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Constitutive Synthesis of a Transport Function Encoded by the *Thiobacillus ferrooxidans merC* Gene Cloned in *Escherichia coli*

TOMONOBU KUSANO,[1,2] GUANGYONG JI,[1]* CHIHIRO INOUE,[2] AND SIMON SILVER[1]

Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60680,[1] and Biotechnology Institute, Akita Prefectural College of Agriculture, Akita 010-04, Japan[2]

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Mercuric reductase activity determined by the *Thiobacillus ferrooxidans merA* gene (cloned and expressed constitutively in *Escherichia coli*) was measured by volatilization of $^{203}Hg^{2+}$. (The absence of a *merR* regulatory gene in the cloned *Thiobacillus mer* determinant provides a basis for the constitutive synthesis of this system.) In the absence of the *Thiobacillus merC* transport gene, the mercury volatilization activity was cryptic and was not seen with whole cells but only with sonication-disrupted cells. The *Thiobacillus merC* transport function was compared with transport via the *merT-merP* system of plasmid pDU1358. Both systems, cloned and expressed in *E. coli*, governed enhanced uptake of $^{203}Hg^{2+}$ in a temperature- and concentration-dependent fashion. Uptake via MerT-MerP was greater and conferred greater hypersensitivity to Hg^{2+} than did uptake with MerC. Mercury uptake was inhibited by *N*-ethylmaleimide but not by EDTA. Ag^{+} salts inhibited mercury uptake by the MerT-MerP system but did not inhibit uptake via MerC. Radioactive mercury accumulated by the MerT-MerP and by the MerC systems was exchangeable with nonradioactive Hg^{2+}.

The bacterial mercury resistance system is complex, consisting generally of about six genes (23). The first three such systems cloned and sequenced from plasmids of gram-negative bacteria (transposon Tn*501* from plasmid pVS1 of *Pseudomonas aeruginosa*, transposon Tn*21* from plasmid R100 of *Shigella flexneri*, and plasmid pDU1358 from *Serratia marcescens* [2, 7, 11, 12, 16]) have the same basic pattern of genes (Fig. 1): the system starts with a divergently transcribed regulatory gene, *merR* (3, 11, 16), whose function represses a basal level of activity in the absence of Hg^{2+} and activates operon transcription from the operator-promoter region in the presence of inducing concentrations of Hg^{2+} (3, 15, 18, 20). After the operator-promoter region, which binds the MerR protein and RNA polymerase (18, 20), comes a series of structural genes: *merT*, determining an integral inner membrane transport protein; *merP*, determining a periplasmic mercury-binding protein that is thought to transfer mercuric ions to MerT; and *merA*, which is the determinant of the cytoplasmic flavoprotein mercuric reductase, which reduces ionic Hg^{2+} to volatile Hg^{0} (11, 12, 16, 23). After the *merA* gene, each of these three systems contains a *merD* gene (2, 7), which is thought to play a regulatory role moderating the effects of MerR (2, 17), and an additional URF1 of unknown function (2, 7). The Tn*21* and pDU1358 *mer* operons each contain an extra gene (Fig. 1): pDU1358 has a *merB* gene (7), which governs the synthesis of the 212-amino-acid organomercurial lyase, the enzyme that cleaves the phenylmercury C-Hg bond (thus conferring resistance to organomercurials); while the Tn*21* *mer* operon contains the *merC* gene (12), which determines a 140-amino-acid polypeptide that is thought to be involved in transport because of its hydrophobic nature (12, 23). However, Tn*501* without *merC* was indistinguishable in all tests of resistance, uptake, and volatilization of mercury from Tn*21* with *merC*. It was thus a surprise when the *Thiobacillus ferrooxidans mer* DNA sequence contained only the operator-promoter region, *merA*, *merC*, and URF1 located upstream from the promoter in a position comparable to that of *merR* in the other systems (8, 22; C. Inoue and T. Kusano, unpublished data).

