Effect of Heavy Metal Ions on the Growth and Iron-oxidizing Activity of *Thiobacillus ferrooxidans*

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Effect of heavy metal ions on the growth and the iron-oxidizing activity of *Thiobacillus ferrooxidans* were investigated.

Cupric, zinc, cadmium, and chromium ions had no effect on the growth and the iron-oxidizing activity of cell suspensions or cell-free extracts of the bacterium in high concentrations (10^{-3} ~ 10^{-2} M). Lead ion delayed the start of the growth slightly in 10^{-3} M, but it did not inhibit the iron-oxidizing activity of the cells in the concentration. Tin and molybdenum oxide ions inhibited both of them in the concentration above 10^{-3} M.

Mercuric mercurous, and silver ions had the most harmful effect. In the concentration of 10^{-3} M, each of the cations inhibited almost completely both the growth and the iron-oxidizing activity of the cells.

In the experiments with cell-free extracts it was observed that the activity of cytochrome oxidase (cytochrome a_{597}) operating in the iron-oxidizing system of the bacterium was specifically inhibited with mercuric ion in the concentration above 5×10^{-4} M.

Thiobacillus ferrooxidans, an iron-oxidizing chemoautotrophic bacterium, exhibits a remarkable tolerance to most heavy metal cations, and survives and grows in the presence of high concentrations of these cations.¹⁾

However, it has been reported²⁾ that uranium, as the soluble uranyl-ion, is toxic to the bacterium.

As *T. ferrooxidans* can assist in the acid leaching of metals from sulfide ores, it is important to investigate the harmful effects of metal ions on the growth and the iron-oxidizing activity of the bacterium.

The present paper deals with screening of harmful metal ions and the inhibiting mechanism of mercuric ion in the iron-oxidizing system of *T. ferrooxidans*.

MATERIALS AND METHODS

Microorganism and culture methods. The chemoautotrophic iron bacterium used in this study was isolated from acid mine water of a mine at Yanahara using 9 K medium reported by Silverman and Lundgren³⁾ and purified twice with a silica gel plate of the

medium. The bacterium was cultured in the medium under aeration at 27~29°C for 98 hr. The culture was filtered under suction in order to remove the bulk of ferric precipitates. The filtrate was centrifuged with a continuous-flow centrifuge. The harvested cells were suspended in cold deionized water, then a small amount of insoluble iron was removed with low speed centrifugation ($350 \times g$), washed three times with deionized water, and stored in the cold.

In the growth experiments, 100 ml of 9 K medium (with or without the test metal salts) in a 500 ml shaking flask was inoculated with 10 ml of the actively growing culture of the same medium and shaken for 6 days at 28°C. The composition of 9 K medium was as follows: $(\text{NH}_4)_2\text{SO}_4$, 3.0 g; KCl, 0.1 g; K_2HPO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{Ca}(\text{NO}_3)_2$, 0.01 g; distilled water, to 700 ml; 10 N H_2SO_4 , 1.0 ml; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 300 ml of a 14.74% (w/v) solution.

Preparation of cell-free extract. Harvested cells were washed twice with cold 0.1 M Tris- H_2SO_4 buffer (pH 7.0), suspended in the buffer and broken with the sonic treatment (20 kHz 20 min) as described in the previous paper.⁴⁾ Unbroken cells and debris were removed by centrifugation at $13,000 \times g$ for 15 min at 4°C. The supernatant fluid was collected and used as crude cell-free extract.

Assay methods. The ferrous ion was determined

colorimetrically by a modification of the *o*-phenanthroline method.⁵⁾ The iron-oxidizing activity of cell suspensions or cell-free extracts was determined by oxygen uptake caused by the oxidation of ferrous ion in a Warburg manometer. Each vessel contained 3.0 ml of liquid volume plus 0.2 ml of 20% potassium hydroxide in the center well. The gas phase was air and the temperature 30°C. The composition of the reaction mixture was as follows (in 3.0 ml): FeSO₄, 100 μ -moles; β -alanine-sulfuric acid buffer, 200 μ -moles (pH 3.5); cells or cell-free extracts, 1 mg or 17 mg protein, respectively; with or without the test metal salts in the concentrations indicated. Spectrophotometric analysis was carried out with a Shimazu Spectrophotometer (MPS-50).

