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Mercury and Organomercurial Resistances Determined by Plasmids in *Pseudomonas*

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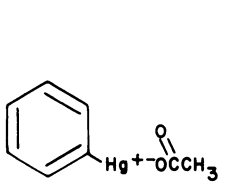
Mercury and organomercurial resistance determined by genes on ten *Pseudomonas aeruginosa* plasmids and one *Pseudomonas putida* plasmid have been studied with regard to the range of substrates and the range of inducers. The plasmidless strains were sensitive to growth inhibition by Hg^{2+} and did not volatilize Hg^0 from Hg^{2+}. A strain with plasmid RP1 (which does not confer resistance to Hg^{2+}) similarly did not volatilize mercury. All 10 plasmids determine mercury resistance by way of an inducible enzyme system. Hg^{2+} was reduced to Hg^0, which is insoluble in water and rapidly volatilizes from the growth medium. Plasmids pMG1, pMG2, R26, R933, R93-1, and pVS1 in *P. aeruginosa* and MER in *P. putida* conferred resistance to and the ability to volatilize mercury from Hg^{2+}, but strains with these plasmids were sensitive to and could not volatilize mercury from the organomercurials methylmercury, ethylmercury, phenylmercury, and thimerosal. These plasmids, in addition, conferred resistance to the organomercurials merbromin, *p*-hydroxymercuribenzoate, and fluorescein mercuric acetate. The other plasmids, FP2, R38, R3108, and pVS2, determined resistance to and decomposition of a range of organomercurials, including methylmercury, ethylmercury, phenylmercury, and thimerosal. These plasmids also conferred resistance to the organomercurials merbromin, *p*-hydroxymercuribenzoate, and fluorescein mercuric acetate by a mechanism not involving degradation. In all cases, organomercurial decomposition and mercury volatilization were induced by exposure to Hg^{2+} or organomercurials. The plasmids differed in the relative efficacy of inducers. Hg^{2+} resistance with strains that are organomercurial sensitive appeared to be induced preferentially by Hg^{2+} and only poorly by organomercurials to which the cells are sensitive. However, the organomercurials *p*-hydroxymercuribenzoate, merbromin, and fluorescein mercuric acetate were strong gratuitous inducers but not substrates for the Hg^{2+} volatilization system. With strains resistant to phenylmercury and thimerosal, these organomercurials were both inducers and substrates.

Mercury and organomercurial resistance determined by plasmids in *Pseudomonas* has been known for several years (1). The original *Pseudomonas aeruginosa* conjugal sex factor FP2 was shown to carry determinants for mercury resistance (10). A separable plasmid conferring mercury resistance as its only known phenotypic marker was found in *Pseudomonas putida* strains with multiple plasmids (1, 2), and a translocation unit, Tn501, containing markers for mercury resistance has recently been identified as movable between plasmids in *P. aeruginosa* (18). Today the best biochemically characterized system for mercury and organomercurial detoxification is that of an unspeciated *Pseudomonas* strain that Tonomura and associates isolated on the basis of phenylmercury resistance (4, 5, 24-27). This bacterial strain has not been shown to harbor a plasmid as yet, although no examples of chromosomally located mercury resistance genes are known in bacterial species. Four years ago, our laboratory (19) reported volatilization of mercury from Hg^{2+} with *P. aeruginosa* strains with plasmids FP2, pMG1, and pMG2, but only later we found that plasmid FP2 carried determinants for volatilization of mercury from organomercurials as well. This paper represents our first attempt to put together our understanding of mercury and organomercurial resistance associated with *Pseudomonas* plasmids in a comprehensive manner. (See Fig. 1 for structures of the organomercurials.)

Although we will equate mercury volatilization with reduction to Hg^0 in this paper, this has not been shown conclusively in every case.



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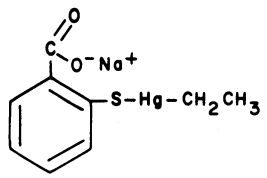

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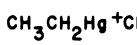

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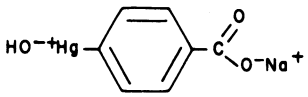
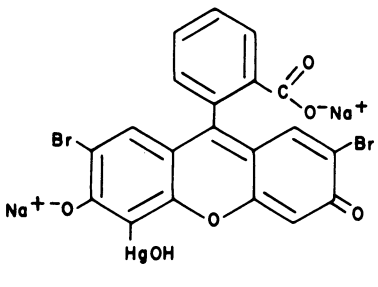
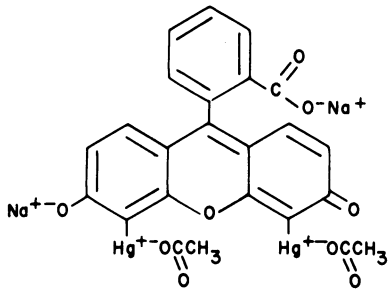


p-HYDROXYMERCURIBENZOATE (pHMB)



MERBROMIN (MERCUROCHROME)



FLUORESCEIN MERCURIC ACETATE (FMA)

FIG. 1. *Structures of the organomercurials.*

In a few cases, the volatile mercury form has been shown to be Hg^0 (5, 7, 11, 21); enzymatic volatilization of mercury in each of many cases requires concomitant oxidation of reduced pyridine nucleotide (5, 7, 28; J. Schottel, submitted for publication).

