Volatilization of Mercury under Acidic Conditions from Mercury-polluted Soil by a Mercury-resistant Acidithiobacillus ferrooxidans SUG 2-2

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Volatilization of mercury under acidic conditions from soil polluted with mercuric chloride (1.5 mg Hg/kg soil) was studied with resting cells of a mercury-resistant strain, Acidithiobacillus ferrooxidans SUG 2-2. When resting cells of SUG 2-2 (0.01 mg of protein) were incubated for 10 d at 30°C in 20 ml of 1.6 mm sulfuric acid (pH 2.5) with ferrous sulfate (3%) and mercury-polluted soil (1 g), which contained 7.5 nmol of Hg, approximately 4.1 nmol of mercury was volatilized, indicating that 54% of the total mercury in the soil was volatilized. The amount of mercury volatilized from the soil was dependent on the concentration of Fe2+ added to the medium. When elemental sulfur, sodium tetrathionate, and pyrite were used as an electron donor for the mercury reduction, 16, 2.4 and 0.84%, respectively, of the total mercury added to the solution were volatilized. The optimum pH and temperature for mercury volatilization were 2.5 and 30°C. Approximately 92% of the total mercury in a salt solution (pH 2.5) with resting cells of SUG 2-2 (0.01 mg of protein), ferrous sulfate (3%) and mercury-polluted soil (1 g) was volatilized by further addition of both resting cells and Fe2+ and by incubating for 30 d at 30°C.

Key words: Acidithiobacillus ferrooxidans; mercury resistance; volatilization of mercury; iron-oxidizing bacterium

Mercury and organomercurial compounds are highly toxic because they have a strong affinity for thiol groups in proteins.1,2 To keep the environment clean, it is important to remove mercury compounds that are bound to the soil. It has been known that bacteria that are resistant to Hg2+ and/or organomercurial compounds have the ability to reduce Hg2+ to volatilizable metal mercury (Hg0).3-5 A wide range of Gram-negative and Gram-positive bacteria have mercury reductases that reduce Hg2+ with NADPH as an electron donor to give Hg0.7-9 The iron-oxidizing bacterium Acidithiobacillus ferrooxidans10 (Thiobacillus ferrooxidans) is one of the most important bacteria for bacterial leaching of sulfide ores.11,12 The bacterium inhabits acid mine drainage containing high concentrations and many kinds of heavy metals. Since mercury, silver, and molybdenum ions markedly inhibit the growth of A. ferrooxidans cells,13-17 it is important to isolate strains of A. ferrooxidans resistant to these heavy metals and clarify the mechanism of resistance to heavy metals. Mercury reductase activity has been found in A. ferrooxidans cells.15,16,18,19 The genes involved in the volatilization of mercury have been cloned and characterized in detail.20-22

We partially characterized the difference between a mercury-resistant and a mercury-sensitive strains of A. ferrooxidans.23 The levels of NADPH-dependent mercury reductase was not significantly different in these strains. Instead, cytochrome c oxidase purified from the resistant strain, Funis 2-1, was more resistant to Hg2+ than that from the sensitive strain, AP19-3.10 Recently, we showed that a newly isolated strain, A. ferrooxidans SUG 2-2, is much more resistant to mercury than the previously reported strain Funis 2-1, and the Fe2+-dependent mercury volatilization activity is present in six A. ferrooxidans strains including strains Funis 2-1 and SUG 2-2.25 Interestingly, the plasma membrane from SUG 2-2 cells, which do not show NADPH-dependent mercury reductase activity, volatilized mercury depending on Fe2+.25 The volatilization activity was markedly activated by the component of the iron oxidation system or rusticyanin, but strongly inhibited by NaCN, suggesting that cytochrome c oxidase, another important component of iron oxidation enzyme system

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of _A. ferroxidans_, plays an important role in the mercury volatilization reaction.