Mutations and deletions in Tn*21* and Tn*501* lacking *merA* mercuric reductase activity but expressing the *merT* plus *merP* transport system (with or without *merC*) confer hypersensitivity to Hg^{2+} and hyperaccumulation of mercuric ions (13–15). This transport activity via the *merT-merP* gene products requires prior induction by mercury salts (13–15) and is lost on deletion of *merT-merP* (4).

The *T. ferrooxidans* mercury resistance system was previously shown to be associated with subcellular mercury volatilization activity (19), but the *Thiobacillus* system differed from that in other gram-negative bacteria in three ways. First, there was no evidence for a plasmid determinant and the genes therefore appeared to reside on the cellular chromosome. (Chromosomal mercury resistance was subsequently established in gram-positive organisms as well [24, 25].) Second, the *Thiobacillus* mercuric reductase system was synthesized constitutively (19). The third difference between the *Thiobacillus* mercury resistance system and those from other gram-negative bacteria was the absence of immunological cross-reaction between antisera prepared against the Tn*21* enzyme and the *Thiobacillus* enzyme (19). At that time, the *Thiobacillus* mercuric reductase was the only one of more than 20 tested enzymes from gram-negative bacteria that failed to cross-react and was in this regard similar to mercuric reductases from gram-positive sources (19; unpublished data).

Shiratori et al. (22) isolated new mercury-resistant *Thiobacillus* strains and found an absence of discernible plasmids in these strains. The constitutive synthesis of mercury-dependent NADPH oxidase activity after cloning into *Escherichia coli* indicated the constitutive synthesis of this system. The *Thiobacillus mer* system was sequenced (8; unpublished data) and surprisingly contains only URF1, the promoter region, *merC*, and *merA* (Fig. 1).

In this study the intact *merC merA Thiobacillus* mercury resistance determinant was compared with a *merT merP merA* system in terms of $^{203}Hg^{2+}$ volatilization activity for both intact whole cells and sonicated subcellular fractions. A

* Corresponding author.

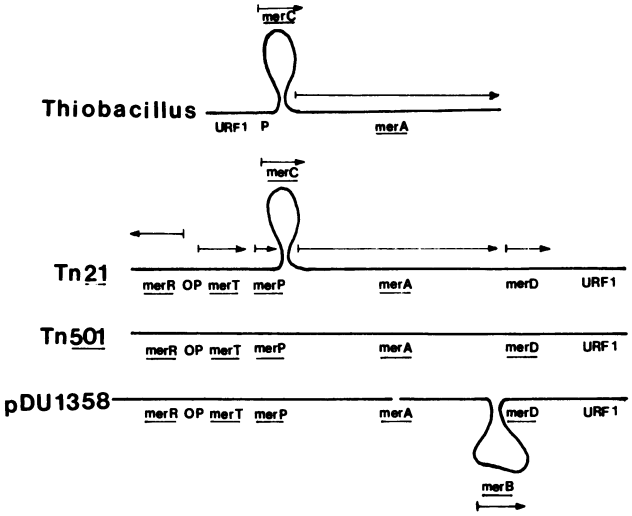

FIG. 1. Diagram of the mercury resistance operons from four sequenced gram-negative sources. Gene mnemonics are defined in the text. Data for Tn*21* and Tn*501* come from references 2, 11, and 12; data for pDU1358 come from references 7 and 16; and data for *T. ferrooxidans* come from references 8 and 22 and C. Inoue et al., unpublished data. OP, Operator-promoter.

subclone containing *merA* alone without *merC* was shown to contain mercuric reductase activity that was cryptic at the whole-cell level. Finally, deletion variants containing the transport genes (*merC* or *merT* plus *merP*) alone without *merA* were tested in $^{203}Hg^{2+}$ uptake experiments, and the properties of the *merC* system were compared with those of *merT* plus *merP*.

MATERIALS AND METHODS

Strains and plasmids. The *E. coli* strains used (C600, DH5α, and MC1061 [21]) are equivalent in terms of mercury sensitivity and resistance (with resistance plasmids). Table 1 lists the plasmids used. Although the mercury resistance determinants originated in *Serratia* (7) and *Thiobacillus* (22) species, they were used here after being subcloned into small vector plasmids in *E. coli*.

