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CHAPTER 25

Plasmid Mediation of Mercury Volatilization and Methylation by Estuarine Bacteria

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The efficiency of microbial volatilization of mercury was examined using bacterial strains isolated from Chesapeake Bay, a major estuary of the eastern seaboard of the United States, and one strain isolated from the Cayman Trench, a deep ocean site. Volatilization of mercury was studied under both aerobic and anaerobic conditions. The mechanism of mercury volatilization was determined to be plasmid mediated in two of the strains tested, but appeared to be chromosomally mediated in one strain of *Pseudomonas fluorescens.* The percent of mercury volatilized under aerobic conditions from media containing 10 mg $HgCl_2$/liter ranged from 21.5–87.2% within 24 h. Incubation under anaerobic conditions resulted in 12.7–78.1% volatilization of the mercury. Mercuric reductases associated with the plasmids were shown to be responsible for volatilization of mercury. Aerobic volatilization of mercury was observed to be more efficient than anaerobic volatilization. Furthermore, methylation of mercury was found to be a phenomenon separate from volatilization, with only two of the bacterial strains proving capable of producing methylmercury under either aerobic or anaerobic conditions. It is concluded that bacteria can mobilize mercury in the marine environment and may be potential agents for recycling mercury from mercury-laden wastes.

INTRODUCTION

Corner and Rigler (1957) established a relationship between presence of bacteria and volatilization of mercury from seawater. Subsequently, *Pseudomonas* K62, a bacterium isolated from soil by Japanese workers (Tonomura et al. 1968a,b), provided the first documentation of a bacterium isolated from the natural environment demonstrating high resistance to a variety of mercury compounds. In addition, the bacterium was able to remove mercury from the culture medium. In later studies, Komura and Izaki (1971) showed that resistance to mercury could be transferred from *Escherichia coli* K12 to mercury-sensitive *E. coli* and *Enterobacter aerogenes* strains. The work of Summers and Lewis (1973) extended these findings by demonstrating that the reduction of mercuric chloride to elemental mercury was plasmid-mediated in laboratory strains of *E. coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa.* Summers and Sugarman (1974) also found that the enzyme, mercuric reductase, was associated with mercury volatilization in *E. coli.* Thus, mercury volatilization processes are beginning to be understood in laboratory strains of bacteria, but little is known about volatilization accomplished by bacterial populations in the aquatic environment.

Spangler et al. (1973) observed that 30 of 207 strains of bacteria isolated from Lake St. Claire were capable of demethylating methylmercury bromide. After induction, 30 of the bacterial strains were found to be capable of volatilizing 50% of the mercury added to the growth medium. Approximately 900 mercury-resistant strains of bacteria were isolated

from Chesapeake Bay by Nelson et al. (1972, 1973), with the predominant genus noted among the mercury-resistant population being *Pseudomonas.* The maximum amount of mercury volatilized by nine of the bacterial strains was 53.7%, using media to which 0.3 mg/liter phenylmercuric acetate was added.

Many estuarine environments are impacted by release of mercury from anthropogenic sources, such as waste disposal, industrial processes, ship maintenance and anti-fouling treatment, and agricultural runoff. In Chesapeake Bay, a major East Coast estuary, the total mercury concn in sediment was found to range from 0.015 to 0.86 mg/kg. As noted above, mercury-resistant bacteria are readily isolated from Chesapeake Bay water and sediment, with significantly high frequency in Baltimore Harbor of Chesapeake Bay (Nelson and Colwell 1975; Austin et al. 1977). In the study reported here, the mechanism(s) involved in mobilization of mercury in the estuarine and marine environment was investigated with the ultimate objective being the development of a microbial process for removing mercury and other heavy metals from effluents entering the natural environment.

