

# Biodegradation of Phenylmercuric Acetate by Mercury-Resistant Bacteria

J. D. NELSON, W. BLAIR, F. E. BRINCKMAN, R. R. COLWELL, AND W. P. IVERSON

Department of Microbiology, University of Maryland, College Park, Maryland 20742, and Inorganic Chemistry Section and Corrosion Section of the National Bureau of Standards, Washington, D. C. 20234

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Selected cultures of mercury-resistant bacteria degrade the fungicide-slimicide phenylmercuric acetate. By means of a closed system incorporating a flameless atomic absorption spectrophotometer and a vapor phase chromatograph, it was demonstrated that elemental mercury vapor and benzene were products of phenylmercuric acetate degradation.

Mercury and its compounds (organic and inorganic) are known to undergo a number of chemical and biological transformations. Microorganisms, in particular the bacteria, have been documented as agents of transformation. Under aerobic conditions, bacteria convert phenylmercuric acetate (PMA) to gaseous mercury,  $Hg^0$  (J. D. Nelson, Jr. and R. R. Colwell, manuscript in press; references 17, 18) and to diphenylmercury (11), and degrade methyl- and ethylmercury to  $Hg^0$ , with the concomitant formation of methane and ethane, respectively (3, 15, 18). Similarly,  $Hg^{2+}$  ion is reduced to  $Hg^0$  by species of *Pseudomonas*, enteric bacteria, and *Staphylococcus aureus* (7, 10, 14, 16). Methyl- and dimethylmercury are formed from  $Hg^{2+}$  anaerobically by aquarium sediments, *Clostridium cochlearium*, and extracts of methanogenic bacteria (6, 15, 17, 19) and aerobically by *Neurospora crassa* (8).

A number of PMA resistant bacteria were isolated from water and sediment samples as part of an ecological study of the cycling of mercury in Chesapeake Bay (12). The metabolism of PMA by selected strains of bacteria was investigated to achieve an understanding of bacterial mercury resistance, as well as to assess the role of bacteria in the transformations of mercury in the natural environment.

## MATERIALS AND METHODS

**Isolation, cultivation, and identification of mercury-resistant bacteria.** Cultures of mercury tolerant bacteria were isolated by spreading suitable dilutions of water and sediment onto a solid selective medium containing PMA and  $HgCl_2$  (12). The basal medium, which was designed to reduce the precipitation of mercury by sulfur-containing compounds, consisted of 2.0 g of glucose, 5.0 g of Casamino Acids

(Difco), and 1.0 g of yeast extract (Difco) per liter of artificial estuarine salts solution (ES) containing 10.0 g of  $NaCl$ , 2.3 g of  $MgCl_2 \cdot 6H_2O$ , and 0.3 g of  $KCl$ . The broth was adjusted to pH 7.3 and autoclaved at 121 C for 15 min. A solid medium was prepared by adding 20.0 g of agar (Difco) per liter of broth. Freshly prepared ethanolic and aqueous solutions of PMA and  $HgCl_2$ , respectively, were diluted 1,000-fold into sterile basal broth or sterile, melted agar. The pure cultures which were isolated during the study were identified as to genus (1, 13) and maintained on basal agar slants.

**Radioisotope experiments.** Bacterial cultures were grown in 2.0 ml of broth containing 0.3 ppm of PMA at 20 C with agitation in a temperature-controlled gyrorotary shaker to approximately equivalent turbidities (24-48 h) and washed once in 0.01 M potassium phosphate-buffered ES (PES; pH 7.0). The pellets were resuspended in 0.9 ml of PES in screw capped tubes and 100  $\mu$ liters of  $^{203}Hg$ -labeled PMA ( $5.75 \times 10^6$  counts per min per  $\mu$ g) in 95% ethanol was added, giving a final concentration of 0.4 ppm of PMA. The tubes were incubated at 25 C, with agitation. To access the loss of  $Hg$ , 100- $\mu$ liter samples were withdrawn and added directly to 10 ml of liquid scintillation cocktail consisting of 8.23% naphthalene; 1.02% 2,5-diphenyloxazole; 0.051% [1,4-bis[2-(5-phenyloxazole)]-benzene] in a solution of toluene; 1,4 dioxane; and ethylene glycol monoethyl ether (1:3:3). Sample radioactivity was measured in a liquid scintillation spectrometer, and efficiency of counting was determined from a channels ratio versus quench curve. Corrections for isotopic decay were made for samples held for prolonged incubation.

