Isolation of Potassium Chromate-tolerant Bacterium and Chromate Uptake by the Bacterium

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A potassium chromate-tolerant bacterium was isolated from activated sludge, and the bacterium was identified as *Pseudomonas ambigua* G-1. The bacterium tolerated up to 2000 ppm of Cr^{6+} , 1700 ppm of Cu^{2+} and 200 ppm of Cd^{2+} , but did not tolerate Hg^{2+} . Chemical analysis indicated 86.5% uptake in the soluble fraction and 13.5% uptake in the insoluble fraction of cells. Chromate uptake distribution in the soluble fraction indicated 28.9% in microsomal fraction and 78.1% in supernatant fraction. Chromate distribution in the insoluble fraction showed 61% in lipid-fraction and 21% in polyphosphate-polysaccharide-fraction. Chromate inhibited the syntheses of protein, DNA in soluble fraction and RNA in microsomal fraction.

Environmental pollution due to chromium scrap and waste water containing chromium has recently become a problem. Among chromium compounds, it has been known that chromate is very toxic to life. The reason for the toxicity appears due to chromate pass easily through biological membranes.

For removal of chromate from waste water, microorganisms might be useful. For this purpose, it is necessary to find chromate-tolerant microorganisms.

Only *Pseudomonas* sp. has been isolated as a chromate-tolerant bacterium,¹⁾ but detail data have not yet been reported. We have previously conducted some studies on the isolation and distribution of heavy metals in the tolerant microorganisms.^{2~6)}

This paper describes the isolation and identification of a new potassium chromate-tolerant bacterium from activated sludge and the chromate uptake mechanism of the bacterium.

MATERIALS AND METHODS

Chemical. The highest grade of potassium chromate (K_2CrO_4) was used throughout this experiment.

Determination of total chromium. Samples were digested in HNO_3 by boiling. The digest was diluted

with water and was analyzed by a Hitachi Atomic Absorption Spectrophotometer 208 at 359.9 nm with 10mA of electric discharge using air-acetylene as flame.

Determinations of protein, RNA and DNA. Determinations of protein, RNA and DNA were carried out by the methods of Folin-Ciocalteu,⁷⁾ orcinol⁸⁾ and indole,⁹⁾ respectively.

Disc electrophoresis. Electrophoresis of polyacrylamide gel (7.5% acrylamide) was carried out in Tris-glycine buffer (pH 8.6) with a current of 3 mA per tube (0.5×7 cm) for 120 min at 5°C. Protein and chromium were stained in 1% Amido Black 10 B and 0.25% diphenyl carbazide, respectively.

Isolation of K_2CrO_4 -tolerant bacterium. Isolation of K_2CrO_4 -tolerant bacterium was performed by the agar plate method [composition: 1% polypeptone, 0.5% meat extract, 0.5% NaCl, 2% agar and K_2CrO_4 (Cr^{6+} , 500 ppm), pH 7.1]. Each 10 ml of molten agar medium was poured into a petri-dish (9 cm in diameter), and after being solidified, an aliquot (0.2 ml) of activated sludge suspended in 5 ml of distilled water was rubbed on the surface of the plate with a sterile glass rod and incubated at 30°C for 24 hr. After being incubated, colonies appearing were picked up with a sterile wire, and each colony was transferred several times to fresh agar plate to get a purified bacterium. The purity of the isolated bacterium, was determined by observations under both the light microscope and transmission electron microscope.

Identification of K_2CrO_4 -tolerant bacterium. Taxo-

nomical studies of the isolated K_2CrO_4 -tolerant bacterium were carried out according to Breed *et al.*,¹⁰⁾ and Komagata.¹¹⁾

Cultivation and fractionation of bacterial components.
Cultivation of bacteria and fractionation of bacterial

components were carried out by the method indicated in Scheme 1 and Fig. 1, that is, 100 ml of medium composed of 1% polypeptone, 0.5% meat extract and 0.5% NaCl, pH adjusted to 7.1, and a loopful of the K_2CrO_4 -tolerant bacterium from stocked culture were cultivated at 30°C for 24 hr and then the broth was centrifuged at

SCHEME 1. Cultivation Method and Procedure for Fractionation of Components of Cells.

