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Mercuric Reductase Enzyme from a Mercury-Volatilizing Strain of *Thiobacillus ferrooxidans*

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Cell-free mercury volatilization activity (mercuric reductase) was obtained from a mercury-volatilizing *Thiobacillus ferrooxidans* strain, and the properties of intact-cell and cell-free activities were compared with those determined by plasmid R100 in *Escherichia coli*. Intact cells of *T. ferrooxidans* volatilized mercury at pH 2.5, whereas cells of *E. coli* did not. Cell-free enzyme preparations from both bacteria functioned best at or above neutral pH and not at all at pH 2.5. The *T. ferrooxidans* mercuric reductase was a soluble enzyme that was dependent upon added NAD(P)H. The enzyme activity was stable at 80°C, required an added thiol compound, and was stimulated by EDTA. Antisera against purified mercuric reductases from transposon Tn*501* and plasmid R831 (which inactivated mercuric reductases from a wide range of enteric and pseudomonad strains) did not inactivate the enzyme from *T. ferrooxidans*.

Thiobacillus ferrooxidans is a chemoautotroph which grows under very acid conditions by oxidizing reduced sulfur compounds to SO_4^{2-} and Fe^{2+} to Fe^{3+} (19). *T. ferrooxidans* has assumed considerable interest in recent years because of its unusual growth conditions and because of its ability to carry out "acid leaching" of low-grade copper (and uranium)-containing ores (1), accounting for perhaps 15% of total copper production in the United States. Although resistant to iron, acidity, and some heavy metals, *T. ferrooxidans* is exceedingly sensitive to silver and mercury salts (6, 7, 14). It is thought that the presence of such heavy metals in ores might limit leaching activity under field conditions (1, 6, 22). Olson et al. (15) recently reported the isolation of *T. ferrooxidans* BA-4, which was adaptively resistant to inorganic Hg^{2+} at concentrations up to 5 μM and which volatilized mercury as Hg^0 during growth over periods of 40 to 250 h at 29°C. We asked whether *T. ferrooxidans* BA-4 contained a mercuric reductase enzyme that carries out the reduction of Hg^{2+} to Hg^0, such as has been reported in a wide range of gram-negative and gram-positive bacteria (16–18, 21, 23, 24). In other bacteria, the presence of a mercuric reductase enzyme has been associated with the presence of plasmids or transposons (4, 16, 23, 24). Although small cryptic plasmids have been found with some *T. ferrooxidans* strains (10, 11), no plasmids with selectable functions are currently known in this species, and efforts to identify a plasmid in strain BA-4 were unsuccessful (15). The identification of a selectable marker on either a plasmid or a transposon would open up the experimental methods of recombinant DNA technology for this important industrial microorganism (9).

MATERIALS AND METHODS

T. ferrooxidans BA-4 was grown as described before (15) on 9K liquid medium (19) at pH 2.5 with 150 mM $FeSO_4$ as the sole energy source. Determinations of cell growth (by phase-contrast microscopy) and utilization of Fe^{2+} were made as described before (15). Previously studied mercuric resistance determinants from plasmid R100 in *E. coli* K-12 (12, 13) and transposon Tn*501* in *Pseudomonas aeruginosa* PAO9501(pVS1) (2) were used as controls for whole-cell and cell-free enzyme. Two forms of the R100 determinants were used, the low-copy-number plasmid R100 in strain DB1406 (D. Berg, personal communication) and a high-copy-number variant called pDU1003, which has the mercuric resistance region of R100 cloned into a derivative of plasmid pBR322 (N. NíBhriain, personal communication).