**Bioreactor experiments.** For the vapor phase analysis of PMA metabolizing cultures, six plates of basal medium containing 0.3 ppm of PMA were streaked from cultures on 0.3-ppm PMA slants and were immediately placed in a hermetically sealed glass bioreactor (volume, approximately 4 liters) (Fig. 1). A blank consisting of six uninoculated plates was also run. At intervals, the valve to the reactor was opened, and the atmosphere was flushed at the rate of

4 liters/min through the BOD bottle,  $V_2$ , and the volume  $V_D$  (atomic absorption [AA] cell and associated pump and flowmeter) for 1 min. The output from the dual-beam AA spectrophotometer was recorded, and the peak height was measured with respect to dry air. This reading constituted the mercury vapor ( $Hg^0$ ) output of the cultures. Analysis for total mercury ( $Hg_{TOTAL}$ ), e.g.,  $Hg^0$  plus any other volatile mercurial metabolites, immediately followed by closing the valve to the bioreactor, whereupon the Hg in  $V_2$  plus  $V_D$  was absorbed by pumping the residual atmosphere for 5 min through a fritted glass sparger in a second BOD bottle ( $V_1$ ) containing 100 ml of the oxidizing solution (5 ml of 5.0%  $K_2S_2O_8$ , 5 ml of 35.0%  $HNO_3$ , 5 ml of 5.0%  $H_2SO_4$ , and 0.25 ml of 5.0%  $KMnO_4/100$  ml of solution) used in the Hatch-Ott procedure (5). The frit was removed, and 5 ml of 1.5%  $NH_2OH \cdot HCl$  and 5 ml of 10.0%  $SnCl_2$  were added. Immediately after the addition of the  $SnCl_2$ , the frit was reintroduced, and  $Hg^0$  formed was pumped through volumes  $V_2$ ,  $V_D$ , and  $V_1$  (volume above the absorbing solution) at 4 liters/min. The peak height was again recorded. Values representing either  $Hg^0$  or  $Hg_{TOTAL}$  concentrations were obtained from calibration curves constructed using least-squares straight line fits, based on computer analyzed data (standard amounts: 0, 10, 20, 50, 100, 200, and 500 ng of  $Hg^{2+}$ ). These concentrations were converted to total quantities of Hg present in the reactor (legend, Fig. 1). The bioreactor and contents (including controls) were incubated at ambient tem-

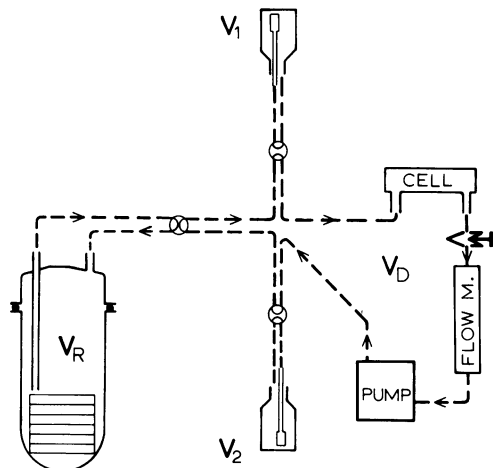


Fig. 1. Flow diagram of atmosphere from bioreactor for AA analysis of mercury: volume  $V_R = 4,084$  ml,  $V_D = 204$  ml,  $V_1 = 190$  ml, and  $V_2 = 290$  ml. In analysis for  $Hg^0$  the initial mercury concentration ( $[Hg^0]$ ) in the reactor =  $[Hg'](V_R + V_D + V_2)/V_R = [Hg'](1.12)$  where  $[Hg']$  is concentration measured in the AA cell during flushing. The total amount of  $Hg^0$  contained in the reactor at the time =  $[Hg'](1.12)(4,084)$  ng. In analysis for total mercury  $Hg_{TOTAL}$  the corresponding total Hg concentration in the reactor =  $[Hg''](V_D + V_1 + V_2)(V_D + V_2 + V_R)/(V_D + V_2)V_R = [Hg''](1.55)$  where  $[Hg'']$  is the concentration of total Hg determined after regenera-

perature ( $20 \pm 2$  C) and continuously exposed to the same level of laboratory fluorescent lighting. Preliminary observations indicated that light increased  $Hg^0$  production slightly from the inoculated plates, but not from the uninoculated plates. A temperature increase of 10 C had less effect upon the production of  $Hg^0$  than light.

