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Ferrous Iron-Dependent Volatilization of Mercury by the Plasma Membrane of *Thiobacillus ferrooxidans*

KENJI IWAHORI,[1] FUMIAKI TAKEUCHI,[2] KAZUO KAMIMURA,[3] AND TSUYOSHI SUGIO[1]*

Science and Technology for Energy Conversion,[1] Administration Center for Environmental Science and Technology,[2] and Department of Biological Function, Faculty of Agriculture,[3] Okayama University, Tsushima Naka, Okayama 700-8530, Japan

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Of 100 strains of iron-oxidizing bacteria isolated, *Thiobacillus ferrooxidans* SUG 2-2 was the most resistant to mercury toxicity and could grow in an Fe^{2+} medium (pH 2.5) supplemented with 6 μM Hg^{2+}. In contrast, *T. ferrooxidans* AP19-3, a mercury-sensitive *T. ferrooxidans* strain, could not grow with 0.7 μM Hg^{2+}. When incubated for 3 h in a salt solution (pH 2.5) with 0.7 μM Hg^{2+}, resting cells of resistant and sensitive strains volatilized approximately 20 and 1.7%, respectively, of the total mercury added. The amount of mercury volatilized by resistant cells, but not by sensitive cells, increased to 62% when Fe^{2+} was added. The optimum pH and temperature for mercury volatilization activity were 2.3 and 30°C, respectively. Sodium cyanide, sodium molybdate, sodium tungstate, and silver nitrate strongly inhibited the Fe^{2+}-dependent mercury volatilization activity of *T. ferrooxidans*. When incubated in a salt solution (pH 3.8) with 0.7 μM Hg^{2+} and 1 mM Fe^{2+}, plasma membranes prepared from resistant cells volatilized 48% of the total mercury added after 5 days of incubation. However, the membrane did not have mercury reductase activity with NADPH as an electron donor. Fe^{2+}-dependent mercury volatilization activity was not observed with plasma membranes pretreated with 2 mM sodium cyanide. Rusticyanin from resistant cells activated iron oxidation activity of the plasma membrane and activated the Fe^{2+}-dependent mercury volatilization activity of the plasma membrane.

Thiobacillus ferrooxidans is an acidophilic, chemolithotrophic, iron-oxidizing bacterium that uses energy produced by oxidation of reduced sulfur compounds and ferrous iron. This bacterium is one of the most important bacteria for bacterial leaching of sulfide ores. To solubilize metal ions from sulfide ores much more efficiently, isolation of *T. ferrooxidans* strains which are resistant to heavy metal ions seems to be important. It has been reported that *T. ferrooxidans* is sensitive to Hg^{2+}, Ag^{+}, and MoO_4^{2-} (5). The properties of mercuric reductase, a flavoenzyme that reduces Hg^{2+} to less toxic Hg^0 with NADPH as an electron donor, have been studied actively with a wide range of gram-negative and gram-positive bacteria (1, 13–15). Mercuric reductase activity has also been found in *T. ferrooxidans* cells (3, 11, 12). The genes involved in volatilization of mercury have been cloned and characterized in detail (4, 6, 8, 11).

Recently, we partially characterized the difference between mercury-resistant and mercury-sensitive strains of *T. ferrooxidans*. The levels of NADPH-dependent mercuric reductase were not significantly different in these strains. Instead, purified cytochrome *c* oxidase from resistant strain Funis 2-1 was more resistant to Hg^{2+} than purified cytochrome *c* oxidase from a sensitive strain was (20). To explain the remarkable mercury resistance observed, we proposed that both a mercury-resistant cytochrome *c* oxidase and a cytosolic NADPH-dependent mercuric reductase, not the latter alone, function in resistant cells (20).

In this study, a more mercury-resistant strain (SUG 2-2) was obtained from 100 new strains of iron-oxidizing bacteria, and we show that the mercury volatilized by resting cells of *T. ferrooxidans* SUG 2-2 was activated in the presence of Fe^{2+}. Although the plasma membrane of *T. ferrooxidans* SUG 2-2 did not have NADPH-dependent mercury reductase activity, the membrane had the ability to volatilize Hg^0 from a salt solution containing Fe^{2+}. Fe^{2+}-dependent mercury volatilization activity with plasma membranes was inhibited by sodium cyanide and activated by the blue copper protein rusticyanin.

