Toxicity and Bioaccumulation of Hexavalent Chromium in Green Paramecium, *Paramecium bursaria*

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The effects of hexavalent chromium on the cell growth of green paramecium, *Paramecium bursaria* (*P. bursaria*), were investigated in this study. Two strains (MB-1 and F_1 generation) of *P. bursaria* were incubated in lettuce media supplemented with different concentrations of potassium dichromate under LL (24 hr light), LD (12 hr light : 12 hr dark) and DD (24 hr dark) conditions, and the IC_{50} values were obtained. The IC_{50} 7-day value showed that the toxicity of potassium dichromate was light-sensitive in both strains of *P. bursaria*. The results of the toxic effect of chromium on the cell shape of *P. bursaria* (BWK-4) showed that the body ratio of *P. bursaria* increased, even if the cells were incubated for 24 hr with 0.5 μM potassium dichromate solution, indicating that the cell shape of *P. bursaria* is very sensitive to potassium dichromate. The average amount of chromium accumulated in green paramecium ranged from 1.72 to 15.5 pg Cr/cell in a time- and concentration-dependent manner. This finding indicates the possibility of the use of *P. bursaria* as a biomonitor and bioaccumulator for chromium contaminants in aquatic environments. The experiment with electrical stimuli into the culture of *P. bursaria* indicated that cells accumulated at the negative electrode, suggesting that *P. bursaria* carried a positive charge. Thus, because the positive charge does not cause any significant absorption of chromium into the cells, *P. bursaria* may develop a variety of mechanisms for chromium accumulation in the cell. Further research studies are required to elucidate the mechanism of chromium uptake in *P. bursaria*.

Key words —— toxicity, bioaccumulation, chromium, IC_{50}, *Paramecium bursaria*

INTRODUCTION

The increasing trend towards combining industrial and municipal wastes for treatment in sewage plants increases the possibility of contamination of the influent by metal ions. Because microorganisms are key components for the decomposition of organic materials, the effect of metal toxicity on microorganisms has received attention in recent years.[1)] It is well-known that heavy metals are toxic to most microorganisms at certain concentrations and often cause serious damage in biological waste treatment plants. It has been suggested that the heavy metal concentration produced a certain level of mortality in the culture and visible behavioral changes in the cells, particularly slower movement compared to control.[2)] However, the mechanisms of their toxic effects are not well elucidated.

In natural water, chromium exists in its two environmentally important oxidation states, chromium (III) and chromium (VI). Chromium (III) is rather benign and is readily adsorbed on soil particles, whereas the chromium (VI), as the most toxic species, is not readily adsorbed.[3)] Hexavalent chromium compounds are being used in a wide variety of commercial processes; therefore, the unregulated disposal of the chromium-containing effluent in both developing and developed countries has led to the contamination of soil, sediment and surface and ground water.[4)] In trace amounts, chromium (III) is considered an essential nutrient for numerous organisms, but in higher amounts, it is toxic and mutagenic.[5)]

The impact of heavy metals on the environment

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and their slow addition through the food chain have promoted many research studies aimed at developing alternative, efficient and low cost systems for wastewater purification.[6] Various conventional methods for removing dissolved heavy metals, such as chemical precipitation/using ion exchange resins or chemical oxidation/reduction are expensive and may not always be feasible.[7] That is why the use of microbial biomass is considered more seriously.[8,9]