Interference by other gaseous metabolites in AA detection of Hg was evaluated for benzene,  $CO_2$ , and ammonia by directly injecting known quantities into a bioreactor through a septum. In results to be reported elsewhere, studies have been conducted on Chesapeake Bay isolates, as well as anaerobic bacteria from other sources, with regard to characterization of reactive metabolites. Here, volatile metabolites were slowly purged with dry air from a bioreactor containing isolate 244 into cold traps on a glass Hg-free, greaseless high vacuum system. Following trap-to-trap fractionation of the condensed fractions, these were identified by infrared and mass spectrometry; principal products were Hg,  $CO_2$ , and  $NH_3$ . In other cases, ethanol, acetone, and organometallic products have been observed.

From a consideration of the amount of non-mercury metabolites added to produce the same AA detector response, a relative sensitivity scale was established for the bioreactor system. Thus, to achieve the same peak height from the AA spectrophotometer, the following relative amounts of absorbant were required in the bioreactor:  $Hg^0:C_6H_6:NH_3 \approx 1:7,990:5,840$  (weight basis) or  $Hg^0:C_6H_6:NH_3 \approx 1:20,540:68,800$  (molar basis), employing the  $Hg^0$  procedure. The AA detector gave no response to a pure atmosphere of  $CO_2$ . After absorption and regeneration in the  $Hg_{TOTAL}$  procedure, only slight elevation occurred in detector response for reagent blanks ( $\sim 10\%$ ) for  $NH_3$  over a 10-fold concentration range, whereas for benzene a substantial (76%) elevation occurred over a fivefold concentration range. These results are to be compared with typical standard error of  $\pm 10$  to 14% for reagent blanks calculated from fitted calibration curves. For more nearly comparable amounts of benzene relative to those observed for Hg (vide infra), little or no elevation for the reagent blanks was observed.

The atmosphere above several of the isolates was examined by vapor phase chromatography at a point (96-99 h) in the course of the growth period just prior to an Hg determination. A research-grade dual-column instrument fitted with a flow splitter for simultaneous thermocouple and flame ionization detection (FID) was employed. Through a small bore (18 gauge) Teflon tube inserted directly into the bioreactor to the quiescent atmosphere just over the agar plates, a batch sample of 2.0 ml was withdrawn into a gas syringe immediately before a regular  $Hg^0$  and  $Hg_{TOTAL}$  analysis run. These samples were injected into the chromatograph operating under the following conditions (3): isothermal at 50 C,  $N_2$  carrier flow at 30 ml/min, 10% Apiezon L on 80/100 Supelcoport in

tion from the absorbing solution in volume ( $V_1 + V_2 + V_D$ ). The initial amount of  $Hg_{TOTAL}$  in the reactor =  $[Hg''](1.55)(4084)$  ng.

0.225 in by 6 ft (approximately 0.57 cm by 1.828 m) stainless steel columns. Bioreactor samples were calibrated against 2.0-ml benzene-air mixtures from which cross-integration of thermocouple and FID peak areas yielded a limit of detection (FID) estimated to be 0.01 ppm (volume) for benzene in air (retention time =  $146 \pm 4$  s for all runs).

## RESULTS

**Degradation of labeled PMA.** Of the nearly 900 cultures of mercury-resistant, estuarine bacteria which have been isolated in the course of the microbial ecology study, the majority (approximately 60%) were identified as *Pseudomonas* species (12). A group of nine representative cultures was selected and screened for the ability to degrade PMA. The cultures were grown in a medium containing 0.3 ppm of PMA, a concentration of Hg approximating that found in the Chesapeake Bay sediments sampled (12). Washed, resting-cell suspensions in PES were prepared, and  $^{203}\text{Hg}$ -labeled PMA (final concentration, 0.4 ppm PMA) was added at time zero. Samples were then withdrawn for counting (Table 1). The levels of mercury in the cultures 244, 127, 72, 132, and 119 decreased significantly during incubation. The losses could not be explained by adsorption onto the vessel walls (unpublished results) or by sampling errors. Furthermore, it has been shown that in the case of strain 244, previous exposure to PMA was necessary for the release of Hg (J. D. Nelson, Jr., and R. R. Colwell, in press).

