Volatilization of Mercury by an Iron Oxidation Enzyme System in a Highly Mercury-resistant *Acidithiobacillus ferrooxidans* Strain MON-1

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A highly mercury-resistant strain *Acidithiobacillus ferrooxidans* MON-1, was isolated from a culture of a moderately mercury-resistant strain, *A. ferrooxidans* SUG 2-2 (previously described as *Thiobacillus ferrooxidans* SUG 2-2), by successive cultivation and isolation of the latter strain in a Fe^{2+} medium with increased amounts of Hg^{2+} from 6 μM to 20 μM. The original strain SUG 2-2 grew in a Fe^{2+} medium containing 6 μM Hg^{2+} with a lag time of 22 days, but could not grow in a Fe^{2+} medium containing 10 μM Hg^{2+}. In contrast, strain MON-1 could grow in a Fe^{2+} medium containing 20 μM Hg^{2+} with a lag time of 2 days and the ability of strain MON-1 to grow rapidly in a Fe^{2+} medium containing 20 μM Hg^{2+} was maintained stably after the strain was cultured many times in a Fe^{2+} medium without Hg^{2+}. A similar level of NADPH-dependent mercury reductase activity was observed in cell extracts from strains SUG 2-2 and MON-1. By contrast, the amounts of mercury volatilized for 3 h from the reaction mixture containing 7 μM Hg^{2+} using a Fe^{2+}-dependent mercury volatilization enzyme system were 5.6 nmol for SUG 2-2 and 67.5 nmol for MON-1, respectively, indicating that a marked increase of Fe^{2+}-dependent mercury volatilization activity conferred on strain MON-1 the ability to grow rapidly in a Fe^{2+} medium containing 20 μM Hg^{2+}. Iron oxidizing activities, 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) oxidizing activities and cytochrome c oxidase activities of strains SUG 2-2 and MON-1 were 26.3 and 41.9 μl O_{2} uptake/mg/min, 15.6 and 25.0 μl O_{2} uptake/mg/min, and 2.1 and 6.1 mU/mg, respectively. These results indicate that among components of the iron oxidation enzyme system, especially cytochrome c oxidase activity, increased by the acquisition of further mercury resistance in strain MON-1. Mercury volatilized by the Fe^{2+}-dependent mercury volatilization enzyme system of strain MON-1 was strongly inhibited by 1.0 mM sodium cyanide, but was not by 50 μM rotenone, 5 μM 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), 0.5 μM antimycin A, or 0.5 μM myxothiazol, indicating that cytochrome c oxidase plays a crucial role in mercury volatilization of strain MON-1 in the presence of Fe^{2+}.

**Key words:** iron-oxidizing bacterium; *Acidithiobacillus ferrooxidans*; mercury resistance; mercury reduction; cytochrome c oxidase

*A. ferrooxidans* inhabits drainage in acid mines and plays a crucial role in the bacterial leaching of sulfide ores. The value of *A. ferrooxidans* in bacterial leaching may be due to the ability of this bacterium to oxidize both ferrous ion (Fe^{2+}) and reduced-sulfur compounds. *A. ferrooxidans* strains that have a high iron-oxidizing activity in an environment with many kinds of and high concentrations of heavy metals are required for microbiological leaching of low grade ores. It has been known that *A. ferrooxidans* cells are in general resistant to many heavy metals including iron, copper, zinc, and nickel, but sensitive to mercury, silver, molybdenum, and tungsten. We have reported the growth inhibition of *A. ferrooxidans* cells by mercury, silver, molybdenum, and tungsten and clarified inhibition sites for these toxic metals. Among these toxic metals mercury is highly toxic for almost all organisms because they have a strong affinity for thiol groups in proteins. The bacteria that are resistant to Hg^{2+} and/or organomercurial compounds have the ability to volatilize metal mercury (Hg^0) from inorganic and organic mercurial compounds. A wide range of Gram-negative and Gram-positive bacteria have mercury reductase that reduce Hg^{2+}.

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with NADPH as an electron donor. A. ferrooxidans cells have mercury reductase activity and the genes involved in the volatilization of mercury have been cloned and characterized in detail.

