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Bacterial Degradation and Utilization of Merbromine and Fluorescein Mercuric Acetate

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Fluorescein mercuric acetate (FMA) and Merbromine (MB) like other organomercurials are potent inhibitors of growth and metabolic activities of microorganisms (Summers and Silver 1978). Resistance to organomercurials such as phenylmercuric acetate (PMA), thimersol, methylmercuric chloride (MMC), ethylmercuric chloride (EMC), methoxyethyl mercuric chloride (MEMC) of broad-spectrum Hg-resistant bacteria (Summers and Silver 1978) is due to the activities of two enzyme systems namely organomercurial lyase which degrades C-Hg bond of organomercurials to liberate Hg^{2+}. Thereafter mercuric reductase catalyzes its reduction to Hg° (Summers and Silver 1978; Robinson and Touvinen 1984). Hg-resistant bacteria (both narrow-spectrum and broad-spectrum) are resistant to FMA and MB and these chemicals are gratuitous inducers of both mercuric reductase and organomercurial lyase yet the enzymatic degradation of these two Hg-compounds are not yet documented (Summers and Silver 1978; Schottel 1978; Fox and Walsh 1982; Nucifora *et al* 1989). Nonpermeability of these organomercurials to cytoplasmic membrane has been reported for cases of bacterial resistance to these chemicals (Summers and Silver 1978; Robinson and Touvinen 1984).

We have isolated two broad-spectrum Hg-resistant bacterial strains, *Bacillus pasteurii* DR_2 and *Klebsiella pneumoniae* KR_2, from a Hg-polluted water source which could degrade $HgCl_2$, PMA and thimersol enzymatically (Pahan *et al* 1990). The former strain DR_2 also can utilize different aromatic hydrocarbons as sole sources of carbon (Pahan *et al* 1991). Here we report the enzymatic degradation of FMA and MB by these two bacterial strains and utilization of FMA by the former strain as sole source of carbon.

MATERIALS AND METHODS

All chemicals and reagents used in this study were of analytical grade (E. Merck, Darmstadt, Germany). FMA, MB and NADPH

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(tetrasodium salt) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

All the Hg-resistant bacterial strains used in this study were isolated from different water sources (Pahan *et al* 1990). Cell-free extracts (c.f.e.) of these organisms were prepared following the procedure of Summers and Silver (1972). Hg induced cells (induced 3X with 10 µM $HgCl_2$) were disrupted mechanically in a mortar-pestle with sea-sand at 4°C. Disrupted cells were suspended in cold 50 mM sodium phosphate buffer (pH 7.35) and centrifuged at 15,000 x g for 30 min at 4°C . Most of the Hg^{2+}-reductase and organomercurial lyase activities were precipitated with 0-50% $(NH_4)_2SO_4$ at 4°C (Pahan *et al* 1990) and the precipitate was dissolved in a minimum volume of the same cold buffer containing 0.25 mM reduced glutathione (GSH). Samples were then dialysed overnight against the same buffer at 4°C. The dialysates were used to study the degradation of FMA and MB. To assay the degradation reaction of FMA and MB, 10-100 µL of c.f.e. along-with 5 mM Na_2EDTA, 2 mM $MgCl_2$ and 1 mM thiol compouund was first incubated for different intervals (½ hr, 1 hr, 1½ hr, 2 hr and 2½ hr) with 30 µM FMA or MB in a total volume of 1 mL made by 50 mM sodium phosphate buffer (pH 7.35). The assay was started by adding 0.15 mM NADPH and its oxidation was monitored at 340 nm. To study the effects of thiol compounds on degradation reaction, each of sodium thioglycollate, β-mercaptoethanol, cysteine, glutathione and dithiothreitol was used separately. Protein was determined by the method of Lowry *et al* (1951).

For studies of utilization of FMA and fluorescein as sole sources of carbon, the bacterial cells were grown overnight in a synthetic medium (NH_4Cl - 1.0 g; $MgSO_4.7H_2O$ - 0.13 g; $Na_2HPO_4.2H_2O$ - 6.0 g; KH_2PO_4 -3.0 g; glucose 4 g and water 1 L) containing 30 µM benzene. The next morning the bacterial culture was aseptically diluted 100 times with sterile glucose-free synthetic media containing different concentrations (0-300 µM) of FMA as the carbon source in different flasks. Three flasks per dose were used. All flasks were placed on a rotary shaker (200 rpm) at 32°C. Total viable count was determined by the agar plate method from suitable portions of culture taken out aseptically and diluted serially after 24 hrs of growth. An average of six separate counts was made. The overnight bacterial culture was also similarly diluted 100 times with sterile synthetic media containing 200 µM of FMA or fluorescein as the carbon source in different flasks. Control flask containing the organism received neither glucose nor these compounds. Flasks were placed in the rotary shaker and at different hours of growth, bacterial count was determined as mentioned earlier. To determine the concentration of FMA and fluorescein in the supernatant at different intervals of growth, cells were harvested as mentioned earlier. A suitable portion of supernatant was diluted with same buffer, and concentration

of FMA and fluorescein was determined in a fluorescence Spectrophotometer, Model F-3010, Hitachi, Japan, (excitation at 238 nm; emission at 517 nm) using a standard curve drawn by known concentrations of FMA or fluorescein.