**Production of  $\text{Hg}^0$  from PMA.** Having demonstrated the loss of Hg from PMA-containing bacterial suspensions, it was necessary to speculate the Hg in the vapor phase. Vapor phase analysis of cultures was done by placing freshly streaked agar plate cultures in a closed system equipped with an on-line AA detector (Fig. 1). The flameless AA detector is insensitive to volatile mercury compounds such as methylmercury chloride or dimethylmercury, hence analysis for presence of these materials as gaseous metabolites at low levels depended on an intermediate concentration-reduction step to generate corresponding elemental Hg (2, 4, 9). A wide range of  $\text{Hg}^0$  and total Hg production from PMA (Fig. 2, 3) was observed. Some of the cultures (strains 119, 244, and 72) produced an initial pulse of  $\text{Hg}^0$  and total Hg, which may have been due to breakdown of PMA bound to the inoculum. It is evident from the multiple peaks produced by isolates 119 and 244 that the atmosphere sampling procedure substantially dilutes the quiescent atmosphere above the culture plates. Both a reduction in the sample

TABLE 1. *Metabolism of phenylmercuric acetate by mercury-resistant bacteria*

Isolate no.	Generic identification	Percent radioactivity remaining <sup>a</sup>	
		1 h <sup>b</sup>	4 days <sup>c</sup>
244 <sup>d,e</sup>	<i>Pseudomonas</i> sp.	52.0	46.3
187 <sup>e</sup>	<i>Pseudomonas</i> sp.	94.8	97.8
94 <sup>e</sup>	<i>Pseudomonas</i> sp.	88.3	82.8
127 <sup>e</sup>	<i>Pseudomonas</i> sp.	82.6	67.2
72	<i>Arthrobacter</i> sp.	94.8	70.9
132	<i>Citrobacter</i> sp.	94.6	73.8
85	<i>Enterobacter</i> sp.	92.3	95.6
21	<i>Vibrio</i> sp.	98.2	98.3
119	<i>Flavobacterium</i> sp.	42.7	48.7

<sup>a</sup> PMA (0.1 ml) in 95% ethanol, final concentration =  $0.4 \mu\text{g}$  of PMA ( $5.75 \times 10^4$  counts per min per  $\mu\text{g}$ ), was added to 0.9 ml of PES buffer (pH 7.0) containing approximately equal quantities of cells. The suspensions were incubated at 25 C, and 100- $\mu\text{l}$ iter samples were withdrawn.

<sup>b</sup> With aeration.

<sup>c</sup> Stationary.

<sup>d</sup> Isolate 244 was incubated 2 days.

<sup>e</sup> Isolates 244, 187, 94, and 127 were *Pseudomonas* sp. types I, II, III, and IV, respectively (13).

volume withdrawn and an adherence to sampling at regular intervals are necessary. Although corrections were utilized to normalize the determination of the concentration of both  $\text{Hg}^0$  and total Hg, the results indicated that substantial amounts of sample were lost through plating out of the mercury on the reactor-detector surfaces or inefficiency of the absorption procedures, or both. As a result, the concentrations of total Hg were less than those of  $\text{Hg}^0$  in almost all the measurements. The differences between  $\text{Hg}^0$  and  $\text{Hg}_{\text{TOTAL}}$  cannot be used to reliably evaluate production of volatile mercury compounds such as dimethylmercury, but a comparison of the ratios of the two values over the entire growth period reveals a monotonic relationship. This is indicative of the absence of important amounts of organomercurials, unless they are fortuitously generated at a rate very similar to that for  $\text{Hg}^0$  production. For example, with two isolates displaying divergent patterns for Hg production (Fig. 2), 119 yields  $\text{Hg}^0/\text{Hg}_{\text{TOTAL}} = 2.01 \pm 0.11$  (31 observations), whereas 72 gives  $\text{Hg}^0/\text{Hg}_{\text{TOTAL}} = 2.65 \pm 0.93$  (26 observations). The results are also consistent with minimal interference with AA mercury readings by volatile non-mercurial metabolites. Nonetheless, detection of benzene at low concentrations produced by the isolates was noted.

