Toxicity of Cadmium Particle Dust in Bacterial Cells

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Received January 20, 1999; Accepted April 12, 1999

When Thiobacillus intermedius 13-1, Escherichia coli JM109, and Agrobacterium radiobacter IFO12665b1 were cultured in LB liquid medium containing 0.03 g or 0.1 g of cadmium particle dust, growth was strongly inhibited. Exposure of cells to cadmium particle resulted in an increase in pH and concentration of ammonium ions. No cadmium particle attachment to the outer wall of T. intermedius or cell wall disruption was observed by transmission electron microscopy.

Key words: cadmium dust; Thiobacillus; bacteria; particle; toxicity

The largest source of heavy metal dust, such as cadmium, and fumes released to the environment is burning of fossil fuels, including coal and oil, and the incineration of municipal waste material. Cadmium can cause a number of adverse health effects such as emphysema, while having no known beneficial effects. Recently, Furst, A. et al. investigated the carcinogenic action of nickel and cadmium powder in the same rat. Powders of nickel and cadmium metals were compared for their relative carcinogenicity when injected in muscles of legs in rats.2)

On the other hand, bacteria have highly specific resistance to heavy metals. The basic biochemical mechanisms of such resistances as well as the molecular biology and genetics that determine and govern them has been explained.3,4) There are many papers concerning resistance mechanisms of bacteria to cadmium ion and other heavy metal ions. Cadmium resistance in Gram-positive bacteria such as Staphylococcus aureus, Bacillus subtilis,5) and Lactobacillus plantarum6) is thought to involve a plasmid-coded cadmium efflux system or chromosomally encoded reduced cadmium uptake. Cadmium resistance results from lower cellular cadmium accumulation, which in turn comes from plasmid-encoded energy-dependent cadmium efflux pump.7)

Strains of Thiobacillus intermedius 13-1 isolated from the corroded concrete of sewers have been found to be resistant to several heavy metals.8) A cadmium-inducible, high molecular-weight, heavy metal-binding protein was isolated from cadmium exposed cells of T. intermedius. The molecular weight of protein, which contained abnormal levels of histidine residues and a low level of cysteine residues, was about 150 kDa and bound 6.5 atoms of cadmium per molecule protein. Since the resistance of T. intermedius for cadmium coincided with increased levels of the heavy metal, the heavy metal-binding protein that was induced by cadmium ions may be important in metal resistance.9) However, the various reports that have described the resistance mechanisms of bacteria have concentrated on the ion or soluble forms and not the insoluble forms of heavy metals. Recently, we have found that T. intermedius, but not Agrobacterium radiobacter IFO12665b1 (a crown gall bacteria which produces an extrapoly saccharide), was adsorbed aggregetly onto the surface of cadmium particles.10) Consequently, in this study, the behavior of T. intermedius, E. coli JM109 and A. radiobacter in the presence of insoluble forms of heavy metals was examined. E. coli was used as a common control strain in a wide range of experiments with cadmium- and zinc-sensitive strains.12) In this paper, we report the toxicity of cadmium particle dust in these bacterial cells. Furthermore, we discuss the mechanisms of the toxicokinetics of insoluble cadmium particles against these bacterial cells.

Agrobacterium radiobacter was obtained from the Institute for Fermentation, Osaka, Japan. Thiobacillus intermedius was isolated from corroded concrete by Morinaga et al.13) This experiment used cadmium particles (Nakalai Tesque, Inc., Kyoto, Japan) with geometric mean diameter 10 to 50 μm as particle dust. The cadmium particles were in the elemental form, not a salt form. All reagents were analytical grade.

T. intermedius, E. coli, and A. radiobacter were cultured in Luria-Bertani (LB) medium at 30°C on a rotatory shaker at 150 rpm. Cells were harvested by centrifugation at 3,000 x g for 10 min and washed twice with distilled water. The cells were resuspended in a small volume of distilled water and adjusted to 3 x 10^7 cells per ml. For each cell type, cultured bacterial cells in the exponential growth phase (50 μl) were added to 5 ml of LB liquid medium in a test tube containing 0.03 g or 0.1 g of cadmium particles and then cultured at 30°C on a rotatory shaker at 150 rpm for 42 h. During cultivation, growth was evaluated by measuring the optical density of the upper phase of the culture medium at 550 nm. All subsequent experiments were done under exactly the same conditions. Figure 1 shows the effects of cadmium particles on cell growth in LB liquid medium containing T. intermedius, E. coli, or A. radiobacter. Cell growth differed significantly in the presence of cadmium particles compared to the controls. Cadmium particles inhibited growth to 30.8% and 18.9% of the control of T.