A. ferrooxidans SUG 2-2 was isolated as a mercury-resistant strain among one hundred A. ferrooxidans strains isolated from natural environments. Strain SUG 2-2 has an ability to volatilize metal mercury from mercury-polluted wastewater and soil under acidic conditions in the presence of ferrous iron. We recently showed that A. ferrooxidans SUG 2-2 has not only NADPH-dependent mercury reductase activity but also Fe\(^{2+}\)-dependent mercury reductase activity in the cells and cytochrome c oxidase purified from strain SUG 2-2 volatilizes mercury in the presence of Fe\(^{2+}\).

In this study, a highly mercury-resistant strain, A. ferrooxidans MON-1 was isolated from a culture of a moderately mercury-resistant strain, A. ferrooxidans SUG 2-2, by successive cultivation and isolation of the latter strain in a Fe\(^{2+}\) medium with increased amounts of Hg\(^{2+}\) from 6 \(\mu\)M to 20 \(\mu\)M. We first show that the Fe\(^{2+}\)-dependent mercury volatilization enzyme system was more important than NADPH-dependent mercury reductase for the acquisition of further mercury resistance in A. ferrooxidans cells and among components of the iron oxidation enzyme system, cytochrome c oxidase activity of strain MON-1 increased accompanied with an increase of mercury resistance.

**Materials and Methods**

**Microorganisms, medium, and growth conditions.** The iron-oxidizing bacteria used in this study were A. ferrooxidans AP19-3(1) and A. ferrooxidans SUG 2-2. Each strain was cultivated at 30°C under aerobic conditions in an Fe\(^{2+}\) medium (pH 2.5) containing 30 g of FeSO\(_4\)-7H\(_2\)O, 3 g of (NH\(_4\))\(_2\)SO\(_4\), 0.5 g of K\(_2\)HPO\(_4\), 0.5 g of MgSO\(_4\)-7H\(_2\)O, 0.1 g of KCl, and 0.01 g of Ca(NO\(_3\))\(_2\). The resting cells were prepared as follows. Each strain of iron-oxidizing bacteria was grown in 70 l of Fe\(^{2+}\) medium under aeration for one week. The culture medium was filtered with a Toyo no.2 filter paper to remove the bulk of the ferric precipitates and then centrifuged with a Hitachi 18PR-52 continuous-flow rotor at 15,000 \(\times\) g and a flow rate of 200 ml/min. Harvested cells were washed three times with 0.1 M \(\beta\)-alanine-SO\(_4^{2-}\) buffer (pH 3.0) before use.

**Analysis of mercury volatilized from culture medium of A. ferrooxidans.** A 50-ml culture flask with a screw cap contained 19 ml of Fe\(^{2+}\) medium (pH 2.5) with 1.0 or 5.0 \(\mu\)M Hg\(^{2+}\) and 1 ml of an active seed culture of A. ferrooxidans added. A small test tube containing 2 ml of a KMnO\(_4\) solution was inserted in the 50-ml culture flask to trap the Hg\(^{2+}\) volatilized from the culture medium. The KMnO\(_4\) solution used (100 ml) was composed of a 10-ml solution containing 0.6 g of KMnO\(_4\), 5 ml of concentrated H\(_2\)SO\(_4\), and 85 ml of deionized water. After the culture medium was aerated by shaking at 30°C and 100 rpm, the concentration of Hg\(^0\) trapped in the KMnO\(_4\) solution was measured by cold-vapor atomic absorption spectroscopy.

**Mercury reductase activity.** The reaction mixture (2.5 ml) contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.2 mM MgSO\(_4\)-7H\(_2\)O, 1 mM \(\beta\)-mercaptoethanol, 0.2 mM NADPH, 1.5 mg bovine serum albumin, 0.1 mM HgCl\(_2\), and cytosols prepared from A. ferrooxidans strains SUG 2-2 and MON-1. The cytosol was prepared by centrifugation of a cell extract at 105,000 \(\times\) g for 1 h. After the reaction mixture was incubated at 37°C for 60 min, the reaction was started by the addition of NADPH. The activity was measured by the rate of oxidation of NADPH by monitoring the decrease of absorbance at 340 nm.