Materials and Methods

Cultures. Pseudomonas sp. 244, *Bacillus* sp. F96, *P. fluorescens* F63, *P. fluorescens* B69, and *P. fluorescens* B1, previously isolated and identified by Nelson et al. (1972, 1973), Allen et al. (1977), and Austin et al. (1977) and *Pseudomonas* sp. S58 and *Acinetobacter* sp. W45, both of which were isolated on estuarine salts agar (NaCl, 10 g; $MgSO_4 \cdot 7H_2O$, 4.75 g; KCl, 0.18 g; proteose peptone, 10 g; yeast extract, 1 g; 1 liter distilled H_2O, and pH adjusted to 7.0) and identified by the method of Stanier et al. (1966), were included in this study. The bacterial strain CC2B, included in the study, was isolated on modified Yamada medium (Yamada and Tonomura 1972), a medium containing 15 mg $HgCl_2$, 0.18 g KCl, and 4.75 g $MgSO_4 \cdot 7H_2O$ per liter. An as yet unidentified gram-negative rod isolated from Cayman Trench sediment by Dr. K. Owada (unpubl. data) was also included in the set of strains examined in this study. All isolates were maintained on Yamada medium containing 10 μg $HgCl_2$/ml, which was used for total viable counts when supplemented with 2% agar (Difco Laboratories, Detroit, MI).

Mercury volatilization and methylation. Bacterial cultures examined for volatilization and methylation of mercury were inoculated into Yamada medium (Yamada and Tonomura 1972) containing 10 μg/ml. The inoculated media were incubated at 21 C, after which cultures in exponential growth were diluted 1:100 in fresh $HgCl_2$-containing medium. Samples were withdrawn at 0, 24, and 48 h for determination of total mercury by atomic absorption spectrophotometry and total viable counts by dilution and spread plating on modified Yamada medium. Samples for total mercury and methylmercury analysis were stored at -70 C until analyzed. At the end of the volatilization experiments, cultures were centrifuged at 15,000 rpm for 20 min. The supernatant and pellet fractions were separated and frozen for subsequent extraction to determine final concn of methylmercury. Determination of anaerobic volatilization and methylation was accomplished as described above, except that all flasks were purged with nitrogen before inoculation and throughout the experiments, which were terminated at 48 h.

Mercury analysis. Total mercury was measured using a Perkin Elmer Atomic Absorption Spectrophotometer Model 460 (Norwalk, CT) equipped with a graphite furnace.

Methylmercury was extracted from the supernatant and bacterial pellet (wet weight) by a modification of the Westöo method (Olson and Cooper 1974) and was measured using the Zee-man Isotope Shift Atomic Absorption Spectrophotometer (Hadeishi and McLaughlin 1971).

Plasmid screening. A modification of the 0.7% agarose gel technique was used to screen for presence of plasmids (Meyers et al. 1976).

Preparation of deoxyribonucleic acid (DNA). Cultures were grown for 24 h in 30-ml volumes of modified Yamada broth as described above. Cleared lysates were prepared following the method of Guerry et al. (1973) and incubated for 1 h at 37 C with 10 μg/ml ribonuclease (Calbiochem, La Jolla, CA). Following ribonuclease treatment, the lysates were extracted twice with Tris (50 mM):saturated phenol and three times with chloroform:iso-amyl alcohol (50:1). Sodium acetate at a concn of 3 M was added to the extracts to yield a final concn of 0.3 M Na-acetate. When examined in a spectrophotometer, the extracts gave ratios of $OD_{260}/OD_{280} > 2.0$. Two volumes of 95% ethanol were added and the samples were stored overnight at -4 C. The DNA preparations were centrifuged at 48,000 × *g* for 30 min at -10 C, the supernatant removed and the samples dried under nitrogen. The DNA was redissolved in 0.2 ml TES buffer (0.05 M NaCl, 0.005 M EDTA, 0.03 M Tris, pH 8.0) and analyzed immediately by gel electrophoresis.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed using a vertical slab gel apparatus (Aquabogua Machine and Repair Shop, Aquabogua, NY), the dimensions of which were 15.9 × 19.0 × 0.13 cm. Four milliliters of 7% acrylamide gel provided a support medium for the agarose gel; 6.9 ml of acrylamide solution (acrylamide 15 g; bisacrylamide 0.75 g in 100 ml distilled water) were mixed with 1.5 ml Na-acetate electrophoresis buffer (0.04 M tris; 0.002 M EDTA; 0.02 M Na-acetate adjusted to pH 8.0 with acetic acid), 7.95 ml distilled water, 150 μl ammonium persulfate, and 10 μl TEMED. The acrylamide gel was allowed to solidify for approx. 20 min and 0.7% agarose (w/v) was dissolved in the Na-acetate buffer (65 C) and poured into the slab, with a 12-tooth comb inserted immediately thereafter. The gel was allowed to stand for 30 min, after which it was removed and the slab was attached to the gel apparatus. The gel was equilibrated for 30 min (50 volts). Prior to loading DNA samples, the DNA preparations were mixed with 10% (v/v) bromophenol blue dye (62% glycerol, 2.5% sodium dodecyl sulfate, 1% bromophenol blue) and the electrophoresis was run at room temp in Na-acetate buffer at 100 volts until the dye reached the interface between the acrylamide layers and agarose, which was approx. 3 to 4 h.