Cells were harvested by centrifugation at 6,000 rpm for 10 min. Most of the precipitated iron was previously removed from *T. ferrooxidans* cultures by allowing the iron precipitate to settle and then decanting the cells, by filtration through Whatman filter paper no. 1, or by both procedures. The cells from 200 to 2,000 ml of *T. ferrooxidans* cultures were washed and suspended in 2 to 5 ml of 9K salts (19) without added $FeSO_4$ or in 5 mM sodium phosphate buffer (pH 7.0), if the intention was to disrupt the cells. *E. coli* and *P. aeruginosa* strains were grown on broth, induced, and harvested as previously described (2, 17). The washed cells were disrupted with a French pressure cell (American Instrument Co., Silver Spring, Md.) at 20,000 lb/in^2 twice for *T. ferrooxidans* and at 14,000

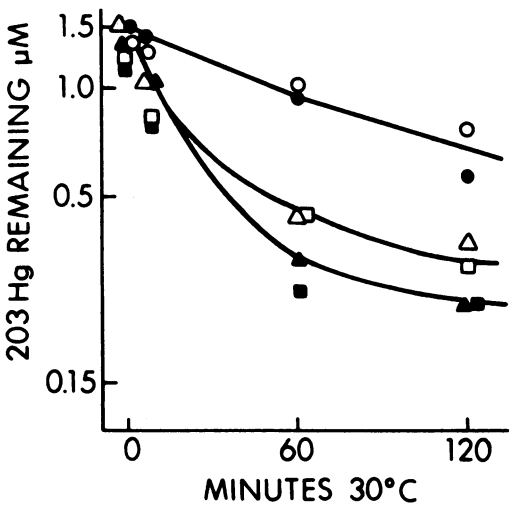

FIG. 1. Volatilization of mercury by intact cells of *T. ferrooxidans* at different pH levels. Cells pregrown on 5 μM Hg^{2+} (induced; closed symbols) or cells grown without added mercury (uninduced; open symbols) were washed, suspended in 9K salts, and added to 0.2 ml of pH 7.0 sodium phosphate buffer (○,●), to 9K salts at pH 2.5 (△,▲), or to pH 2.5 9K salts with added $FeSO_4$ (□,■). The assay medium contained 1.5 μM $^{203}Hg^{2+}$ and 2.7×10^8 induced cells or 3.1×10^8 uninduced cells in a total volume of 0.2 ml. Samples (25 μl) were removed periodically to assay remaining radioactivity.

once for *E. coli*. Disrupted cell preparations were "cleared" in a Eppendorf model 5414 Microfuge at 13,000 rpm for 2 min.

Mercury volatilization assays were as reported by Schottel (17) in an assay mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM disodium EDTA, 0.2 mM $MgSO_4$, 1 mM β-mercaptoethanol, 200 μM NADPH, 0.5 mg of bovine serum albumin per ml, and $^{203}Hg^{2+}$, generally at 2 to 5 μM. Deviations from this mixture are indicated in specific experiments. The preparation of preimmune rabbit serum and antisera prepared against purified mercuric reductase from transposon Tn*501* (2, 20) and plasmid R831 (17) will be described elsewhere (T. G. Kinscherf and S. Silver, manuscript in preparation).

RESULTS

Our first goal was to reduce the time scale for volatilization of 3 μM Hg^0 from the 40 to 80 h described in reference 15 to the minutes used with other bacteria (12, 17). Figure 1 shows that *T. ferrooxidans* cells, washed and resuspended at concentrations 10 times greater than those obtained during growth, volatilized mercury over a 2-h period more quickly at pH 2.5 than at pH 7.0. The addition of 150 mM $FeSO_4$ accelerated the rate of mercury volatilization slightly in this experiment and not at all in other similar experiments. It appears as if adequate intracellular energy reserves occur within the cells. The difference in volatilization rate between growth pH (2.5) and the assay buffer (pH 7.0) was also small and not reproducible from experiment to experiment. The reproducible conclusion was that *T. ferrooxidans* could volatilize mercury under nongrowing conditions at either pH. Cells grown in the absence of Hg^{2+} volatilized mercury at close to the same rate as did cells grown on mercury-containing medium (Fig. 1). The background mercury level in 9K medium was less than 0.5 nM Hg as determined by flameless atomic absorption spectroscopy. This would not have been sufficient to induce synthesis of mercury-volatilizing activity in previously studied bacteria (2, 13, 16, 23).