MATERIALS AND METHODS

Bacteria and media. *P. aeruginosa* plasmids were all in sublines of the thoroughly studied PAO strain of Holloway. Substrain PU21 (*ilvB112 leu-1 str^r rif^r*; 8) without a resistance plasmid and substrain PU21 with plasmids R3108, FP2, pMG1, and pMG2 were obtained from G. A. Jacoby, and the original sources of these strains are given in references 7 to 9 and 16. PAO9501 (*ade-36 leu-8 rif-1 chl-3*) without plasmid or with plasmid pVS1, R26, or RP1-74, PAO1670 (*ade-36 leu-8 rif-1 chl-3*) without a plasmid or with plasmid RP1, FP2, R931-1, R933, or R38, and PAO9505 (*arg-18 chl-2 rif-4*) without a plasmid or with plasmid pVS2 were all obtained from V. A. Stanisich. The auxotropic and antibiotic resistance markers on the chromosomes of these four PAO substrains should not affect our studies of the mercury and organomercurial resistances. These nine plasmids were described in references 17 and 18, which also give their origins. RP1 differs from the other plasmids in that it does not have a determinant for mercury and organomercurial resistance. Plasmid RP1-74 is a hybrid plasmid containing the 6.6×10^6-dalton translocation unit Tn501 from plasmid pVS1 inserted into the tetracycline resistance locus of plasmid RP1 (18). *P. putida* strain AC10 and strain AC77 harboring the OCT-MER plasmid aggregate (1, 2) were obtained from A. M. Chakrabarty. *Pseudomonas* strain K62, which has been extensively studied in Tonomura's laboratory (4, 5,

23-27), was obtained from the Agricultural Research Service, Peoria, Ill., as NRRL B-40109.

Three media were used. Tryptone broth contained 8 g of tryptone (Difco) and 5 g of NaCl per liter of deionized water. Luria broth contained 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract (Difco) per liter of deionized water plus 1 g of glucose per liter added after sterilization. Salts buffer contained 50 mM Na-PO_4 buffer (pH 7.4) plus 0.5 mM ethylenediamine-tetraacetic acid, 0.2 mM $MgCl_2$, and 1 mM 2-mercaptoethanol.

Materials. Radioactive $^{203}Hg(NO_3)_2$ and $^{14}CH_3HgCl$ were obtained from New England Nuclear Corp., Boston, Mass. ^{203}Hg-labeled phenylmercuric acetate, methylmercuric chloride, and ethylmercuric chloride were obtained from ICN Pharmaceuticals, Inc., Irvine, Calif. Nonradioactive organomercurials were obtained as follows: merbromin and thimerosal from K and K Laboratories, Inc., Plainview, N.Y.; phenylmercuric acetate from Eastman Organic Chemicals, Rochester, N.Y.; *p*-hydroxymercuribenzoate (pHMB) and fluorescein mercuric acetate from Sigma Chemical Co., St. Louis, Mo.; and methylmercuric chloride from Ventron Corp. Alfa Products, Danvers, Mass. Scintillation fluid consisted of 2 liters of toluene, 1 liter of Triton X-100, and 16.5 g of preblended dry fluor 2a40 from Research Products International Corp., Elk Grove Village, Ill.

Radioactive mercury volatilization assays. Radioactive mercury volatilization assays were conducted as described in the accompanying paper (28) except that the cells were grown at 32°C, although the volatilization assays in buffer were carried out at 37°C. Nonradioactive mercury volatilization assays were done by atomic absorption spectroscopy, using cells grown at 32°C in Luria broth, induced by addition of 10 μM Hg^{2+} at a cell turbidity of 75 Klett units followed by an additional 75 min of incubation. The cells were harvested by centrifugation, washed once in an equal volume of Luria broth to remove residual Hg^{2+}, and suspended again in the original volume of fresh Luria broth. After addition of 5 μM organomercurial, 0.8-ml samples were removed to 35% HNO_3. The organomercurial content remaining was determined by the Hatch-Ott (6) procedure involving acid hydrolysis with concentrated HNO_3-H_2SO_4, oxidation by $KMnO_4$, destruction of excess permanganate with hydroxylamine, and, finally, reduction of the mercury to metallic Hg^0 by addition of $SnCl_2$. The Hg^0 was sparged into the mercury vapor cell of a Perkin-Elmer Corp. mercury kit attached to a Perkin-Elmer model 305 atomic absorption spectrometer.

Resistance to Hg^{2+} and organomercurials was determined both on petri dishes and in liquid cultures. Petri dish disk assays of resistance are described in the accompanying paper (28). For inhibition of growth of liquid cultures, Hg^{2+} or organomercurials were added to late-log-phase cultures in broth, and continued growth was monitored with the Klett colorimeter. Occasionally with merbromin, the ultimate growth yield was determined from turbidity in a Zeiss PMQII spectrophotometer after subtracting the contribution of the dye to the optical density.

RESULTS

Mercury and organomercurial resistance. Table 1 contains a summary of the mercury and organomercurial resistance and volatilization results we have obtained. In all cases, broth and petri dish resistance tests gave consistent results, although different amounts of mercuri-

TABLE 1. *Pseudomonas resistance and volatilization of Hg^{2+} and various organomercurial substrates*[a]

Strain	Hg^{2+}	PMA	Thimerosal	EMC	MMC	pHMB	Merbromin	FMA
P. aeruginosa[b]								
PAO	S NV	S NV	S NV	S NV	S NV	S NV	S NV	S NV
PAO(R3108)	R V	R V	R V	R V	R V	R NV	R NV	R NV
PAO(FP2)	R V	R V	R V	R V	R V	R NV	R NV	R NV
PAO(R38)	R V	R V	R	R V	R V	R	R	R
PAO(pVS2)	R V	R V	R	R V	R V	R	R	R
PAO(RP1)	S NV	S NV	S	S NV	S NV	S	S	S
PAO(pMG1)	R V	S NV	S NV	S NV	S NV	R NV	R NV	R
PAO(pMG2)	R V	S NV	S NV	S NV	S NV	R NV	R NV	R NV
PAO(R26)	R V	S NV	S	S NV	S NV	R	R	R
PAO(R933)	R V	S NV	S	S NV	S NV	R	R	R
PAO(RP1-74)	R V	S NV	S	S NV	S NV	R	R	R
PAO(R931-1)	R V	S NV	S	S NV	S NV	R	R	R
PAO(pVS1)	R V	S NV	S	S NV	S NV	R	R	R
P. putida	S NV	S NV	S V	NV	S NV	S	S NV	S
P. putida (MER)	R V	S NV	S NV	NV	S NV	R NV	R NV	R NV

[a] R, Resistance; S, sensitivity; V, volatilization; NV, no volatilization. PMA, Phenylmercuric acetate; EMC, ethylmercuric chloride; MMC, methylmercuric chloride; FMA, fluorescein mercuric acetate.

