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Enhanced Elimination of $HgCl_2$ from Natural Water by a Broad-Spectrum Hg-Resistant *Bacillus pasteurii* Strain DR2 in Presence of Benzene

K. Pahan, J. Chaudhuri, D. Ghosh, R. Gachhui, S. Ray, A. Mandal

Department of Biochemistry, University of College of Science, 35 Ballygunge Circular Road, Calcutta 700 019, India

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Mercury (Hg) compounds are extensively used in agriculture as seed-dressers and pesticides, in hospitals as disinfectants (Summer and Silver 1978), in sewage treatment and in industries including pulp and paper and chloro-alkali industries as chemical catalysts. Several thousand millions of tons of mercury are dispersed into the environment every year (Summers and Silver 1978). Rain water washes mercury from soils and rocks. In aquatic environments sediments are the richest deposits of mercury compounds (Summers and Silver 1978). The mutagenicity and teratogenicity of these Hg-containing compounds are well-documented (Mix 1986; Summers and Silver 1978).

In recent years hydrophobic organic chemicals have contaminated numerous aquifers. Gasoline from underground storage tanks, petroleum products from refineries and chlorinated solvents from cleaning operations in various industries are some examples of sources of groundwater contamination. In addition, there is always the risk of new contamination from accidental spills and discharges. Many of these compounds are also carcinogenic and enhancers of mutagenicity (Mix 1986; Sato *et al.* 1983). Both mercury compounds and hydrophobic aromatic compounds have contaminated several aquatic environments and caused damage to the indigenous biota (Cocchieri *et al.* 1993). The potential damage to water bodies necessitates that attention be directed to developing methods for removing these contaminants from aquifers.

Several Gram positive and negative bacteria can effectively detoxify mercury compounds through the sequential action of mercuric reductase and organomercurial lyase as reviewed by Summers and Silver (1978). In many cases bacterial resistance of mercury compounds is plasmid-mediated (Schottel *et al.* 1974; Silver and Misra 1988). Aromatic compounds are also degraded and utilized as a sole source of carbon by different plasmid-bearing bacterial strain (Dong *et al.* 1992). Of the many options available for the remediation of polluted water sources,

Correspondence to: A. Mandal

exploitation of microorganisms to scavenge these pollutants has been suggested in many studies (Bury and Miller 1993; Summers and Silver 1978).

We previously isolated a broad-spectrum, Hg-resistant *Bacillus pasteurii* strain (DR2) that volatilized Hg-compounds, including organomercurials from its growth media and utilized, different aromatic compounds as the sole source of carbon (Pahan *et al.* 1990; Pahan *et al.* 1991a). This strain can also degrade and utilize fluorescein mercuric acetate and merbromine (Pahan *et al.* 1992). Its growth was also stimulated in the presence of benzene and phenylmercuric acetate due to facilitated transport of the nutrients across the cell wall (Pahan *et al.* 1993a). The objective of the present study was to utilize the dual characteristics of the organism, elimination of Hg-compounds and utilization of aromatic compounds, in natural conditions. We report the increased rate of the elimination of $HgCl_2$ by this strain from natural river water in the presence of organic compounds.

MATERIALS AND METHODS

All chemicals and reagents used in this study were of analytical grade (E. Merck, U.K.). Water samples were collected from the river Ganges which is reported to contain 0.11±0.01 ppm mercury (Pahan *et al.* 1990b). Bacterial strain *B. pasteurii* DR2 was grown in nutrient broth media containing 10 μM $HgCl_2$ and 30 μM benzene. $HgCl_2$ was added to induce mercuric reductase and organomercurial lyase (Pahan *et al.* 1990a) and benzene was added to stimulate growth (Pahan *et al.* 1993a). Flasks were kept overnight in a rotary shaker at 200 rpm at 32°C. This bacterial culture was diluted 1:100 with sterile river water to a total volume of 200 ml to maintain 1.7 X 10^7 bacterial cells/ml. After sterilization of the water, solutions of benzene and petroleum ether (boiling range 60°-80°C) were added to separate flasks to a concentration of 1 mM. Solutions of these aromatic compounds were made in 90% ethanol and these solutions were not sterilized. The water sample was sterilized by autoclaving after cotton plugging. Control flasks containing the organisms only were also run. To some flasks $HgCl_2$ was added at a concentration of 10 μM and PMA at 30 μM. A similar overnight culture was also diluted with sterile river water which received after sterilization, different concentrations of benzene (1 to 10 mM). All the flasks were kept in a B.O.D. incubator at 20°C. At intervals of several days portions of the samples were taken out aseptically and diluted serially with sterile potassium phosphate buffer (0.1 M, pH 7.0) and bacterial counts were determined by spreading the definite volumes of the diluted bacterial suspension on a nutrient agar. An average of six separate determinations were taken in each case. The bacterial culture treated with 10 μM $HgCl_2$ was added to a final concentration of 100 μM. Benzene and petroleum ether was added to separate sterile flasks at 1 mM concentration. Control flasks were