* Corresponding author. Mailing address: Science and Technology for Energy Conversion, Okayama University, Tsushima Naka, Okayama 700-8530, Japan. Phone: 81-86-251-8306. Fax: 81-86-251-8306. E-mail: Sugio@cc.okayama-u.ac.jp.

MATERIALS AND METHODS

Microorganisms, medium, and growth conditions. Ninety-four strains of iron-oxidizing bacteria isolated from streams and soils in Japan and the United States and *T. ferrooxidans* ATCC 13661, ATCC 14119, ATCC 19859, ATCC 21834, ATCC 23270, and ATCC 33020 from the American Type Culture Collection were used in this study. To isolate iron-oxidizing bacteria, stream water or soil samples were incubated at 30°C under aerobic conditions in Fe^{2+} medium (pH 2.5) containing (per liter) 30 g of $FeSO_4 \cdot 7H_2O$, 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, and 0.01 g of $Ca(NO_3)_2$ (16). When the Fe^{2+} in the culture medium was oxidized, samples were plated on 1.0% gellan gum plates containing (per liter) 30 g of $FeSO_4 \cdot 7H_2O$, 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, 0.01 g of $Ca(NO_3)_2$, and 0.3 g of yeast extract. Rusty colonies appearing on the plate were picked. This process was repeated more than three times, and the final isolates were preserved on Fe^{2+} medium (pH 2.5) and used throughout this study. The method used for large-scale production of cells has been described previously (18).

Growth rate. After cultivation in Fe^{2+} medium (pH 2.5) at 30°C, cells were separated from the particles of ferric hydroxide by filtering with a no. 5B Toyo paper filter. The numbers of cells in the filtrates were counted with a microscope and hemacytometer (Kayagaki Irika Kogyo Co., Ltd., Tokyo, Japan) after dilution with 0.1 N sulfuric acid when necessary.

Analysis of mercury volatilized from *T. ferrooxidans* culture medium. A 50-ml culture flask with a screw cap contained 19 ml of Fe^{2+} medium (pH 2.5) supplemented with 0.7 M Hg^{2+} and 1 ml of an active seed culture of *T. ferrooxidans*. 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_2SO_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.

Analysis of mercury volatilized by resting cells and the plasma membranes of *T. ferrooxidans*. Each of several 50-ml flasks with screw caps contained a reaction mixture plus 2 ml of a $KMnO_4$ solution as described above. The gas phase was air, and the reaction mixture was rotated at 100 rpm at 30°C. The reaction

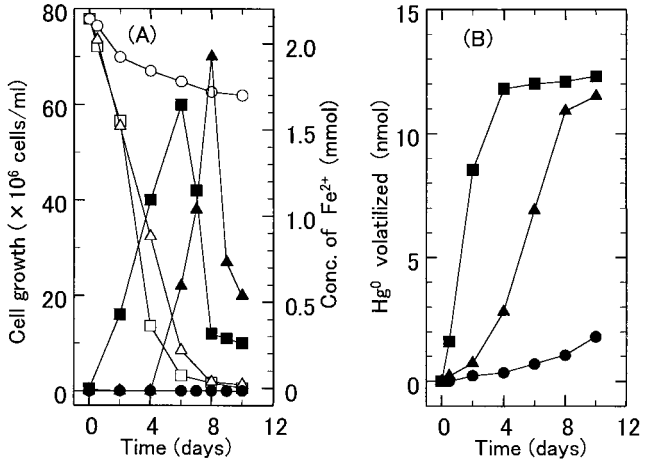

FIG. 1. Volatilization of metal mercury from Fe^{2+} medium containing 0.7 M Hg^{2+} by *T. ferrooxidans* strains. (A) Cell growth and amount of Fe^{2+} remaining in Fe^{2+} medium (pH 2.5) containing 0.7 μM Hg^{2+}. Symbols: ■, ▲, and ●, cell growth of *T. ferrooxidans* SUG 2-2, Funis 2-1, and AP19-3, respectively; □, △, and ○, amount of Fe^{2+} remaining with *T. ferrooxidans* SUG 2-2, Funis 2-1, and AP19-3, respectively. (B) Mercury volatilized in Fe^{2+} medium (pH 2.5) containing Hg^{2+} (0.7 μM). Cultures of *T. ferrooxidans* SUG 2-2 (■), Funis 2-1 (▲), and AP19-3 (●) were examined.