[b] *P. aeruginosa* plasmids were all tested in substrains of the standard PAO strain (17); plasmids R3108, pMG1, and pMG2 were in strain PU21 (8); plasmid FP2 was tested both in PU21 and in substrain 1670; plasmids RP1, R38, R933, and R931-1 were also in substrain 1670; plasmids RP1-74, R26, and pVS1 were in substrain 9501; and plasmid pVS2 was in substrain 9505.

als give the best distinctions between resistant and sensitive strains under different conditions of testing. With disk tests as described in Materials and Methods, 200 nmol of Hg^{2+}, 50 nmol of methylmercuric chloride, 50 nmol of ethylmercuric chloride, 20 to 50 nmol of phenylmercuric acetate, 200 nmol of thimerosal, 1,000 nmol of pHMB, 1,000 to 2,000 nmol of merbromin, and 500 to 1,000 nmol of fluorescein mercuric acetate allowed the classification for both *P. aeruginosa* and *P. putida* in Table 1. Although in most cases the distinction between resistance and sensitivity was very clear-cut, in the case of pHMB with *P. aeruginosa*, plasmid R3108 conferred very strong resistance, whereas plasmids pMG1, pMG2, and FP2 conferred intermediate resistance levels—clearly more resistant than the plasmidless PAO strain but less resistant than PAO(R3108). This may have something to do with the "semiconstitutive" functioning of plasmid R3108 as described below. In this paper, we are not showing the results of disk assays for organomercurial resistance, as we have published comparable data both for *Escherichia coli* (13) and *Staphylococcus aureus* (28) elsewhere. In tryptone broth growth experiments, 5 to 15 μM Hg^{2+}, 15 to 25 μM phenylmercuric acetate, 10 μM thimerosal or above, and 50 μM pHMB gave clear distinctions between sensitive and resistant strains. Figure 2 shows data for tryptone broth liquid cultures. One can note that merbromin is much less toxic than inorganic Hg^{2+} (although different strains were used in the two experiments in Fig. 2). Merbromin resistance is clear-cut, and as little as 10 μM merbromin was growth inhibitory for the plasmidless *P. aeruginosa*, whereas 400 μM merbromin was not inhibitory for the strain with plasmid R3108. Tonomura's *Pseudomonas* strain was somewhat more sensitive to merbromin than were the plasmid-containing *P. aeruginosa* or *P. putida* (Fig. 2A and added data). However, we were not using ideal growth conditions for this organism. The MER plasmid in *P. putida* confers strong clear-cut resistance to Hg^{2+}, as previously reported (2). With strain AC77 harboring the OCT-MER plasmid aggregate (2), we never observed resistance to phenylmercury acetate such as Chakrabarty and Friello (2) reported for another strain, AC28, containing three plasmids, OCT, K, and MER.

Mercury volatilization. Two hypotheses have been put forward for mechanisms of mercury and organomercurial resistance. One is a difference in binding or uptake of the mercurial between the resistant and sensitive strains (27). The other is that resistant bacteria have enzyme systems capable of degrading the organomercurials and converting Hg^{2+} to volatile Hg^0 (1, 4, 5, 14, 21). As described in detail in reference 12 and in our companion paper (28),

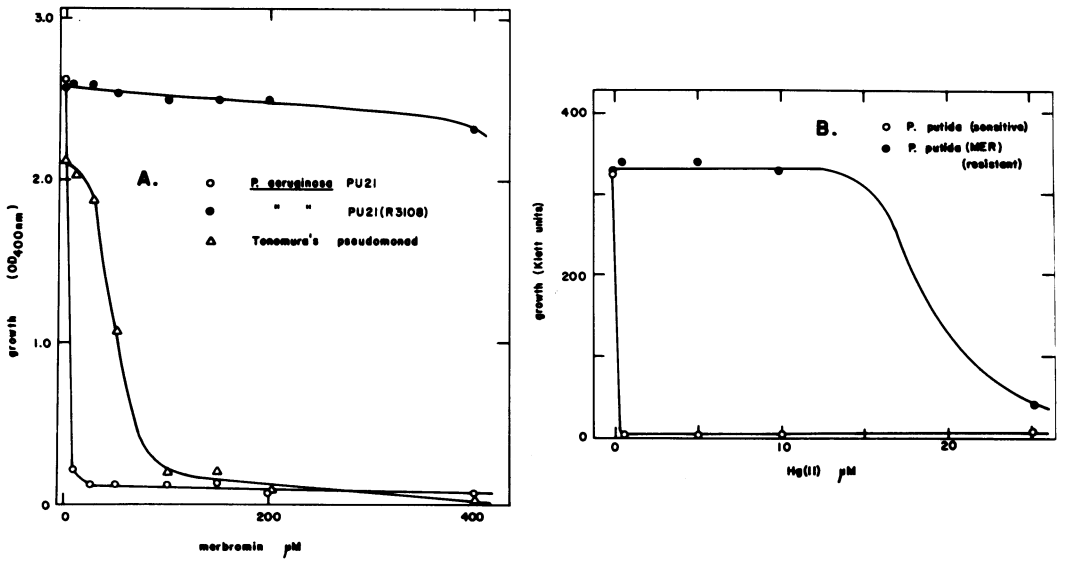


FIG. 2. *Growth inhibition by merbromin and by Hg^{2+}. (A) P. aeruginosa strains PU21 and PU21(R3108) and Pseudomonas strain K62 were inoculated from overnight cultures into tryptone broth containing merbromin and grown with aeration by shaking for 8 h at 37°C. Growth was measured by turbidity at 400 nm in a Zeiss spectrophotometer after subtracting the absorbance due to merbromin. (B) P. putida strains AC10 and AC77(MER) were grown for 11 h with aeration at 37°C in tryptone broth with added $HgCl_2$. Turbidity was determined in a Klett colorimeter with a no. 54 green filter.*

