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Volatilization of Mercury by Resting Mercury-Resistant Bacterial Cells

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The mercuric ion reduction system encoded by the Hg^{2+} inducible mer operon confers bacterial resistance to mercuric ion. The mer A gene product which is a FAD-containing enzyme catalyzes the reduction of Hg^{2+} to volatile elemental mercury with the help of intracellular thiols and NADPH as a cofactor (Schottel 1974; Summers and Silver 1978; Fox and Walsh 1982; Misra 1992).

Our earlier studies have shown that growing cells of different mercury-resistant bacteria reduce Hg^{2+} compounds to Hg(O) (Ray et al. 1989; Pahan et al. 1990a; Gachhui et al. 1989). We have also shown the effect of thiol compounds and flavins on mercury-degrading enzyme activities in mercury-resistant bacteria (Pahan et al. 1990b). Here we report that resting cells of mercury-resistant bacteria survive in a buffer system for several hours, synthesize inducible mercury-degrading enzymes and volatilize mercury from a mercury-containing buffer system. We know of no information regarding studies of mercury-degrading enzymes in resting mercury-resistant bacterial cells.

MATERIALS AND METHODS

All chemicals and reagents used in this study were of analytical grade. NADPH was purchased from Sigma Chemicals, St. Louis, MO, USA.

A broad-spectrum mercury-resistant Azotobacter sp. SS_2 was isolated from soil at the agricultural farm, Giridi, India, and was identified in our laboratory following Bergey's Manual of Determinative Bacteriology, Ninth Edition. This strain was grown aerobically in nutrient broth.

Azotobacter sp SS_2 was inoculated in 40 ml of nutrient broth and incubated at 32°C overnight with shaking under air. Overnight culture of bacterial cells was diluted 1:10 with sterile nutrient broth to a final volume of 100 ml. The organisms were

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grown for 4 hr on a rotary shaker (200 rpm) at 32°C. The early log-phase cells were then harvested by centrifugation at 6000 g for 10 min at 4°C and washed 3 times with sterile 50 mM phosphate buffer (pH 7.4). The bacterial cells were then suspended in sterile phosphate buffer (pH 7.4).

Total viable counts were determined by the agar plate method from a suitable portion of culture removed aseptically and diluted serially at different times during the time of incubation.

For the determination of Hg-volatilization from mercury-containing buffer, control flasks received 20 µM $HgCl_2$ without organisms. Four control flasks and four experimental flasks were shaken on a rotary shaker (200 rpm) for up to 12 hr. Twenty-five ml of the culture were withdrawn from the experimental flasks at intervals of 4, 6, 8, 10 and 12 hr. The cells were then harvested by centrifugation at 5000 g for 10 min at 4°C and washed three times with demineralized water. Weighed amounts of these wet cells of each flask were separately taken in 50 ml Erlenmeyer flask and digested to bring all the mercury into ionic form. Similarly, 1 ml of the supernatant after each cell harvesting from the 25 ml culture of each experimental flask and 1 ml of the control buffer containing $HgCl_2$ were separately digested. Mercury content of each set was determined by the cold-vapor atomic absorption spectrometric technique (Bradenberger and Bader 1967; Pahan et al. 1991) as follows : Contents of the flasks were separately digested with equal volumes of concentrated HNO_3 and concentrated H_2SO_4 at 60-70°C for 2 hr. Each digestion mixture was then diluted with distilled water followed by the addition of $KMnO_4$ (5% w/v) and the mixture was kept overnight at room temperature. On the next day 5% hydroxyiamine hydrochloride was added until all the brown MnO_2 and excess $KMnO_4$ were reduced. Mercury content was measured by cold vapor atomic absorption spectrometry. Pure $HgCl_2$ (Sigma Chemicals) was used for calibration of the instrument Mercury Analyzer (MA 5800 ECIL, Hyderabad, India), a cold vapor atomic absorption spectrometer. The level of detection of the instrument is in the range of 10-200 ng Hg in the sample. The percentage recovery of the method is 98%. Cell-free extracts of mercury-resistant organisms were prepared following the procedures of Summers and Silver 1972. After thawing, the wet cells were suspended in cold 50 mM sodium phosphate buffer (pH 7.4) by sonication at 4°C. The distrupted cell suspensions were centrifuged at 15000 x g for 30 min at 4°C. Mercuric reductase activity was determined by measuring Hg^{2+}-induced NADPH oxidation spectrophotometrically at 340 nm. The reaction mixture contained 5 mM Na_2EDTA, 2 mM $41gCl_2$, 1 mM sodium thioglycolate, 33 µM $HgCl_2$ and 50-150 µM NADPH in 50 mM sodium phosphate buffer (pH 7.4). A suitable volume of 10-100 µl cell-free extracts was 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. In the

Table 1. Volatilization of mercury from $HgCl_2$-containing phosphate buffer (pH 7.35) by mercury-resistant resting cells of *Azotobacter* sp SS_2.