mixture used for the measurement of mercury volatilization with resting cells was composed of a salt solution (pH 2.5) (20 ml), resting cells of *T. ferrooxidans* (1 mg of protein), 0.7 μM $HgCl_2$, and 25 mM ferrous sulfate. The reaction mixture used for the measurement of mercury volatilization with plasma membranes was composed of a salt solution (pH 3.8) (10 ml), plasma membranes of *T. ferrooxidans* SUG 2-2 (50 μg of protein), 0.7 μM $HgCl_2$, and 1 mM ferrous sulfate. The salt solution used contained (per liter) 3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of KCl, and 0.01 g of $Ca(NO_3)_2$. The concentration of Hg^0 trapped in the $KMnO_4$ solution was measured by cold-vapor atomic absorption spectroscopy.

Preparation of plasma membranes and rusticyanin. Cells of *T. ferrooxidans* SUG 2-2 grown in Fe^{2+} medium (pH 2.5) at 30°C for 1 week were washed three times with 0.1 M potassium phosphate buffer (pH 7.5), disrupted by sonication for 15 min with a sonicator (model INSONATOR 201M; Kubota Co., Tokyo, Japan), and centrifuged at 12,000 × *g* for 10 min to remove cell debris. The cell extract obtained was centrifuged at 105,000 × *g* for 60 min to obtain the plasma membrane fraction. Rusticyanin was prepared by the method described previously (2).

Protein content. Protein content was determined by the method of Lowry et al. (9) with crystalline bovine serum albumin as the standard.

RESULTS

Volatilization of mercury from Fe^{2+} medium by mercury-resistant or -sensitive *T. ferrooxidans* strains. Screening 100 iron-oxidizing bacterial strains, including six *T. ferrooxidans* strains from the American Type Culture Collection, for resistance to Hg^{2+} was done. *T. ferrooxidans* SUG 2-2, isolated from hot spring water from Aomori Prefecture, Japan, was the strain most resistant to Hg^{2+} toxicity among the strains tested and could grow in Fe^{2+} medium supplemented with 6 μM Hg^{2+}. This strain gave a cell yield of 6.0×10^7 cells/ml after 6 days of cultivation in Fe^{2+} medium (pH 2.5) supplemented with 0.7 μM Hg^{2+} (Fig. 1A). SUG 2-2 volatilized 86% of the total mercury (14 nmol) added to the culture medium after 4 days of cultivation (Fig. 1B). *T. ferrooxidans* Funis 2-1 (10, 20) volatilized 78% of the total mercury after 8 days of cultivation. In contrast, *T. ferrooxidans* AP19-3 (7, 17) could not grow in Fe^{2+} medium supplemented with 0.7 μM Hg^{2+} and volatilized only 10% of the total mercury added to the medium after 10 days of cultivation (Fig. 1). Neither Funis 2-1 nor AP19-3 could grow in Fe^{2+} medium supplemented with 6 μM $HgCl_2$.

Effects of ferrous iron on the amount of mercury volatilized from resting cells. The mercury reductase activity of SUG 2-2 cells was determined with NADPH as an electron donor and cytosol prepared from the strain as an enzyme source. The NADPH-dependent mercury volatilization activity of the cytosol of strain SUG 2-2 was nearly the same as that of Funis 2-1 (data not shown). From our results, it seems that strain SUG 2-2 has another enzyme system to detoxify mercury. The role of the iron-oxidizing system of SUG 2-2 in mercury detoxification by the strain was studied. The amount of mercury volatilized from a 10-ml salt solution (pH 2.5) containing resting cells of SUG 2-2 and 0.7 μM Hg^{2+} was 20% of the total amount of mercury (14 nmol) added to the reaction mixture after 3 h of incubation. The amount of mercury volatilized by the resting cells increased approximately threefold when 25 mM Fe^{2+} was added, and approximately 62% of the total mercury added was volatilized. The amount of mercury volatilized by SUG 2-2 cells increased in proportion to the concentration of Fe^{2+} added to the reaction mixture and also in proportion to the cell concentration (data not shown). To clarify whether *T. ferrooxidans* strains other than SUG 2-2 also have Fe^{2+}-dependent mercury volatilization activity, the same experiments were done with resting cells of nine additional iron-oxidizing bacterial strains with or without 25 mM Fe^{2+} (Fig. 2). After 60 min of incubation, resting cells of Funis 2-1 volatilized 34 and 14% of the total amount of mercury (14 nmol) added to the reaction mixture in the presence and in the absence of 25 mM Fe^{2+}, respectively, indicating that Funis 2-1 as well as SUG 2-2 has Fe^{2+}-dependent mercury volatilization activity. *T. ferrooxidans* ATCC 33020 from the American Type Culture Collection volatilized mercury in the presence of Fe^{2+}. In comparison, the amounts of metal mercury volatilized by *T. ferrooxidans* AP19-3 and ATCC 13661 were less or the same with or without Fe^{2+}. As shown in Fig. 2, the day after each of the strains exhibited the maximum cell number in Fe^{2+} medium containing 0.7 μM Hg^{2+}, testing was carried out. The results indicate that the strains which had higher Fe^{2+}-dependent mercury volatilization activities were more resistant to Hg^{2+} toxicity than the strains which had lower activities. The Fe^{2+}-dependent mercury volatilization activities were completely inhibited by pretreating strain SUG 2-2 and Funis 2-1 cells with 5 mM NaCN for 15 min, suggesting that cytochrome *c* oxidase of *T. ferrooxidans* is involved in the Fe^{2+}-dependent mercury volatilization reaction.