For all previously studied species, mercury volatilization activity was due to a mercuric reductase enzyme that was released from disrupted cells as a soluble enzyme (5, 8, 12, 17, 23). *T. ferrooxidans* BA-4 contained a similar enzyme activity (Fig. 2). Disrupted cells showed five times more rapid mercury volatilization than did intact cells, and this activity was entirely associated with the cell-free supernatant fluid. Taking the pellet from the 13,000-rpm centrifu-

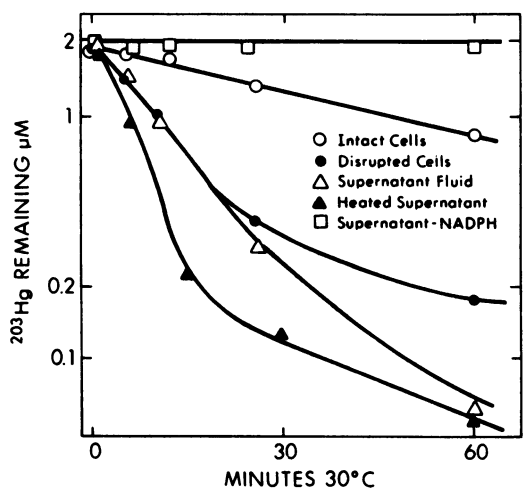

FIG. 2. Intact-cell and cell-free activities. *T. ferrooxidans* cells grown with 5 μM Hg^{2+} were centrifuged, washed, and suspended in 50 mM sodium phosphate buffer (pH 7.0) at 2×10^{10} cells per ml (○). Cells were disrupted by passage through a French pressure cell at 20,000 lb/in^2 twice (●). The disrupted cells were centrifuged at 13,000 rpm for 2 min to yield the cell-free supernatant fluid (△). That fluid was heated at 63°C for 20 min (▲). All assays were started by the addition of 5×10^8 cell equivalents to 0.2 ml of assay mixture (see the text) containing 2 μM $^{203}Hg^{2+}$ and 200 μM NADPH, except for the assay without NADPH (□).

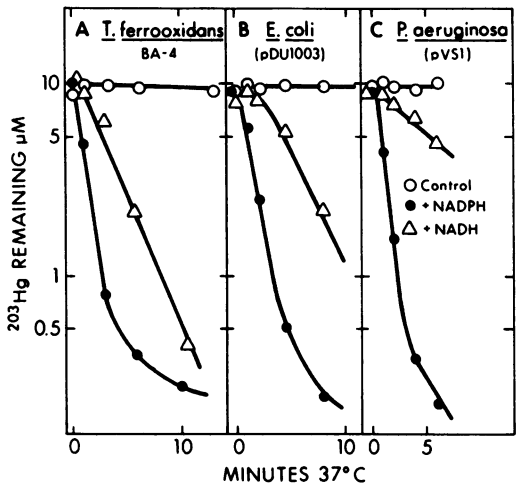

FIG. 3. NADPH and NADH dependency of cell-free activities. Cells of *T. ferrooxidans* BA-4, *P. aeruginosa* PAO9501(pVS1), or *E. coli* C600(pDU1003) were induced by preexposure to 5 μM Hg^{2+} during growth, centrifuged, and washed with and resuspended in 50 mM sodium phosphate buffer (pH 7.0). The cell suspensions were disrupted in a French pressure cell. Samples (25 μl) of centrifuged, heated (63°C) cell-free supernatant fluids or dilutions of the supernatant fluids were added to 0.2 ml of assay mixture containing 10 μM ^{203}Hg and either no pyridine nucleotide cofactor (○) or 100 μM NADPH (●) or NADH (△).