each of the nearly 100 plasmid-determined systems for Hg^{2+} resistance we have studied involved volatilization of mercury from the medium. Figure 3 shows the properties of mercury volatilization with the MER plasmid in *P. putida*. When 5 μM $^{203}Hg^{2+}$ was added to a culture of the mercury-sensitive strain AC10, more than 80% of the mercury remained for 5 h. The 20% loss was probably non-biological and similar to that seen in the absence of bacteria in reference 21. More than 80% of the ^{203}Hg present became bound to the sensitive cells (Fig. 3A). With cells harboring plasmid MER, there was a rapid loss of more than 90% of the ^{203}Hg from the culture. The cells bound most of the low level of mercury that remained. An experiment similar to that in Fig. 3 but with *P. aeruginosa* strains PU21 and PU21(R3108), and with phenylmercury instead of inorganic Hg^{2+}, was shown in a previous report (13).

To examine the volatilization of mercury from radioactive mercurials in greater detail, we harvested mercury-exposed cells from broth and incubated them for short periods in an assay buffer devised for subcellular enzymatic studies (12). Figure 4 shows some representative data with *P. putida* and *P. aeruginosa*. Reference 28 contains similar data for *S. aureus*. With each and every plasmid-bearing strain we have studied, the mercury volatilization system is inducible by exposure to Hg^{2+} or organomercurials. Figure 4A shows that exposure to 10 μM Hg^{2+} caused a more than 10-fold increase in rate of ^{203}Hg volatilization, whereas the addition of 10 μM phenylmercury or pHMB did not. We will return to the question of the range of inducers below. *P. putida* (MER), whether induced for mercury volatilization or not, could not cause the volatilization of ^{203}Hg from phenylmercury, ethylmercury, or methylmercury (Table 1; data not shown) or the volatilization of [^{14}C]methane from $^{14}CH_3Hg^+$ (Fig. 4B). Plasmid RP1, which was the only plasmid we studied that does not confer resistance to inorganic mercury, also does not confer the ability to volatilize mercury from $^{203}Hg^{2+}$ (Table 1). Seven plasmids in *P. aeruginosa* are listed in Table 1 that are like the MER plasmid in *P. putida* in that they confer resistance to and the volatilization of mercury from Hg^{2+} but neither resistance to nor volatilization of mercury from the organomercurials phenylmercury, methylmercury, and ethylmercury. We refer to these as "narrow spectrum" with regard to their resistance patterns to organomercurials. They do confer resistance to three organomercurials in Fig. 1: pHMB, merbromin, and fluorescein mercuric acetate. However, we

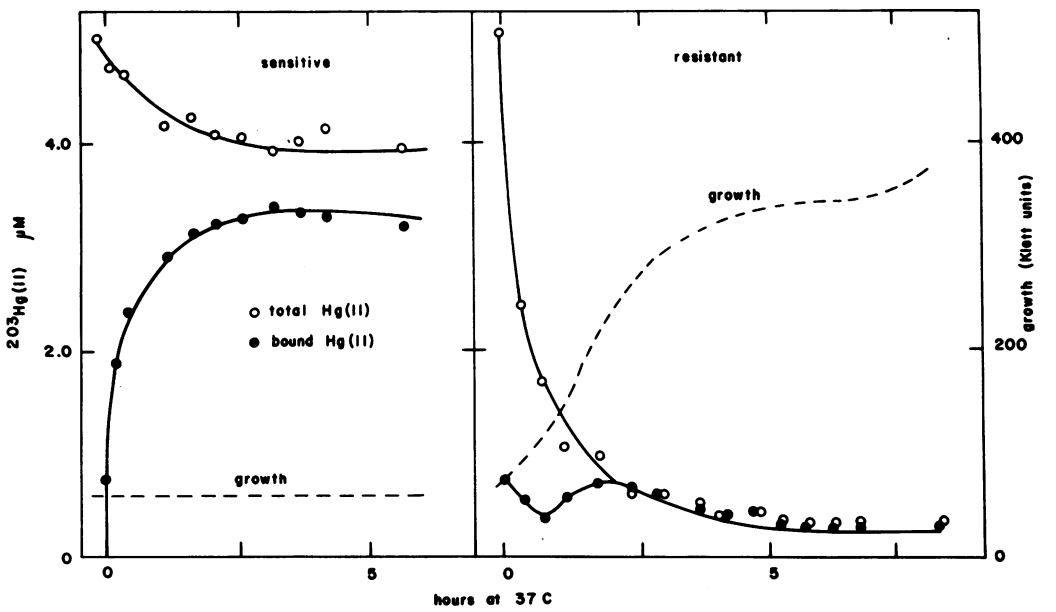


FIG. 3. *Binding and volatilization of mercury by P. putida. Strains AC10 and AC77 were grown at 37°C in tryptone broth to a turbidity of 60 Klett units.* $^{203}HgCl_2$ *(5 μM) was added, and* ^{203}Hg *remaining in the culture was followed by periodic removal of samples into scintillation fluid.* ^{203}Hg *bound to cells was determined by membrane filtration (Millipore Corp.) of 0.1-ml samples, followed by washing with broth and counting of the filters in scintillation fluid. Growth was measured as turbidity with a Klett colorimeter (dotted lines).*