Paramecium bursaria (*P. bursaria*) is a unicellular organism, which is widely distributed in aquatic environments. One green paramecium possesses several hundred green algae, which are morphologically very similar to algae in the genus *Chlorella*. Because *P. bursaria* can utilize the photosynthetic products supplied by endosymbiotic algae as nutrients,[10] the photosynthetic products enable *P. bursaria* to remain alive under a starvation condition for food organisms. Therefore, the culture of green paramecia is much easier than that of other organisms including other protozoa and mammalian cell lines. With these advantages, the use of paramecia can permit a quicker and more convenient evaluation of the toxicity of various polluting chemicals.[11] In addition, ciliates are important components of the aquatic food chain[12] and play an essential role in the purification processes of both aerobic and anaerobic biological wastewater treatment systems.[13,14] In spite of the important roles played by the ciliated protozoa in the ecology of freshwater ecosystems, little is known about the accumulation efficiency of heavy metals by ciliates. A few papers describing the toxicity and effect of chromium on ciliates have been published.[1,14] In this study, the toxicity to and bioaccumulation of chromium (VI) into green paramecium, *P. bursaria*, is analyzed and discussed.

MATERIALS AND METHODS

Stains and Culture —— Three strains of *P. bursaria* syngen 1, MB-1 (mating type I, Shiga Prefecture, Japan), BWK-4 (mating type IV, Shiga Prefecture, Japan) and F_1 generation (immaturity; described below) were used in this study. The strains, MB-1 and BWK-4 were derived from a single cell collected from Lake Biwa (Shiga Prefecture, Japan). The F_1 generation was newly produced by hybridization with two stocks, BWK-4 and KN-21. Stock KN-21 (mating type III) was collected from Kinokawa River (Wakayama Prefecture, Japan). Those stains were cultured in lettuce infusion, containing the bacteria *Klebsiella pneumoniae* (*K. pneumoniae*) as food, and growing under light (L) dark (D) condition at 23°C following the methods previously described by Hosoya.[15]

Heavy Metal Salt —— Potassium dichromate ($K_2Cr_2O_7$) (Katayama Chemical, Japan) was used as source of chromium (VI). The stock solution (10 mM) was made and kept in refrigerator at 4°C until use.

Determination of Growth Rate and IC_{50} Value —— To determine the effect of chromium (VI) on the cell growth of *P. bursaria*, the cells were cultured in 12-well microplates (flat bottom and polystyrene-treated plates, Asahi Glass Co. Ltd., Japan). Each well was filled with 2 ml fresh lettuce infusion containing different concentrations of potassium dichromate (0–1000 μM) without *K. pneumoniae*, and each culture was started at an initial density of 1000 cells/ml. To determine the chronic toxicity, *P. bursaria* (MB-1) cells were incubated for 1, 3, 5 and 7 days under LD condition with (0.01–100 μM) or without potassium dichromate. Next, to determine the IC_{50} 7-day value, two stains of *P. bursaria* (MB-1 and F_1 generation) were incubated for 7 days under the LL, LD and DD conditions. After incubation, the number of *P. bursaria* in each well was counted under a binocular microscope (Model C-DS, Nikon, Japan). The IC_{50} values defined as the concentrations of substances required for 50% inhibition of the proliferation of cells were determined as a parameter for the toxicity of model chemical pollutants.

Cell Shape of *P. bursaria* —— To investigate the effect of chromium (VI) on the cell shape of *P. bursaria*, the BWK-4 strain was cultured in lettuce infusion with different concentrations (0–3 μM) of potassium dichromate under LD condition as previously described. After incubation, the cells were fixed in 10% formalin and photographs were taken by a Nomarski differential interference contrast (DIC) microscope (Optiphot, Nikon) equipped with a digital camera (COOLPIX 900, Nikon).

Chromium Accumulation by Cells —— To quantify the chromium accumulation in the cells, *P. bursaria* (F_1 generation) was cultured in lettuce media supplemented with potassium dichromate (0–160 μM) at an initial density of 1000 cells/ml and incubated for 3 to 7 days. After incubation, the cell number was counted. The samples with or without cells were filtered by membrane filters (5 μm pore, ADVANTEC, Japan), and the membranes were

washed 3 times with fresh lettuce infusion to remove the unbound chromium. After drying the filters at room temperature, they were treated overnight with 2 ml of 0.1 N nitric acid (Sigma-Aldrich, Japan). After centrifugation, an aliquot of the supernatant was used to measure the chromium concentration by an atomic absorption spectroscope (AA-220Z, Varian Inc., Australia), equipped with a GTA-96 graphite tube atomizer and a hollow cathode lamp for chromium detection, according to the manufacturer's standard protocol. Chromium standard solution (Wako Pure Chemical, Japan) was used for calibration.

