to be not more resistant to other heavy metal agents such as cupric sulfate and mercuric chloride than sensitive one, but more resistant to chromium trioxide and ferric chloride. Both strains had similar resistance to lead acetate and zinc nitrate.

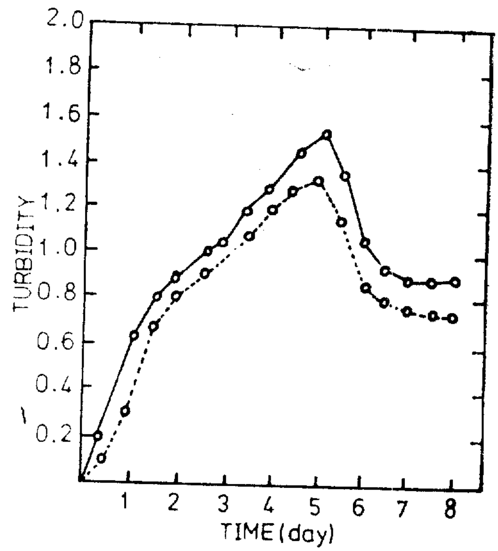

Fig. 1. Growth curves of cadmium ion-resistant and cadmium ion-sensitive cultures in nutrient broth: sensitive strain(……); resistant strain(—)

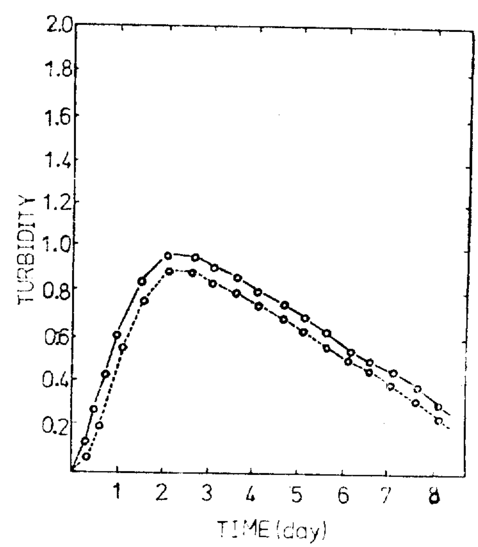

Fig. 2. Growth curves of cadmium ion-resistant and cadmium ion-sensitive cultures in tryptic soy broth: sensitive strain(……); resistant strain(—)

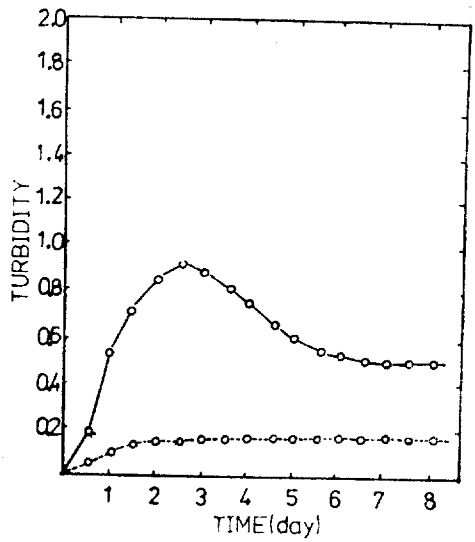

Fig. 3. Growth curves of cadmium ion-resistant and cadmium ion-sensitive cultures in synthetic medium: sensitive strain(……); resistant strain(—)

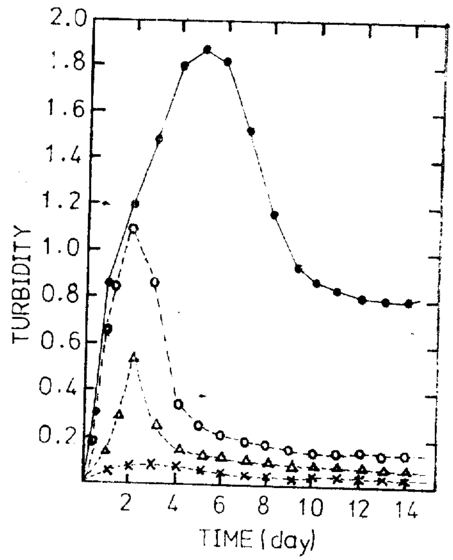

Fig. 4. Growth response of cadmium ion-sensitive strain with time. Concentration of cadmium ions (ppm): (●) 0; (○) 100; (△) 400; (×) 700.

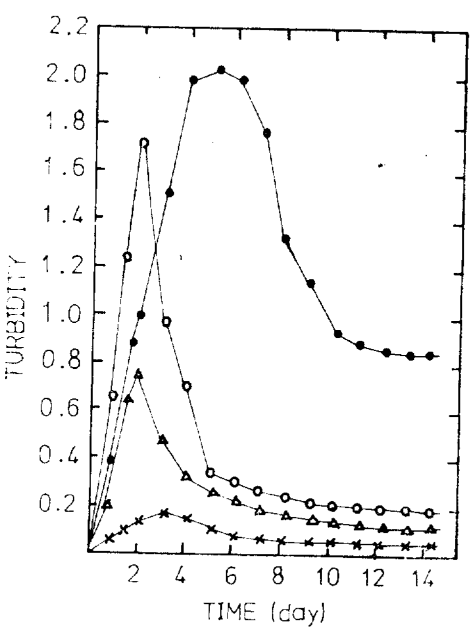

Fig. 5. Growth response of cadmium ion-resistant strain with time. Concentration of cadmium ions (ppm): (●) 0; (○) 100; (△) 400; (×) 700.

