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Elimination of Mercury and Organomercurials by Nitrogen-Fixing Bacteria

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Bacteria isolated from mercury-polluted environments are often resistant to mercuric ions (Hg^{2+}) and organomercurials. Plasmids determining mercury resistance have been well characterized in gram-negative system. However, in *Staphylococcus aureus* mercury resistance has been found to be chromosomally determined (Misra 1992a). The known mechanism of bacterial Hg^{2+}-resistance is detoxification of the toxic Hg^{2+} by its enzymatic transformation by mercuric reductase to Hg (o). Organomercurial lyase mediates the degradation of organomercurial compounds to Hg^{2+} (Schottel 1978; Misra 1992a).

Mercury and organomercurial resistances have been studied in different bacterial genera (Schottel et al. 1974; Pahan et al. 1995). There is little information on Hg-resistance in N_2-fixing soil bacteria (Ghosh et al. 1996). however, in many developing countries, including India, mercury pollution is still a problem because Hg-based pesticides and fungicides are still used routinely as seed-dressers in agriculture to control soil-borne and seed-borne fungal diseases. Volatilization of Hg from laboratory media by mercury-resistant bacteria containing low levels of mercury has been reported by several workers (Komura et al. 1971; Nakamura et al. 1990; Furukawa and Tonomura 1972). It is interesting to note that N_2-fixing, Hg-resistant soil isolates could volatilize Hg from medium containing very high amounts of $HgCl_2$. In the present paper we report the volatilization patterns of five N_2-fixing bacterial strains, the effect of different inducers on mercuric reductase, and the pattern of substrate utilization by organomercurial lyase.

In the presence of a low concentration of $HgCl_2$, enzymatic detoxification is sufficient to combat the adverse situation created by the presence of Hg^{2+} ions. In the presence of a high concentration of $HgCl_2$, intracellular sequestration by Hg^{2+} binding components may play an additional role in counteracting Hg-toxicity (Silver 1992).

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MATERIALS AND METHODS

Mercury-resistant, N_2-fixing bacterial strains were isolated from soil collected from agricultural farms of West Bengal and Bihar, India and identified in this laboratory following Bergey's Manual of Determinative Bacteriology, 9th edition (1994). The culture was maintained in a solid nitrogen-free Winogradsky's glucose agar medium (Bergey's Manual of Determinative Bacteriology, 9th edition, 1994).

The minimum inhibitory concentration (MIC) values of $HgCl_2$and other organomercurials, including phenylmercuric acetate (PMA,) p-hydroxymercuric benzoate (pHMB), fluorescein mercuric acetate (FMA), thimersol (Tm), and merbromine (Mb), towards these organisms were determined using a filter paper disc (4mm diameter) and nutrient agar plate (Schottel et al. 1974).

For the Hg-volatilization experiment, 3.333 mg of $HgCl_2$ were added to 200 ml of nutrient broth in control flasks which received no organisms. In the experimental flasks an overnight culture of the bacterial cells was diluted 1:10 with sterile nutrient broth to a final volume of 200 ml and 3.333 mg of $HgCI_2$ were added. The organisms were grown up to 12 hr on a rotary shaker (200 rpm) at 32°C; the control flask was similarly shaken. The cells were then harvested by centrifugation at 6000 x g for 10 min at 0-4°C and washed 3 times with deionized water. A weighed amount of wet cells, 1 ml of the supernatant after cell harvesting and 1 ml of control medium containing $HgCl_2$, were placed in separate 50-ml volumetric flasks and digested to bring all the mercury into the ionic form. Mercury concentrations of the samples were determined quantitatively with a cold vapor atomic absorption spectrometer (MA 5800D, ECIL, Hyderabad, India) (Bradenberger and Bader 1967).

Hg-reductase activity was determined spectrophotometrically at 340 nm in the cell-free extracts by measuring Hg^{2+}-induced NADPH oxidation (Komura et al. 1971). Most of the Hg-reductase activity in the supernatant was precipitated with 30-50% (NH_4), SO_4 at 4°C and the precipitate was dissolved in minimum volume of 50 mM phosphate buffer containing 0.25 mM Na_2EDTA and then dialyzed overnight at 4°C. The dialyzates were used for the assay of Hg-reductase activities. In the control set, NADPH oxidation by the cell-free extract without any $HgCl_2$ was monitored under the same condition. The reaction mixture contained 5mM Na_2EDTA, 2 mM $MgCl_2$, 1mM sodium thioglycolate, 30 µM $HgCl_2$ and 50-150 µM NADPH in 50 mM sodium phosphate buffer (pH-7.4). A suitable volume of 10-100µl cell-free extracts were used to follow the reaction kinetics uniformly for 5 min in a final volume of 1 ml. The reaction mixture was preincubated for 10 min at 32°C and then the reaction was started by adding enzyme and NADPH. Organomercurial lyase activity was also determined by the same procedure using different organomercurial compounds used as substrates in the reaction mixture. Protein content was measured following the method of Lowry et al.(1951).