Detection of plasmids. Following electrophoresis, each gel was soaked in a solution of ethidium bromide (Sigma, St. Louis, MO), 0.4 μg/ml, for 30 min at 4 C. Records were prepared using a Polaroid Model MP4 Camera.

Determination of the molecular weight of the plasmids. Plasmids of known molecular weight (MW) were obtained through the courtesy of Dr. E. Lederberg, Plasmid Reference Center, Stanford University, Palo Alto, CA. The plasmids employed were Sa (23 megadaltons), RP4 (34 megadaltons), and R1 (62 megadaltons) (Meyers et al. 1976).

Curing experiments. Three different curing agents were used. Curing by acridine orange was accomplished as described by Willetts (1967). Cells were grown overnight in Yamada broth containing 2 μg/ml mitomycin C (Calbiochem., La Jolla, CA). Cells in stationary phase were used in the UV-curing experiments as follows. The cells were twice centrifuged at 9750 × *g* for 15 min, the supernatant removed, and the cells, at a concn of approx. 10^8 cells/ml, resuspended in estuarine salts solution (NaCl, 10 g; $MgSO_4 \cdot 7H_2O$,4.7 g; KCl, 0.18 g; distilled H_2O, 1 liter). The suspensions were placed in quartz tubes and exposed to UV light for 45 min (Ultra Violet Products, San Gabriel, CA, Model UVS-54) at 460mW/cm^3 at 15.24 cm from the UV source. Control suspensions were treated in the same manner except that they were not exposed to UV light. Following curing, the cells were plated onto solid modified Yamada medium, without added mercury. When growth was visible, the plates were replicated onto modified Yamada medium containing Hg (at a concn of 10 μg/ml) or without added Hg. Colonies which grew on plates with no Hg added but failed to grow in the presence of mercury were restricted, according to the above method. The presence of cured cells was confirmed by inoculating cured cell suspensions into tubes containing Yamada broth with and without added mercury.

Results and Discussion

Aerobic and anaerobic volatilization patterns for strain CC2B, *P. fluorescens* B1, *Pseudomonas* sp. 244, *Pseudomonas* sp. S58, the unidentified gram-negative rod No. 69, *P. fluorescens* F63, and *Bacillus* sp. F96 are shown in Figs. 1 and 2. Bacterial growth patterns for these isolates are given in Table 1. Results of experiments quantitating growth and mercury volatilization by *P. fluorescens* B69 and *Acinetobacter* sp. W45 and their cured derivatives are given in Figs. 3 and 4. As seen in Figs. 1-4, all of the bacterial strains included in this study volatilized mercury under aerobic conditions.