Bacterial cells were grown in synthetic media containing either 200 µM FMA or fluorescein as sole sources of carbon for 24 hrs on a rotary shaker (200 rpm). The cells were harvested and washed 3X with 50 mM sodium phosphate buffer (pH 7.35). The washed cells were suspended in the same buffer and oxygen consumption was measured polarographically in a Gilson Model 5\6 oxygraph, (Gilson Medical Electronics, Villevs-le-bel, France) by using these two compounds as sole sources of carbon following the method of Spain and Nishino (1987).

RESULTS AND DISCUSSION

Figs 1 and 2 show that c.f.e. of two broad-spectrum Hg-resistant bacterial strains *K. pneumoniae* KR_2 and *B. pasteurii* DR_2 carried out FMA and MB induced oxidation of NADPH indicating the enzymatic degradation of these organomercurials. With the increase in the incubation period of the c.f.e. with these Hg-compounds, the rate of the degradation of MB and FMA also increased. In case of MB, the enhancement of O.D. change was found upto 1½ hr to 2 hr and in case of FMA, it was found upto 2½ hr. On 5 min incubation, in both the cases, there was almost negible O.D. change. C.f.e. of these bacterial strains without prior induction with $HgCl_2$ failed to degrade these mercurials indicating the inducible nature of this enzymatic degradation. Again the presence of thiol compounds in the reaction mixture increased the degradation rate of these Hg-compounds. Among five different thiol compounds used, GSH was the best in stimulating the degradation rate of both the compounds (data not shown). It is interesting to note that c.f.e. of a narrow-spectrum Hg-resistant bacterial strain, *E. coli* ACR_2, was a poor degrader of both FMA and MB. However, *B. pasteurii* DR_2 degraded FMA much better than *K. pneumoniae* KR_2 whereas the latter strain degraded MB better than the former strain. This work was undertaken to monitor the degradation of MB and FMA by Hg-resistant bacterial strains. Results clearly indicated the involvement of both organomercurial lyase and Hg^{2+}-reductase in these bacterial strains and these enzymes are probably needed to degrade MB and FMA. It was reported earlier (Pahan *et al* 1990) that GSH was the best thiol compound in stimulating the activities of both Hg^{2+}-reductase and organomercurial lyase in these bacterial strains. Here it was also found that a thiol compound, preferably GSH, was needed for optimum rate of degradation of these organomercurials.

It is evident from Fig 3 that with increasing concentrations of FMA used as sole source of carbon, cell number of *B. pasteurii* DR_2 gradually increased up to 200 µM compared

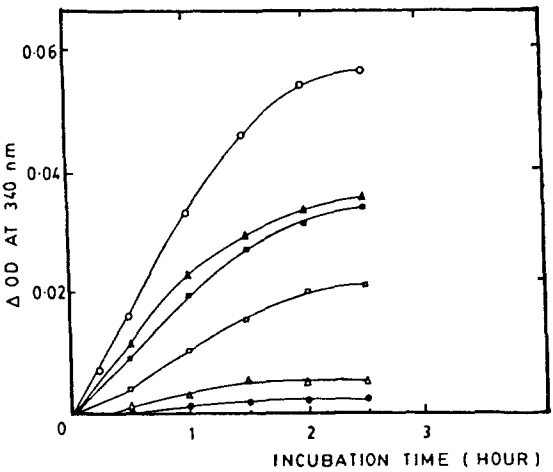

Figure 1. FMA-dependent NADPH oxidation by some Hg-resistant bacterial strains. O—O CFE (≡ 0.42 mg protein) of *B. pasteurii* DR_2 with thiol compound and ▲—▲ without thiol compound; ■—■ CFE (≡ 0.45 mg protein) of *K. pneumoniae* KR_2 with thiol compound and □—□ without thiol compound; Δ—Δ CFE (≡ 0.48 mg protein) of *E. coli* ACR_2 with thiol compound; ●—● without CFE (control).

to control set (without any carbon source). So 200 µM of FMA and fluorescein was used throughout the study.