**Fe\(^{2+}\)-dependent mercury volatilization activity.** Each of several 50-ml flasks with screw caps contained a reaction mixture plus 2 ml of a KMnO\(_4\) solution described above. The gas phase was air, and the reaction mixture rotated at 100 rpm at 30°C. The reaction mixture used for the measurement of Fe\(^{2+}\)-mercury volatilization activity was composed of water acidified with sulfuric acid (20 ml), resting cells of A. ferrooxidans (1 mg of protein), mercuric chloride (14-140 nmol), and ferrous sulfate (100 \(\mu\)mol). After the reaction mixture was aerated by shaking at 30°C and 100 rpm, the concentration of Hg\(^0\) trapped in the KMnO\(_4\) solution was measured by cold-vapor atomic absorption spectroscopy.

**Iron oxidizing activity.** The activities of iron oxidation by A. ferrooxidans cells was measured by the amount of oxygen uptake due to Fe\(^{2+}\) oxidation with a biological Oxygen Monitor (Yellow Spring Instrument Co., inc., Ohio USA). The composition of the reaction mixture used to measure the iron-oxidizing activity was as follows: resting cells (0.1 mg of protein), Fe\(^{2+}\) (100 \(\mu\)mol), and 0.1 M \(\beta\)-alanine-SO\(_4^{2-}\) buffer (pH 3.0) (2.5 ml). The total volume was 3.0 ml. The reaction mixture except for ferrous iron was first incubated at 30°C for 10 min. The reaction was started by adding ferrous iron into this reaction mixture.

2,3,5,6-tetramethyl-p-phenylenediamine oxidizing activity. The activities of 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) oxidation by A. ferrooxidans cells was measured by the amount of oxygen uptake, due to TMPD oxidation, with a biological Oxygen Monitor (Yellow Spring Instrument Co.,
inc., Ohio USA). The composition of the reaction mixture used to measure the TMPD-oxidizing activity was as follows: resting cells (0.1 mg of protein), TMPD (100 µmol), and 0.1 M β-alanine-SO₄²⁻ buffer (pH 3.0) (2.5 ml). The total volume was 3.0 ml. The reaction mixture except for TMPD was first incubated at 30°C for 10 min. The reaction was started by adding TMPD to this reaction mixture.

**Cytochrome c oxidase activity.** The activity of cytochrome c oxidase was measured at 550 nm as the absorbance decrease with the oxidation of the reduced mammalian cytochrome c (Sigma, from horse heart) at 30°C using Shimadzu UV-1200 spectrophotometer. The reaction mixture contained plasma membranes from *A. ferrooxidans* strain AP19-3, SUG 2-2, and MON-1 (0.25 mg), reduced cytochrome c (0.4 mg) and sodium phosphate buffer (pH 5.5, 2.25 ml). The total volume of the reaction mixture was 2.5 ml. The reduced cytochrome c was prepared by the reduction of cytochrome c by the reduction of cytochrome 1 amount of enzyme that catalyzed the oxidation of 1 µmol of reduced cytochrome c in one min at 30°C. The plasma membrane was prepared as follows. A cell-free extract was prepared from the resting cells by sonication in 0.1 M sodium phosphate buffer (pH 7.5) at 100 W for 10 min and then centrifugation at 12,000×g for 10 min to remove cell debris. The extract was centrifuged at 105,000×g for 1 h to obtain the plasma membrane.

**Protein content.** Protein content was measured by the method of Lowry et al. with crystalline bovine serum albumin as the standard.