gation of disrupted cells and running it through the French pressure cell twice again yielded a cell-free preparation with about 20% of the activity of the initial cell-free supernatant fluid and a residual disrupted cell pellet that had less than 1% of the activity of the first supernatant fluid. We do not know whether the 20% enzyme activity represents enzyme loosely associated with the bacterial membrane or the relative difficulty of disrupting *T. ferrooxidans* in the French pressure cell. Previous attempts to identify a membrane-associated form of the enzyme did not succeed (17, 21). As with mercuric reductases from other gram-negative cells (Kinscherf and Silver, in preparation), the mercuric reductase activity from *T. ferrooxidans* was stimulated by heating the cell-free enzyme at 63°C for 20 min (Fig. 2). This is presumably due to precipitation of heat-sensitive proteins that bind Hg^{2+} in competition with the mercuric reductase enzyme. As with other cell-free mercuric reductase enzymes (17), activity was totally dependent on added NADPH (Fig. 2). Although NADPH was the preferred cofactor, NADH stimulated mercury volatilization as well (Fig. 3). In this regard the *Thiobacillus* enzyme was quite similar to those from plasmid R100 in *E. coli* and transposon Tn*501* in *P. aeruginosa* (Fig. 3).

Since *T. ferrooxidans* grows at very low pH, it was of interest to compare the intact-cell and cell-free activities for pH dependency. *E. coli* R100 cells and cell-free enzyme were included as controls (Table 1). In this experiment, the cells differed from those in Fig. 1 in that they were washed and resuspended in pH 7.0 phosphate buffer and they volatilized 80 to 90% of the added 3 μM $^{203}Hg^{2+}$ within 75 min in an exponential fashion. The *Thiobacillus* intact cells volatilized mercury at 63% of their maximum rate in 9K salts (pH 2.5). The *E. coli* cells showed no detectible activity in this medium. Cell-free enzyme preparations from both organisms showed a broad pH dependency with maximum activity at slightly alkaline pH. This is as expected for an intracellular enzyme such as mercuric reductase (17), since the intracellular pH of the *T. ferrooxidans* is near neutral (3), just like that of less acid-tolerant bacteria.

The mercuric reductase of *T. ferrooxidans* was very heat stable, surviving 10 min of incubation at temperatures up to 80°C (Fig. 4). If anything, the *Thiobacillus* mercuric reductase was slightly more heat resistant than the enzyme from *E. coli* plasmid R100. Heat resistance is a characteristic of the mercuric reductases from gram-negative bacteria (Kinscherf and Silver, in preparation); this distinguishes these enzymes from the mercuric reductases from gram-positive microorganisms (*Staphylococcus* and *Bacillus*), which are inactivated by temperatures above 60°C. In this respect the *Thiobacillus* enzyme appeared like a typical gram-negative organism mercuric reductase.

Mercury volatilization by the crude cell-free *Thiobacillus* preparations showed Michaelis-Menten kinetics with a K_m of about 15 μM Hg^{2+}, indistinguishable from the K_m for prepa-

TABLE 1. Effect of pH on cellular and subcellular mercuric reductase activity

pH	Mercuric reductase activity (% of maximum)[a]			
	T. ferrooxidans BA-4		*E. coli* C600(pDU1003)	
	Cells	Cell-free extract	Cells	Cell-free extract
2.5	63	<1	<1	<1
4.6	75	25	100	38
5.7	75	25	100	50
6.9	83	42	100	100
8.1	100	100	100	100

[a] The maximum activities were in each case 0.11 (*E. coli*) and 0.028 (*T. ferrooxidans*) nmol/min per 10^9 cells for intact cells and 0.74 (*E. coli*) and 0.17 (*T. ferrooxidans*) nmol/min per 10^9 cell equivalents for supernatant fluids of heated, French press-disrupted cells.