also run; one flask contained only sterile river water, another flask contained only organisms and no $HgCl_2$ and the third flask contained only $HgCl_2$. In similar experiments, $HgCl_2$-treated bacterial cultures were also diluted 1:100 with non-sterile river water containing 1 mM benzene and 100 μM $HgCl_2$. Respective control flasks were also run. All the flasks were incubated at 20°C and the mercury content was measured using cold vapour atomic absorption spectrometry (Bradenberger and Bader 1967). Contents of the flasks were digested with equal volumes of concentrated HNO_3 and concentrated H_2SO at 60°-70°C for 2 hr. The 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% hydroxylamine hydrochloride was added until all the brown MnO_2 and excess $KMnO_4$ were reduced. Mercury content was measured by cold vapour atomic absorption spectrometry.

RESULTS AND DISCUSSION

The survival of the organisms in natural water is a prerequisite for its ability to remove toxicants from the system. In our earlier studies, we found that Hg-detoxifying enzymes of Hg-resistant bacterial strains isolated from different aquatic environments were most stable at 20°C which is the ambient temperature of the water bodies (Pahan *et al.* 1993b). In these studies the flasks containing the bacterial strains were kept at 20°C for several days. From Fig. 1A and 1B it is clear that in sterile river water this bacterial strain survived for 30 days. The presence of 1 mM benzene or petroleum ether increased its survival rate compared to the control. As *B. pasteurii* DR2 utilized aromatic compounds as the sole source of carbon from its growth medium (Pahan *et al.* 1991a), this increase in growth in sterile river water in the presence of benzene and petroleum ether was probably due to the utilization of these compounds as nutrients. The number of bacteria increased significantly in the presence of these compounds after 7 days (Fig. 1A) and was not significantly affected in the presence of either 100 μM $HgCl_2$ or 30 μM PMA after 7 days. Conversely, the presence of PMA increased the bacterial number (Fig. 1B). This increase in growth of the bacterial strain in the presence of PMA may have been due to the facilitated transport of nutrients through the cell membrane (Pahan *et al.* 1993a), to the utilization of benzene moiety released from PMA through the action of organomercurial lyase present in the organism (Pahan *et al.* 1991b), or both. However, with the increase in incubation period, the bacterial number gradually decreased. Increasing concentration of benzene in the range of 1.6 mM in sterile river water stimulated the gradual increase in the number of organisms after 15 days, indicating that this compound was being used as a nutrient (Fig. 2). Higher concentrations of benzene were inhibitory. This Hg-ressistant aquatic isolate volatilized Hg-compounds from its growth medium and utilized