**Results**

**Isolation of strain MON-1 from a culture of *A. ferrooxidans* SUG 2-2**

The mercury-sensitive strain *A. ferrooxidans* AP19-3 cannot grow in a Fe²⁺ medium containing 0.7 µM Hg²⁺, but can grow in a Fe³⁺ medium containing 0.6 µM Hg²⁺ after a lag time of 24 days. By contrast, a moderately mercury-resistant strain, *A. ferrooxidans* stain SUG 2-2, grew in a Fe²⁺ medium containing 6 µM Hg²⁺ with a lag time of 22 days, but could not grow in a Fe³⁺ medium containing 10 µM Hg²⁺. The processes to isolate a highly mercury-resistant strain MON-1 compared with strain SUG 2-2 are described below. Eight times successive cultivations of strain SUG 2-2 in a Fe²⁺ medium containing 6 µM Hg²⁺ shortened the lag time to 4 days, from which strain SUP-1 was newly isolated as a single colony on a 1.0% gellan gum plate containing ferrous sulfate and salts. Strain SUP-1, thus obtained, could grow in a Fe²⁺ medium containing 20 µM Hg²⁺ with a lag of 10 days. Five successive cultivations of strain SUP-1 in a Fe²⁺ medium containing 20 µM Hg²⁺ shortened the lag time to 4 days, from which strain MON-1 was isolated as a single colony on a 1.0% gellan gum plate containing ferrous sulfate and salts. Strain MON-1 grew in a Fe³⁺ medium containing 20 µM with a lag time of 2 days, slightly grew in a Fe³⁺ medium containing 40 µM Hg²⁺, but could not in a Fe²⁺ medium containing 80 µM Hg²⁺. Strain MON-1 maintained an ability to grow rapidly in a Fe²⁺ medium containing 20 µM Hg²⁺ after successive cultivation of the strain in a Fe³⁺ medium without Hg²⁺. When grown in a Fe³⁺ medium (pH 2.5) containing 3% ferrous sulfate and salts at 30°C, strain MON-1 gave cell yields ranged from 4–5×10⁶ cells/ml. Growth rate and cell yield of strain MON-1 in the Fe²⁺ medium were lower than those of strains AP19-3 and SUG 2-2 (Fig. 1).

**Volatilization of metal mercury from a Fe²⁺ medium containing mercuric chlorides**

Cell growth and the amount of mercury volatilized from a medium were measured by the cultivation of strains AP19-3, SUG 2-2, and MON-1 in a Fe²⁺ medium (pH 2.5) containing ferrous sulfate (3%), salts and mercuric chloride (5.0 µM). Strong growth inhibition was observed in the case of strain SUG 2-2.
Fig. 2. Cell Growth and Volatilization of Metal Mercury from a Fe$_2^+$ Medium Containing A. ferrooxidans Strains and 5 µM Hg$_2^+$. A. ferrooxidans strains AP19-3 (○), SUG 2-2 (△), and MON-1 (●) were grown in a 3% ferrous sulfate medium (pH 2.5) containing 5 µM Hg$_2^+$ at 30°C. Ferrous ion concentrations in the media inoculated with A. ferrooxidans strains AP19-3 (○), SUG 2-2 (△) and MON-1 (●) were also measured. Since strain SUG 2-2 could volatilize 12 nmol of mercury when cultured in a Fe$_2^+$ medium (pH 2.5) containing ferrous sulfate (3%), salts, and mercuric chloride (0.7 µM) for 4 days, it is suggested that the mercury volatilization system of strain SUG 2-2, but not MON-1 cells, was inhibited by 5 µM Hg$_2^+$. A mercury-sensitive strain, A. ferrooxidans AP19-3, did not grow in a Fe$_2^+$ medium (pH 2.5) containing ferrous sulfate (3%), salts and mercuric chloride (5.0 µM) and as a result, metal mercury was not volatilized from the medium (Fig. 2A and 2B).

**NADPH-dependent mercury reductase activity**

It has been known that a wide range of Gram-negative and Gram-positive bacteria have mercury reductases that reduce Hg$^{2+}$ with NADPH as an electron donor. NADPH-dependent mercury reductase activity was measured with cell extracts of strains SUG 2-2 and MON-1 grown in a Fe$_2^+$ medium (pH 2.5) without Hg$^{2+}$. Mercury reductase activity measured by the oxidation of NADPH increased in proportion to the concentration of cell extracts from both strains (data not shown). NADPH-dependent mercury reductase activities of strains SUG 2-2 and MON-1 were 0.026 and 0.023 ΔA$_{340}$ nm/mg/min, respectively. NADPH-dependent mercury reductase activities of SUG 2-2 and MON-1 measured by the amount of mercury volatilized from the same reaction mixture were 26.4 and 34 nmol Hg volatilized/mg/3 h, respectively. These results indicate that strains SUG 2-2 and MON-1 have a similar level of NADPH-dependent mercury reductase activity in spite of the difference in strains mercury resistance.