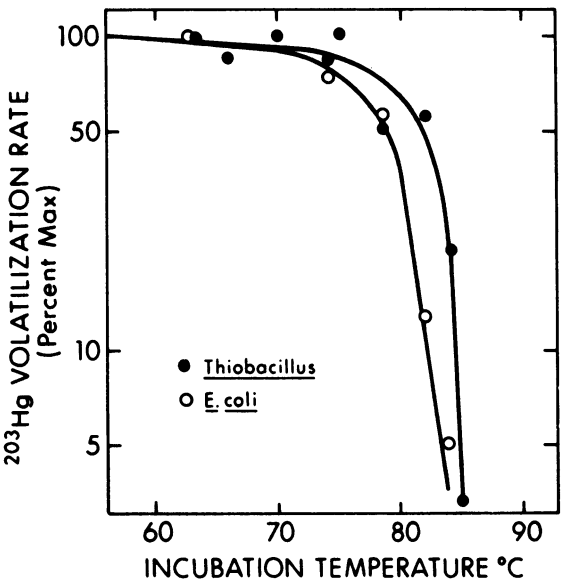

FIG. 4. Temperature sensitivity of the mercuric reductase from *T. ferrooxidans*. Samples (50 μl) of centrifuged supernatant from French press-disrupted *T. ferrooxidans* BA-4 (●) or *E. coli* C600(pDU1003) (○) were incubated at the indicated temperature for 10 min and subsequently assayed for the rate of ^{203}Hg volatilization with complete assay mixture containing 3 μM $^{203}Hg^{2+}$.

rations from *E. coli* C600(pDU1003) and *P. aeruginosa* PAO9501(pVS1) in the same experiment (data not shown). The V_{max} values of the cell-free preparations were more difficult to compare, since the *E. coli* and *P. aeruginosa* crude enzyme preparations generally lost about 90% of enzyme activity upon freeze-thawing and overnight storage at −20°C, whereas the *Thiobacillus* enzyme activity was stable to repeated freeze-thaw cycles (data not shown). However, the activity of *Thiobacillus* preparations was perhaps 10% as high as that for preparations of strain DB1406, with the low-plasmid-copy-number plasmid R100, and less than 1% of the V_{max} values with high-gene-copy-number strains C600(pDU1003) and PAO9501(pVS1) (on a per-cell basis; data not shown). The gene dosage effects on the level of mercuric reductase enzyme in other species have been reported (12). The volatilization of 50% of the 10 μM Hg in 1 min (Fig. 3A) by a cell-free *T. ferrooxidans* preparation might be compared with the 40 h for volatilizing 50% of 3 μM Hg under growing conditions (15). The main reason for this difference lies in the cell densities used. The growth experiments were initiated with about 10^7 cells per ml (15).

The mercuric reductase of *T. ferrooxidans* BA-4 required the presence of a thiol compound (Table 2), with β-mercaptoethanol being perhaps a little more effective than cysteine, dithiothreitol, or thioglycolate. The optimum concentration of β-mercaptoethanol was around 0.5 mM for both the *Thiobacillus* enzyme and that from plasmid R100 (Fig. 5). With enzyme from both species, higher mercaptoethanol concentrations were suboptimal, and the highest concentrations tested actually inhibited the *Thiobacillus* preparation to a value about half of that with no added thiol compound (Fig. 5). We do not know whether the lack of comparable inhibition with the *E. coli* preparation indicates a subtle difference between the enzymes or a difference in amounts of crude enzyme preparations used in these tests. EDTA was required for maximum enzyme activity. Without added EDTA the enzyme rate was about 10% of that obtained with 5 mM EDTA, which was the optimum concentration for both the *E. coli* and *T. ferrooxidans* enzymes (data not shown).

The mercuric reductase activity of *T. ferrooxidans* was not inactivated by antiserum prepared against the mercuric reductase from transposon Tn*501* (Fig. 6), which originated in *P. aeruginosa* (20), or antiserum prepared against mercuric reductase from plasmid R831 (data not shown), which originated in a *Serratia marcescens* strain (16). These antisera completely inactivated the mercuric reductase from plasmid R100 (Fig. 6) and those from over 20 otherwise unrelated mercuric resistance plasmids that have been transferred into *E. coli* or *P. aeruginosa* (Kinscherf and Silver, in preparation). The antisera did not inactivate the mercuric reductase from gram-positive bacteria. Both the preimmune serum and the antiserum inhibited the *Thiobacillus* mercuric reductase activity by about two-thirds (Fig. 6). This is apparently a nonspecific effect, since the effect of the preimmune serum was also seen with the *E. coli*