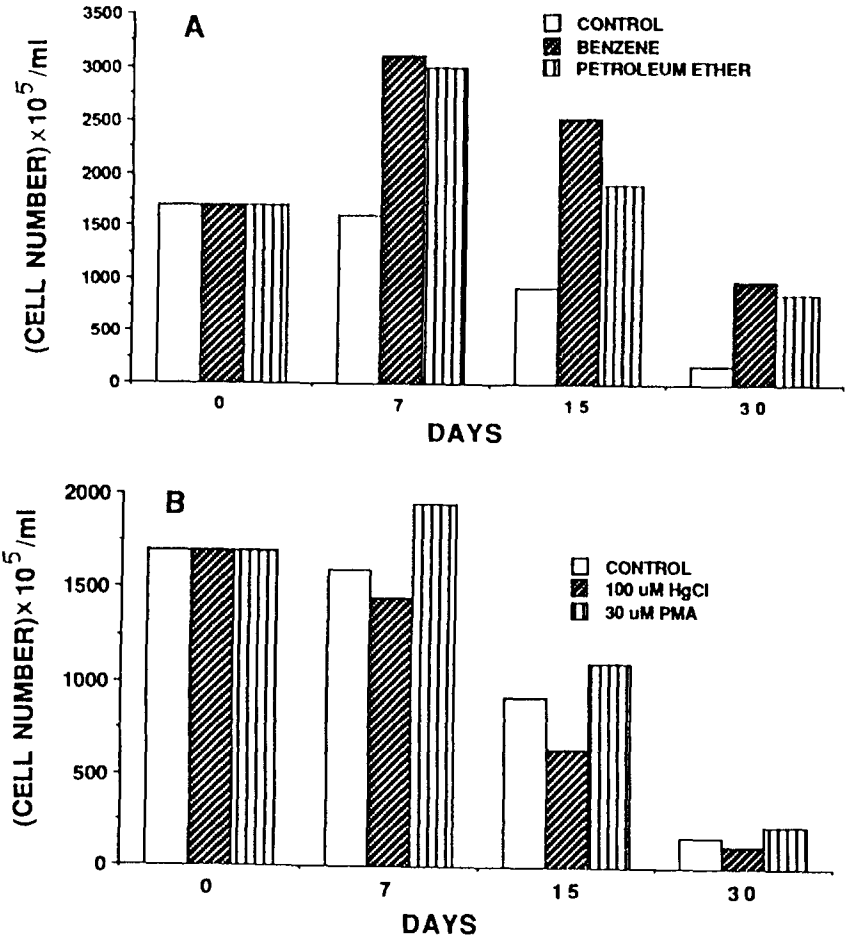

Figure 1. Survival of *B. pasteurii* DR2 in sterile river water (A) in presence of benzene and petroleum ether; and (B) in presence of $HgCl_2$ and phenylmercuric acetate. Initially 3.4 X 10^9 bacterial cells were added to 200 ml sterile river water to maintain 1.7 X 10^7 cells/ml. The results are the average of two separate determinations.

aromatic compounds as the sole source of carbon (Pahan *et al.* 1991a). Figure 3 shows that this bacterial strain was able to eliminate $HgCl_2$ from sterile river water as compared to the control experiment and it is also noteworthy that the presence of benzene and petroleum ether at 1 mM concentration enhanced this rate of mercury volatilization. Addition of the same concentration of benzene or petroleum ether to the control in absence of the organism did not increase the rate volatilization (data not shown). Hg-volatilization rate was slightly higher in the presence of benzene than petroleum ether and this volatilization from sterile water indicates that it was mediated by only this organism. Similar experiments were conducted with non-sterile river water where the

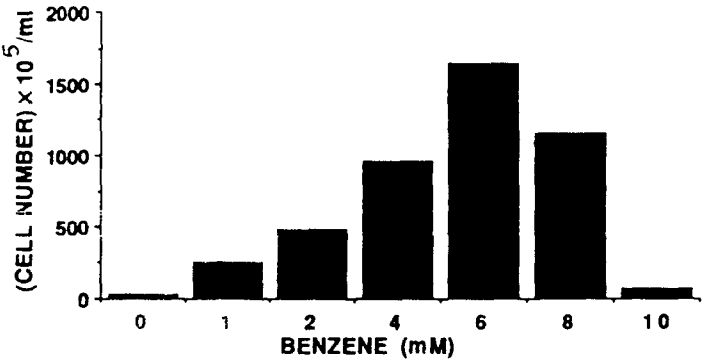

Figure 2. Effect of graded concentrations of benzene on the survival of *B. pasteurii* DR2 in sterile river water. Initially 3.4 X 10^9 bacterial cells were taken in 200 ml sterile river water to maintain 1.7 X 10^7 cells/ml and bacterial counts were determined after 15 days. The data presented are the average of two separate determinations.