**Fe$^{2+}$-dependent mercury volatilization activity**

We recently showed that A. ferrooxidans SUG 2-2 has not only NADPH-dependent mercury reductase activity but also Fe$^{2+}$-dependent mercury reductase activity in the cells and the latter activity was important for strain SUG 2-2 to grow rapidly in a Fe$_2^+$ medium containing 0.7 µM Hg$^{2+}$ and volatilize mercury from the medium. Fe$^{2+}$-dependent mercury volatilization activity was measured in 20 ml of water acidified with sulfuric acid (pH 2.5) containing resting cells grown in a Fe$_2^+$ medium (pH 2.5) without Hg$^{2+}$ (1.0 mg) and 140 nmol HgCl$_2$ by incubating for

![Graph](image-url)
Volatilization of Mercury by Iron Oxidase in *A. ferrooxidans*

3 hours at 30°C. The amounts of mercury volatilized from reaction mixtures were 27 nmol for strain MON-1 (Fig. 3C). Further addition of 100 μmol ferrous sulfate to the reaction mixture enhanced the amount of mercury volatilized from the reaction mixture 2.5 fold. In contrast, 1.2 fold of enhancement was observed in the case of strain SUG 2-2 (Fig. 3C). Fe²⁺-dependent volatilization of mercury was found when strain SUG 2-2 cells were incubated in a 20 ml of acidic water containing 14 or 100 nmol Hg²⁺ (Fig. 3A and 3B). However, the amount of mercury volatilized from the reaction mixture containing resting cells of strain SUG and 140 nmol Hg²⁺ markedly decreased by contrast to the case of strain MON-1 (Fig. 3C), suggesting that Fe²⁺-dependent volatilization activity of SUG 2-2 was inhibited by 140 nmol Hg²⁺.

Iron-oxidizing activity and cytochrome c oxidase activity of *A. ferrooxidans* strains AP19-3, SUG 2-2, and MON-1

Iron-oxidizing activity was measured with resting cells of *A. ferrooxidans* strains AP19-3, SUG 2-2, and MON-1. The activity of strain MON-1 was approximately 2.6- and 1.6-fold higher than those of strain AP19-3 and SUG 2-2 (Fig. 4A). These results correspond well with the results that a high Fe²⁺-dependent mercury volatilization activity was observed in strain MON-1 compared with strains AP19-3 and SUG 2-2 (Fig. 3). We recently showed that cytochrome c oxidase purified from strain SUG 2-2 volatilizes mercury in the presence of Fe²⁺. Cytochrome c oxidase is one of the most important components of the iron oxidation enzyme system in *A. ferrooxidans*. Therefore, cytochrome c oxidase activity was compared among resting cells of strains AP19-3, SUG 2-2, and MON-1 by measuring the oxidation rate of 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD). TMPD oxidizing activity of strain MON-1 was approximately 4.9- and 1.6-fold higher than that of strain AP19-3 and SUG 2-2 (Fig. 4B). Cytochrome c oxidase activities were also measured by the oxidation rate of reduced cytochrome c with plasma membranes prepared from strains AP19-3, SUG 2-2, and MON-1. The activity of strain MON-1 was 2.8-fold higher than that of strain SUG 2-2 (Fig. 4C).