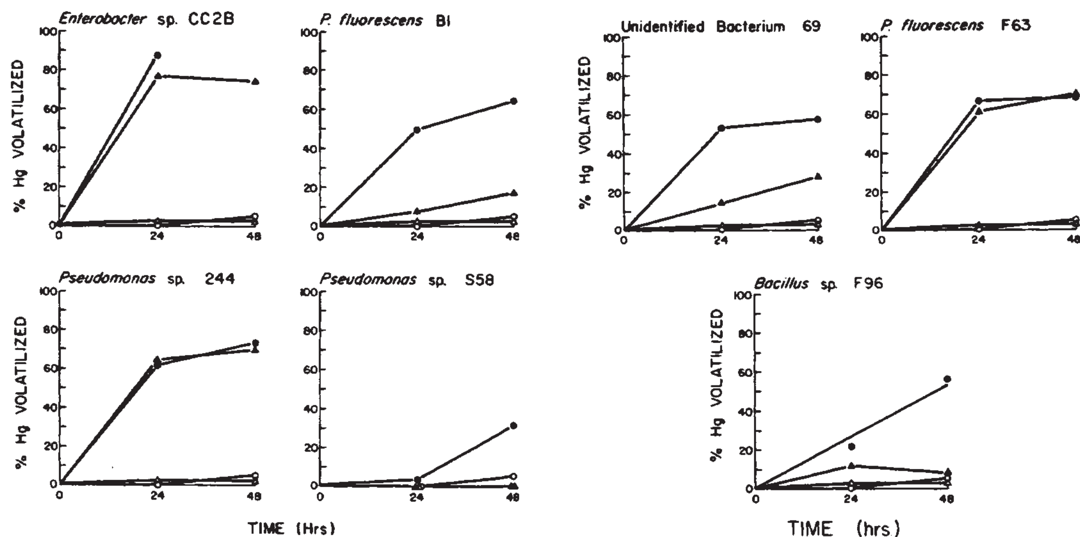

Fig. 1. Aerobic and anaerobic volatilization of mercuric chloride by marine bacteria. ● aerobic volatilization, ▲ anaerobic volatilization, ○ aerobic control, △ anaerobic control.

Fig. 2. Aerobic and anaerobic volatilization of mercuric chloride by marine bacteria. ● aerobic volatilization, ▲ anaerobic volatilization, ○ aerobic control, △ anaerobic control.

TABLE 1. *Growth of mercury volatilizing bacteria under aerobic and anaerobic conditions*

	Aerobic			Anaerobic		
	Time (hours)			Time (hours)		
Bacterial strain	0	24	48	0	24	48
Bacillus sp. F96	$\times 10^7$	1.9×10^9	4.9×10^9	$\times 10^4$	$\times 10^4$	$\times 10^5$
P. fluorescens F63	1.3×10^8	2.0×10^9	$\times 10^{10}$	1.1×10^6	2.2×10^6	3.5×10^7
Pseudomonas sp. S58	4.6×10^5	3.9×10^6	2.7×10^8	6.4×10^5	2.5×10^5	6.7×10^5
Isolate 69	7.0×10^7	1.1×10^9	4.6×10^9	7.3×10^5	5.1×10^5	4.3×10^5
P. fluorescens B1	7.5×10^7	2.9×10^9	6.2×10^9	2.6×10^6	1.1×10^7	2.8×10^8
Pseudomonas sp. 244	3.9×10^5	6.2×10^8	1.6×10^9	3.9×10^5	2.2×10^6	3.5×10^7
Isolate CC2B	5.9×10^6	9.7×10^8	9.7×10^9	3.0×10^7	5.3×10^8	5.9×10^8

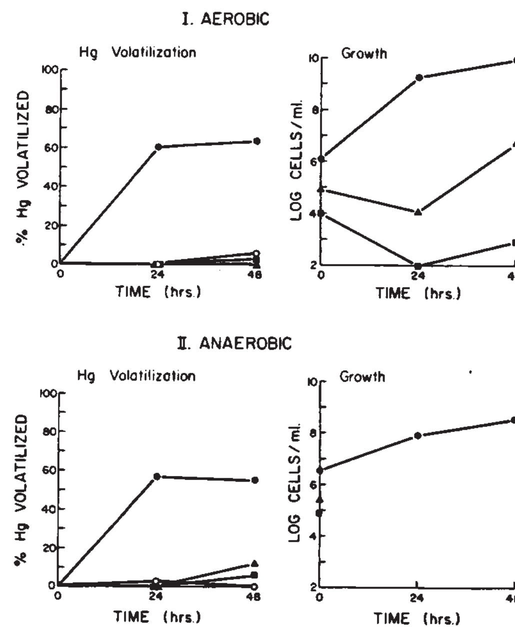

Fig. 3. Aerobic and anaerobic volatilization of mercuric cholride by *Pseudomonas fluorescens* B69 and cured derivative strains. ● B69, ■ cured strain B69A, ▲ cured strain B69F, O control.