To construct a plasmid containing *Thiobacillus merC* under control of its own promoter, plasmid pTM503 (22), containing the intact *Thiobacillus mer* determinant, was digested with the restriction enzymes *Sal*I and *Sma*I. After the *Sal*I end was filled with Klenow fragment DNA polymerase I and blunt end ligation was done, the deletion plasmid pTMC503 containing *merC* (but only part of *merA*) was transformed into *E. coli* DH5α.

TABLE 1. Plasmids

Plasmid	Description	Reference or source
pUC18	Vector, sensitive	21
pTM314	Cloned *Thiobacillus merA* and *merC* in pUC18	22
pTMC503	Cloned *Thiobacillus merC* in pUC18	This study
pTM504	Cloned *Thiobacillus merA* in pUC18	22
pHG103	Intact pDU1358 *mer* system in pBR322	7
pGN106	*merA* deletion mutant of *mer* of pHG103	G. Nucifora, unpublished data

Radioactive mercury volatilization experiments. Experiments with 5 μM $^{203}Hg^{2+}$ were carried out as previously described (9, 15). Cells were grown in LB broth to a turbidity of about 50 Klett units at 37°C. After an additional 1 h of incubation with (induced) or without (uninduced) 1 μM Hg^{2+}, the cells were harvested by centrifugation at 4,000 × *g* for 10 min, washed once with 50 mM sodium phosphate buffer (pH 7.4), and suspended in the same buffer at 12.5 mg (dry weight) per ml. One half of the suspended cells was used as a whole-cell preparation, and the other half was sonicated in an Ultratip Labsonic sonicator (Lab-line Instruments, Inc., Melrose Park, Ill.) for 2 min (intermittently) at full power with cooling on ice. After the removal of cell debris by centrifugation for 15 min in an Eppendorf microfuge, the supernatant fluids were used as a crude cell-free enzyme (9). A portion (25 μl) of whole cells or cell-free enzyme was added to 0.2 ml of the assay mixture (9, 15) containing 5 μM $^{203}Hg^{2+}$ and without (whole-cell) or with (cell-free) 0.2 mM NADPH. Samples (25 μl) were removed so radioactivity could be counted periodically during shaking at 200 rpm at 37°C.

Radioactive mercury uptake experiments. *E. coli* cells were grown in LB broth to a turbidity of 50 Klett units; they were induced for *mer* operon function by the addition of 0.2 μM Hg^{2+} for 30 min and then an additional 0.2 μM Hg^{2+} and another 30 min of growth at 37°C. After the cells were harvested by centrifugation and washed once with LB broth plus 100 μg of chloramphenicol per ml, the cells were suspended at a final turbidity corresponding to 0.125 mg (dry weight) per ml in LB broth containing chloramphenicol. Radioactive $^{203}Hg^{2+}$ was added at 5 μM, and 1-ml samples were filtered (0.45-μm-pore-diameter nitrocellulose filters) at the indicated times and washed with 10 ml of LB broth.

RESULTS AND DISCUSSION

Measurements of disk inhibition zones (Fig. 2) showed differences between the resistance level conferred by the cloned *Thiobacillus mer* determinant and that of plasmid pDU1358. In addition, the hypersensitivity of the pDU1358 *merT* plus *merP* system was greater than that for *merC* alone (Fig. 2). The slightly increased sensitivity to mercuric ions governed by *merC* (compared with cells with the vector plasmid) is best contrasted with the slight resistance with the complete *Thiobacillus mer* system (Fig. 2). These differences were reproducible and must be accounted for by the results of physiological measurements.

When volatilization of radioactive $^{203}Hg^{2+}$ was compared with the cell-free fractions from *E. coli* containing the *Thiobacillus mer* system and that from pDU1358, the rates of mercury loss were found to be approximately equal (Fig. 3B). However, the activity from the *Thiobacillus* system was synthesized constitutively, whereas that from pDU1358 was made inducibly. Intact cells volatilized mercury somewhat more slowly than did cell-free preparations (Fig. 3A), indicating that the transport function is probably rate limiting with the intact cells (13–15).