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intermedius after 32 h in the presence of 0.03 g and 0.1 g of cadmium particle, respectively, and completely inhibited growth of A. radiobacter and E. coli in the presence of both 0.03 g and 0.1 g of cadmium particles. These results indicate that under these culture conditions, T. intermedius is more resistant to cadmium particles than E. coli and A. radiobacter.

A 300-μl sample of each bacterial cell suspension (3 × 10^8 cells/ml) was added to tubes containing 0.1 g of cadmium particles which had been washed twice with distilled water of 10 volumes of the cell suspension. The mixture was vigorously shaken with a vortex mixer for 10 min at room temperature. The aqueous phase of samples was then diluted 10^4 to 10^7 times with sterile distilled water. A series of LB agar plates were spread with 100 μl of each diluted sample. After a 24-h incubation at 30°C, the number of colonies formed was counted. Growth was expressed relative to growth of bacteria that had not been exposed to cadmium particles (control plates). All platings were done in duplicate. In the liquid medium, the frequency of exposure of bacterial cells to cadmium particles was quite low because the weight of the particles caused them to sediment to the tube bottom during culture shaking. However, in this experiment, the bacterial cells were well exposed to cadmium particles for 10 min. The viability of bacterial cells so exposed to insoluble cadmium particles was compared to those of controls which were not exposed to cadmium. The viability of cadmium-exposed T. intermedius, E. coli, and A. radiobacter was significantly decreased to 0.49%, 0.50%, and 0.50% of the controls, respectively, suggesting that the bacteria are extremely sensitive to cadmium particles in this condition. Comparison of cadmium particle exposure time and viability of T. intermedius, E. coli, and A. radiobacter indicated that within even 60 seconds of exposure, 90 to 99% of the viability was lost (data not shown).

Each bacterial cell suspension (2.0 × 10^6 cells in 100 μl of sterile water), respective amino acid solutions (10 μmol of glycine, serine, threonine, asparagine, alanine, valine, arginine, lysine, or aspartic acid in 100 μl of sterile water), or protein solution (200 μg of bovine serum albumin in 100 μl of sterile water) was added to 4.2 ml of distilled water containing 0.1 g of cadmium particles, which had been washed twice with 100 volumes of distilled water. The mixture was then vortexed vigorously for 1 min at room temperature. The pH of the aqueous phase was measured using a pH meter (TOA model HM-5S) before and after the additional of these solutions. When 0.1 g of cadmium particles was added to T. intermedius, E. coli, and A. radiobacter cell suspensions in distilled water (2.0 × 10^6 cells), the pH of the mixture with cadmium particles increased from 7.80 to 8.40, 7.88 to 8.51, and 8.00 to 9.10, respectively. This increase in pH also occurred when 0.1 g of cadmium particles was mixed with 10 μmol of free amino acids and 200 μg of commercial protein as follows: neutral amino acids, glycine (pH 6.70 to 9.30), serine (pH 6.90 to 9.20), threonine (pH 6.70 to 9.25), asparagine (pH 6.70 to 9.20), alanine (pH 6.70 to 9.25), and valine (pH 6.70 to 10.20); basic amino acids, arginine (pH 10.20 to 11.30) and lysine (pH 6.70 to 9.20); and an acidic amino acid, aspartic acid (pH 3.50 to 6.80); and bovine serum albumin (pH 7.10 to 8.85). As for the pH change, it didn’t happen only because of suspending cadmium particles in distilled water. The elevation of the pH wasn’t observed when a cellular suspension was added to the aluminum, iron, nickel, molybdenum, lead, or zinc particles in the procedure, which was the same as the procedure in which a cellular suspension was added to the cadmium particles. Aluminum (80–160 μm), iron (10–135 μm), nickel (2–4 μm), molybdenum (4–30 μm), lead (8–60 μm), and zinc (2–8 μm) were used as pure elements, not their salt forms.

An increase in pH indicates an increase in hydroxide ion. We examined whether ammonium ion was released
after addition of bacterial cells to cadmium particles. *T. intermedius* cells harvested from 1 to 5 ml of overnight culture by centrifugation at 3,000 × g for 10 min were washed with 3 ml of distilled water and resuspended in 1 ml of distilled water. Each cell suspension (1 ml, pH 6.0) and respective supernatant, which was used as the blank, was added to 0.1 g of cadmium particles that had been washed with 3 ml of distilled water. The mixture was vortexed vigorously for 10 min at room temperature and then a 50 µl sample of the mixture supernatant after centrifugation at 3,000 × g for 5 min was analyzed for ammonium ion concentration. Ammonium ion was measured colorimetrically using a modification of the Conway method as described by Okuda et al.14) Cell number had already been clear from the value of the optical density, and was calculated from the value of the optical density for the number of cells. An absorbance of 1 at 550 nm occurs at 2 × 10⁹ cells per ml. As shown in Fig. 2, when 26.9 × 10⁹ cells of *T. intermedius* were added to 0.1 g of cadmium particle and vortexed, 80.9 pmol of ammonium ion was detected. The concentration of ammonium ions detected was dependent on the number of bacterial cells present. This suggested that ammonium ion was liberated from the bacterial cell surface upon exposure to cadmium particles. Ammonium ion wasn’t detected in the control without addition of the cadmium particle or the cells at all.

