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Volatilization of Fluorescein Mercuric Acetate by Marine Bacteria from Minamata Bay

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Some bacteria that live in a mercury-polluted environment are resistant to mercury compounds (Nelson and Colwell 1975 ; Nakamura *et al.* 1988). A majority of these mercury-resistant bacteria have been found to volatilize organic as well as inorganic mercury compounds into elemental mercury vapor by means of their enzymes (Olson *et al.* 1979 ; Robinson and Tuovinen 1984). One compound, fluorescein mercuric acetate (FMA) has long been in use as a disinfectant in hospitals; yet, there has been little definitive information on bacterial resistance to this compound. Some plasmid-bearing strains of *Pseudomonas species* (Clark *et al.* 1977) and *Staphylococcus aureus* (Weiss *et al.* 1977) have been found to be resistant, and clinical isolates of *Klebsiella pneumoniae* (Kono *et al.* 1985) were recently found to degrade FMA into the end products of metallic mercury sediment and fluorescein. In these cases, resistance to FMA was not attributable to bacterial volatilization.

Minamata Bay has been heavily polluted by mercury, which has caused methylmercury poisoning in humans, called Minamata disease (Kutsuna 1968 ; Irukayama 1977). Sediments from the Bay still contain high concentrations of mercury (Kumamoto Prefectural government 1973 ; Kumagai and Nishimura 1978). The percentage of mercury-resistant bacteria in the total bacterial count is higher in these sediments than in those of other marine environments (Nakamura *et al.* 1986). FMA-pollution, however, has not been reported.

Research into the mechanism of bacterial resistance to FMA will not only add to our general understanding of the ability of certain bacteria to resist mercury, but will also help in defining the role bacteria play in the mercury cycle of a mercury-polluted environment. The purpose of the present study is to determine the mechanism of resistance to FMA of the FMA-resistant bacteria living in the Bay.

MATERIALS AND METHODS

1432 FMA-resistant bacteria were isolated from Minamata Bay

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sediment and seawater by using 40 μg/mL of FMA in October, 1985, and June, 1986. The isolated strains were classified in accordance with the taxonomic scheme proposed by Shewan *et al.* (1960) and Simidu (1974). Of these, 322 strains were used in this study. Three strains belonging, respectively, to *Bacillus, Moraxella,* and *Pseudomonas sp.* were used for further study on the mechanism of resistance to FMA.

The basal medium was artificial sea water medium (modified ZoBell 2216 E medium) as described previously (Nakamura *et al.* 1986). The sedimentation of metallic mercury was observed in a liquid medium containing 40 μg/mL of FMA, after incubation for 48 h at 25°C. The rate of volatilization of FMA by each of the 322 FMA-resistant strains was determined by measuring the amount of mercury loss from a culture medium containing 5 μg/ml of FMA, after incubation for 48 h at 25°C.

The total mercury contents of the medium containing FMA were analyzed by a flameless atomic adsorption spectrophotometer with a mercury analysis vaporizer (Tanaka *et al.* 1974).

Mercury vapor from FMA by the bacterial volatilization was analyzed by gas chromatography-mass spectroscopy. Three FMA-resistant bacterial strains (*Bacillus, Moraxella,* and *Pseudomonas sp.*) were inoculated in a 1/15 M phosphate buffer (pH 7.0) containing 20 μg/mL of thioglycolate, 0.5 mM EDTA, 0.2 mM magnesium acetate, and 80 μg/mL of FMA. After anaerobic incubation for 5 h at 30°C, the vapor phase of the tube was analyzed by gas chromatography-mass spectroscopy (JEOL D-300) following the EI method. Conditions under which mercury vapor was measured were as follows : column temperature, 170°C ; injection temperature, 220°C ; the flow rates of helium gas, 1.0 kg/cm^2. The coiled glass column with Thermo Hg (Shimazu Column Packing) was 2 m in length and 3 mm in diameter.