Response of *P. bursaria* to Electrical Stimuli —— To investigate the mechanism of chromium accumulation in green paramecium, *P. bursaria* (BWK-4) was tested with electrical stimuli. The experiments were performed in a flat bottom tissue culture dish (35 mm) (Corning 430588, U.S.A.) containing a lettuce medium with *P. bursaria*. Two electrodes (+/–) were placed into the culture media, and a low current (1.5 volt) was supplied from a dry cell battery to monitor the response of *P. bursaria* to the electrical stimuli.

RESULTS AND DISCUSSION

Effect of Chromium (VI) on the Cell Growth of *P. bursaria*

The growth of *P. bursaria* (MB-1) in a lettuce medium supplemented with potassium dichromate is shown in Fig. 1. After 1, 3, 5 and 7 days of incubation, the toxicity of chromium was examined on the basis of cell number. The cell numbers decreased tremendously in the presence of 100 μM potassium dichromate within 24 hr, indicating that 100 μM of potassium dichromate shows acute toxicity to the cells.

The light-dependently promoted toxicity is one of the typical features for reactive oxygen generators such as paraquat, methylene blue, haematoporphyrin and chlorophyll. To investigate the light-dependent toxicity, the two stains of *P. bursaria* (MB-1 and F_1 generation) were incubated for 7 days under LL, LD and DD conditions. The growth rate was designated as values relative to the number of cells right after initiation of culture. The IC_{50} 7-day values of the MB-1 and F_1 generation are very similar under LL and LD conditions. However, the IC_{50} 7-day values of both strains under the DD condition

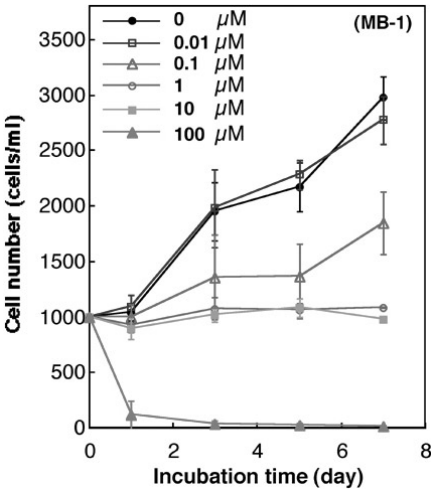

Fig. 1. Effect of Different Concentration of $K_2Cr_2O_7$ on Cell Proliferation of *P. bursaria* (MB-1) under Light and Dark Condition

Showing the cell number after 1, 3, 5 and 7 days of incubation.

are much higher than those under LL and LD conditions, showing that the toxicity of chromium is light-sensitive in the *P. bursaria* (Fig. 2). Takahashi[16] reported the similar observations found in *P. bursaria* under LL, LD and DD conditions in the presence of different concentrations of paraquat.

Chromium (VI) is the most toxic and mutagenic metal ion in biological systems. Although the toxic effects of chromium on microorganisms and invertebrates have been a topic for researchers over the past few decades,[1,14,17,18] less information using *P. bursaria* is available. Tanaka[19] reported that the IC_{50} 5-day value of potassium dichromate ranged from 0.27 to 1.65 μM for *P. bursaria* syngen 1 (KSK-103, mating type-IV) under LD condition. This result suggests that the sensitivity to chromium in *P. bursaria* is similar among the different strains.