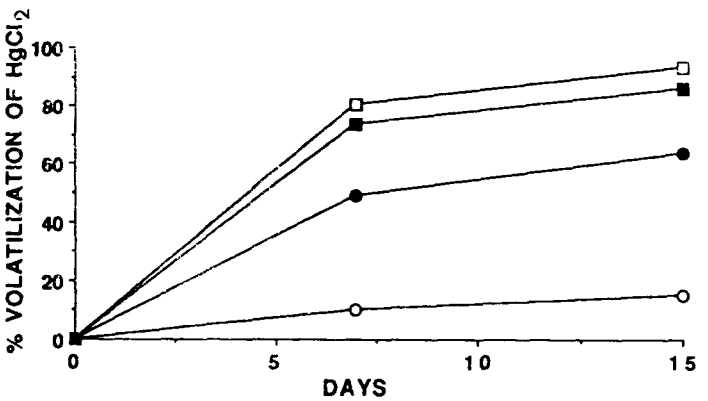

Figure 3. Volatilization of $HgCl_2$ by *B. pasteurii* DR2 from sterile river water in presence of benzene and petroleum ether (O—O, without organism; ●—●, with organism; □—□ with organism and 1 mM benzene; ■—■ with organism and 1 mM petroleum ether). 200 ml sterile river water initially contained 0.025 mg mercury and 100 μM $HgCl_2$ equivalent to 4 mg mercury was added to each flask. 1% (v/v) inoculum was added initially to maintain 1.7 X 10^7 cells/ml. Average of two separate determinations is presented.

addition of 1.7 X 10^7 cells of this bacterial strain per ml of water increased volatilization of $HgCl_2$ (Table 1). Of greater interest is the comparison between the volatilization rate in the presence of this organism and that in the presence of

Table 1. Volatilization of Hg-compounds by *B. pasteurii* DR2 from non-sterile river water in the presence of 1 mM benzene.

Experimental sets		Time in (days)	Amount of mercury present initially (mg)	Final amount of mercury present (mg)	Amount of mercury removed (mg)	% of mercury volatilization
- Hg compounds	Control	7	0.035	0.023	0.012	34.28
- DR2		15	0.035	0.020	0.015	42.85
- Hg compounds		7	0.035	0.019	0.016	45.71
+ DR2		15	0.035	0.011	0.024	68.57
- 100 μM $HgCl_2$	Control	7	4.035	2.025	2.010	49.81
- DR2		15	4.035	1.72	2.315	57.37
- 100 μM $HgCl_2$		7	4.035	1.21	2.825	70.01
+ DR2		15	4.035	0.096	3.039	75.31
+ 100 μM $HgCl_2$	Control	7	4.035	2.040	1.995	49.44
+ 1 mM benzene -DR2		15	4.035	1.785	2.250	55.76
+ 100 μM $HgCl_2$		7	4.035	1.004	3.031	75.11
+ 1 mM benzene +DR2		15	4.035	0.545	3.49	86.49

200 ml non-sterile river water initially contained 0.035 mg mercury. 1% (v/v) inoculum was added initially to maintain 1.7×10^7 cells/ml 100 μM. $HgCl_2$ equivalent to 4 mg mercury was added to respective flasks. Average of two separate determinations is presented.

both organism and 1 mM benzene; the rate of volatilization being higher in the latter case (Table 1). In all cases the rate of volatilization was higher in the first seven days than in the next seven days.

The data presented here demonstrate clearly that this broad-spectrum Hg-resistant, hydrocarbon-utilizing and sporeforming *B. pasteurii* DR2 isolated from water can survive in both sterile and non-sterile river water and at the same time can eliminate $HgCl_2$ from both. The aromatic compounds served as its nutrients and facilitated its survival and the rate of elimination of mercury. Removal of mercury from waste-water and from mercury-polluted soil samples have been studied by several workers (Campanella *et al.* 1986; Rogers and James 1979) and bacteria-mediated removal of aromatic compounds from soil and water have also been reported (Mahmood and Rama Rao 1993; Bury and Miller 1993). Our results indicate that bacteria have the potential of removing both mercury contaminants and organic pollutants. Further experiments are needed to optimize the conditions and to study the pathogenicity of the strain.