*T. intermedius* cultured in LB medium was washed with distilled water and exposed to 0.1 g of cadmium particle for 1 min. A sample of cells was then fixed for transmission electron microscopy with a solution of 0.1% ruthenium red (Sigma, St. Louis, USA) and 2% glutaraldehyde in 0.1 m cacodylate buffer (pH 7.4), stained with 0.25% uranyl acetate in 0.1 m sodium acetate buffer (pH 6.3), dehydrated with ethanol, and embedded in Quetol 651 (Nishin EM, Tokyo, Japan). Finally, the sections were stained with lead acetate and uranyl acetate and examined with an electron microscope (Hitachi H4800MU) at 100 kV. Electron microscopy of a thin section of *T. intermedius* showed that the cell surface had a layered structure (Fig. 3, A). After exposure to cadmium, the layered structure disintegrated (Fig. 3,

**Fig. 2.** Release of Ammonium Ions upon Exposure of *Thiobacillus intermedius* 13-1 to Cadmium Particle.

The line is based upon a regression equation (\(y = 3.131x - 11.429\)) with a statistically significant slope.

**Fig. 3.** Transmission Electron Microscopy of a Thin Section of *Thiobacillus intermedius* 13-1 Vigorously Shaken with or without Insoluble Cadmium Particles.

A: The cell wall of the black edge hemming shown with the arrow is confirmed in the control cell. B: The cell wall shown in the arrow disappears in the cells shaken with the cadmium particles. C: A cell is destroyed soon. An arrow shows that the contents of the cell begins to leak out. Scale bar = 500 nm.
B), and was finally degraded (Fig. 3, C). Tremendous cells have been kept as incomplete form, meaning that the cell wall was degraded by some reaction derived from cadmium particle exposure.

The effects of insoluble cadmium particle dust on T. intermedius, E. coli, and A. radiobacter growth in LB liquid medium were observed for longer-term exposure to low doses (low-level chronic exposure) (Fig. 1), and shorter-term exposure to high doses (high-level acute exposure). All strains were completely inhibited by cadmium exposure upon both low level chronic and high level acute exposure. Consequently, cadmium was a highly toxic heavy metal under both conditions. When the bacterial strains suspended in distilled water were exposed to 0.1 g of cadmium particles, an increase in pH was observed. However, this did not occur upon addition of other insoluble heavy metal particles. It was shown as the reason that the affinity of cadmium particle for the bacterial cells is high, while the affinity of other heavy metal particle is low. A bacterial cell could be adsorbed abundantly on to the cadmium particles in comparison with other heavy metal particles. Cadmium particles are thought to have high reactivity. This increase was also observed upon exposure of 100 mM of various amino acid solutions to cadmium particles. The increase in pH observed upon addition of bacteria to cadmium particles may be due to increases in the hydroxide or basic ion. Since amino acids, protein, and bacterial cell surfaces have amino groups in common and may be highly reactive with cadmium particles, the free ammonium ion concentration was measured. The amount of ammonium ion released by the reaction between T. intermedius cell and cadmium particles was dependent on the number of bacterial cells present (Fig. 2).

Here, based on these results, the following hypothesis is proposed for the mechanism of toxicity of cadmium particles to bacterial cells. Cadmium particles adsorbed to an amino acid (257 µg glycine / 100 mg cadmium particle) and seemed to liberate electrons from the surface, followed by cleavage of the amino group region of the amino acid, resulting in a pH increase. Therefore, these results support the hypothesis that the cadmium particle surface reacts with the amino group in basic amino acids such as lysine and arginine on bacterial cell surface proteins or with ε-diamino acid in peptidoglycan, and then liberates free ammonium ions at room temperature via catalysis by the cadmium particles. Electron microscope observation showed that an outer layer was present on the cell surface, but no visible outer layer was present around cells exposed to cadmium particles, indicating that the cell wall was destroyed (Fig. 3). These phenomena also support the hypothesis that insoluble cadmium particles catalytically cleave amino group complexes on the surface of bacterial cells, resulting in change or destruction of the cell surface structure, which in turn leads to cell death.

References