_A. ferroxidans_ has unique properties different from many heterotrophic bacteria. Namely, _A. ferroxidans_ is an obligate acidophile and has an activity to volatilize mercury under acidic conditions. The bacterium is an obligate chemolithotroph and does not require organic compounds as an energy or carbon source for cell growth and cell maintenance. Moreover, _A. ferroxidans_, in general, is resistant to heavy metals except the following the four heavy metal compounds, mercury chloride, silver nitrate, sodium molybdate, and sodium tungstate. Considering these unique properties, an _A. ferroxidans_ strain resistant to mercury seems to be useful, especially under acidic conditions, for the removal of mercury from mercury-polluted soils in which low concentrations of organic compounds, but high concentrations of heavy metals are present. There have been no reports on the volatilization of mercury by a mercury-resistant strain of _A. ferroxidans_ from soil polluted with mercury chloride under acidic conditions. In this work, we first show that mercury-resistant _A. ferroxidans_ SUG 2-2 could volatilize mercury from mercury-polluted soil under acidic conditions, and then, optimize the conditions for mercury volatilization.

**Materials and Methods**

**Microorganisms, medium, and growth conditions.**

The iron-oxidizing bacteria used in this study were _A. ferroxidans_ strains SUG 2-2, Funis 2-1, and AP19-3. These bacteria were cultivated at 30°C under aerobic conditions in ferrous iron medium (pH 2.5) containing 30 g of FeSO₄·7H₂O, 3 g of (NH₄)₂SO₄, 0.5 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of KCl, and 0.01 g of Ca(NO₃)₂ per liter. Resting cells were prepared as follows. Three strains of _A. ferroxidans_ were grown in 70 liters of Fe²⁺ medium (pH 2.5) under aeration, for a week. The culture medium was filtered with Toyo no. 2 filter paper to remove the bulk of the ferric precipitates and then centrifuged using a Hitachi 18PR-52 continuous-flow rotor at 15,000 × g and a flow rate of 200 ml/min. Harvested cells were washed three times with 0.1 M β-alanine-SO₄²⁻ buffer (pH 3.0), and twice with 1.6 mM sulfuric acid (pH 2.5), and used as resting cells in this study.

**Analysis of mercury volatilized from mercury-polluted soil.** The soil polluted with mercuric chloride was obtained from Niigata Prefecture and was used as an example of mercury-polluted soil in this experiment. The mercury-polluted soil (pH 8.9) contained 36.2% moisture and 1.5 mg of Hg per kg of soil. A 50-ml culture flask with a screw cap contained 19 ml of 1.6 mM sulfuric acid, with cell suspension of _A. ferroxidans_ SUG 2-2 (1 ml) and mercury-polluted soil (1 g). A small test tube containing 2 ml of a KMnO₄ solution was inserted in the 50-ml reaction flask to trap the Hg⁶⁺ volatilized from the reaction mixture. The KMnO₄ solution used (100 ml) was composed of 10 ml of solution containing 0.6 g of KMnO₄, 5 ml of concentrated H₂SO₄, and 85 ml of deionized water. After the reaction mixture was shaken at 30°C and 100 rpm, the concentration of Hg⁶⁺ trapped in the KMnO₄ solution was measured by cold-vapor atomic absorption spectroscopy.

**Protein content.** Protein was measured by a biuret method, using crystalline bovine serum albumin as the standard.

**Results and Discussion**

**Volutilization of mercury from mercury polluted soil by resting cells of _A. ferroxidans_ SUG 2-2**

Volutilization of mercury from mercury-polluted soil by resting cells of _A. ferroxidans_ SUG 2-2 was studied in diluted sulfuric acid. The total mercury in 1 g of mercury-polluted soil was 7.5 nmol. When resting cells of SUG 2-2 (0.01 mg of protein) were incubated for 10 d at 30°C in 20 ml of 1.6 mM sulfuric acid (pH 2.5) supplemented with ferrous sulfate (3%) and a mercury-polluted soil (1%), approximately 4.1 nmol of mercury was volatilized accompanied with the oxidation of Fe²⁺ in the reaction mixture, indicating that 54% of the total mercury in the soil was volatilized (Fig. 1). Mercury was not volatilized from the reaction mixture without resting cells, or with the resting cells boiled for 10 min. In these control experiments, the amounts of Fe²⁺ oxidized for 10 d of incubation were quite low compared with that done with active cells.