Metal salts. Forms and concentrations of the metals used in this study were as follows: CdSO₄, $10^{-4} \sim 10^{-2}$ M; ZnSO₄, $10^{-5} \sim 10^{-3}$ M; HgNO₃, $10^{-6} \sim 10^{-3}$ M; HgCl₂, $10^{-5} \sim 10^{-3}$ M; AgNO₃, $10^{-5} \sim 10^{-3}$ M; Pb(NO₃)₂, $10^{-5} \sim 10^{-3}$ M; Na₂MoO₄, $10^{-5} \sim 10^{-2}$ M; SnCl₂, $10^{-5} \sim 10^{-3}$ M; CuSO₄, $10^{-4} \sim 10^{-2}$ M; Cr₂(SO₄)₃, $10^{-4} \sim 10^{-2}$ M. All of them are commercial source of analytical grade.

RESULTS

Effect of metal ions on the growth of the bacterium

Each metal salt was added to 100 ml of 9K medium in the concentrations indicated above. The media were inoculated and cultured for 6 days as described in MATERIALS AND METHODS. At intervals of 24 hr, the number of cells and the quantity of the ferrous ion remained in the media were determined and the values were compared with those in the control (the culture without extra metal).

In the control, the number of cells reached to the maximum (2×10^8 /ml) in 3~4 days after the inoculation and the ferrous ion in the culture medium was completely consumed in the same days. As indicated in Table I, cadmium sulfate, zinc sulfate, copper sulfate, and chromic sulfate did not produce any effect on the growth of the bacterium and on the rate of iron consumption in the culture medium even in high concentrations. Lead nitrate in 10^{-3} M delayed the start of the growth and the iron consumption for 2 days. Sodium molybdate and tin chloride inhibited both of the growth and the iron consumption in the concentration above 10^{-3} M.

Mercuric chloride, mercurous nitrate, and

TABLE I. EFFECT OF METAL SALTS ON THE GROWTH AND IRON-CONSUMPTION IN CULTURE MEDIUM

Concentration of added metal salt (M)	Growth of cells	Iron-consumption
CdSO ₄	$10^{-4} \sim 10^{-2}$	normal
ZnSO ₄	$10^{-5} \sim 10^{-3}$	normal
CuSO ₄	$10^{-4} \sim 10^{-2}$	normal
Cr ₂ (SO ₄) ₃	$10^{-4} \sim 10^{-2}$	normal
Pb(NO ₃) ₂	10^{-4}	delayed 1 day
	10^{-3}	delayed 2 days
	10^{-2}	delayed 1 day
Na ₂ MoO ₄	10^{-4}	normal
	10^{-3}	delayed 1 day
	10^{-2}	inhibited 90%
SnCl ₂	10^{-5}	normal
	10^{-4}	normal
	10^{-3}	inhibited almost completely
AgNO ₃	10^{-5}	delayed 2 days
	10^{-4}	inhibited 90%
	10^{-3}	inhibited almost completely
HgCl ₂	10^{-5}	inhibited 80%
	10^{-4}	inhibited almost completely
	10^{-3}	inhibited almost completely
HgNO ₃	10^{-6}	normal
	10^{-5}	inhibited 90%
	10^{-4}	inhibited almost completely

silver nitrate exhibited the most harmful effect. These salts inhibited the growth of cells almost completely in the concentration below 10^{-3} M. As the growth of the bacterium is not inhibited with anions such as nitrate ion (as potassium nitrate) in 10^{-3} M⁶⁾ or with chlorine ion (as sodium chloride) in 10^{-2} M, the harmful effect of mercuric chloride, mercurous nitrate, and silver nitrate will be attributed principally to mercuric, mercurous, and silver ion, respectively.