Effect of Chromium Toxicity on the Cell Shape of *P. bursaria*

To evaluate the toxic effect of chromium (VI) on the cell shape, *P. bursaria* (BWK-4) was cultured in a lettuce medium with (0.5–3 μM) or without potassium dichromate. In the control, the body ratio (width/length) of *P. bursaria* varied from 0.37 to 0.41. After a 3-day incubation, the body ratio of *P. bursaria* was 0.49, 0.50 and 0.49 in the presence of 0.5, 1 and 3 μM potassium dichromate, respectively (Table 1). Even after 7 days of incubation with 20 μM potassium dichromate, the body ratio was 0.49, suggesting that an incubation longer than

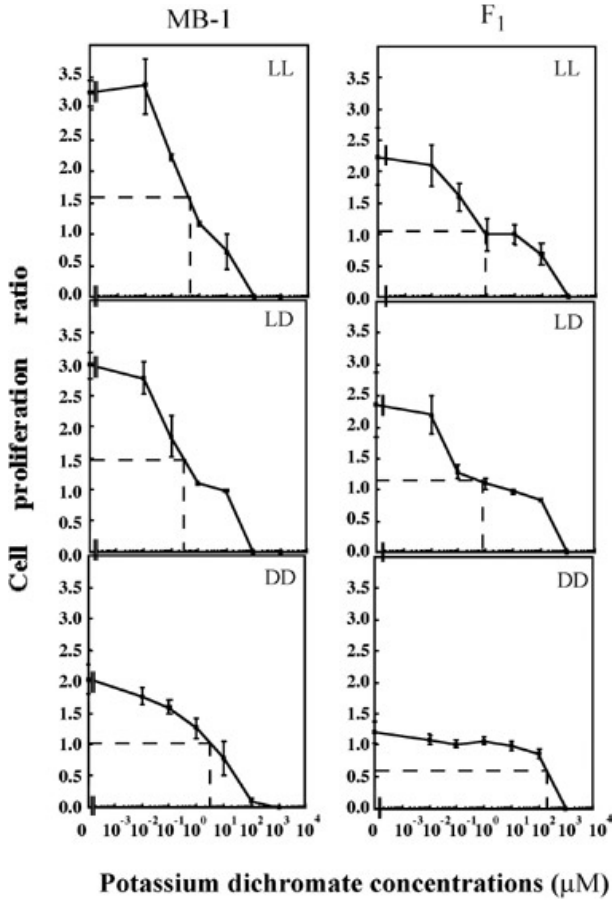

Fig. 2. Estimates of IC_{50} Values for Population Growth of Two Strains (MB-1 and F_1 Generation) of *P. bursaria* after 7 Days Exposure to $K_2Cr_2O_7$ under LL, LD and DD Conditions

3 days and with a concentration of potassium dichromate higher than 3 μM caused no more significant changes in the cell shape of *P. bursaria* (data not shown). The cell shape may depend on the cytoskeletal structures in *P. bursaria*. Several investigations revealed that heavy metals caused several changes in the organization of microtubules, which is one of the major cytoskeletal structures, such as the formation of numerous shorter microtubules.[20] Gennari[21] observed that cadmium ion caused a depolymerization of actin filaments, which is another important cytoskeletal structure. These effects of heavy metal ions on cytoskeletal structures, such as shortening of the microtubule length or depolymerization of microfilaments will disturb the shape of *P. bursaria*. Chromium (VI) ion may affect the microtubules or microfilaments of *P. bursaria*. More research studies are required to elucidate the mechanisms of the chromium ion effects on the microtubules or microfilaments of a *P. bursaria* cell.

Bioaccumulation of Chromium in the Symbiotic *P. bursaria*

To investigate the toxicity of chromium in a symbiotic paramecium, the chromium accumulation of *P. bursaria* was detected using an atomic absorption spectrophotometer. The results show that *P. bursaria* accumulated chromium and the accumulation depends on the time and the chromium concentration (Table 2).