**The effects of pH and temperature on the volatilization of mercury from the mercury-polluted soil**

The optimum pH for mercury volatilization was 2.5, when the mercury-polluted soil was incubated for 10 d in diluted sulfuric acid with resting cells of _A. ferroxidans_ SUG 2-2 (0.01 mg of protein) and ferrous sulfate (3%) (Fig. 2). We did similar experiments with salt solution with different pH values to check whether more mercury is volatilized from the soil than in the experiments with sulfuric acid. The salt solution used for this purpose contained 3 g of (NH₄)₂SO₄, 0.5 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of KCl and 0.01 g of Ca(NO₃)₂ per liter of distilled water. When resting cells of SUG 2-2 (0.01 mg of protein) were incubated for 10 d at 30°C in 20 ml of the salt solution (pH 2.5) with ferrous sulfate (3%) and a mercury-polluted soil (1%), approximately 3.7 nmol of mercury was volatilized. The results indicate that sulfuric acid was slightly better than the salt solution for the mercury volatilization from the mercury-polluted soil.
Volatilization of Mercury by *A. ferrooxidans*

![Graph](image)

**Fig. 1.** Volatilization of Mercury from Mercury-polluted Soil by Resting Cells of *A. ferrooxidans* SUG 2-2.

Mercury-polluted soil used in this study was soil contaminated with mercuric chloride (1.5 mg Hg per kilogram of soil). Volatilization of mercury from mercury-polluted soil was done in 20 ml of 1.6 mm sulfuric acid (pH 2.5) with ferrous sulfate (3%), mercury-polluted soil (1 g), and resting cells of *A. ferrooxidans* SUG 2-2. Symbols: the amount of mercury volatilized ( ■), active cells; ( ●), the cells boiled for 10 min; the amount of Fe$^{2+}$ in 20 ml of 1.6 mm sulfuric acid ( ○), active cells; ( □), the cells boiled for 10 min). Volatilization of mercury from the soil was also done in 20 ml of 1.6 mm sulfuric acid (pH 2.5) with ferrous sulfate (3%), mercury-polluted soil (1 g), but without resting cells of *A. ferrooxidans* SUG 2-2: the amounts of mercury volatilized ( ▲) and Fe$^{2+}$ in 20 ml of 1.6 mm sulfuric acid ( △).

**Fig. 2.** Effects of pH on the Volatilization of Mercury from Mercury-polluted Soil by Resting Cells of *A. ferrooxidans* SUG 2-2.

Volatilization of mercury from mercury-polluted soil was done in 20 ml of acidic water (pH 2.5) (white bar) or salt solution (pH 2.5) (black bar) with ferrous sulfate (3%), mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2. The pH of the solution was adjusted by adding sulfuric acid or sodium hydroxide until it came to the pH indicated in the figure.

...at almost all the pHs tested. These results probably suggest that SUG 2-2 cells did not grow in salt solution with both Fe$^{2+}$ and a mercury-polluted soil (1 g) although they could oxidize the Fe$^{2+}$ in the solution. The pH which gave the maximum mercury volatilization activity corresponded well with the optimum pH for iron oxidation by this bacterium. The pH of mercury-polluted soil used in this experiment is 8.9. However, quite a low level of mercury was volatilized from the soil at pH 9 compared with pH 2.5, probably because the iron oxidation system of *A. ferrooxidans* did not operate at pH 9 and the Fe$^{2+}$, which is required as an electron donor for the reduction of Hg$^{2+}$, was rapidly oxidized chemically at the pH.

The optimum temperature for mercury volatilization was 30°C when mercury-polluted soil was incubated for 10 d in 1.6 mm sulfuric acid with resting cells of SUG 2-2 (0.01 mg of protein) and ferrous sulfate (3%). The effects of temperature on the mercury volatilization from the mercury-polluted soil were also studied in 20 ml of salt solution (pH 2.5). The same optimum temperature and a similar level of mercury volatilization as those done with 1.6 mm sulfuric acid were obtained.

**The effects of the concentration of SUG 2-2 cells on the volatilization of mercury from the mercury-polluted soil**

The amount of mercury volatilized from the soil increased with increasing concentrations of resting cells of SUG 2-2, and the largest mercury volatilization was obtained when 0.01 mg of cell protein was added to the reaction mixture. Addition of ten times more cells (0.1 mg of protein) to the reaction mixture rather decreased the amount of mercury volatilized. We cannot explain the reason why resting cells more than 0.1 mg decreased the amount of mercury volatilization. However, since a rapid oxidation of Fe$^{2+}$ in the reaction mixture was observed in the early step of incubation when 0.1 mg of protein was added to the reaction mixture, we speculate the reason as follows: the Fe$^{2+}$ needed to the reduction of Hg$^{2+}$ was oxidized at the early step of the incubation without reducing Hg$^{2+}$.