Effect of metal ions on the iron-oxidizing activity of intact cells

The following reasons may be considered for the inhibition of the growth with special metal ions: (1) The iron-oxidizing system of the cells is inhibited with the metal ions, and the cells become unattainable to acquire energy for the growth. (2) The carbon dioxide-assimilating system of the cells is inhibited. (3) Both of the above systems and/or other important biological systems are inhibited. The authors examined first the effect of metal ions on the iron-oxidizing system of the cells.

Intact cells obtained in the way described in MATERIALS AND METHODS were suspended in β -alanine-sulfuric acid buffer (5×10^{-2} M, pH 3.5). The effect of metal ions on the iron-oxidizing activity of the cell suspension was examined manometrically.

As the results, cupric, chromic, cadmium, lead, and zinc ions did not inhibit the activity at all in the concentration of 10^{-3} M. Already, it was observed by the authors⁶⁾ that cobalt sulfate and nickel sulfate did not affect the activity in 8.3×10^{-2} M. On the other hand, mercuric, mercurous, silver, tin, and molybdenum oxide ions inhibited the iron-oxidizing activity in definite concentrations (Fig. 1).

The inhibitory action of mercuric, mercurous, and silver ions was very intense and each of them inhibited the iron-oxidizing activity of cells completely in the concentration of 10^{-3} M. The sorts of metals which have inhibitory effect

on the iron-oxidizing activity of intact cells were coincided with those which inhibit the growth of the bacterium. Further, the inhibitory concentrations of the metals to the iron-oxidizing activity were almost identical with

FIG. 1. Effect of Metal Salts on Iron-oxidizing Activity of Cells.

As to the conditions of the reaction, see text.

those to the growth.

Effect of mercuric and silver ions on the iron-oxidizing activity of cell-free extract

The preparing method of the cell-free extracts and the experimental conditions are described in MATERIALS AND METHODS. As indicated in Fig. 2, mercuric and silver ions inhibited the iron-oxidizing activity of the cell-free extracts almost completely in the concentration of 10^{-3} M as in the case of intact cells. From the results, it may be postulated that mercuric or silver ions directly inhibit the iron-oxidizing system of the cells.

Inhibiting mechanism of mercuric ion in the iron-oxidizing system

At the present time, the details of iron-oxidizing enzyme system of *T. ferrooxidans* has not been clarified yet. However, Yates and Nason have⁷⁾ postulated the following me-

FIG. 2. Effect of Mercuric and Silver Ions on Iron-oxidizing Activity of Cell-free Extract.

As to the conditions of the reaction, see text.

chanism for the iron-oxidizing system of the bacterium.

We have also detected cytochrome c_{553} and cytochrome a_{597} in the cell-free extract and in the particulate fraction⁸⁾ of the bacterial cells grown autotrophically on ferrous sulfate. Further, it has been confirmed that these cytochromes in the cell-free extract are reduced with ferrous ion in the absence of oxygen. Then, we have investigated the affecting site of mercuric ion in the iron-oxidizing system. If mercuric ion inhibits the electron transfer between ferric ion and *c* type cytochrome (that is, the inhibition of iron-cytochrome *c* reductase), the oxidation of ferrous ion will not occur, and *c* and *a* type cytochromes will not be reduced in the presence of ferrous ion.

On the other hand, if the electron transfer is blocked between *c* and *a* type cytochromes with mercuric ion, *c* type cytochrome will be reduced, but *a* type cytochrome will not on the addition of ferrous ion. Further, if the blocking site exist between *a* type cytochrome and oxygen, both *c* and *a* type cytochromes will be reduced with ferrous ion.

In such considerations, the inhibiting mechanism of mercuric ion in the iron-oxidizing system was examined. The cell-free extract was used as the iron-oxidizing system. It was aerated prior to the experiment in order to oxidize *c* and *a* type cytochromes sufficiently. The composition of the reaction mixture was as follows: β -alanine-sulfuric acid buffer (pH 3.5), 200 μ moles; cell-free extract, 20 mg

protein; HgCl₂ ($0 \sim 5 \times 10^{-3}$ M, final); FeSO₄, 2 μ moles; total volume, 3.0 ml; temperature, 26°C; incubation time, 20 min.