Effects of inhibitors of the terminal electron transport system and chloramphenicol on Fe²⁺-dependent mercury volatilization activity of *A. ferrooxidans* strain MON-1

The existence of the energy-dependent electron transfer pathway involved in the reduction of pyridine nucleotides which is required for CO₂ fixation in the acidophilic chemolithotrophic bacterium *A. ferrooxidans* has been presented. Cytochrome c in *A. ferrooxidans* cells is reduced by Fe²⁺ and then the electrons from the reduced cytochrome c are transported by a uphill pathway involving cytochrome bc₁ and NADH: coenzyme Q oxidoreductase complex functioning in reverse to NAD⁺. It is supposed that the NADPH required for the reduction of mercury by NADPH-dependent mercury reductase is produced by a transhydrogenase reaction from NADH. An uphill pathway to produce NADH is inhibited by rotenone, antimycin A, and myxothiazol, but not by sodium cyanide. The Fe²⁺-dependent mercury volatilization activity of strain MON-1 was strongly inhibited by 1 mM of sodium cyanide which inhibits the cytochrome c oxidase activity of *A. ferrooxidans* cells, indicating that cytochrome c oxidase was involved in Fe²⁺-dependent mercury volatilization of strain MON-1. By contrast, inhibi-
Fig. 5. Proposed Mechanism of Mercury Reduction by NADPH-Dependent Mercuric Reductase System and Fe$^{2+}$-Dependent Mercury Volatilization Enzyme System in *A. ferrooxidans* Strains SUG 2-2 and MON-1.

Fig. 6. Effects of Inhibitors of Terminal Electron Transport System on Fe$^{2+}$-Dependent Mercury Volatilization Activity of *A. ferrooxidans* Strain MON-1.

Fe$^{2+}$-dependent mercury volatilization activity of resting cells of *A. ferrooxidans* strain MON-1 was measured in the presence of inhibitors of the terminal electron transport system: •, rotenone (50 nM); ■, 2-n-heptyl-4-hydroxy-quinoline-N-oxide (HQNO) (5 μM); ▲, antimycin A (5 μM); ▼, myxothiazol (5 μM); ○, sodium cyanide (1 mM).

Fig. 7. Effects of Chloramphenicol on Fe$^{2+}$-Dependent Mercury Volatilization Activity of *A. ferrooxidans* Strains.

Fe$^{2+}$-dependent mercury volatilization activities of resting cells of *A. ferrooxidans* strains AP19-3 (●), SUG 2-2 (▲), and MON-1 (■) were measured in 20 ml of water acidified with sulfuric acid (pH 2.5) containing resting cells of *A. ferrooxidans* (1 mg of protein), ferrous sulfate (100 μmol), 100 nmol of Hg$^{2+}$, and chloramphenicol (3 mM). Fe$^{2+}$-dependent mercury volatilization activities of *A. ferrooxidans* strains AP19-3 (○), SUG 2-2 (▲) and MON-1 (●) were also measured in 20 ml of water acidified with sulfuric acid (pH 2.5) containing resting cells of *A. ferrooxidans* (1 mg of protein), ferrous sulfate (100 μmol), and 100 nmol of Hg$^{2+}$.

Chloramphenicol completely inhibited the growth of strain MON-1 at 2 mM (data not shown). Chloramphenicol did not inhibit Fe$^{2+}$-dependent mercury volatilization activities of strains SUG 2-2 and MON-1 at 3 mM (Fig. 7), indicating that the Fe$^{2+}$-dependent mercury volatilization reaction was done under the conditions in which *de novo* synthesis of NADP-dependent mercury reductase and cytochrome c oxidase were completely inhibited in the resting cells of MON-1.

Discussion

A highly mercury-resistant strain *A. ferrooxidans* MON-1 was isolated from a culture of a moderately mercury-resistant strain *A. ferrooxidans* SUG 2-2 by successive cultivation and isolation of the latter strain...
in a Fe$^{2+}$ medium with increased amounts of Hg$^{2+}$ ranging from 6 μM to 20 μM. The most remarkable properties of strain MON-1 concerning mercury detoxification was the cell’s possession of high Fe$^{2+}$-dependent mercury volatilization activity. Since similar levels of NADPH-dependent mercury reductase activity was observed in cell extracts from strains Sugio, Sugio, T., Kuwano, H., Negishi, A., Maeda, T., Isolation and some properties of silver ion-resistant iron-oxidizing bacterium, *A. ferrooxidans*. Appl. Environ. Microbiol., 66, 3823–3827 (2000).


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