Ciliates have many advantages as a test organism for investigating environmental pollution.[11,13,14,19,22–24] The study of *P. bursaria* and its interaction with chromium may be useful for bioremediation of chromium-contaminated environments. The aim of the study was to determine the ability of *P. bursaria* to accumulate chromium. Data on the bioaccumulation of heavy metals by invertebrates are available for lead/cadmium in marine protozoan communities,[25] lead/cadmium/copper/zinc in terrestrial invertebrates[26] and organotin in *Artemia franciscana*.[17] However, no data for chromium accumulation by ciliates have been reported. The amount of chromium in the body of *green paramecium* (8.35 pg Cr/cell) in the presence of 80 μM (8.3 mg/l) potassium dichromate is compared with those for other invertebrates with other metals. In the present study, the chromium accumulation in *P. bursaria* is compared to 2.25 ng Sn/animal accumulated by *Artemia franciscana* in the presence of 10 mg/l trimethyltin chloride.[17] To evaluate these data, the approximate volume of both animals was calculated and the volume of metal amount/m^3 was obtained. In the present study, the average length of *P. bursaria* is 115 with 46 μm in diameter (Table 1). Abatzopoulos[27] reported about the average length and diameter of *Artemia franciscana* are 10 and 4 mm, respectively. The approximate amount of metal per volume for artemia and paramecium was calculated to be 1.8×10^{10} pg Sn/m^3 and 4.4×10^{13} pg Cr/m^3, respectively. This suggests that the ability for accumulation of chromium in *P. bursaria* is significantly higher than that for tin in artemia. These results indicate that *P. bursaria* may be used as an active bioaccumulator for other heavy metals. The present work suggested that the potential of the symbiotic *P. bursaria* for the accumulation of chromium (VI) indicates its utility as a bioaccumulator and biomonitor of chromium contamination in freshwater environments.

Table 1. Length, Width and Body Ratio of *P. bursaria* (BWK-4)

Day	$K_2Cr_2O_7$ concentration (μM)					
	Control (0)			0.5		
	Length (μm)	Width (μm)	Body ratio[a)]	Length (μm)	Width (μm)	Body ratio
0	113 ± 9.8	42 ± 4.0	0.37	—	—	—
	$n = 35$					
1	122 ± 17.6	50 ± 9.0	0.41	105 ± 7.3	46 ± 3.6	0.44
	$n = 30$			$n = 38$		
2	109 ± 7.8	44 ± 4.0	0.40	108 ± 11.3	51 ± 5.1	0.47
	$n = 34$			$n = 36$		
3	115 ± 9.5	47 ± 5.4	0.41	101 ± 10.2	49 ± 5.2	0.49
	$n = 32$			$n = 34$		
Average	115	46				
Day	$K_2Cr_2O_7$ concentration (μM)					
	1			3		
	Length (μm)	Width (μm)	Body ratio	Length (μm)	Width (μm)	Body ratio
0	—	—	—	—	—	—
1	103 ± 10.0	49 ± 5.0	0.48	102 ± 7.3	47 ± 3.5	0.46
	$n = 38$			$n = 34$		
2	105 ± 8.1	51 ± 5.0	0.49	103 ± 8.9	49 ± 4.2	0.48
	$n = 40$			$n = 36$		
3	106 ± 10.3	53 ± 4.1	0.50	100 ± 7.3	49 ± 4.1	0.49
	$n = 34$			$n = 34$		

a) Body ratio calculated as width/length.