* Basal medium: 1% polypeptone; 0.5% meat extract; 0.5% NaCl; pH 7.1.

FIG. 1. Sequential Extraction Method for Insoluble Fraction.

13,000 $\times g$ for 10 min to separate the cells from broth. Thirty mg of the growing cells was further cultivated in the same medium with K_2CrO_4 (Cr^{6+} , 250 ppm) at 30°C for 36~48 hr. The broth was centrifuged at 13,000 $\times g$ for 10 min. After the collecting cells were washed with water and centrifuged at 13,000 $\times g$ for 10 min, cells were suspended into water (10 ml) and disrupted with a sonicator (20 KC, 20 min). The homogenate was centrifuged at 15,000 $\times g$ for 10 min to separate the soluble fraction from insoluble fraction. The soluble fraction was centrifuged at 105,000 $\times g$ for 120 min to separate the microsomal fraction from supernatant fraction. After the microsomal fraction was suspended into water, NaCl was added to the fraction to a final concentration of 0.14 M. After 5% sodium dodecyl sulfate was added to the fraction to a final concentration of 0.41%, an equal volume of phenol saturated with water was added to the fraction. After being stirred for 30 min, the mixture was centrifuged at 1700 $\times g$ for 10 min to separate the phenol layer from water layer. The water layer was extracted once more with phenol. To the water layer which still contained some phenol, ethyl ether was added, and the mixture was shaken to remove a bit of phenol remained in the water layer, and then the water layer was concentrated *in vacuo*. On the other hand, an equal volume of ethanol was added to the phenol layer and after stirring for 1 hr, a precipitate was obtained by centrifugation at 13,000 $\times g$ for 10 min. The collecting precipitate was washed with ethanol and suspended into water. Next, the supernatant fraction was applied to a silicic acid column (Mallinckrodt, 2.7 $\times$ 15 cm) to separate the nucleic acid from protein. Nucleic acid was not adsorbed on this column, whereas protein was adsorbed. So, the column was washed with 10 mM Tris-HCl buffer (pH 7.3) containing 0.3 M NaCl to remove the nucleic acid. The washed solution was used as the nucleic acid fraction. The protein adsorbed on this column was eluted from the column with 1.5 N NH_4OH . Finally, the insoluble fraction was further divided into

six components by the method shown in Fig. 1.

RESULTS

Taxonomical characteristics of K_2CrO_4 -tolerant bacterium

The bacterium was 0.6 $\times$ 1.1 μm in size, rod shape and had a polar flagellum, as shown in Fig. 2. Table I summarizes the morphological and physiological characteristics of the bacterium. From these results, this bacterium was identified and named *Pseudomonas ambigua* G-1.

TABLE I. TAXONOMICAL STUDIES OF THE ISOLATED BACTERIUM

Morphological characteristics	
Rods, 0.6 $\times$ 1.1 μm , motile; monotrichous; Gram stain, negative; spore, not formed	
Cultural and physiological characteristics	
Nutrient agar colonies: Circular; smooth; entire; pale brown; convex	
Nutrient agar slant: Moderate growth; spreading	
Gelatin: No liquefaction	
Bromo cresol purple: Acid; coagulation; decoloring	
Nitrate reduction: Positive	
Soluble pigment production: Negative	
Growth at 37°C: Grew readily	
Cellulose hydrolyzation: Negative	
Utilization of <i>p</i> -hydroxybenzoate: Positive	
Utilization of urea: Positive; alkaline	
Utilization of sugar: Positive but did not form gas from glucose and lactose	

Tolerance-concentration of bacterium to heavy metallic ions

Tolerance-concentrations of the bacterium to other heavy metallic ions were measured.

FIG. 2. Electron Micrograph of the Isolated Bacterium. Bar, 1 μm .

FIG. 3. Cross Tests of the Chromate-tolerant Bacterium to Heavy Metallic Ions. The gradient agar plate method was used for determining the degree of tolerance.