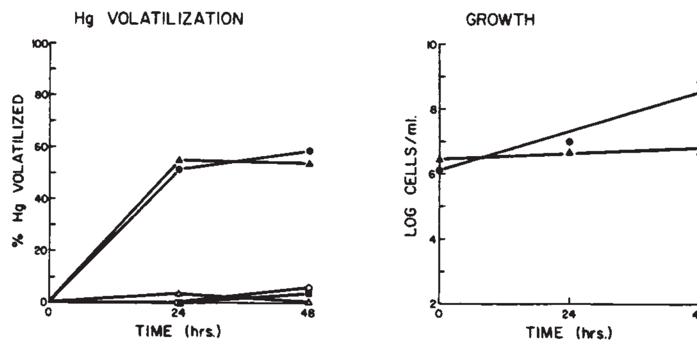

Fig. 4. Aerobic and anaerobic volatilization of mercuric chloride and growth of *Acinetobacter* sp. W45 and cured derivative strain. ● W45-aerobic, ▲ W45-anaerobic, ■ cured derivative strain W45-aerobic and anaerobic, O aerobic control, Δ anaerobic control.

Five of the nine bacterial strains were identified as members of the genus *Pseudomonas*, confirming the findings of Nelson et al. (1973) that *Pseudomonas* spp. not only are the predominant organisms among those bacteria demonstrating resistance to mercury but also play a significant role in the mobilization of mercury in the estuarine environment. *Pseudomonas* sp. 244 volatilized 62.7% of added mercury aerobically within 24 h. *Pseudomonas* sp. S58 was able to volatilize 30.8% of the mercury in the medium after incubation for 48 h. Interestingly, *P. fluorescens* B1, F63, and B69 volatilized similar amounts of mercury. Although some variation in volatilization of mercury was observed among the several *Pseudomonas* spp., there was less variation within the strain cluster of *P. fluorescens.*

Aerobic volatilization was found to be more efficient than anaerobic volatilization in all strains examined, except Pseudomonas sp. 244. Aerobic and anaerobic volatilization was found to be approx. the same in the case of *Pseudomonas* sp. 244. Under aerobic conditions, as much as 87.2% of the mercury was volatilized within 24 h by this isolate. Only two of the strains that were examined, i.e., *Pseudomonas* sp. S58 and *Bacillus* sp. F96, volatilized less than 50% of the mercury in the medium. In certain instances, anaerobic conditions acted to retard severely the volatilization capacity of the bacteria. *P. fluorescens* strain B1 and *Bacillus* sp. F96, both active mercury volatilizers under aerobic conditions, were not capable of volatilizing mercury anaerobically.

The rate at which mercury was removed from the culture medium was rapid, with most of the mercury being released within 24 h. Rapid rates of volatilization have been reported by other investigators (Summers and Silver 1972; Schottel et al. 1974; Clark et al. 1977; Weiss et al. 1977).

Two strains, *Pseudomonas* sp. S58 and *Bacillus* sp. F96, yielded different patterns of mercury volatilization. In the case of *Pseudomonas* sp. S58 (Fig. 1), the volatilization capacity of the organism was found to be limited. From comparisons of growth and volatilization (Table 1 and Fig. 1), resistance to mercury appeared to be inducible, with little volatilization occurring during the first 24 h after transfer to fresh medium containing mercury, followed by release of 30% of the added mercury within the next 24 h. Volatilization was accompanied by negligible growth during the first 24 h, with good growth noted at 48 h.