Table 2. Chromium Accumulation by *P. bursaria* (F_1 Generation) in Lettuce Media Supplemented with Potassium Dichromate

$K_2Cr_2O_7$ concentrations (μM)	Cell number after incubation (cells/ml)[a)]	Duration of incubation (day)	Total amount of Cr in the pellet (ng)[b)]	Amount of Cr/cell (pg)
0	1231 ± 108.3		0.78	0.32
5	1181 ± 71.7	3	4.06	1.72
10	981 ± 41.9		5.02	2.56
20	994 ± 46.7		8.42	4.24
0	2720 ± 318.9		0.52	0.1
5	1419 ± 182.0	7	6.04	2.13
10	1292 ± 273.2		10.4	4.04
20	1148 ± 209.9		14.7	6.42
0	2742 ± 343.4		0.88	0.16
40	1200 ± 251.0	7	18.8	8.26
80	1031 ± 118.2		17.2	8.35
160	897 ± 51.5		27.9	15.5

a) 2000 cells in 2 ml of lettuce infusion were incubated without or with various concentrations of chromium. *b*) Total amount of Cr was calculated in the pellet of cells obtained from the total volume (2 ml) of culture.

Response of the *P. bursaria* to Electrical Stimuli

To determine the electrical charge in *P. bursaria*, two electrodes were placed in the paramecium culture medium, and the response of *P. bursaria* to electrical stimuli was monitored. On supplying a low electrical current, the paramecia were swimming towards the negative electrode, and a large number of cells were concentrated around the negative electrode. After removing the electrodes from the culture media, the swarming of *P. bursaria* gradually disappeared from the negative electrode (Fig. 3). The results show that green paramecia carry a positive

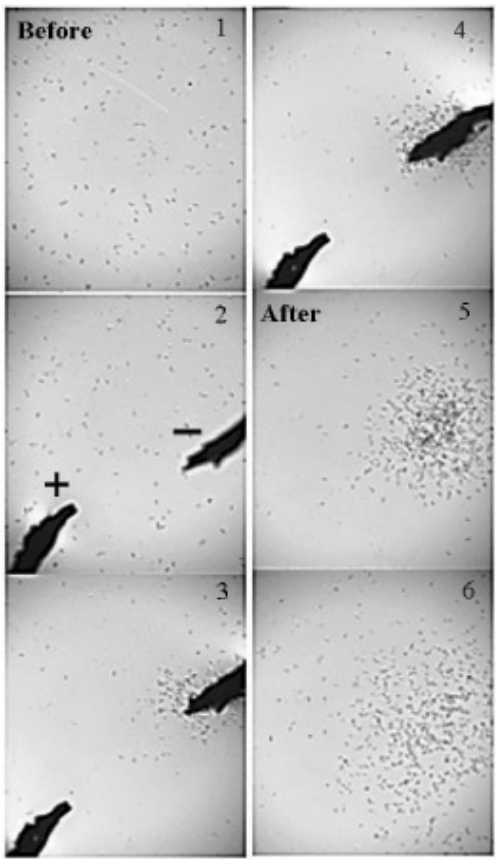

Fig. 3. *P. bursaria* Shows a Response to Electrical Charge when Two Electrodes (+/–) are Placed in the Paramecium Culture Medium

Panel 1. *P. bursaria* is normally swimming in the culture media. Panel 2. Two electrodes are placed in the paramecium culture medium. Panels 3 and 4. Paramecia are swimming toward the negative electrode and swarming near the negative electrode end. Panels 5 and 6. Two electrodes are removed from the paramecium culture medium and the swarming of *P. bursaria* near the negative electrode gradually disappeared.

(+) charge. Wichterman[28] reported that the paramecium showed the same charge. Because chromium ion is positively charged, its accumulation in the *P. bursaria* is not caused by electromagnetic interaction, suggesting that the *P. bursaria* may develop a variety of mechanisms for chromium uptake and metabolisms such as the binding of chromium (VI) to DNA or proteins. A metalloproteome is known to be a set of proteins that has metal-binding capacity by being metalloproteins or by having metal-binding sites. A different metalloproteome may exist for each metal.[29] Therefore, characterization of metalloproteomes provides information relating to the cellular accumulation of heavy metals. Salnikow[30] described that Cr (III) was directly involved in the formation of DNA-protein complexes in intact cells. However, information on a Cr (VI) binding protein has not been published. Future research should be aimed at elucidating the characterization of chromium (VI) binding proteins.

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