Ferrous Iron-Dependent Volatilization of Mercury by the Plasma Membrane of _Thiobacillus ferrooxidans_

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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 Fe2+ medium (pH 2.5) supplemented with 6 μM Hg2+. In contrast, _T. ferrooxidans_ AP19-3, a mercury-sensitive _T. ferrooxidans_ strain, could not grow with 0.7 μM Hg2+. When incubated for 3 h in a salt solution (pH 2.5) with 0.7 μM Hg2+, 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 Fe2+ was added. The optimum pH and temperature for mercury volatilization activity were 2.5 and 30°C, respectively. Sodium cyanide, sodium molybdate, sodium tungstate, and silver nitrate strongly inhibited the Fe2+-dependent mercury volatilization activity of _T. ferrooxidans_. When incubated in a salt solution (pH 3.8) with 0.7 μM Hg2+ and 1 mM Fe2+, 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. Fe2+-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 Fe2+-dependent mercury volatilization activity of the plasma membrane.

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 Hg0 from a salt solution containing Fe2+. Fe2+-dependent mercury volatilization activity with plasma membranes was inhibited by sodium cyanide and activated by the blue copper protein rusticyanin.

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 Fe2+ medium (pH 2.5) containing (per liter) 30 g of FeSO4·7H2O, 3 g of (NH4)2SO4, 0.5 g of MgSO4·7H2O, 0.1 g of KCl, and 0.01 g of Ca(NO3)2 (16). When the Fe2+ in the culture medium was oxidized, samples were plated on 1.0% gellan gum plates containing (per liter) 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 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 Fe2+ 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 Fe2+ 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 (Kagayaki 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 Fe2+ medium (pH 2.5) supplemented with 0.7 μM Hg2+ and 1 ml of an active seed culture of _T. ferrooxidans_. A small test tube containing 2 ml of a KMnO4 solution was inserted in the 50-ml culture flask to trap the Hg2+ volatilized from the culture medium. The KMnO4 solution used (100 ml) was composed of a 10-ml solution containing 0.6 g of KMnO4, 5 ml of concentrated H2SO4, and 85 ml of deionized water. After the culture medium was aerated by shaking at 30°C and 100 rpm, the concentration of Hg0 trapped in the KMnO4 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 KMnO4 solution as described above. The gas phase was air, and the reaction mixture was rotated at 100 rpm at 30°C. The reaction
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 mM HgCl₂ 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 Fe²⁺ 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 Fe²⁺ 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 Fe²⁺-dependent mercury volatilization activity, the same experiments were done with resting cells of nine additional iron-oxidizing bacterial strains with or without 25 mM Fe²⁺ (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 Fe²⁺, respectively, indicating that Funis 2-1 as well as SUG 2-2 has Fe²⁺-dependent mercury volatilization activity. T. ferrooxidans ATCC 33020 from the American Type Culture Collection volatilized mercury in the presence of Fe²⁺.

In comparison, the amounts of metal mercury volatilized by T. ferrooxidans AP19-3 and ATCC 13661 were less or the same with or without Fe²⁺. As shown in Fig. 2, the day after each of the strains exhibited the maximum cell number in Fe²⁺-medium containing 0.7 mM HgCl₂, testing was carried out. The results indicate that the strains which had higher Fe²⁺-dependent mercury volatilization activities were more resistant to Hg²⁺ toxicity than the strains which had lower activities. The Fe²⁺-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 Fe²⁺-dependent mercury volatilization reaction.

Characteristics of Fe²⁺-dependent mercury volatilization activity. The optimum pH and temperature for Fe²⁺-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 Fe²⁺-dependent mercury volatilization activity were studied with resting cells of SUG 2-2 (Table 1). Na₂MoO₄ and Na₂WO₄ 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. Fe²⁺-dependent mercury volatilization activity was measured with plasma membranes from strain SUG 2-2 cells. The plasma membranes did not have merciruc reductase activity when NADPH was used as an electron donor. To decrease the amount of reduced compounds in the plasma membrane, KMnO₄ 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 Fe²⁺, 50 μg of plasma membranes prepared from SUG
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 \(\mu\)mol 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\(^a\)

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>Conc. (mM)</th>
<th>Remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>NiSO(_4)</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>CuSO(_4)</td>
<td>10</td>
<td>82</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td>10</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>78</td>
</tr>
<tr>
<td>CdSO(_4)</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>(C(_2)HO(_2))(_2)Pb</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>ZnSO(_4)</td>
<td>10</td>
<td>72</td>
</tr>
<tr>
<td>MnSO(_4)</td>
<td>10</td>
<td>69</td>
</tr>
<tr>
<td>Na(_2)MoO(_4)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Na(_2)WO(_4)</td>
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<td>1</td>
</tr>
<tr>
<td>SnCl(_2)</td>
<td>10</td>
<td>104</td>
</tr>
<tr>
<td>MgSO(_4)(_2)</td>
<td>10</td>
<td>73</td>
</tr>
<tr>
<td>Fe(_2)(SO(_4))(_3)</td>
<td>10</td>
<td>69</td>
</tr>
</tbody>
</table>

\(^a\) 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.
mercury, suggesting that the membranes still had Fe²⁺-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.

**DISCUSSION**

Bacteria resistant to mercury usually have a cytosolic NADPH-dependent mercuric reductase which catalyzes the reduction of soluble Hg²⁺ to volatile Hg⁰ 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 *Psuedomonas,* *Staphylococcus,* *Bacillus,* and *Serratia* (1, 13, 15). The iron-oxidizing chemolithotrophic bacterium *T. ferrooxidans* also has mercuric reductase to detoxify Hg²⁺ (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 Fe²⁺ medium containing Hg²⁺ (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 Fe²⁺-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 Hg²⁺ at concentrations up to 5 μM was slightly accelerated by the addition of 150 mM FeSO₄. The level of Fe²⁺-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 Fe²⁺ medium (pH 2.5) containing 0.7 μM Hg²⁺. This strongly suggests the possibility that both Fe²⁺-dependent mercury volatilization and cytosolic NADPH-dependent mercury volatilization play a role in detoxification of Hg²⁺ in many strains of *T. ferrooxidans.* According to the level of Fe²⁺-dependent mercury volatilization activity, the *T. ferrooxidans* strains isolated can be categorized into two groups, one containing the strains possessing a high level of Fe²⁺-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 Fe²⁺-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 Fe²⁺-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 Fe²⁺-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 Fe²⁺-dependent mercury volatilization activity. The Fe²⁺-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 O₂ consumed/mg per min for the resting cells and the plasma membranes, respectively.

This paper shows that there is a novel Fe²⁺-dependent mercury volatilization activity in six strains of *T. ferrooxidans,* including strain SUG 2-2. To clarify the mechanism of the Fe²⁺-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+}.

REFERENCES