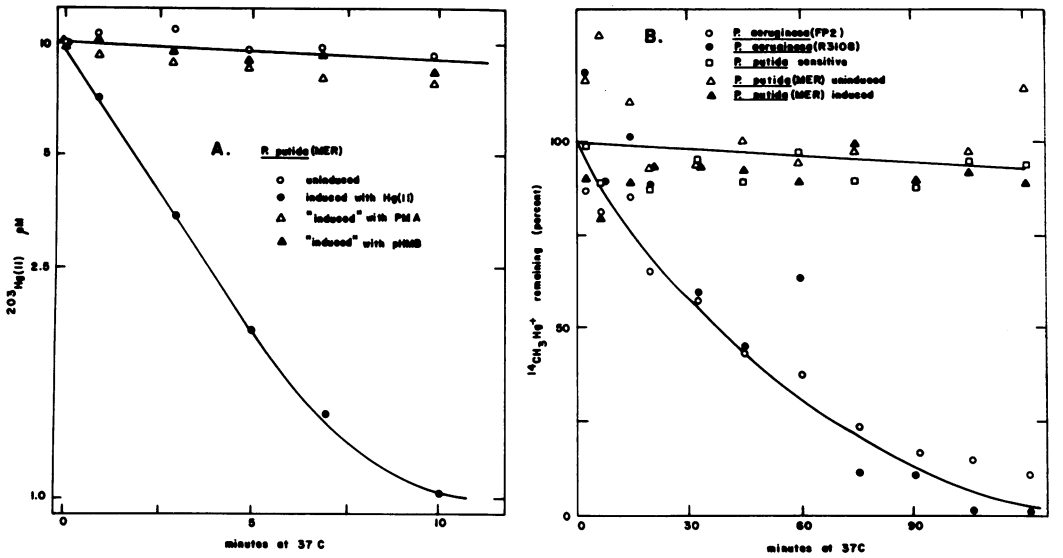


FIG. 4. *Volatilization of ^{203}Hg from mercuric chloride and of ^{14}C from [^{14}C]methylmercury chloride. Cells were grown in Luria broth at either 32°C (A) or 37°C (B) and induced by addition of 10 μM Hg^{2+}, 10 μM phenylmercuric acetate (PMA), or 10 μM pHMB (A) or 10 μM Hg^{2+} (B), followed by an additional 75 min of incubation. Cells, harvested by centrifugation and concentrated in buffer, were diluted to an equivalent of 125 Klett units of turbidity in buffer containing 10 μM $^{203}Hg^{2+}$ (A) or 0.57 μM $^{14}CH_3Hg^+$ (B). Samples were periodically removed to scintillation fluid.*

have not detected volatilization of mercury from these compounds with plasmid-bearing *Pseudomonas* strains. The narrow-spectrum plasmids include the hybrid plasmid RP1-74 that contains the mercury-resistance translocation segment from plasmid pSV1 incorporated into plasmid RP1 (18). *P. aeruginosa* strains PAO(R3108), PAO(FP2), PAO(R38), and PAO-(pVS2), however, showed a "broad-spectrum" ability to volatilize mercury from organomercurials. In Fig. 4B, the volatilization of $^{14}CH_4$ with strains PAO(FP2) and PAO(R3108) is shown. Similar experiments demonstrated the volatilization of ^{203}Hg from ^{203}Hg-labeled phenylmercury, methylmercury, and ethylmercury (Table 1; data not shown). Volatilization of mercury from phenylmercury was also demonstrated by direct volatilization into the atomic absorption spectrometer. Preliminary results of this nature were presented in two symposium papers (13, 14). of the four organomercurials we have tested, but for which we lack radioactive compounds, only thimerosal was quantitatively lost from cultures of *P. aeruginosa* strains PAO(FP2), PAO(R3108) (Fig. 5A), PAO(R38), and PAO(pVS2) (Table 1; data not shown). Plasmidless sublines of *P. aeruginosa* PAO (see Materials and Methods) and *P. putida* and strains with any of the seven narrow-spectrum plasmids did not cause volatilization of mercury from thimerosal (Fig. 5A; Table 1; data not shown). Table 2 contains quantitative data comparing volatilization rates with Hg^{2+}-induced cells under standard conditions. The rate of volatilization of mercury from $^{203}Hg^{2+}$ was invariably greater than was the rate of volatilization of mercury from organomercurials. Under comparable conditions, the rate of mercury volatilization from phenylmercury or ethylmercury was greater than that from methylmercury. The same order of relative enzyme activities was found with subcellular enzyme preparations from a broad-spectrum, plasmid-containing *E. coli* (J. L. Schottel, submitted for publication).

None of the 11 mercury-resistant plasmid-bearing *P. aeruginosa* strains we have studied volatilized mercury from merbromin, pHMB, or fluorescein mercuric acetate (Fig. 5B, C, and D; additional data not shown), although all of these strains were resistant to all three of these organomercurials (Fig. 2; Table 1).

There was a problem with our preliminary reports (13, 14), where we presented data showing volatilization of mercury from merbromin and pHMB into the atomic absorption spectrometer. Because of the extraordinary sensitivity of the atomic absorption spectrometer, we were measuring "full scale" the volatilization of a few percent of the total organomercurial added. This low-level volatilization could be accounted for by contamination of the organo-