Varying levels of benzene were identified in

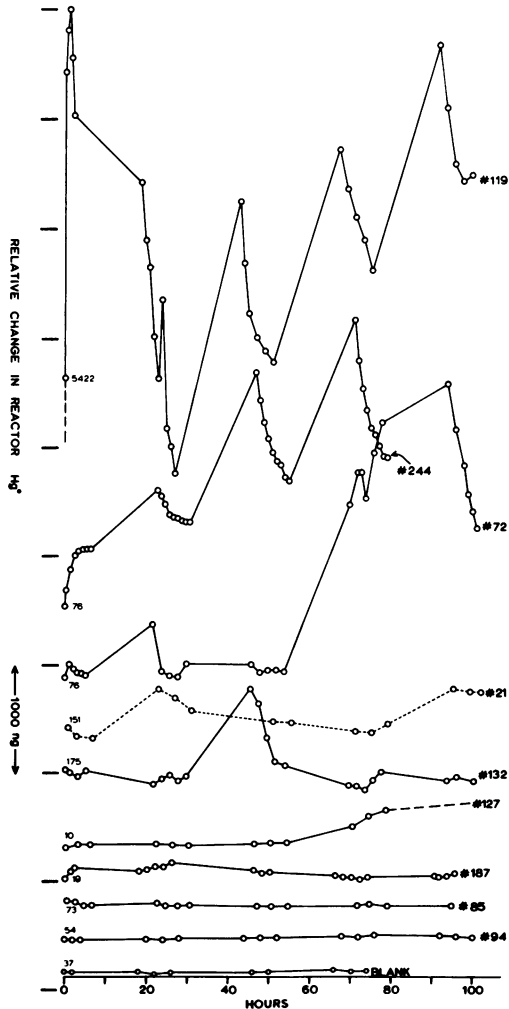


FIG. 2. The change in mercury contained in the bioreactor atmosphere as a function of time is plotted for formation of  $Hg^{\circ}$  from nine bacterial isolates from the Chesapeake Bay, each growing on six agar plates of basal medium containing 0.3 ppm PMA. The diameter of the open circles represents standard deviation from the mercury calibration curves used.  $Hg$  values plotted here are not additively corrected for portions of reactor atmosphere removed at each sampling period. Note that to avoid overlapping, the individual curves are not referred to a common zero on the ordinate, but the 1,000 ng intervals shown on that scale provide an indication of the relative change in the amount of  $Hg$  present above each isolate referred to a control blank (uninoculated plates).

the bioreactor atmospheres, along with independently determined  $Hg^{\circ}$  content, as follows: isolate 72, 0.19 ppm of benzene (1,584 ng of  $Hg^{\circ}$ ); 132, 0.02 ppm of benzene (100 ng of  $Hg^{\circ}$ ); 187, 0.67 ppm of benzene (63 ng of  $Hg^{\circ}$ );

244 at 48 h, 0.12 ppm of benzene (793 ng of  $Hg^{\circ}$ ); and 244 at 143 h, 0.91 ppm of benzene (318 ng of  $Hg^{\circ}$ ). Assuming that each benzene concentration reflects the total amount in the entire reactor volume ( $V_R$ ), an upper limit can be readily derived for the molar ratio of benzene to  $Hg^{\circ}$  present. For the isolates examined, (nmole of benzene)/(nmole of  $Hg^{\circ}$ ) = 4.4 (72), 7.2 (132), 389 (187), 5.5 (244 at 48 h), and 105 (244 at 143 h). An accumulation of benzene over  $Hg^{\circ}$  was anticipated, since the sensitivity experiments (Materials and Methods) showed the latter product to plate out far more extensively than the hydrocarbon. Consideration on a molar