Assuming that the *Thiobacillus merC* gene provides an alternative transport pathway to the quantitatively stronger *merT* plus *merC* system previously characterized (13–15), the deletion of *merC* would be expected to result in cryptic cells that contain mercuric reductase but cannot volatilize mercury. *E. coli* cells with the *Thiobacillus merA* but no transport system did not volatilize ^{203}Hg when tested as

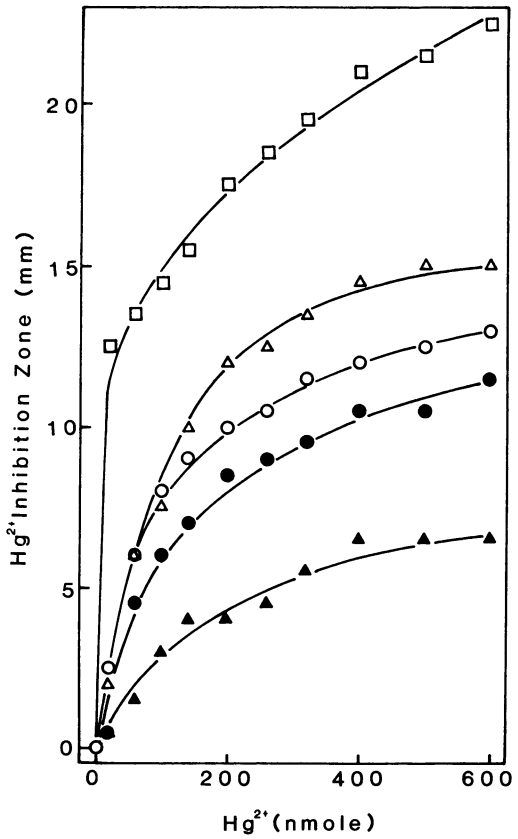

FIG. 2. Disk inhibition zones for resistant and hypersensitive clones. Resistance of the *Thiobacillus mer* determinant cloned into *E. coli* compared with the *merT merP* system of plasmid pDU1358 is shown. $Hg(NO_3)_2$ was added to paper disks placed on LB agar petri dishes spread with 0.1 ml of overnight cultures of the indicated *E. coli* strain: ○, DH5α(pUC18), vector plasmid, sensitive; ●, DH5α (pTM314), *Thiobacillus merA* plus *merC*; △, DH5α(pTMC503), *Thiobacillus merC* only; ▲, C600(pHG103), intact pDU1358 *mer* system, resistant; and □, MC1061(pGN106) *merA* deletion of pHG103, hypersensitive. After overnight growth at 37°C, the diameter of the inhibition zone (minus the 6.5-mm disk diameter) was measured.