Characteristics of Fe^{2+}-dependent mercury volatilization activity. The optimum pH and temperature for Fe^{2+}-dependent mercury volatilization activity of strain SUG 2-2 cells were pH 2.3 and 30°C, respectively. These values are the same as the optimum pH and temperature values for the iron oxidase of *T. ferrooxidans* SUG 2-2 cells (data not shown). The effects of heavy metal ions on Fe^{2+}-dependent mercury volatilization activity were studied with resting cells of SUG 2-2 (Table 1). Na_2MO_4 and Na_2WO_4 inhibited the activity more than 80%. Silver ions also markedly inhibited the activity at 5 mM. These compounds strongly inhibited both iron oxidase and cytochrome *c* oxidase activities of *T. ferrooxidans* (5, 10, 19).

Volatilization of mercury by the plasma membrane of *T. ferrooxidans* SUG 2-2. Fe^{2+}-dependent mercury volatilization activity was measured with plasma membranes from strain SUG 2-2 cells. The plasma membranes did not have mercuric reductase activity when NADPH was used as an electron donor. To decrease the amount of reduced compounds in the plasma membrane, $KMnO_4$ was added to the membrane until reduced type *c* and *a* cytochromes in the membrane were nearly completely oxidized. In the presence and absence of 1 mM Fe^{2+}, 50 μg of plasma membranes prepared from SUG