TABLE 2. Dependency of *Thiobacillus* mercury volatilization on thiol compounds[a]

Thiol	Enzyme rate (nmol/min per 10^9 cell equivalents)
None	0.03
Mercaptoethanol	0.42
Dithiothreitol	0.17
Thioglycolate	0.21
Cysteine	0.35

[a] Thiol compounds were added at 1 mM to assay buffer containing 2 μM $^{203}Hg^{2+}$, and cleared, disrupted-cell supernatant fluid from 6.5 × 10^8 cells was added to initiate the volatilization reaction. The initial rate of mercury loss was calculated.

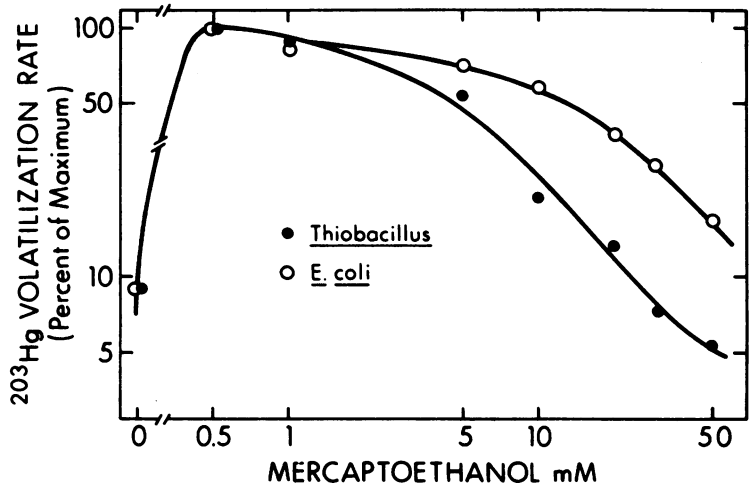

FIG. 5. Mercaptoethanol dependence of *T. ferrooxidans* BA4 (●) and *E. coli* C600(pDU1003) (○) mercuric reductase. Centrifuged cell-free supernatant fluids were tested in assay medium with 2 μM $^{203}Hg^{2+}$ and β-mercaptoethanol as indicated.

enzyme (Fig. 6), and a doubling of the amount of preimmune serum incubated with the enzyme (from 25 to 50 μl) did not cause further inhibition (data not shown) as would have been expected with an antibody-antigen reaction.

The immunological difference between the *T. ferrooxidans* enzyme and that from plasmid R100 and transposon Tn*501* is the primary one we have found to date. The only other significant difference was that crude enzyme preparations from *Thiobacillus* were completely stable to repeated freezing (−20°C) and thawing, whereas comparable preparations from *E. coli* C600(pDU1003) and *P. aeruginosa* PAO9501(pVS1) lost 90% of activity in one freeze-thaw cycle (data not shown).