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REFERENCES

Bradenberger H, Bader H (1967) Determination of nanogram levels of mercury in solution by flameless atomic absorption technique. Atomic Absorption Newsletter 6 : 101-103

Bury SJ, Miller CA (1993) Effect of miceller solubilization on biodegradation rates of hydrocarbons. Environ Sci Technol 27 : 104-110

Campanella L, Cardarella E, Ferriand T, Petronio (1986) Mercury removal from petrochemical wastes. Wat Res 20 : 63-66

Cocchieri RA, Prete UD, Arness A, Giuliano M, Roncioni A (1993) Heavy metals and polycyclic aromatic hydrocarbons in marine organisms from the Ionian sea (Italy). Bull Environ Contam Toxicol 50 : 618-625

Dong FM, Wang LL, Wang CM, Cheng JP, He ZQ, Sheng ZJ, Shen RQ (1992) Molecular cloning and mapping of phenol degradation genes from *Bacillus stearothermophilus* FDTP-3 and their expression in *Escherichia coli*. Appl Environ Microbiol 58 : 2531-2535

Mahmood SK, Rama Rao P (1993) Microbial abundance and degradation of polycyclic aromatic hydrocarbons in soil. Bull Environ Contam Toxicol 50 : 486-491

Mix MC (1986) Cancerous diseases in aquatic animals and their association with environmental pollutants : critical literature review. Mar Environ Res 20 : 1-141

Pahan K, Ray S, Gachhui R, Chaudhuri J, Mandal A (1990a) Effect of thiol compounds and flavins on mercury and organomercurial degrading enzymes in mercury resistant aquatic bacteria. Bull Environ Contam Toxicol 44 : 216-223

Pahan K, Gachhui R, Ray S, Chaudhuri J, Mandal A (1990b) Characteristics of mercury resistant bacteria from West Bengal rivers. Ind J Microbiol 30 : 35-44

Pahan K, Ray S, Gachhui R, Chaudhuri J, Mandal A (1991a) Volatilization of mercury compounds and utilization of aromatic hydrocarbons by a broad-spectrum mercury resistant *Bacillus pasteurii* strain. Bull Environ Contam Toxicol 46 : 591-598

Pahan K, Gachhui R, Ray S, Chaudhuri J, Mandal A (1991b) A PMA degrading constitutive organomercurial lyase in a broad-spectrum mercury resistant *Bacillus pasteurii* strain DR2. Ind J Expt Biol 29 : 1147-1149

Pahan K, Ray S, Gachhui R, Chaudhuri J, Mandal A (1992) Bacterial degradation and utilization of merbromine and fluorescein mercuric acetate. Bull Environ Contam Toxicol 48 : 421-427

Pahan K, Gachhui R, Ray S, Chaudhuri J, Mandal A (1993a) Stimulatory effect of phenylmercuric acetate and benzene on the growth of a broad-spectrum mercury-resistant strain of *Bacillus pasteurii*. J Appl Bacteriol 74 : 248-252

Pahan K, Gachhui R, Ray S, Chaudhuri J, Mandal A (1993b) Heat sensitivity of mercuric ion and organomercurial degrading enzymes of aquatic mercury-resistant bacteria. World J Microbiol Biotechnol 9 : 180-183

Rogers RD, James CM (1979) Factors influencing the volatilization of mercury from soil. J Environ Quality 8 : 255-260

Sato T, Momma T, Ose Y, Ishikasa T, Kato K (1983) Mutagenicity of Nagara River sediment. Mut Res 118 : 257-267

Schottel J, Mandal A, Clark D, Silver S, Hedges RW (1974) Volatilization of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. Nature (London) 251 : 335-337

Silver S, Misra TK (1988) Plasmid-mediated heavy metal resistances. Ann Rev Microbiol 42 : 717-743

Summers AO, Silver S (1978) Microbiol transformation of metals. Ann Rev Microbiol 32 : 637-672