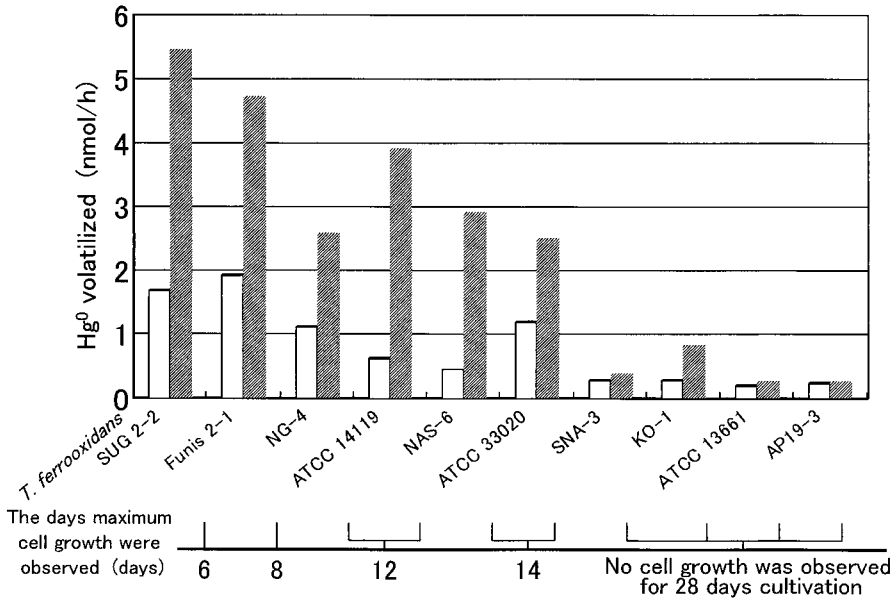

FIG. 2. Fe^{2+}-dependent mercury volatilization activities of resting cells of 10 *T. ferrooxidans* strains. The mercury volatilization activities of *T. ferrooxidans* strains were measured in 20 ml of salt solution (pH 2.5) containing resting cells (1 mg of protein), 0.7 μM Hg^{2+}, and 25 mM Fe^{2+} (striped bars). The activities were also measured in 20 ml of salt solution (pH 2.5) containing resting cells and Hg^{2+} but no Fe^{2+} (open bars). The days on which maximum cell growth was observed in Fe^{2+} medium containing 0.7 μM Hg^{2+} are shown for the 10 strains of *T. ferrooxidans*.

2-2 cells volatilized 48 and 12%, respectively, of the total mercury (7 nmol) added to a 10-ml reaction mixture after 5 days of incubation at 30°C (Fig. 3). Mercury was not volatilized in a reaction mixture containing Hg^{2+}, Fe^{2+}, and plasma membranes boiled for 10 min. Since the plasma membranes prepared from *T. ferrooxidans* SUG 2-2 cells had low iron-oxidizing activity, we added the blue copper protein rusticyanin to the reaction mixture. Rusticyanin isolated from SUG 2-2 cells activated the iron-oxidizing activity of plasma membranes 4.5-fold (data not shown). Fe^{2+}-dependent mercury volatilization activity markedly increased in the presence of 0.1 mg of rusticyanin (Fig. 4). In the presence of 0.1 mg of rusticyanin, plasma membranes of SUG 2-2 cells volatilized 1.7 and 0.2 nmol of mercury in 10-ml reaction mixtures containing 7 nmol of Hg^{2+} in the presence and in the absence of 1 mM Fe^{2+}, respectively, after 2 days of incubation. However, since only 2.2 nmol of mercury was volatilized after 5 days of incubation, 7 nmol of $HgCl_2$ and 10 μmol of Fe^{2+} were added to the reaction mixture every 3 days (Fig. 5). Further additions of $HgCl_2$ and Fe^{2+} to the reaction mixture resulted in further volatilization of the

TABLE 1. Effects of heavy metal ions on the mercury volatilization activity of *T. ferrooxidans* SUG 2-2[a]

Heavy metal	Concn (mM)	Remaining activity (%)
None		100
$NiSO_4$	10	72
$CuSO_4$	10	82
$AgNO_3$	10	0.0
	5	0.1
	1	24
	0.1	63
	0.05	78
$CdSO_4$	10	90
$(CH_3COO)_2Pb$	10	67
$ZnSO_4$	10	72
$MnSO_4$	10	69
Na_2MoO_4	10	15
Na_2WO_4	10	1
$SnCl_2$	10	104
$MgSO_4$	10	73
$Fe_2(SO_4)_3$	10	69

[a] Volatilization activity was measured in a reaction mixture (20 ml) containing resting cells of strain SUG 2-2 (1 mg of protein), ferrous iron (25 mM), and heavy metal ions. One hundred percent of remaining activity was 5.4 nmol/mg of protein/h at 30°C.

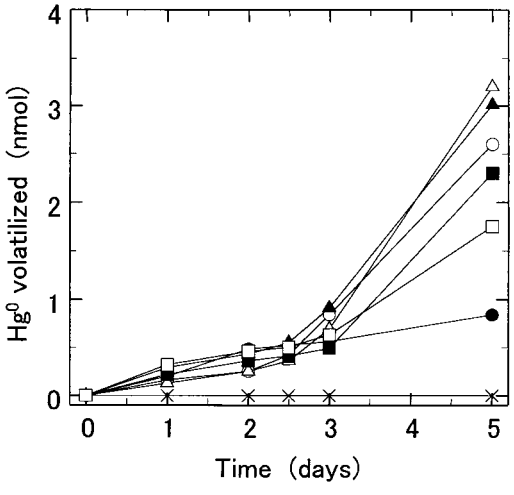

FIG. 3. Effects of Fe^{2+} concentration on the mercury volatilization activity of plasma membranes of *T. ferrooxidans* SUG 2-2. The Fe^{2+}-dependent mercury volatilization activity was measured in 10 ml of salt solution (pH 3.8) containing 50 μg of plasma membranes, 0.7 μM Hg^{2+} and Fe^{2+} (○, 0.01 mM; ▲, 0.2 mM; △, 1 mM; ■, 3 mM; and □, 5 mM). The activities were also measured in 10 ml of salt solution containing 50 μg of plasma membranes and 0.7 μM Hg^{2+} but no Fe^{2+} (●) and in 10 ml of salt solution containing 50 μg of plasma membranes boiled for 10 min, 0.7 μM Hg^{2+}, and 1 mM Fe^{2+} (×).