**The effects of concentration of mercury-polluted soil on the mercury volatilization**

The amount of mercury volatilized from mercury-polluted soil increased in proportion to the amount of mercury-polluted soil added to 1.6 mm sulfuric acid and the largest mercury volatilization was obtained when 1.0 g of polluted soil was added. However, a large amount of soil, more than 1 g, rather inhibited the mercury volatilization reaction. The amounts of mercury volatilized from 1.6 mm sulfuric acid containing 3.0, 5.0, and 10 g of mercury-polluted soil were 80, 36, and 28% of that obtained in the experiment with 1 g of the soil respectively.

**The effects of concentration of Fe$^{2+}$ on the volatilization of mercury from the mercury-polluted soil**

The amount of mercury volatilized from the mercury-polluted soil was dependent on the concentra-
Volatilization of mercury from mercury-polluted soil was done in 20 ml of salt solution (pH 2.5) containing mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2 (0.01 mg of protein), and each of the following electron donors: ferrous sulfate, 0.2–5% (■); elemental sulfur, 0.05–3% (●); sodium tetrathionate, 0.01–0.2% (▲), and pyrite, 0.03–3% (■). Volatilization of mercury from mercury-polluted soil was also done in 20 ml of 1.6 mM sulfuric acid (pH 2.5) containing mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2 (0.01 mg of protein) and ferrous sulfate, 0.2–5% (●).

**Fig. 3.** Effects of Electron Donors on the Volatilization of Mercury from Mercury-polluted Soil by Resting Cells of *A. ferrooxidans* SUG 2-2.

Volatilization of mercury from mercury-polluted soil was done in 20 ml of salt solution (pH 2.5) containing mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2 (0.01 mg of protein), and each of the following electron donors: ferrous sulfate, 0.2–5% (■); elemental sulfur, 0.05–3% (●); sodium tetrathionate, 0.01–0.2% (▲), and pyrite, 0.03–3% (■). Volatilization of mercury from mercury-polluted soil was also done in 20 ml of 1.6 mM sulfuric acid (pH 2.5) containing mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2 (0.01 mg of protein) and ferrous sulfate, 0.2–5% (●).

**Fig. 4.** Mercury Volatilization Activity of Mercury Resistant or Sensitive Strain of *A. ferrooxidans*.

Volatilization of mercury from mercury-polluted soil was done in 20 ml of salt solution (pH 2.5) with ferrous sulfate (3%), mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* (0.01 mg of protein); ■, SUG 2-2; ●, Funis 2-1; ▲, AP19-3.

**Fig. 4.** Mercury Volatilization Activity of Mercury Resistant or Sensitive Strain of *A. ferrooxidans*.

Volatilization of mercury from mercury-polluted soil was done in 20 ml of salt solution (pH 2.5) with ferrous sulfate (3%), mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* (0.01 mg of protein); ■, SUG 2-2; ●, Funis 2-1; ▲, AP19-3.

Volatilization of mercury from mercury-polluted soil was done in 20 ml of salt solution (pH 2.5) containing mercury-polluted soil (1 g), resting cells of *A. ferrooxidans* SUG 2-2 (0.01 mg of protein) and ferrous sulfate, 0.2–5% (●).

Mercury volatilization activities of mercury resistant and sensitive strains of *A. ferrooxidans*

Mercury resistant strains, *A. ferrooxidans* SUG 2-2 and Funis 2-1, but not a mercury-sensitive strain *A. ferrooxidans* AP19-3, have the Fe²⁺ dependent mercury volatilization activity and the activity of SUG 2-2 is higher than that of Funis 2-1. Therefore, it is interesting to compare the activities of mercury volatilization from mercury-polluted soil among mercury-resistant and sensitive strains. The largest amount of mercury was volatilized from the soil when it was incubated with a mercury-resistant strain SUG 2-2 (Fig. 4). In contrast, the smallest amount of mercury was volatilized when the soil was incubated with a mercury-sensitive strain, AP19-3.