RESULTS AND DISCUSSION

All the soil samples isolated from different agricultural farms were mostly semidried, loosely textured and slightly acidic (pH-6.4-6.5) except the sample from a sugarcane farm which was slightly alkaline (pH-7.1) and the soil sample from a black gram farm was neutral (pH-7.0). In all samples, the ratio of total number of Hg-resistant, N_2-fixing organisms to the number of N_z-fixing organisms was low, ranging from 1:83 to 1:1200. These samples contained high levels of mercury in the range of 112-190 ng/g soil samples. The mercury content of unpolluted soil was found to be 10-20 ng/g soil (data not shown). It is significant that the total viable count of Hg-resistant, N_2-fixing bacteria increased with the increase in the mercury content of the soil. It has been reported by other workers also that resistant bacteria are prevalent in environments enriched with toxic compounds (Misra 1992b). From these soil samples a large number of mercury-resistant bacterial strains belonging to the genera, *Azotobacter* and *Beijerinckia* were isolated. All the mercury-resistant isolates were resistant to $HgCl_2$ and the organomercurials PMA, thimersol, pHMB, FMA and merbromine. Mercury-resistance in these bacterial strains was also associated with antibiotic-resistant properties (data not shown) (Schottel et al. 1974).

Table 1. Pattern of mercury-resistance properties, Hg-volatilization from $HgCl_2$ containing nutrient broth and specific activity of Hg-reductase in some mercury-resistant, N_2-fixing bacteria.

Strain No.	MIC value of $HgCI_2$	% of Hg volatilization after 12 hr incubation	Total Hg bond per gram cell mass (mg)	Specific activity value of Hg reductase enzyme
Azotobacter sp SS_2	300	79.92	0.015	0.225
Azotobacter sp S_6	200	72.37	0.034	0.156
Beijerinckia sp SSG_1	100	68.5	0.248	0.053
Azotobacter sp S_1	50	55.25	0.015	0.022
Azotobacter sp GR_2	25	10.81	0.180	0.012

Specific activity expressed in µmol NADPH oxidized per min per mg enzyme protein.

Table 1 represents the pattern of Hg-volatilization by five mercury-resistant, N_2-fixing bacteria. From the mercury volatilization experiment it was found that some amount of mercury always remained bound to the cellular constituent even after several washings with deionized water. In our experimental conditions we used 62 µM $HgCl_2$, which was significantly higher than the concentration used by other workers. Capacity for Hg-volatilization by these bacteria varied according to their minimum inhibitory concentration towards mercury compounds (Table 1). In the presence of low concentration of mercury compounds, Hg-resistant bacteria could detoxify the toxic Hg^{2+} by completely eliminating all the Hg from the bacterial system. In the presence of high concentration of $HgCl_2$, however, intracellular sequestration by metal-binding components may also take place (Silver 1992). This result supports work of Gachhui et al. (1991) who showed that at high concentrations of mercury, free reduced glutathione levels and glutathione reductase activity were increased within the cell. It is also evident from our work that Hg-resistant bacteria have limited capacity for Hg-volatilization.

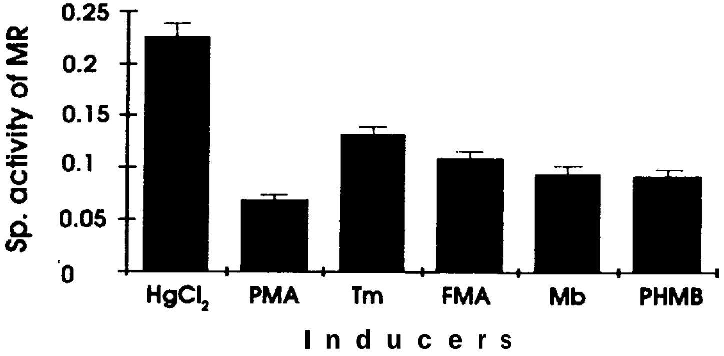

Figure 1. Effect of different agents on the induction of mercuric reductase (MR) activity (mean ±SD; n=4) in *Azotobacter chroococcum* SS_2.