DISCUSSION

It was reasonable to assume there was as much mercury in the resistant cells as in the sensitive cells. The mercury taken up was vaporized in the resistant cells and it seemed likely that the vaporization provided a mechanism for resistance to mercuric chloride, though the precise mechanism of the vaporization was unknown(16). But there was no evidence that the cadmium taken up was vaporized in the resistant cells. The total uptake of cadmium by cadmium-sensitive cells was about 15 times that found with the resistant organisms and cadmium ions taken up by the bacteria were likely to have bound to some structure within the cell rather than absorbed adventitiously to the surface(6). In this case, cadmium ions were more concentrated inside the

Table 4. Effects of other heavy metals on cadmium ion-sensitive and cadmium ion-resistant strain Heavy metal Sensitive strain

Heavy metal	Sensitive	strain	Resistant	strain
	Concent. of chemical(ppm)	No. of survivor/ml	Cencent. of chemical(ppm)	No. of survivor/ml
Chromium trioxide	0.01	8.1×10^8	6.0	6.0×10^8
	0.02	6.2×10^8	8.0	5.5×10^8
	0.06	4.9×10^8	10.0	5.3×10^8
	0.10	3.1×10^8	20.0	4.5×10^8
Cupric sulfate	0.40	8.1×10^8	0.20	4.1×10^8
	0.60	6.1×10^8	0.40	2.6×10^8
	0.80	4.0×10^8	0.60	1.6×10^8
	1.0	2.1×10^8	0.80	1.1×10^8
Ferric chloride	10.0	8.0×10^8	20.0	7.5×10^8
	14.0	6.1×10^8	24.0	6.0×10^8
	18.0	3.2×10^8	28.0	4.2×10^8
	20.0	1.6×10^8	30.0	2.1×10^8
Lead acetate	0.60	7.1×10^8	0.60	8.1×10^8
	0.80	4.1×10^8	0.80	6.4×10^8
	1.0	2.2×10^8	1.0	3.7×10^8
Mercuric chloride	0.40	7.1×10^8	0.20	4.1×10^8
	0.60	6.5×10^8	0.40	2.6×10^8
	0.80	4.0×10^8	0.60	1.6×10^8
	1.0	2.1×10^8	0.80	1.1×10^8
Zinc nitrate	6.0	4.7×10^8	6.0	4.6×10^8
	8.0	3.1×10^8	8.0	3.2×10^8
	10.0	1.4×10^8	10.0	2.2×10^8
	20.0	1.4×10^8	20.0	1.7×10^8

* No. inoculum: 8.7×10^8/ml

cells in the sensitive strain than in the resistant one. It is also assumed that the resistant strain may reduce the accumulation of cadmium by an unknown mechanism. Various hypotheses can be raised in attempts to understand biochemical patterns showing resistance to cadmium ions. The first possibility is that the resistant cells are able to inactivate the inhibitor such as cadmium ions passing through the cell wall by degradative enzyme modification. Second is the possibility that the resistant cells are less permeable to cadmium ions than the sensitive cells. Recently this possibility was suggested again by Chin and Goldstein(2).

Growth curves of cadmium ion-sensitive andresistant strains cultivated on nutrient broth and trytic soy broth were very similar, but on the synthetic medium those of both strains had difference. This physiological difference is an important evidence for mutant possessing resistance to heavy metal. There were no differences of growth curves between cadmium ion-resistant cell and sensitive one in the medium containing various concentrations of cadmium ions with time. Rapid autolysis took place both in the sensitive cells and in the resistant cells. The induction of autolysis was due to a toxidity, and was different depending upon the codncentrations of their addition in the media and exerted inhibitory action on cell growth.

From the results reported in this paper it was conceivable that resistant cells to camium ions showed only slight mutagenic action although some of chemicals especially such as ferrous ions were known to be potent mutagens.

Two suggestions may be made: Specific biological changes or adaptations on cell surface induced an acquisition of resistance and this may be spontaneous protection from permeation of chemicals into cell. Or resistance was arisen by mutation and the resistant cell had changes phenotypically but showed diversity in effect. The resistant bacteria used in this experiment generally meant the former because they reduced the initial level of resistance after grown on a nutrient agar slant, but they were able to keep resistance on cultivation higher than that of control.

From these two bases, it is essential to make clear whether or not the sensitive cells are able to acquire greater resistance to cadmium ions when they moved to higher concentrations of cadmium ions; and whether or not cadmium ions can reach their DNA when cadmium ions were contacted with bacterial cells. These will make a contribution to the confirmation of the former or the latter.

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적 요

부산근교의 공업지역에서 유출되는 폐수의 sludge로 부터 카드뮴 내성균을 분리하여 동정한 결과 Citrobacter로 판명되었다. 카드뮴 내성균과 카드뮴 감수성균은 nutrient broth와 tryptic soy broth 배지내에서 유사한 증식곡선을 나타내었으나 합성배지내에서는 명백한 증식차이를 나타내었다. 카드뮴 내성균은 경미한 mutagenic action을 가지고 있으나 내성균의 증식시에 카드뮴 이온과의 접촉이 없으면 카드뮴 이온에 대한 원래의 저항성은 감소되는 것 같다.

카드뮴 내성균은 구리(황산동)와 수은(염화 제 2 수은)과 같은 중금속에는 감수성균보다 내성이 작았으나, 크롬(3 산화 크롬)과 철(염화 제 2 철)에는 감수성균보다 큰 내성을 나타내었다.

또 납(초산납)이나 아연(질산아연)에는 유사한 감수성을 나타내어, 카드뮴 화합물을 제외한 각종 중금속 화합물에는 선택적 감수성을 보였다.

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