Experimental sets	Wet weight of the cell (gm)	Total mercury bound by cells (μg)	Total mercury bound per gram cell mass (μg)	Total mercury retained in 25 ml buffer after volatilization (μg)	Total mercury lost from 25 ml buffer after volatilization (μg)	% of volatilization
Control (without organism) 12 hours	-	-	-	124.63	11.172	8.22
Azotobacter sp SS_2						
4 hr	0.0749	24.4	325.767	103.75	7.65	5.63
6 hr	0.0666	15.475	232.357	59.5	60.825	44.79
8 hr	0.0716	15.475	216.131	39.65	80.675	59.40
10 hr	0.1034	17.85	172.63	29.75	88.2	64.94
12 hr	0.105	17.85	170	29.75	88.2	64.94

25 ml buffer initially contained 135.8 μg mercury. Average of four separate determination is presented.

control set, NADPH oxidation by the cell-free extract without any $HgCl_2$ was monitored under the same conditions. Protein content was measured following the method of Lowry et al. 1951.

RESULTS AND DISCUSSION

Table 1 shows the volatilization of mercury from $HgCl_2$-containing phosphate buffer (50 mM, pH 7.34) by mercury-resistant resting cells of Azotobacter sp SS_2. Uninduced resting cells induced by 20 µM $HgCl_2$ volatilized only 5.63% Hg from the buffer after 4 hr. 'The percentage of mercury volatilization gradually increased with time and reached a maximum level after 8 hr. The percentage of mercury volatilization as well as specific activity of mercuric reductase significantly increased during 4 to 6 hr. Mercuric reductase activity was first detected after 4 hr. The specific activity value of the enzyme reached a maximum after 8 hr and then the activity of the enzyme decreased during 10 and 12 hr of incubation. The amount of mercury which was bound per gram cell mass was maximum at 4 hr and then the value gradually decreased during 12 hr of incubation. In our assay system we detected that duration taken for induction of Hg-reductase was not less than 4 hr. These data could be correlated with the results of the growth curve of mercury-resistant organisms (data not shown). Uninduced cells after mercury treatment took 3 hr more lag phase of growth than the mercury-induced control cells which were later grown in the presence of $HgCl_2$. For the induction of mer operon, the inducer Hg^{2+} is to be transported with the help of the transport proteins mer P, mer T and mer C within the cell and thus switching on the synthesis of mercury-resistant protein components (Misra 1992). The decrease in the activity of mercuric reductase after 8 hr may have been due to the cell death in the presence of mercury (Table 2).

Tabale 2. Induction of mercuric reductase in resting cells of Azotobacter sp SS_2 with time.

Hours of incubation of the resting cells in buffer system	Specific activity of Hg^{2+}-reductase
4	**2.35**
6	**9.35**
8	**18.21**
10	11.20
12	**8.80**

Sp activity expressed in n mol. NADPH oxidised per min per mg enzyme protein.

Table 3. Viable count of resting *Azotobacter* sp SS_2 incubated in the presence of buffer.

Hours of incubation of the resting cells in buffer	Control cell number without $HgCl_2$	Cell number with $HgCl_2$	% survival of control cells	% survival of $HgCl_2$-treated cells
0 hr	1.52×10^{10}	1.915×10^{10}	100	100
4 hr	1.44×10^{10}	1.52×10^{10}	94.73	79.37
6 hr	1.32×10^{10}	1.32×10^{10}	86.84	68.92
8 hr	1.11×10^{10}	1.15×10^{10}	73.02	60.05
10 hr	0.95×10^{10}	0.86×10^{10}	62.5	44.90
12 hr	0.92×10^{10}	0.65×10^{10}	60.52	33.94

The average of four separate determinations is presented.

It was found that only 33.94% of the cells survived after 12 hr in buffer containing 20 μM $HgCl_2$. The decreased activity of mercuric reductase after 12 hr may also have been due to the denaturation of the enzyme after 12 hr incubation in phosphate buffer. As these organisms can volatilize mercury compounds from the buffer in the resting condition, it is clearly evident from our experimental data that even in conditions that are not suitable for growth of the Hg-resistant bacteria) they would be able to detoxify mercury compounds. This experiment shows that resting mercury-resistant bacteria may serve as a tool for mercury scavanging.

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