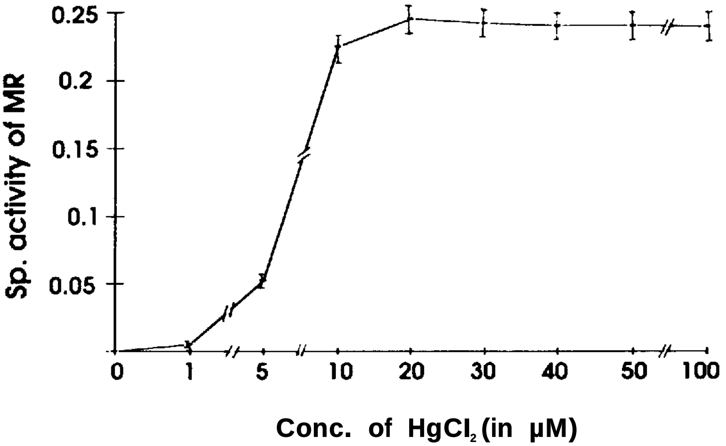

Figure 2. Effect of graded concentration of $HgCl_2$ as inducer for the induction of mercuric reductase (MR) (mean±SD; n=4) In *Azotobacter chroococcum* SS_2

Figure 1 shows specific activity level of mercuric reductase of *Azotobacter chroococcum* SS_2, a broad spectrum, mercury-resistant, nitrogen-fixing soil bacterium, with different inducers. $HgCl_2$ was the best inducer for mercuric reductase activity of this organism. Figure 2 represents the effect of graded concentrations of $HgCI_2$ on the induction of mercuric reductase activity in *Azotobacter chroococcum* SS_2. The optimum concentration for the induction of this enzyme was 20 µM $HgCI_2$. However, no significant difference in enzyme activity was obtained by varying the $HgCI_2$ concentration from 20-100 µM. This indicates that for attaining an optimum level of the enzyme in the mercury-resistant organism SS_2, a suitable concentration of $HgCI_2$ was needed. This also supported our previous experiment of Hg volatilization, where it was shown that even in the presence of a higher concentration of the inducer $HgCl_2$, the percentage of mercury volatilization did not increase. However, the cells could survive even at this high concentration of mercury.

Table 2. Specific activity of organomercurial lyase from *Azotobacter chroococcum* SS_2 in presence of different mercury compounds as inducers and substrates.

Mercury compounds	MIC (n moles /cup)	Inducer (10 µM)	Sp. activity of organomercurial lyase using different substrates					
			PMA	FMA	Tm	Mer	pHMB	MMC
Merbromine Mb	100	Mb	6.65	22.2	6.65	12.39	-	-
Thimersol Tm)	12.5	Tm	15.52	30.32	18.5	10.2	12.22	10.60
p-hydroxy mercuric benzoate (PHMB)	100	pHMB	17.52	28.5	11.68	-	11.68	11.67
Fluorescein mercuric acetate (FMA)	200	FMA	4.78	33.58	4.78	-	-	-
Phenylmercuric acetate (PMA)	20	PMA	20.2	28.79	22.5	-	9.59	14.39
Mercuric chloride	300	$HgCl_2$	15.2	35.3	28.0	14.4	12.86	19.26

Specific activity expressed in nmole of NADPH oxidized per mg enzyme protein.

Table 2 shows the effects of different inducers on organomercurial lyase activity of Hg-resistant *Azotobacter chroococcum* SS_2. Although organomercurial lyase activity was induced with different mercury compounds, e.g., $HgCl_2$, PMA, pHMB, thimersol, FMA and merbromine (Mb), the pattern of induction of lyase was not similar. PMA-induced organomercurial lyase could not utilize pHMB and MMC as substrates. The pattern of inducibility of lyase by FMA-induced cells was interesting as it showed that merbromine, pHMB and MMC were not utilized as substrates by organomercurial lyase induced by FMA. This type of wide-range substrate specificity may have been selected by the pressure of mercury in the mercury-polluted environment as suggested by Nakamura et al. (1990). Thus, the involvement of more than one organomercurial lyase with different substrate specificity cannot be ruled out. Tezuka and Tonomura (1976) reported the presence of two organomercurial lyase in *Pseudomonas* K62 with different substrate specificity.

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