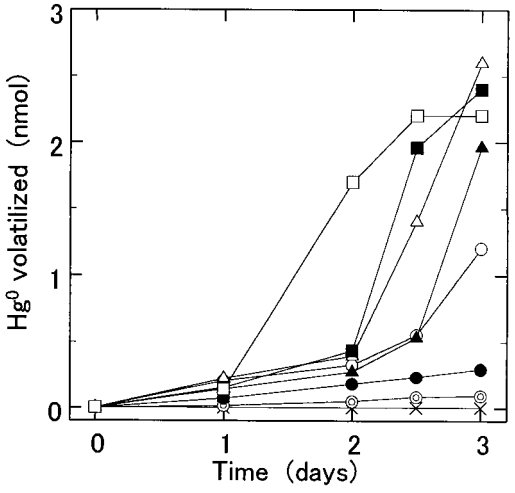

FIG. 4. Effects of rusticyanin on the Fe^{2+}-dependent mercury volatilization activity of *T. ferrooxidans* SUG 2-2 cells. The Fe^{2+}-dependent mercury volatilization activity was measured in 10 ml of salt solution (pH 3.8) containing 50 μg of plasma membranes, 1 mM Fe^{2+}, 0.7 μM Hg^{2+}, and rusticyanin (▲, 0.01 mg; △, 0.02 mg; ■, 0.05 mg; and □, 0.1 mg). The activities were also measured in 10 ml of salt solution containing 50 μg of plasma membranes, 1 mM Fe^{2+}, and 0.7 μM Hg^{2+} (○), in a salt solution containing 50 μg of plasma membranes and 0.7 μM Hg^{2+} (●), in a salt solution containing 1 mM Fe^{2+}, 0.7 μM Hg^{2+}, rusticyanin (0.1 mg of protein), and boiled plasma membranes (×), and in a salt solution containing 1 mM Fe^{2+}, 0.7 μM Hg^{2+}, and rusticyanin (0.1 mg of protein) but no plasma membranes (◎).

mercury, suggesting that the membranes still had Fe^{2+}-dependent mercury volatilization activity after 6 days of incubation. After 8 days of incubation and two more additions of mercury, the plasma membranes of SUG 2-2 cells volatilized 5 nmol of mercury in the reaction mixture. In contrast, only 1.5 nmol of mercury was volatilized when the membranes were incubated without a further addition of mercury. Mercury was not volatilized in the reaction mixture containing plasma membranes boiled for 10 min.

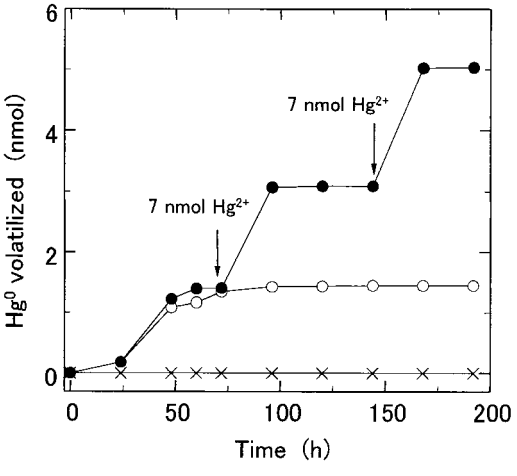

FIG. 5. Effects of further addition of $HgCl_2$ to the reaction mixture on Fe^{2+}-dependent mercury volatilization activity. The Fe^{2+}-dependent mercury volatilization activity was measured in 10 ml of salt solution (pH 3.8) containing 50 μg of plasma membranes, 1 mM Fe^{2+}, 0.7 μM Hg^{2+}, and rusticyanin (0.1 mg of protein). Hg^{2+} (7 nmol) and Fe^{2+} (10 nmol) were added to the reaction mixture every 3 days (●). The Hg^{2+} volatilized was also measured in a 10-ml reaction mixture to which $HgCl_2$ was not added (○). The arrows indicate the times at which $HgCl_2$ (7 nmol) was added to the reaction mixture. The Fe^{2+}-dependent mercury volatilization activity was also measured in 10 ml of salt solution (pH 3.8) containing 50 μg of plasma membranes boiled for 10 min, 1 mM Fe^{2+}, 0.7 μM Hg^{2+}, and rusticyanin (0.1 mg of protein) (×).