Volatilization of mercury has not been reported for *Bacillus* spp. From the data accumulated during the course of this study, it is clear that, under aerobic conditions, *Bacillus* sp. F96 volatilized mercury. Differences in cell wall permeability (Schaechter and Santomassion 1962) between gram-negative and gram-positive bacteria may explain the higher resistance of some gram-positive bacteria to mercurial compounds. Cell wall permeability can affect the rate at which mercury enters a cell and, therefore, will influence the pattern of volatilization, but growth does not appear to be a factor because no significant difference was observed among the growth patterns of the *Bacillus* spp. and those of the other bacterial strains tested.

Pseudomonas fluorescens strain B1 (Table 2) was the only bacterial strain in which plasmids could not be detected. Some of the difficulties in detecting plasmids are: (1) large plasmids (100 megadaltons) will not penetrate 0.7% agarose gel; (2) DNA bands localized in the same region of the chromosomal band will not be observed; and (3) breakage of large plasmids during extraction will often be interpreted as loss of the plasmid.

Mercury volatilization was observed to be independent of growth rate and increase in cell

TABLE 2. *Molecular weight of plasmids present in mercury volatilizing bacteria*

Isolate	Molecular Weight (megadaltons)
Bacillus sp. F96	89.9
P. fluorescens F96	1.6
	4.5
Pseudomonas sp. S58	53.5
Isolate 69	109.2
P. fluorescens B1	plasmid not observed
Pseudomonas sp. 244	3.2
	6.6
Isolate CC2B	2.3
	3.3
	6.6
P. fluorescens B69	18.5
	34.3
	39.5
P. fluorescens (cured strain B69A)	18.5
	39.5
P. fluorescens (cured strain B69F)	plasmid not observed
Acinetobacter sp. W45	2.2
	4.7
	81.9
Acinetobacter sp. W45 (cured strain)	2.2
	81.6

number (Figs. 1-4 and Table 1). In general, aerobic growth patterns for the bacterial strains examined showed a mean increase of two logs, while under anaerobic conditions the majority of bacterial strains tested demonstrated less than a one log increase in viable cells. For example, *P. fluorescens* B1 increased in cell numbers by two logs over the 48-h anaerobic experiment, but total volatilization under anaerobic conditions was found to be less than 20% compared to 65% under aerobic conditions of growth. *Pseudomonas* sp. 244, which demonstrated an increase of two logs in cell number, volatilized 71.3% of the mercury in the medium after incubation under anaerobic conditions for 48 h. Interestingly, only *P. fluorescens* F63 and unidentified strain CC2B were able to produce methylmercury under aerobic and anaerobic conditions, respectively. The inability of these isolates to produce methylmercury suggests that a separate group of microorganisms, other than *Pseudomonas* spp., are responsible for the methylation of mercury in the natural environment. This conclusion is supported by results of other investigators who have isolated and examined bacterial strains capable of methylating mercury (Vonk and Sijpesteijn 1973; Hamdy and Noyes 1975; Reich and Olson 1977). The majority of the bacteria shown to methylate mercury have been identified as members of the Enterobacteriacae.

Using mitomycin C, cured derivatives were obtained from *Acinetobacter* sp. W45 and *P. fluorescens* B69 (Table 2 and Fig. 5). All three of the curing agents failed to induce loss of one or more plasmids in the six bacterial strains tested (Table 2). The lack of success with curing treatments suggest that the plasmids harbored were extremely stable. Thus, mercury resistance may well be a relatively stable trait, as well as common, in bacteria found in the estuarine and marine environments, especially where mercury resistant bacterial populations abound.