RESULTS AND DISCUSSION

Three FMA-resistant strains (*Moraxella, Pseudomonas, and Bacillus sp.*) did not produce the precipitate of metal mercury in a liquid culture containing 40 μg/mL of FMA, after incubation for 48 h at 25°C, as reported in the case of *Klebsiella pneumoniae* (Kono *et al.* 1985). The amount of mercury loss from a liquid culture containing 80 μg/mL of FMA during incubation time was then determined using the same three strains (Fig. 1). The mercury content of the culture decreased within 24 h and then remained virtually unchanged for at least another 72 h.

Figure 2 shows the mass-spectrograms for elemental mercury vapor (Hg^0); for the vapor produced from a 1/15 M phosphate buffer (pH 7.0) containing 80 μg/mL of FMA, 20 μg/mL of thioglycolate, 0.5 mM EDTA, 0.2 mM magnesium acetate, by FMA-resistant *Pseudomonas sp.*; as well as for the vapor produced from a 1/15 M phosphate buffer containing 20 μg/mL of $HgCl_2$ instead of 80 μg/mL of FMA by the same strain. The mass-spectrograms of vapor produced by the other two FMA-resistant strains were the same as those in the case of

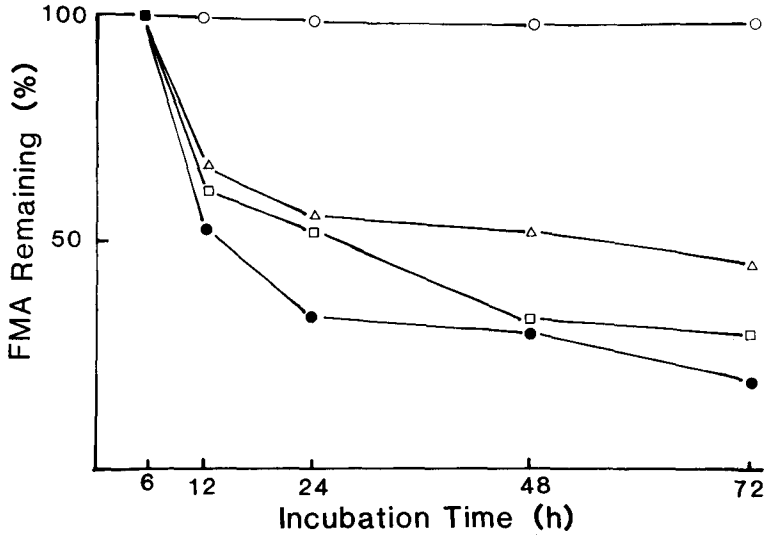

Figure 1. Loss of FMA from an artificial seawater medium containing 80 µg/mL of FMA. Symbols : -○-, Uninoculated control ; -△-, *Moraxella sp* ; -□-, *Pseudomonas sp* ; -●- , *Bacillus sp.*

Pseudomonas sp. It can be seen that mass-spectrograms for the vapors produced by the bacteria are the same as that of elemental mercury vapor (Hg^0); which is readily identified by the characteristic ratios of the six naturally occurring isotopes (Summers *et al.* 1972). From these results, the vapor from both liquid cultures was identified as elemental mercury vapor (Hg^0).

Although clinical isolates of *Klebsiella pneumoniae* have been found to degrade FMA into the end products of metallic mercury sediment and fluorescein (Kono *et al.* 1985), bacterial volatilization of the compound had not been yet reported. However, the amount of mercury in a liquid culture containing originally 80 µg/mL of FMA decreased in the cases of three FMA-resistant strains (*Moraxella , Pseudomonas , and Bacillus sp.*) from Minamata Bay, moreover without metal mercury precipitate being produced. Vapors produced from liquid cultures containing either mercury chloride or FMA by FMA-resistant bacteria were identified as elemental metal mercury (Hg^0).