In the control (without HgCl₂), the amount of ferrous ion in the reaction mixture was oxidized completely in 10 min, and the heights of absorption peaks at 553 and 597 nm were increased at first and then decreased during the reaction (Fig. 3). This indicates that *c* and *a* type cytochromes are reduced with ferrous ion and reoxidized with oxygen after the completion of iron oxidation.

FIG. 3. Time Course of the Change in Absorption Spectrum of the Control Mixture.

A, before the addition of ferrous ion; B, immediately after the addition of ferrous ion; C, after 5 min; D, after 10 min; E, after 15 min.

Therefore, 20 min after the addition of ferrous sulfate, the oxidation-reduction states of *c* and *a* type cytochromes in the reaction mixtures were analyzed spectrophotometrically marking the heights of their α -peaks (at 553 and 597 nm, respectively). As indicated in Fig. 4, both of the α -peaks were low in the mixture added with mercuric chloride in the concentrations below 10^{-4} M. On the other hand, both *c* and *a* type cytochromes are remained in reduced state in the mixtures added with mercuric chloride in the concentrations above 5×10^{-4} M (Fig. 4). The quantities of ferrous ion remained in the mixtures were also analyzed. As indicated in Fig. 5, ferrous ion had been oxidized almost completely in

FIG. 4. Effect of Mercuric Chloride on the Redox States of c and a Type Cytochromes.

As to the conditions of the reaction, see text.

FIG. 5. Quantities of Ferrous Ion Remained in the Reaction Mixtures.

the mixtures added with mercuric chloride in the concentrations below 10^{-4} M. On the other hand, 30, 70, and 80% of ferrous ion remained in the mixtures added with mercuric chloride in the concentrations of 5×10^{-4} , 10^{-3} , and 5×10^{-3} M, respectively.

From the results, it will be seen that mercuric ion in the concentration below 10^{-4} M does not inhibit the action of iron-oxidizing system, and c and a type cytochromes of the system return to oxidized state after the completion of iron oxidation. However, mercuric ion in the concentration above 5×10^{-4} M inhibits the iron oxidation, and both c and a type cytochromes remain in the reduced state. The pattern of this effect of mercuric ion on the iron-oxidizing system is analogous to that of cyanide (Fig. 6). Cyanide also inhibits the iron

FIG. 6. Effect of Potassium Cyanide on the Redox States of c and a Type Cytochromes.

The reaction conditions were identical with that of Fig. 4, except mercuric chloride was replaced with potassium cyanide.

oxidation and is well known as a specific inhibitor to cytochrome oxidase.

On the other hand, high concentrations of cupric ion did not inhibit the iron oxidation, and c and a type cytochromes in each reaction mixture were reoxidized after the completion of iron oxidation (Fig. 7).

From these results, it may be postulated that

FIG. 7. Effect of Copper Sulfate on the Redox States of c and a Type Cytochromes.

The reaction conditions were identical with that of Fig. 4, except mercuric chloride was replaced with copper sulfate.

mercuric ion specifically inhibits the autooxidizability of the terminal cytochrome oxidase (cytochrome a_{597}) in the iron-oxidizing system of *T. ferrooxidans* as in the case of cyanide.

DISCUSSION

Reactions of heavy metal ions with biological system are diverse and complicated. For example, it has been observed that heavy metal cations cause plasmolysis in heterotrophic microorganisms⁹⁾ and uranium is reversibly bound in sites of the cell wall or cytoplasmic membrane which locate carriers for transport of organic compounds in yeasts.¹⁰⁾ Further, Tuovinen and Kelly²⁾ has been investigated the growth inhibition of *T. ferrooxidans* with uranyl-ion and supposed that uranyl-ions may presumably be bound in the bacterium in a manner similar to that in yeasts. Therefore, it is difficult to explain simply the reason for the growth inhibition of *T. ferrooxidans* which is caused by even a single metal ion. However, as one of the reasons for the growth inhibition

with mercuric ion, the authors postulate the specific inhibitory action of the cation upon the terminal cytochrome oxidase in the iron-oxidizing system of the bacterium.

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