The gradient concentration method for heavy metallic ions similar to the drug tolerance method of Bryson and Szybalski¹²⁾ was used to determine tolerance to heavy metallic ions. The bacterium tolerated up to 2000 ppm of Cr^{6+} , 1700 ppm of Cu^{2+} and 250 ppm of Cd^{2+} , but did not tolerate Hg^{2+} , as shown in Fig. 3. Next, a tolerant test to Cr^{6+} was carried out using liquid cultures which contained four different concentrations of Cr^{6+} in medium. The bacterium grew up to 750 ppm of Cr^{6+} , but at over 250 ppm, growth was slow, and the maximum growth level was low compared with the control, as shown in Fig. 4.

Uptake and distribution of chromate into cells with cultivation time

The uptake of chromate by intact cells with incubation time was examined in the presence of chromate. The amount of chromate uptake into cells and in soluble fraction increased with

FIG. 4. Relationship between Cr^{6+} -Concentration and Growth Change with Time.

Cr^{6+} -tolerant bacterium was used. ○—○, 0 ppm of Cr^{6+} ; ●—●, 250 ppm; □—□, 500 ppm; ■—■, 750 ppm.

incubation time, but the amount in insoluble fraction increased only slightly with incubation time, as shown in Fig. 5. Next, in order to check the distribution of chromate uptake into

FIG. 5. Growth Curve and Distribution of Chromium.

Experiment was carried out using cells grown with Cr^{6+} . $\square$ — $\square$, cells; $\bullet$ — $\bullet$, chromium in cultural fluid; $\circ$ — $\circ$, chromium in cells; $\triangle$ — $\triangle$, chromium in soluble fraction; $\triangle$ — $\triangle$, chromium in insoluble fraction.

cells, the experiment described in Scheme 1 was carried out. The ratio of chromate uptake into

soluble fraction to that of insoluble fraction was about 6.5:1. The distribution of chromate into the soluble fraction was tested further. The ratio of chromate uptake into microsomal fraction to that of supernatant fraction was about 1:3.6, as shown in Table II. An experiment was carried out with the microsomal fraction; that is, the microsomal fraction was divided into two components, nucleic acid and protein, by the phenol extraction method. The results showed that chromate was present in both nucleic acid and protein, as shown in Table III. A further experiment was carried out with the supernatant fraction; that is, the supernatant fraction was divided into two components, nucleic acid and protein, by silicic acid column chromatography. The results showed that chromate was also present in both nucleic acid and protein, as shown in Table IV. On

TABLE II. UPTAKE OF CHROMIUM BY INTACT CELLS

Fraction	Chromium		Components					
	mg	%	Protein		RNA		DNA	
			mg	%	mg	%	mg	%
Cultural fluid (I)	18.3	83.2	—	—	—	—	—	—
Cells (II)	3.7	16.8	—	—	—	—	—	—
Soluble (II-1)	3.2	86.5	37.3	100	11.2	100	4.0	100
Insoluble (II-2)	0.5	13.5	—	—	—	—	—	—
Microsomal (II-1-A)	0.7	21.9	10.4	28.0	3.6	32.0	0.6	15.0
Supernatant (II-1-B)	2.5	78.1	26.9	72.0	7.6	68.0	3.4	85.0

Note; cultivation was carried out in the basal medium with chromium (Cr^{6+} , 22 mg).

TABLE III. DISTRIBUTION OF CHROMIUM IN VARIOUS COMPONENTS OF MICROSOMES FRACTIONATED BY PHENOL EXTRACTION

Fraction	Chromium		Protein		RNA		DNA	
	mg	%	mg	%	mg	%	mg	%
Microsomal(II-1-A)	0.70	100	10.4	100	3.6	100	0.6	100
Nucleic acid(II-1-A-a)	0.23	32.9	0.9	8.7	3.2	88.9	0.4	66.7
Protein(II-1-A-b)	0.47	67.1	9.5	91.3	0.4	11.1	0.2	33.3

TABLE IV. DISTRIBUTION OF CHROMIUM IN VARIOUS COMPONENTS OF SUPERNATANT FRACTIONATED BY SILICIC ACID COLUMN CHROMATOGRAPHY