**Effects of further addition of Fe²⁺ and/or resting cells to salt solution on the volatilization of mercury from mercury-polluted soil**

When mercury-polluted soil (1 g) was incubated for 10 d in salt solution (pH 2.5) with resting cells (0.01 mg of protein) and ferrous sulfate (3%), only 49% of the total mercury added to the reaction mixture was volatilized, suggesting that a part of the Fe²⁺ added was oxidized by molecular oxygen without reducing Hg²⁺ or that the activity of resting cells needed for the volatilization of mercury was lost during the long incubation time. To check these points, we studied the effects of further addition of Fe²⁺ and/or resting cells to the salt solution on the amount of mercury volatilized from the mercury-polluted soil. Further addition of ferrous iron or resting cells slightly increased the amount of mercury volatilized from the soil compared with the experiments done without further addition of cells or Fe²⁺ (Fig. 5). However, addition of both Fe²⁺ and resting cells markedly increased mercury volatilization, with the result that 92% of the total mercury added to the medium was volatilized after 30 d of incubation.

It was found that *A. ferrooxidans* strain SUG 2-2,
Volatilization of Mercury by *A. ferrooxidans*

![Graph: Effect of Further Addition of Fe$^{2+}$ and/or Resting Cells of *A. ferrooxidans* SUG 2-2 to Salt Solution on the Volatilization of Mercury from Mercury-polluted Soil.](image)

Volatilization of mercury from mercury-polluted soil was done without further addition of resting cells and/or Fe$^{2+}$ in 20 ml of salt solution (pH 2.5) with ferrous sulfate (3%), mercury-polluted soil (1 g), and resting cells (0.01 mg of protein) (○). Volatilization of mercury from the soil was done by newly adding resting cells (0.01 mg of protein) and/or Fe$^{2+}$ (2.2 mmol) at the time shown with an arrow: ▲, addition of resting cells; ●, addition of both cells and Fe$^{2+}$.

resistant to mercuric ion, volatilized mercury from the soil contaminated with mercuric chloride under acidic conditions. The best conditions obtained for mercury volatilization from the mercury-polluted soil in 20 ml of sulfuric acid was as follows: the concentration of resting cells of *A. ferrooxidans* SUG 2-2, 0.01 mg of protein; pH of the reaction mixture, 2.5; incubation temperature, 30°C; and electron donor for Hg$^{2+}$ reduction, ferrous sulfate (3%). When mercury-polluted soil was incubated for 10 d under the best conditions for mercury volatilization, approximately 54% of the total mercury was volatilized. The chemical form of mercury in the mercury-polluted soil is now not clear. However, it seems to be mercuric chloride, but not mercury sulfide (HgS) because metallic mercury was not volatilized from HgS by incubating SUG 2-2 cells in 1.6 mM sulfuric acid with HgS and Fe$^{2+}$ (pH 2.5). Although mercury chloride newly added to the mercury-polluted soil attached tightly to the soil and thus volatilization rate was delayed, it was volatilized by the action of SUG 2-2 cells under the acidic conditions. Interestingly, further addition of both resting cells and Fe$^{2+}$ increased the amount of mercury volatilized from the reaction mixture and 92% of the total mercury added to the medium was volatilized after 30 d of incubation. These results suggest that in order to volatilize almost all the mercury in mercury-polluted soil, further addition of both cells and Fe$^{2+}$ are absolutely needed probably because an activity of resting cells to volatilize mercury was inactivated during the incubation of cells with mercury or only a part of the electrons from the Fe$^{2+}$ added to an acidic water was used for the reduction of Hg$^{2+}$. Most of the Fe$^{3+}$ added to the solution seems to be consumed to reduce molecular oxygen via the ion oxidation electron transport system of this bacterium.

The Fe$^{2+}$ dependent mercury volatilization activity of *A. ferrooxidans* SUG 2-2 is higher than that of Funis 2-1. In contrast, a mercury sensitive strain of *A. ferrooxidans* AP19-3 does not have the Fe$^{2+}$-dependent mercury volatilization activity although it has the NADPH-dependent mercury reductase activity. In this report we showed that the order of three strains to give a large amount of mercury volatilization from mercury-polluted soil was: strains SUG 2-2, Funis 2-1, and AP19-3. The results suggest that the activity of mercury volatilization from mercury-polluted soil by *A. ferrooxidans* correspond with the levels of the Fe$^{2+}$-dependent mercury volatilization activity in the cells.

References