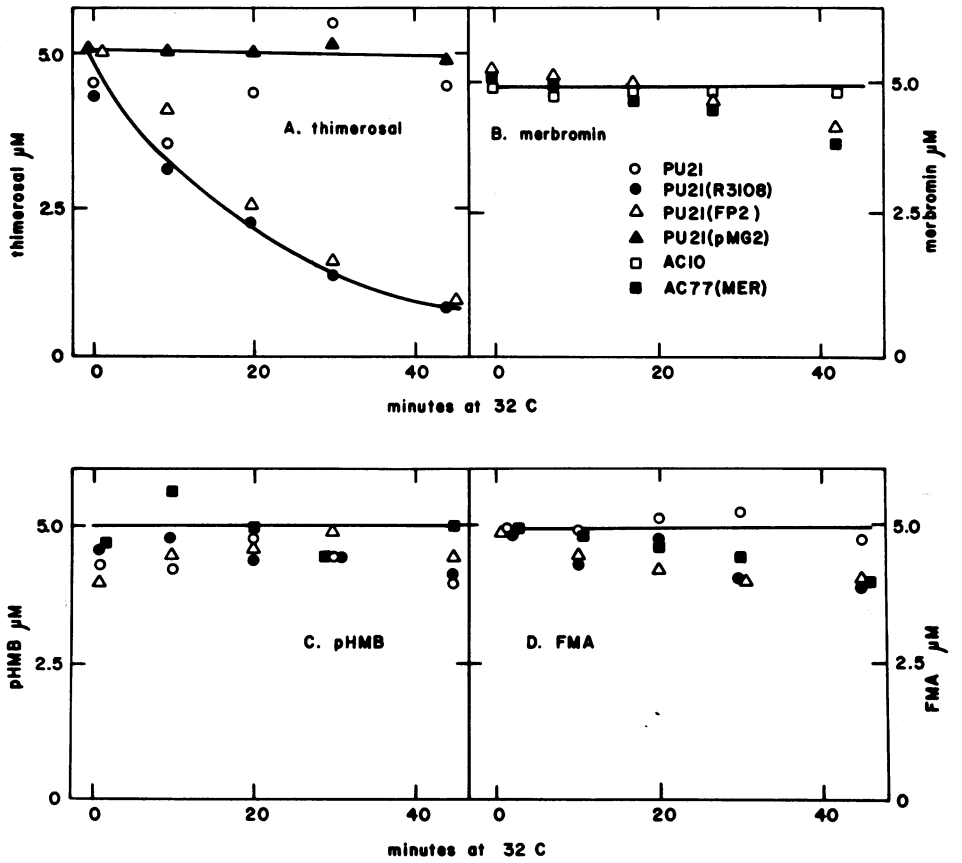


FIG. 5. *Organomercurials determined by atomic absorption spectroscopy. P. aeruginosa and P. putida were grown at 32°C in Luria broth to a Klett turbidity of 75 and induced by addition of 10 μM Hg^{2+} and an additional 75 min of exposure. After harvesting the cells by centrifugation, they were washed once with Luria broth to remove remaining inducer and suspended in fresh broth. Organomercurials were added at 5 μM, and during incubation with gentle aeration at 32°C, 0.8-ml samples were periodically removed and added to 5 ml of 35% HNO_3. Organomercurial content was determined by the Hatch-Ott (6) procedure, using atomic absorption spectroscopy. FMA, Fluorescein mercuric acetate.*

TABLE 2. *Mercury and organomercurial volatilization rates*[a]

Strain	Rate of volatilization (nmol/min per mg [dry wt] of cells)				
	Hg^{2+}	Phenylmercury	Methylmercury	Ethylmercury	Thimerosal
P. aeruginosa					
PU21	≤0.07	≤0.01	≤0.002	≤0.001	≤0.1
PU21(FP2)	1.9	0.49	0.09	0.32	0.475
PU21(R3108)	1.9	0.70	0.25	0.59	0.475
PU21(pMG1)	1.7	≤0.02	≤0.01	≤0.006	
PU21(pMG2)	1.5	≤0.01	≤0.01	≤0.006	≤0.1
P. putida					
AC10	≤0.1	≤0.01	≤0.004	≤0.005	
AC77(MER)	1.5	≤0.01	≤0.004	≤0.005	≤0.1

[a] Standardized buffer assay conditions with 1 μM $^{203}Hg^{2+}$, ^{203}Hg-phenylmercuric acetate, ^{203}Hg-methylmercuric chloride, or ^{203}Hg-ethylmercuric chloride, or 5 μM thimerosal (nonradioactive).

mercurials with a few percent of inorganic mercury, and this explanation was supported by Hatch-Ott (6) analysis showing a few percent mercury that could be reduced by $SnCl_2$ in the absence of acid hydrolysis (unpublished data). Another source of mercury volatilization in these experiments, even with bacteria with no ability to degrade the organomercurials, is the slow chemical hydrolysis of mercury from the organomercurial followed by the bacterial reduction of released Hg^{2+} to volatile Hg^0. We believe that this process is responsible for the gradual loss of mercury from cultures containing merbromin and fluorescein mercuric acetate in Fig. 5B and D. Loss of mercury from merbromin has been more pronounced in additional experiments with tryptone broth cultures (data not shown). Finally, there was the least suggestion of phenylmercury volatilization activity with *P. putida* strain AC77(MER) in some experiments. However, the maximum rates of volatilization were no more than 1 to 2% of those with strains PAO(FP2) and PAO-(R3108). Nevertheless, the distinction between degraded organomercurials (Fig. 4B and 5A) and undegraded organomercurials (Fig. 5B, C, and D) is clear-cut (summarized in Table 1).

Inducibility of volatilization activity. Volatilization of mercury from Hg^{2+} and organomercurials was inducible by addition of Hg^{2+} or organomercurials to strains with the mercury resistance plasmids. Bacteria not previously exposed to mercury or mercurials had low volatilization rates which increased upon induction (Fig. 4). Organomercurials as well as Hg^{2+} were inducers for the mercury volatilization systems (Fig. 6). Phenylmercury (Fig. 6A and B) and thimerosal (Fig. 6B) were poor but significant inducers of mercury volatilization activity with strains with plasmids for which phenylmercury and thimerosal were not substrates (12, 20; Table 1). Phenylmercury and thimerosal were more effective inducers (rates more comparable to those achieved with Hg^{2+} induction) with the broad-spectrum organomercurial resistance plasmids R3108 and FP2 (Fig. 7) than with narrow-spectrum plasmids (Fig. 6). Merbromin and pHMB, which were not degraded by any of the plasmid-bearing strains (Fig. 5; Table 1), were highly effective inducers (Fig. 6A and 7; additional data). Merbromin and pHMB were effective inducers with *P. putida* (MER) as well as with the strains of *P. aeruginosa* (data not shown). Although all the plasmid-determined mercury volatilization systems increased in activity upon induction, a low-level, "micro-constitutive" activity was found with some plasmid-bearing strains. This activity ranged from below detection limits with comparable systems in *E. coli* to a few percent of maximum activities with *P. putida* (MER) (Fig. 4A and 6B) and *P. aeruginosa*