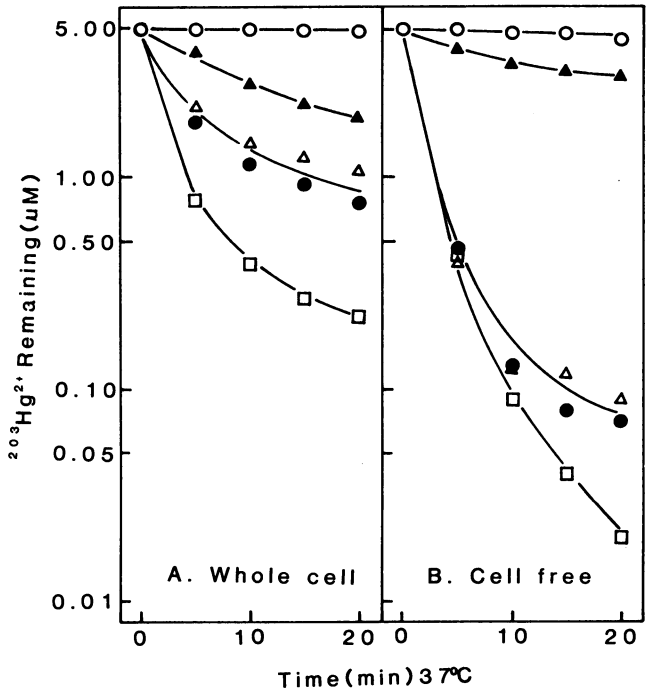

FIG. 3. Volatilization of $^{203}Hg^{2+}$ determined by cloned *Thiobacillus mer* operon: whole cells versus cell-free activity. Whole cells of *E. coli* strains DH5α(pTM314), induced (△) or uninduced (●) (*Thiobacillus merC merA* system); MC1061(pHG103), induced (□) or uninduced (▲) (intact pDU1358 *mer* system); or DH5α(pUC18) (○) (sensitive vector control) were grown in LB broth with or without 1 μM Hg^{2+} as indicated in Materials and Methods. Cells were centrifuged, washed, and divided in half. (A) One portion was assayed for the volatilization of 5 μM $^{203}Hg^{2+}$ with intact cells, and (B) another portion was assayed after cell disruption by sonication.

whole cells (Fig. 4) but showed volatilization activity in cell-free preparations. Introduction of *merC* in *trans* to a mercury-sensitive *E. coli* containing pTM504 (*merA* alone) resulted in the regaining of mercury resistance (data not shown).

Hypersensitivity to Hg^{2+} with *merA* mutants of Tn*21* and Tn*501* is associated with hyperuptake of mercury salts (13–15). Results show that this is also the case for the pDU1358 mercury transport system (Fig. 5). The conditions used in the current work are slightly different from those of earlier experiments (13–15), and the results are somewhat more striking. The *Thiobacillus merC* system also confers hyperuptake (Fig. 5) but does so much less quantitatively than does the system with *merT* plus *merP* (Fig. 5). While the pDU1358 $^{203}Hg^{2+}$ uptake activity was synthesized inducibly, that for the *Thiobacillus* system was made constitutively (Fig. 5), as expected.

The *merC* system from *T. ferrooxidans* was characterized and compared with the *merT* plus *merP* system of pDU1358 with regard to physiological properties and inhibitors. Both systems were temperature dependent, functioning better at 37°C than at 25 or 4°C (data not shown). Since *N*-ethylmaleimide inhibits membrane transport systems involving critical exposed thiols (5) and the mercury transport system is expected to contain these groupings (23), *N*-ethylmaleimide inhibition was determined. Both systems were sensitive to *N*-ethylmaleimide (data not shown), although Nakahara et al. (14) could not demonstrate this inhibition with the Tn*21* system under slightly different conditions. EDTA (at 1 mM) did not inhibit $^{203}Hg^{2+}$ uptake.

The most significant qualitative difference found between the *merC* and the *merT merP* systems was the sensitivity of the latter (but not the former) to Ag^+ ions (Fig. 6). Ag^+ salts were recently used to inhibit cell-free mercuric reductase activity (9). Although Ag^+ inhibited $^{203}Hg^{2+}$ accumulation, Ag^+ did not induce loss of previously accumulated mercury (Fig. 7). An analysis of MerC- versus MerT-plus-MerP-mediated $^{203}Hg^{2+}$ uptake gave approximate Michaelis-Menten kinetics constants. The less active *Thiobacillus* MerC system had a K_m of about 3 μM Hg^{2+} and a V_{max} of 0.2 μmol/min per g (dry weight) of cells, while the pDU1358 MerT plus MerP system had a K_m of more than 20 μM and a V_{max} of more than 5 μmol/min per g (dry weight) of cells (data not shown).

The $^{203}Hg^{2+}$ accumulated by *merC* or *merT merP* cells was exchangeable with excess nonradioactive mercury (Fig. 7). Whereas nonradioactive Hg^{2+} exchanged for radioactive mercury with both transport systems, the addition of excess Ag^+ either was without effect (Fig. 7B) or resulted in the