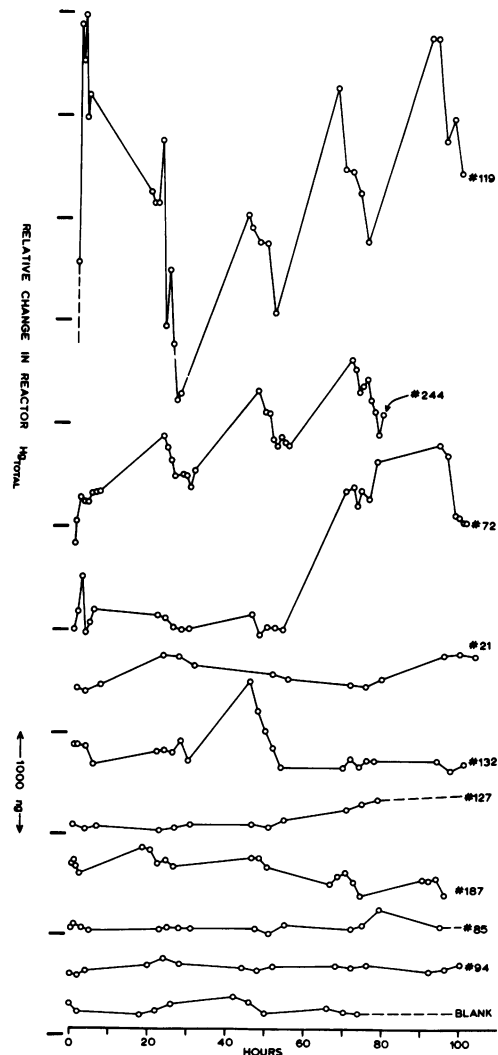


FIG. 3. Same as Fig. 2, except that the change in mercury contained in the bioreactor atmosphere as a function of time is plotted for formation of  $Hg_{TOTAL}$ .

basis of the  $C_6H_6/Hg^0$  ratios for the several isolates so examined suggests that previously postulated (3, 11, 18) simple reductive cleavage mechanisms involving the sigma benzenoid-metal bond may be important in the processes observed in the present study.

### DISCUSSION

Bacterial decomposition of mercury compounds resulting in formation of mercury in the gaseous elemental state has been reported by several investigators (J. D. Nelson and R. R. Colwell, manuscript in press; references 7, 10, 14, 15, 16, 17, 18). This phenomenon is not only important in providing a mechanism of resistance to this heavy metal, but it may have special significance in the mobilization of mercury in the aquatic environment. It has been shown that mercury-resistant, aerobic, heterotrophic bacteria are present in Chesapeake Bay water, sediment, and plankton. These bacteria are most prevalent in areas in which ambient levels of total mercury are highest (12). The results obtained in this study show that a number of these organisms are capable of decomposing an organomercury compound with the concomitant formation of  $Hg^0$  and benzene. These findings in conjunction with the work of others (14-17) considerably extend unequivocal demonstrations of specific bacterial  $Hg^0$  production. The cultures examined in this study showed a varied tolerance to  $HgCl_2$  (unpublished results), and, not surprisingly, different degrees of PMA metabolism. Some of the cultures were apparently inactive under the experimental conditions used. However, the mode of resistance of the non-mercury volatilizing bacteria may arise from an alternative metabolic pathway such as the cleavage of PMA with the formation of a nonvolatile form of mercury and benzene, as suggested by the analytical results, or possibly through the induced or fortuitous production of sulfhydryl compounds which "neutralize" or immobilize mercury via mercaptide formation. Another possible explanation may be that these microorganisms are impervious to mercury; i.e., the compounds do not enter the cells. Experiments in progress should resolve this point.

Generally, the present work shows that more precise analytical methods are required for full molecular characterization of both elemental and organomercury metabolites at concentrations eliminating requirements for usual batch or destructive reconcentration procedures. Recent demonstrations (2, 4, 9) of a vapor phase chromatograph coupled to both FID and AA detectors in tandem appeared promising in view

of the analytical results of the present work. Such a system has been incorporated into the basic modular bioreactor apparatus used in these laboratories and has already proven effective in a preliminary examination of an anaerobic Chesapeake Bay culture producing both  $Hg^0$  and dimethylmercury at different rates.

The information obtained from the laboratory experiments leads to the question of the relationship of degradative-reductive processes to methylation and overall cycling of mercury in the natural environment. In situ experiments which have been designed expressly to clarify this relationship and to determine whether reactions observed under laboratory conditions occur in nature are now underway.

### ACKNOWLEDGMENTS

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