Fraction	Chromium		Protein		RNA		DNA	
	mg	%	mg	%	mg	%	mg	%
Supernatant(II-1-B)	2.5	100	26.9	100	7.6	100	3.4	100
Nucleic acid(II-1-B-a)	1.22	48.8	2.4	8.9	6.3	82.9	3.2	94.1
Protein (II-1-B-b)	1.28	51.2	24.5	91.1	1.3	17.1	0.2	5.9

TABLE V. DISTRIBUTION OF CHROMIUM IN INSOLUBLE FRACTION (II-2)

Fraction	Chromium %
Polyphosphate-polysaccharide	21
Lipid	67
Poly- β -hydroxybutyrate	0
Mucopeptide	7
Polysaccharide	2
Lipopolysaccharide-polysaccharide	3

the other hand, chromate distribution in the insoluble fraction was tested.

Chromate was distributed 67% in lipid fraction and 21% in polyphosphate-polysaccharide fraction, as shown in Table V.

Comparison of components of bacterial cells with or without chromate

A comparison was made of components of bacterial cells with or without chromate. A large decrease of protein was observed in soluble fraction with chromate compared with the case without chromate, and some decreases were observed in RNA in the microsomal fraction with chromate. A decrease in DNA was also observed in the soluble fraction, as shown in Table VI.

Distribution of chromate in protein of supernatant fraction

A considerable amount of chromate was distributed in protein of the supernatant fraction. The protein component containing chromate was checked by the disc electrophoretic method using 7.5% polyacrylamide gel. Disc electrophoresis was initially carried out using the supernatant preparation from cells cultivated with or without chromate by the usual method.

Some differences were observed between

proteins with or without chromate; that is, some protein components were not detected in the preparation with chromate compared with that without chromate, as shown in Fig. 6.

FIG. 6. Disc Electrophoresis of Supernatant Preparations from Cells Cultivated with or without Chromium.

(a) Without chromium; (b) with chromium. Protein was stained with Amido Black 10B.

Next, disc electrophoresis was carried out for protein prepared from the chromate preparation. In this case, protein stain and chromate stain in diphenyl carbazide were used. The main protein containing chromate was detected at the same position as bromophenol blue dye and another minor protein containing chromate was detected near the top of gel, as shown in Fig. 7.

FIG. 7. Disc Electrophoresis of Supernatant Preparations from Cells Cultivated with Chromate.

(a) Stained with Amido Black 10B; (b) stained with diphenylcarbazine. Arrows show bands containing chromium.

TABLE VI. COMPARISON OF COMPONENT QUANTITY WITH OR WITHOUT CHROMIUM

Fraction	Without chromium						With chromium							
	Protein		RNA		DNA		Chromium		Protein		RNA		DNA	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
Soluble (II-1)	62.2	100	15.0	100	12.0	100	3.2	100	37.3	100	11.2	100	4.0	100
Microsomal(II-1-A)	17.6	28.3	7.2	48.0	1.6	13.3	0.7	21.9	10.4	27.9	3.6	32.1	0.6	15.0
Supernatant(II-1-B)	44.6	71.7	7.8	52.0	10.4	86.7	2.5	78.1	26.9	72.1	7.6	67.9	3.4	85.0

DISCUSSION

Although much research has been done on mercury-effects on microorganisms and some works done on cadmium-effects on microorganisms, there has only been the report of Shimada¹⁾ on chromate-tolerant bacterium. We have already conducted some studies on de-pollution of the complex heavy metal system by biological treatment. In this paper, we isolated and identified a chromate-tolerant bacterium, and some similarities are apparent between the distribution of chromate and mercury in the soluble fraction of cells, but different patterns existed among the distributions of chromate, mercury and cadmium in insoluble fraction of cells; that is, chromate was distributed mainly in the lipid fraction, whereas mercury was distributed in the mucopolypeptide fraction and, cadmium was distributed in the polyphosphate-polysaccharide fraction. Some differences were found between component of cell with or without chromate by disc electrophoresis; that is, in cells with chromate, some proteins were not detected. Two kinds of proteins were found to combine with chromate.

Studies are in progress on the preparation of these proteins containing chromate, the interaction of chromate to nucleic acid and the

toxicity of chromate on cells.

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