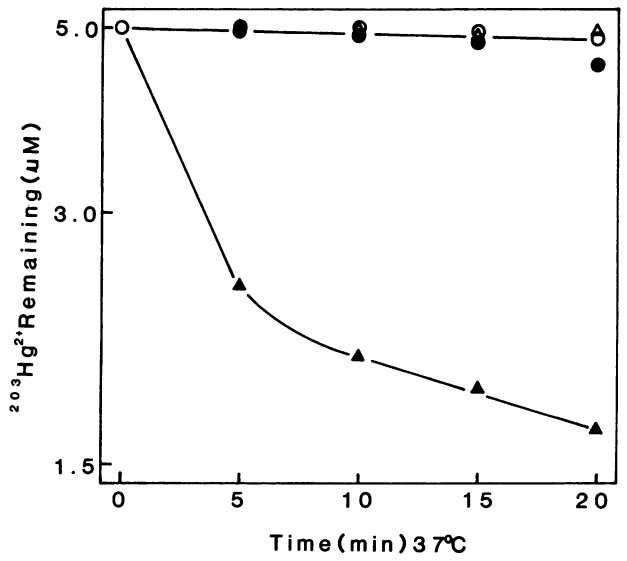

FIG. 4. Volatilization of $^{203}Hg^{2+}$ by intact cells (△) or cell-free preparations (▲) of *E. coli* DH5α(pTM504) harboring the *Thiobacillus merA* gene in the absence of a mercury transport system. Cells (○) and cell-free sonicate (●) from sensitive cells with pUC18 vector are shown. Conditions were as described in Materials and Methods and as shown in Fig. 3.

inhibition of subsequent uptake but no loss of $^{203}Hg^{2+}$ already accumulated (Fig. 7C).

It is puzzling why alternative Hg^{2+} uptake pathways exist in mercuric resistance determinants of gram-negative bacteria and why they are distributed in different systems as they are. Of the three sequenced systems found originally in enterics and pseudomonads, only that of R100 contained the *merC* gene, and that had no discernible phenotype in the presence of *merT* plus *merP* (2, 12). The two MerC amino acid sequences from plasmid R100 (12) and from the *T. ferrooxidans* chromosome (unpublished data) are identical at 56% (78 of 140) shared positions, somewhat less than the 79% shared amino acids for their mercuric reductase sequences (8, 12). There is a somewhat weaker but highly significant sequence homology between MerC and the MerT transport protein of plasmid R100 (26% amino acid identities [30 of 116 shared positions] [unpublished data]). The *T. ferrooxidans* system lacks the more effective *merT* plus *merP* system (8). The sequencing and transport results now clarify and explain the colony-blotting DNA-DNA hybridization analyses (1, 6) which showed that *merC* determinants were frequently found in mercury-resistant strains, especially in *Escherichia* isolates from environmentally polluted sources. Yet our studies have not provided an explanation of why *merC* exists sometimes as a supplement and sometimes as an alternative to *merT* plus *merP*. In mercury resistance systems of gram-positive bacteria, there are transport genes rather different from those of gram-negative bacteria (10, 24) which also cause hypersensitivity to Hg^{2+} in the absence of MerA (R. A. Laddaga and T. K. Misra, personal communications).

In summary, the work described here adds to the recent reports (8, 22) that the *T. ferrooxidans* mercury resistance system functions constitutively in *E. coli*. The *Thiobacillus merC* gene determines a mercury transport activity that was measured as slight hyperuptake of Hg^{2+} in cells lacking mercuric reductase and by a slight increase in sensitivity by those cells, compared with that of control mercury-sensitive