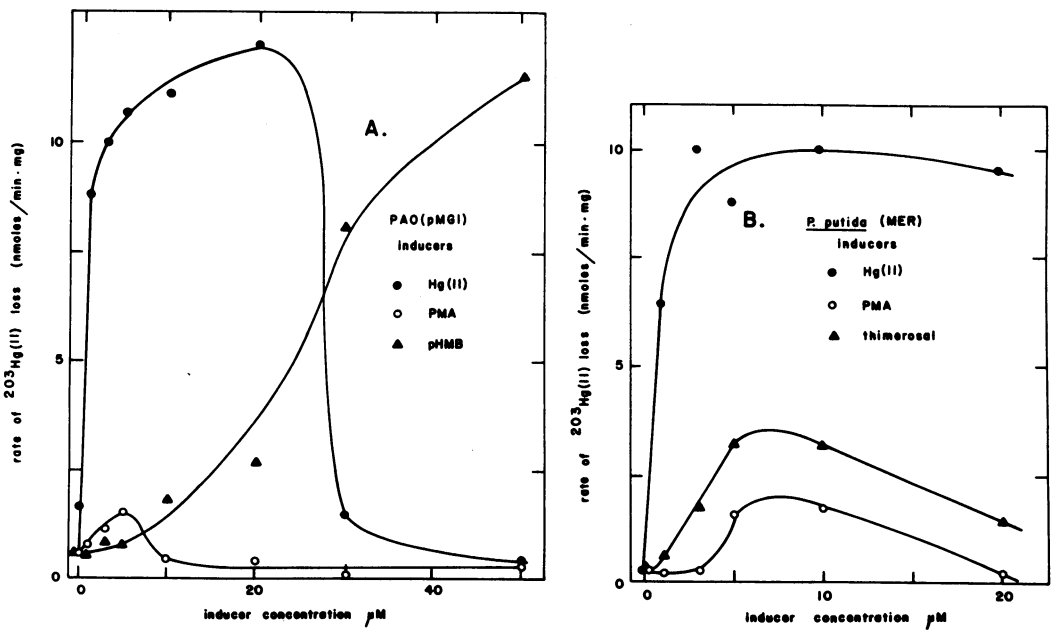


FIG. 6. *Induction of Hg^{2+} volatilization activity. P. aeruginosa PU21(pMG1) and P. putida AC77 were grown at 32°C in Luria broth, "induced," harvested, and assayed as in Fig. 4A. PMA, Phenylmercuric acetate.*

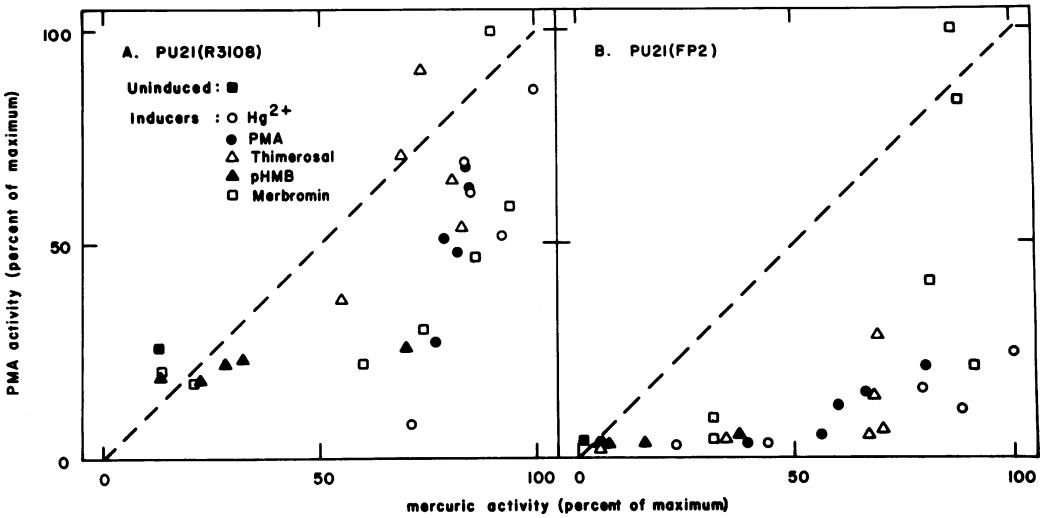


FIG. 7. *Coordinate induction of mercury volatilization activities from Hg^{2+} and phenylmercury. Each point represents the percentage of the maximum activity rate of volatilization of mercury from 8 μM $^{203}Hg^{2+}$ or 0.7 μM ^{203}Hg-phenylmercuric acetate (PMA). These maximum activities were: PU21(FP2), 11.3 nmol/min per mg (dry weight) with $^{203}Hg^{2+}$ and 0.212 nmol/min per mg with ^{203}Hg-PMA; PU21(R3108), 10.2 nmol/min per mg with Hg^{2+} and 0.053 nmol/min per mg with PMA.*

PU21(FP2) (Fig. 7B) up to a high of 15 to 20% of maximum activity with strain PU21(R3108) (Fig. 7A). This high semiconstitutive activity with PU21(R3108) has been a reproducible result over several years.