DISCUSSION

Bacteria resistant to mercury usually have a cytosolic NADPH-dependent mercuric reductase which catalyzes the reduction of soluble Hg^{2+} to volatile Hg^0 with NADPH as an electron donor. Studies to clarify the characteristics of mercury reductase and its gene structure have been performed with various bacteria, including members of the genera *Pseudomonas*, *Staphylococcus*, *Bacillus*, and *Serratia* (1, 13, 15). The iron-oxidizing chemolithotrophic bacterium *T. ferrooxidans* also has mercuric reductase to detoxify Hg^{2+} (8, 11, 12). Recently, it was shown that both mercuric reductase and cytochrome *c* oxidase, but not mercuric reductase alone, function in mercury-resistant strain Funis 2-1 of *T. ferrooxidans* when the strain grows in Fe^{2+} medium containing Hg^{2+} (20).

In this report, it is shown that a newly isolated strain, *T. ferrooxidans* SUG 2-2, is much more resistant to mercury than the previously reported strain Funis 2-1 and that Fe^{2+}-dependent mercury volatilization activity is present in six *T. ferrooxidans* strains, including strain SUG 2-2. Olson et al. (11, 12) reported that the rate of mercury volatilization by *T. ferrooxidans* BA-4 which was adapted to Hg^{2+} at concentrations up to 5 μM was slightly accelerated by the addition of 150 mM $FeSO_4$. The level of Fe^{2+}-dependent mercury volatilization activity found in the six strains of *T. ferrooxidans* corresponded well with the level of mercury resistance of these strains, which was estimated by the growth rate in Fe^{2+} medium (pH 2.5) containing 0.7 μM Hg^{2+}. This strongly suggests the possibility that both Fe^{2+}-dependent mercury volatilization and cytosolic NADPH-dependent mercury volatilization play a role in detoxification of Hg^{2+} in many strains of *T. ferrooxidans*. According to the level of Fe^{2+}-dependent mercury volatilization activity, the *T. ferrooxidans* strains isolated can be categorized into two groups, one containing the strains possessing a high level of Fe^{2+}-dependent mercury volatilization activity and the other containing the strains possessing a low level of activity.

Involvement of an iron oxidation enzyme system in the Fe^{2+}-dependent mercury volatilization reaction of *T. ferrooxidans* SUG 2-2 cells is supported by the following findings. (i) Plasma membranes prepared from strain SUG 2-2 cells did not have NADPH-dependent mercuric reductase activity but had Fe^{2+}-dependent mercury volatilization activity. Plasma membranes boiled for 10 min did not have mercury volatilization activity. (ii) Rusticyanin purified from strain SUG 2-2 cells enhanced both iron-oxidizing activity and the Fe^{2+}-dependent mercury volatilization activity. (iii) NaCN, which strongly inhibits the iron oxidase activity of cells and the cytochrome *c* oxidase activity of plasma membranes, completely inhibited the Fe^{2+}-dependent mercury volatilization activity. The Fe^{2+}-dependent mercury volatilization activity measured with plasma membranes was very low compared with that of the resting cells. This is probably because the components needed for iron oxidation, for instance, rusticyanin and soluble cytochrome *c* oxidase, were lost during preparation of plasma membranes by disruption of the resting cells with a sonicator. The iron-oxidizing activities measured at pH 3.8 were 11.4 and 0.14 μl of O_2 consumed/mg per min for the resting cells and the plasma membranes, respectively.

This paper shows that there is a novel Fe^{2+}-dependent mercury volatilization activity in six strains of *T. ferrooxidans*, including strain SUG 2-2. To clarify the mechanism of the Fe^{2+}-dependent mercury volatilization reaction more precisely, it is

important to answer the following question precisely: is cytochrome *c* oxidase alone or cytochrome *c* oxidase plus other components of the iron oxidation enzyme system involved in the mercury volatilization reaction? Our preliminary experiments show that cytochrome *c* oxidase partially purified from *T. ferrooxidans* SUG 2-2 volatilizes mercury in the presence of Fe^{2+}.

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