Table 1. Multiplication of *B. pasteurii* DR_2 in the presence of FMA and fluorescein as sole sources of carbon

Experimental sets	Number of bacteria present per ml after	
	12 hrs	24 hrs
200 µM FMA	7.52×10^7	2.15×10^8
200 µM Fluorescein	1.22×10^8	4.08×10^8
Control (without FMA or fluorescein)	2.80×10^7	3.00×10^7

1.7×10^7 cells/ml was added initially

Table 1 shows that growth of *B. pasteurii* DR_2 is supported by FMA and fluorescein as the sole source of carbon. The control flask contained no FMA and fluorescein and the growth of the bacterial strain was continued upto 24 hrs. Higher cell

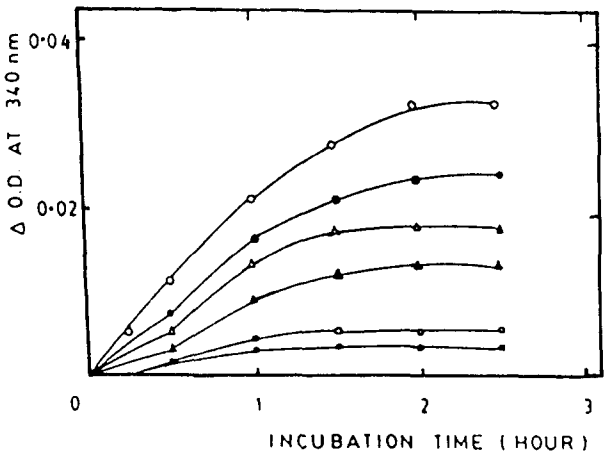

Figure 2. MB-dependent NADPH oxidation by some Hg-resistant bacterial strains. O—O CFE (≡ 0.45 mg protein) of *K. pneumoniae* KR_2 with thiol compound and ●—● without thiol compound; Δ—Δ CFE (≡ 0.42 mg protein) of *B. pasteurii* DR_2 with thiol compound and ▲—▲ without thiol compound; □—□ CFE (≡ 0.48 mg protein) of *E. coli* ACR_2 with thiol compound; ■—■ without CFE (control).

Table 2. Utilization of FMA and fluorescein by *B. vasteurii* DR_2

Experimental sets	Amount (μM) present in the supernatant after				% Utilized after 24 hrs
	0 hr	4 hrs	12 hrs	24 hrs	
FMA without DR_2	200	200	200	192	4
FMA with DR_2	200	180	130	70	65
Fluorescein without DR_2	200	200	194	190	5
Fluorescein with DR_2	200	164	107	28	81

number in presence of fluorescein than in presence of FMA indicated that the former compound served as a better utilizable material for the organism. The organism could utilize 61% and 76% of FMA and fluorescein respectively at the end of 24 hrs (Table 2). Suspensions of two different types of washed cells pre-exposed to FMA and fluorescein oxidised these two substrates (Table 3). However, the rate of oxidation

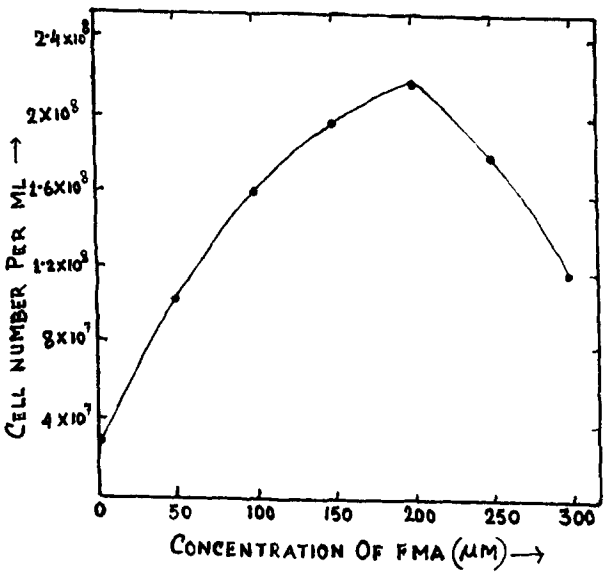

Figure 3. Multiplication of *B. pasteurii* DR_2 in presence of different concentrations of FMA as sole source of carbon after 24 hrs. 1.7 X 10^7 cells ml^{-1} was added initially.

Table 3. Oxygen consumption by washed cells of *B. pasteurii* DR_2

	Rate (nmol/min/mg of protein) of oxygen consumption by washed cells after growth with	
Substrates	FMA	Fluorescein
FMA	23	20
Fluorescein	31	36

of fluorescein was higher than that of FMA. That the organism could utilize fluorescein better than FMA is substantiated by its stimulated growth in the presence of fluorescein (Table 1). *K. pneumoniae* KR_2 degraded FMA enzymatically but it was unable to utilize it as sole source of carbon. Although both the bacterial strains *B. pasteurii* DR_2 and *K. pneumoniae* KR_2 degraded MB but none of them was able to utilize it as the sole soource of carbon.

B. pasteurii DR_2 utilizes different aromatic hydrocarbons as sole sources of carbon (Pahan *et al* 1991) and plasmid involvement in such degradation by other bacterial strains

was also reported (Friello and Chakraborty 1976; Kiyohara *et al* 1983). So utilization of FMA was mainly due to the release of fluorescein moeity possibly through the action of organomercurial lyase which was then utilized as sole source of carbon.

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