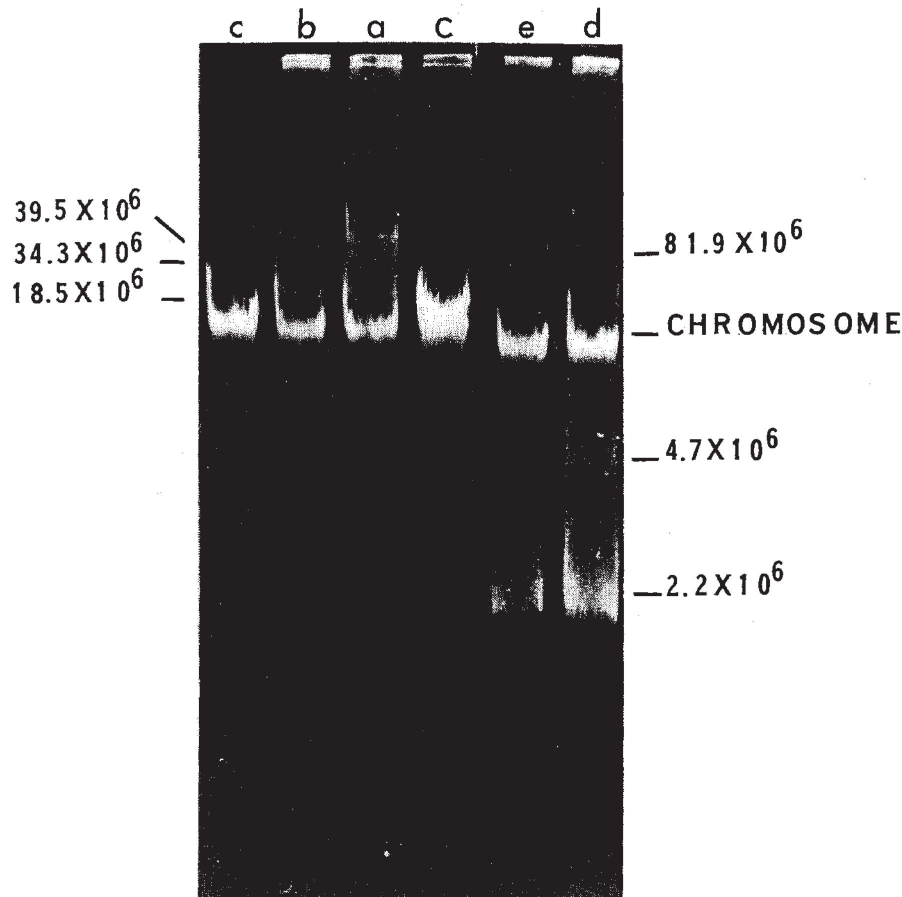

Fig. 5. Plasmids harboured by *P. fluorescens* B69, *Acinetobacter* sp. W45 and their cured derivative strains.
C - *E. coli* W3110 chromosomal DNA
a - *P. fluorescens* B69
b - *P. fluorescens* B69 cured strain B69A
c - *P. fluorescens* B69 cured strain B69F
d - *Acinetobacter* sp. W45
e - *Acinetobacter* sp. W45 cured derivative W45A
The molecular weight in daltons for each plasmid is presented on the figure.

Both *Pseudomonas fluorescens* B69 and *Acinetobacter* sp. W45 were found to produce mercuric reductase, whereas the cured derivatives of the two strains did not produce the enzyme (Silver, pers. comm.). Thus, confirmatory evidence that mercury volatilization and resistance are plasmid mediated and the enzyme for reduction of the mercury is associated with the plasmid was obtained for the two bacterial strains. Under aerobic conditions, the cured derivatives of strain B69 (B69A and B69F) were found to be resistant to mercury after incubation for 48 h, but no mercury was volatilized from the culture. Therefore, it would appear that a subpopulation of resistant cells was selected, but this population exhibited a detoxification mechanism other than volatilization, a phenomenon not noted in the cured derivative of *Acinetobacter* sp. W45 (Fig. 4).

In conclusion, mercury-resistant bacterial isolates have been shown to be efficient in volatilizing mercury. Because these bacteria occur in large numbers and are distributed widely in the estuarine and marine environment, they appear to be significant in mobilizing inorganic mercury, notably under aerobic conditions which, in the normal situation, predominates in Chesapeake Bay water. Mobilization of mercury by estuarine bacteria could be harnessed for detoxification of a mercury-polluted environment by developing the microbial system for removal of mercury from effluents prior to discharge, thereby preventing movement of mercury further up the food chain.

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