With plasmids in *E. coli* (Schottel, submitted for publication), *S. aureus* (28), and *Pseudomonas* strain K-62 (24), it is now known that separate enzymes are involved first in the hydrolysis of organomercurials such as phenylmercury to benzene plus Hg^{2+}, followed by the reduction of Hg^{2+} to volatile Hg^0. We have not carried out subcellular enzyme tests with the plasmid-containing *Pseudomonas* strains, since the same system is being thoroughly studied with other organisms. However, since volatilization of mercury from Hg^{2+} appears separable from the activity with organomercurials, one can ask whether the induction of these two activities is "coordinate" or not. Coordinate induction has been considered indicative of genes under common regulatory control. Figure 7 shows the induction of activity toward Hg^{2+} and phenylmercury with strains PU21(FP2) and PU21(R3108). Although both activities were inducible and although all five inducers tested induced both activities, the induction patterns did not appear to be strictly coordinate with either strain. (Coordinate induction would lead to all points in Fig. 7 lying on the diagonal lines in the figure.) In particular, pHMB appeared to preferentially induce mercury volatilization from Hg^{2+}, whereas thimerosal and merbromin under some conditions preferentially induced the degradation of phenylmercury (i.e., points above the diagonal lines). These results should be compared with the *S. aureus* experiments summarized in Fig. 6 of the accompanying paper (28), where pHMB also preferentially induced the activity toward Hg^{2+}, especially with a mutant plasmid, and where merbromin preferentially induced activity toward phenylmercury. The result that most points in experiments such as shown in Fig. 7 lie significantly below the diagonal coordinate line indicates some regulation beyond that of a simple operon. The scatter of such experimental data has discouraged us from testing specific, more complex hypotheses. The scatter of data in Fig. 7 is not due to poorly defined rates of volatilization, since as seen in Fig. 4 and in comparable data in reference 28, the precision of determining a rate is quite high. This lack of quantitative reproducibility between different induction experiments has also prevented us from determining whether there are differences in inducer patterns between the broad-spectrum plasmids FP2 and R3108.

DISCUSSION

The basic properties of mercury and organomercurial resistance associated with volatilization of mercury from mercury and mercurials in *P. aeruginosa* and *P. putida* are basically similar to those systems in *E. coli* (12–15, 20, 21) and *S. aureus* (28). We will not repeat here the pertinent discussion in the next paper (28),

but rather will consider the differences among the systems in *S. aureus*, *E. coli*, and *Pseudomonas*.

Until very recently we thought that mercury resistance R factors fell into the two classes that we had found in a survey of plasmids in *E. coli* (12): narrow-spectrum plasmids that determined resistance to and volatilization of mercury from Hg^{2+} and broad-spectrum plasmids that determined resistance to and volatilization of mercury from both Hg^{2+} and a range of organomercurials. These two classes are readily apparent from the results summarized in Table 1. We have seven independent examples of narrow-spectrum plasmids in Table 1. These plasmids also confer resistance to the three organomercurials pHMB, merbromin, and fluorescein mercuric acetate, from which we have never observed mercury volatilization with *Pseudomonas* or *S. aureus* (28) strains. An additional genetic determinant(s) must be involved in these resistances, although we have never observed resistances to these three organomercurials separate from mercury resistance. pHMB presents a problem for us, since it is degraded by Tonomura's pseudomonad (24). Furthermore, some *E. coli* plasmids confer the ability to volatilize mercury from pHMB (A. A. Weiss, J. L. Schottel, D. L. Clark, R. G. Beller, and S. Silver, *In* D. Schlessinger, ed., *Microbiology—1978*, in press). It is unclear from looking at the structures in Fig. 1 as to why phenylmercury should be a substrate for degradation, whereas the *p*-carboxyl group in pHMB would protect this compound from degradation. It appears that the hydrolytic enzyme(s) of some organisms has a different and broader substrate range than that of others. The broad-spectrum organomercurial resistance plasmids of *P. aeruginosa*, of which class there are four representatives in Table 1, confer additional resistances to the organomercurials phenylmercury, ethylmercury, methylmercury, and thimerosal as well as the ability to volatilize mercury from these compounds. One might note from the structures in Fig. 1 that thimerosal is ethylmercurithiosalicylate, and the ethylmercury portion of this molecule may be accessible for enzymatic hydrolysis. Among the broad-spectrum organomercurial resistance plasmids, those in *E. coli* have the broadest spectrum of organomercurials that are degraded, including pHMB. The broad-spectrum organomercurial resistance plasmids in *P. aeruginosa* fall in the middle, and the comparable plasmids in *S. aureus* have the narrowest spectrum of degraded organomercurials of the three species. With *S. aureus* plasmids (28), phenylmercury and thimerosal were degraded and mercury was volatilized from these compounds. However, with the *S. aureus* plasmid-bearing strains, the rate of ethylmercury degradation was barely detectable, and methylmercury degradation was not detectable (28). There may be more than one organomercurial hydrolase enzyme required to explain these differences. With the soil pseudomonad, Tezuka and Tonomura (24) found two peaks of hydrolytic enzyme activity, apparently with somewhat different substrate ranges (personal communication). Schottel (submitted for publication) has kinetic evidence for two phenylmercury hydrolytic activities with a plasmid-bearing *E. coli* strain. And finally, other factors such as barriers for access for the organomercurials to the hydrolytic enzymes may govern the patterns of resistance and volatilization. We summarized our current understanding of the mercury and organomercurial resistances determined by plasmids in *Pseudomonas*, *S. aureus*, and *E. coli* in greater detail at the recent ASM Conference on Extrachromosomal Elements (A. A. Weiss et al., *In Microbiology—1978*, in press).

We have not as yet determined what is the mechanism of resistance to the three nonhydrolyzed organomercurials in *Pseudomonas*. A permeability barrier to the organomercurials is one possibility. It is clear that even with plasmidless, sensitive strains, much higher concentrations of these three organomercurials were required for inhibition of growth than was the case with the four hydrolyzed organomercurials in Fig. 1 (Fig. 2; additional data).

P. putida harboring the OCT-K-MER plasmid aggregate was patented by the General Electric Company (A. M. Chakrabarty, D. A. Friello, and R. Mylroie, U.S. patent 3,923,597, 2 December 1975) at a time when this strain appeared to bind more mercury than did the comparable plasmidless strain. However, the more thorough analysis in the current work shows that the MER plasmid confers resistance due to volatilization just as do all the other plasmids we have studied. *Pseudomonas* K-62 also was initially thought to be resistant due to a binding difference between it and sensitive strains (27). With inducible enzyme systems with toxic inducers and toxic substrates and with the propensity of mercury and mercurials to bind to cell surface thiol groups, it is not difficult to find conditions of differing growth that provide more cell mass with the resistant strain and, therefore, the erroneous suggestion that the resistance was due to binding differences.

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