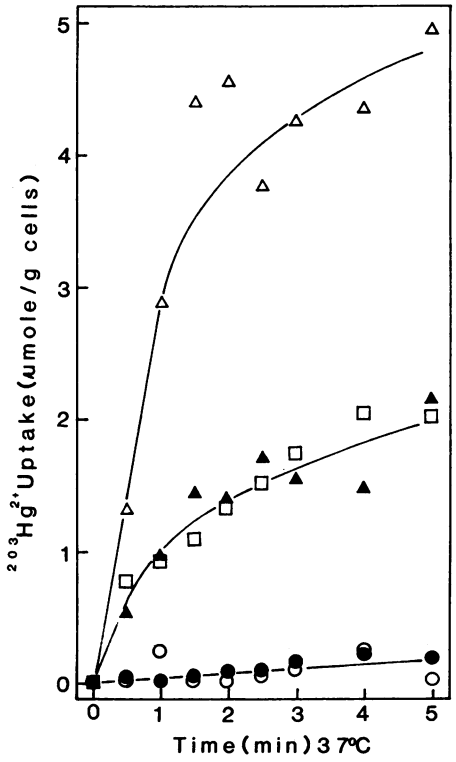

FIG. 5. Hyperuptake of $^{203}Hg^{2+}$ by reductase-negative derivatives of the *Thiobacillus* and pDU1358 *mer* operons. Cells were grown, induced (or not), and harvested as indicated in Materials and Methods. An amount of 5 μM $^{203}Hg^{2+}$ was added, and samples were filtered and washed. *E. coli* strains with plasmids DH5α(pUC18) (sensitive, vector control) (○), MC1061(pGN106) (hypersensitive) (induced [△] or uninduced [●]), and DH5α(pTMC503) (*Thiobacillus merC*) (induced [□] or uninduced [▲]) are shown.

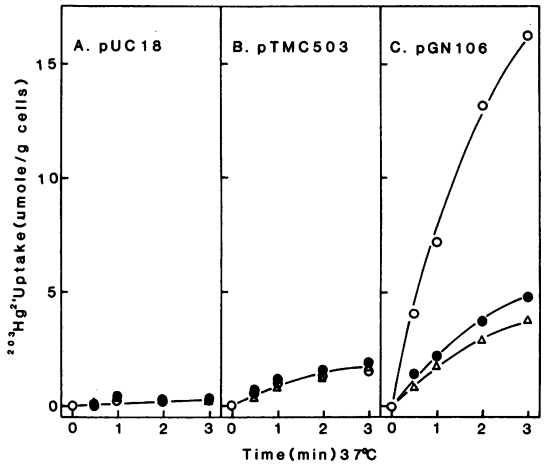

FIG. 6. Inhibition of the pDU1358 but not the *Thiobacillus* mercury uptake system by silver salts. Cells were grown, prepared, and assayed as described in the legend to Fig. 5 in the presence of 10 (●) or 50 (△) μM Ag^{+} added to the uptake assay mixture before the addition of the cells. ○, No Ag^{+} added.

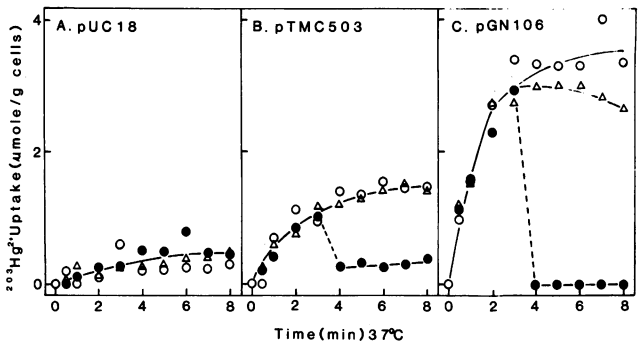

FIG. 7. Exchange of accumulated $^{203}Hg^{2+}$. *E. coli* cells containing (A) the pUC18 vector, (B) the *Thiobacillus merC* system, or (C) the pDU1358 *merT merP* system were grown, induced (C) or uninduced (A and B), and assayed for $^{203}Hg^{2+}$ uptake (○) as described in the legend to Fig. 5. After 3 min of accumulation of 5 μM $^{203}Hg^{2+}$, 500 μM Hg^{2+} (●) or Ag^{+} (△) was added and sampling continued.

cells. Both for hypersensitivity and for transport, the *merC* system was quantitatively less active than was the plasmid pDU1358 system consisting of *merT* plus *merP*. This is consistent with our earlier inability to detect differences in resistance or other functions between Tn*501* (with *merT* and *merP*) and R100 (with additional *merC*) (2, 11, 12). This report concerns *Thiobacillus* genes cloned in *E. coli* rather than in their original host cell. Additional understanding of the *merC* transport system will require a careful analysis of transport and volatilization with *T. ferrooxidans* genes in *T. ferrooxidans*. However, there is currently no method for introducing cloned genes into *T. ferrooxidans*.

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