Since the hypothesis had been made that fluorescein would be one common end product of the process of decomposition of FMA when brought about by FMA-resistant bacteria, tests were performed by gas chromatography-mass spectroscopy according to both the in-beam CI method as described by Kono *et al.*(1985) and the FAB method in order to measure the amount of fluorescein produced. However, none could be detected through either method. This result can be considered due either to the disturbance caused by the salinity of the artificial seawater medium or to the low volatility rate of FMA.

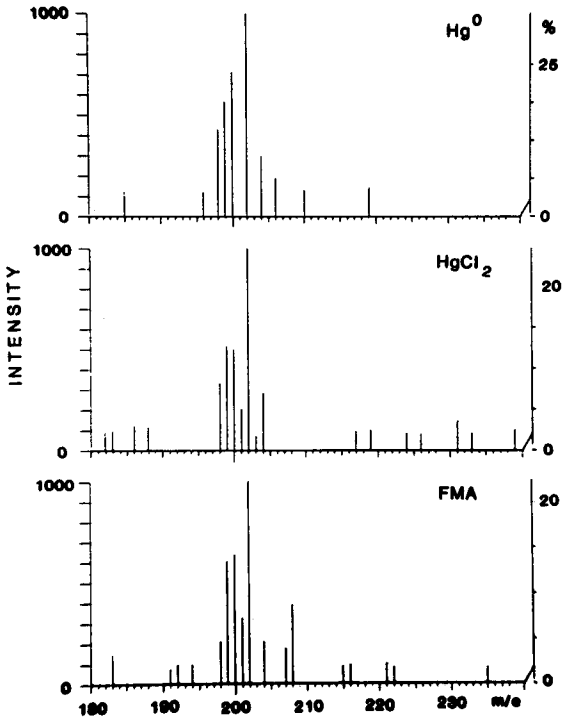

Figure 2. Mass-spectrograms for vaporized metal mercury (Hg^0); and for the vapors produced by a FMA-resistant strains (*Pseudomonas sp.*) when added to a 1/15 M phosphate buffer (pH 7.0) containing 20 μg/mL of thioglycolate, 0.5 mM EDTA, and 0.2 mM magnesium acetate, with either 20 μg/mL of mercury chloride ($HgCl_2$) or 80 μg/mL of FMA (FMA). The FMA-resistant strain was incubated anaerobically for 5 h at 30° C.

The conclusion may therefore be drawn that FMA-resistant bacteria transform both FMA and mercury chloride into Hg^0 through bacterial volatilization. It shows that FMA is very likely transformed into fluorescein, acetic acid and Hg^0 through the volatilization.

The bacterial volatilization of FMA by 322 FMA-resistant strains isolated from Minamata Bay are shown in Table 1. All strains failed to produce mercury precipitate in a liquid culture containing 5 μg/mL of FMA within 48 h. However, of 322 strains, 144 (43.9 %) were able to volatilize the FMA in a similar liquid culture within 48 hr. These strains belong to the following genera : *Bacillus*, *Micrococcus, Corynebacterium, Staphylococcus, Pseudomonas, Moraxella, Vibrio, Enterobacter,* and *Flavobacterium.* The volatilization of FMA was observed in all bacterial genera tested in this study. These results suggest that bacteria more commonly volatilize FMA rather than produce such precipitates.

Table 1. Volatilization of FMA by the FMA-resistant bacteria isolated from Minamata Bay

	No. of Positive Strains (%)	Volatilization Rate
Gram-positive (154[a])		
Bacillus (110)	72 (65.4)	62.5±1.2[b]
Micrococcus (24)	4 (16.7)	54.1±4.4
Corynebacterium (9)	4 (44.4)	43.9±2.2
Staphylococcus (11)	3 (27.3)	46.6±6.6
Gram-negative (168)		
Pseudomonas (77)	35 (45.5)	55.9±2.0
Moraxella (53)	20 (35.7)	59.9±3.0
Vibrio (21)	2 (9.5)	72.8±6.7
Enterobacter (9)	2 (22.2)	58.8±1.8
Flavobacterium (8)	2 (25.0)	50.7±0.9

[a]Number of tested strains
[b]Means loss ± SE

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