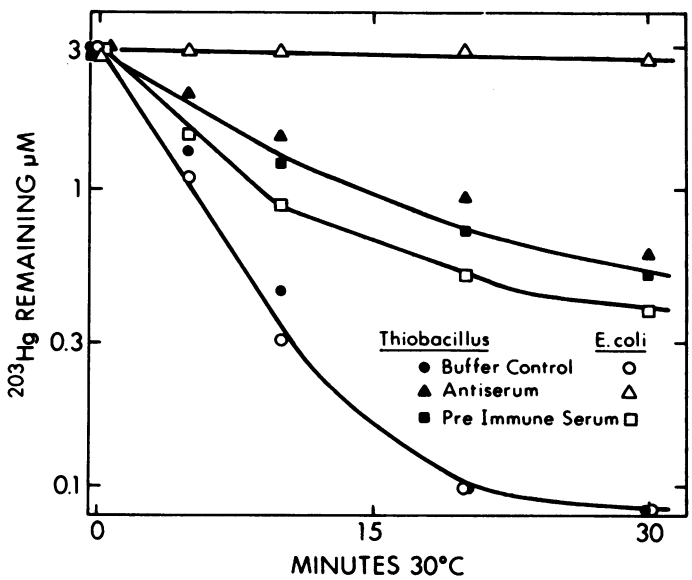

FIG. 6. Effect of antiserum on mercuric reductase from *T. ferrooxidans* and *E. coli*. A 25-μl sample of centrifuged supernatant fluid was added to 25 μl of pH 7.0 buffer (●,○), antiserum prepared against purified mercuric reductase coded by transposon Tn*501* (▲,△), or preimmune rabbit serum (■,□); 10 min later, 0.2 ml of assay medium was added. Closed symbols, Crude enzyme from *T. ferrooxidans* BA-4; open symbols, crude enzyme from *E. coli* C600(pDU1003).

DISCUSSION

This report describes the existence and some basic properties of the mercuric reductase enzyme from a mercury-volatilizing *T. ferrooxidans* strain. There are several reasons to do this, including the basic interest in *Thiobacillus* itself as well as the questions of whether there are more than one mechanism of mercuric resistance and more than one type of mercuric reductase enzyme. *T. ferrooxidans* BA-4 produces a soluble enzyme with basic properties similar to those of the mercuric reductases found in other bacterial species. The enzyme is soluble (not membrane bound) and dependent upon a thiol compound, EDTA, and NAD(P)H for activity. We have previously (17, 18, 21) considered the mercuric reductase enzymes as NADPH specific, based on results both with an *E. coli* enzyme (17) and one from *Staphylococcus aureus* (23) showing little effect of NADH unless added at 50 μM or more, i.e., in considerable excess of that needed to reduce the Hg^{2+} substrate (17). Izaki et al. (8) reached a similar conclusion with another *E. coli* plasmid enzyme, but Furukawa and Tonomura (5) found that both NADPH and NADH could function with a mercuric reductase from a soil pseudomonad. The results in Fig. 3 require a reinterpretation of previous conclusions (17, 21, 23). Although mercuric reductase from all three species tested in the experiment in Fig. 3 worked at a higher rate with NADPH than with NADH, significant activity was found with NADH as well. The *Thiobacillus* enzyme seemed to be no different from other mercuric reductases in this regard, and both the absolute amounts and the ratio of the Hg^{2+} concentration to that of the pyridine nucleotide affected the appearance of NADPH specificity.

The *T. ferrooxidans* mercuric reductase was as heat resistant as other mercuric reductases from gram-negative species, but it was not inactivated by antisera raised against other mercuric reductase enzymes. This lack of immunological cross-reaction is novel, since most other mercuric reductase enzymes from gram-negative bacteria do cross-react with antisera (Kinscherf and Silver, in preparation). Before we can determine additional basic properties of the enzyme, such as molecular weight, subunit composition, and flavin adenine dinucleotide content (4, 5, 8, 17), we will need larger amounts than currently available.

The previous report (15) showed that cells of *T. ferrooxidans* BA-4 grown without mercury lacked volatilization activity. Those cells were derived from the original *T. ferrooxidans* isolate and had never been exposed in the laboratory to Hg^{2+}. The current "uninduced" cells were grown instead for several passages (up to five 10-fold serial dilutions) without added Hg^{2+} and fully retained volatilization activity during that passage. We have confirmed that a culture that originated from strain BA-4 and that has been maintained in the absence of mercury has lost the volatilization activity (data not shown). Thus, *T. ferrooxidans* BA-4 appears constitutive for the synthesis of mercuric reductase and is the first such constitutive strain found after testing more than 150 other gram-negative and gram-positive strains of many species (2, 16, 23, 24). The non-volatilizing substrain derived from BA-4 has apparently lost (been cured of) the mercury-volatilizing activity and might thus be called *T. ferrooxidans* BA-4C.

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