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Mercury Resistant Bacteria Isolated from Sediment

Jack T. Trevors

Department of Environmental Biology, Ontario Agricultural College, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Studies have shown that mercury resistance and mercuric reductase activity in some bacteria may be plasmid-encoded (Meissner and Falkinham, 1984). These researchers isolated a strain of Mycobacterium scrofulaceum, containing a 115 x 10^6 daltons plasmid that encoded for resistance to mercuric chloride ($HgCl_2$). A plasmid cured derivative of the same organism failed to grow in the presence of 100 μM $HgCl_2$, and also displayed no mercuric reductase activity. Other metal-resistant bacteria have also been isolated and studied (Silver, 1981; Marques et al., 1979; Nakahara et al., 1977; Trevors et al., 1985). Haefeli et al. (1984) recently isolated a silver-resistant strain of Pseudomonas stutzeri, which contained a 49.4 x 10^6 daltons plasmid controlling resistance to this metal. This was one of the first reported cases of plasmid-encoded silver resistance in pseudomonads and clearly illustrates the paucity of knowledge that exists on the ecology, physiology, and genetics of metal-resistance.

Other studies on metal-tolerant bacterial populations in natural and metal-polluted soils have been carried out by Duxbury and Bicknell (1983). They suggested in general, Gram-negative bacteria are more metal-tolerant than Gram-positive bacteria. Also, in soils where toxic metal concentrations are very low, bacteria may be able to function without the presence of a metal resistance plasmid. Kelley and Reanney (1984) suggested that translocatable DNA elements encoding for mercury resistance may provide an explanation for the dissemation and maintenance of mercury resistant microbial populations.

In determining heavy metal resistance in bacteria, it is difficult to precisely designate the concentration of heavy metal that is tolerated before an organism is considered resistant. The amount used in the present study was 100 μM. This was based on the findings of Meissner and Falkinham (1984) who identified a number of bacterial strains resistant to 100 μM $HgCl_2$. The isolation of bacterial strains resistant to mercuric chloride is reported in this investigation. Also, the plasmid content of the resistant isolates was examined.

MATERIALS AND METHODS

Sediment samples were collected from the surface 10 cm of the Speed River, Guelph, Ontario, Canada. Samples were stored for no longer than 48 h at 4°C in the dark prior to being used. Serial dilutions of sediment were prepared in sterile distilled water and plated on LB agar (tryptone: 10 g, yeast extract: 5 g, NaCl: 5g, agar: 15 g, distilled water 1000 ml) and supplemented with 100 µM $HgCl_2$. The $HgCl_2$ was filter-sterilized separately, and aseptically added to the LB agar just prior to pouring the plates. Petri plates were incubated at 25°C for 72 h in the dark at which time different colony types were isolated and purified by restreaking at least 5-10 times on LB agar supplemented with 100 µM $HgCl_2$. Pure isolates were then maintained in LB broth containing 100 µM $HgCl_2$, and used in identification, growth, and plasmid isolation experiments.

Isolates were identified using standard morphological and biochemical tests. Cell morphologies and Gram-stain reactions were observed using a Zeiss phase contrast microscope. Identification of the isolates was based on Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974) and Cowan (1979).

Plasmid isolation was carried out using the method described by Trevors (1984). Pure cultures were inoculated into 10 ml of LB broth (supplemented with 100 µM $HgCl_2$) contained in screw-capped test tubes and incubated for 16 h with shaking at 120 rpm at 25°C in the dark. Cells were harvested by centrifuging 1.5 ml of the bacterial culture in a sterile polypropylene centrifuge tube for 1 min at 15,600 x g. The supernatant was discarded and 400 µl of a mixture containing 8% sucrose, 5% Triton X-100, 50 mM EDTA and 10 mM Tris-HCl (pH 8) was added. The tube contents were gently mixed by tapping the centrifuge tube until the cell pellet was completely resuspended. A 50 µl volume of freshly prepared lysozyme solution (10 mg ml^{-1} in 10 mM Tris-HCl, pH 8) was added to each tube, and the contents mixed by gently rolling the tube several times. The tube was placed in a 100°C water bath for 30 seconds, at which time it was removed and centrifuged at 11,500 x g for 10 min at room temperature. The pellet was removed from the bottom of the centrifuge tube using a sterile toothpick.

To the remaining supernatant, 50 µl of cold (4°C) 3M sodium acetate and 420 µl of cold (4°C) isopropanol were added. The tubes were incubated at -20°C for 30 minutes and then centrifuged at 11,500 x g for 15 min at 4°C. The supernatant was carefully decanted and the tube inverted over a clean absorbent paper towel, and allowed to drain for 2-3 min. Fifteen µl of cold TE buffer (50 mM Tris, 1 mM EDTA, pH 8) was added to the tube which was then incubated for 1 h at 4°C in the dark.

Prior to horizontal agarose gel electrophoresis, 15 µl of loading solution (filter-sterilized 40% sucrose, 0.25% bromophenol blue) was added to each tube. A 10 µl sample was pipetted into each agarose well. Electrophoresis was carried out in 0.7% (w/v)

agarose (BRL Laboratories Inc., Burlington, Ont.) in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH adjusted to 8.0 with acetic acid). Constant voltage was applied at 4 V cm^{-1} for 2 1/2 h in a Bio-Rad horizontal gel chamber until the tracking dye had migrated about 8.5 cm. Plasmid bands were stained for 1 h with an aqueous solution of 0.5 ug ml^{-1} ethidium bromide, and visualized using a Spectroline 302 nm ultraviolet transilluminator. Agarose gels were photographed using a MP-4 Polaroid camera and type 57 polaroid film. A Kodak Wratten 22 filter was inserted between the ultraviolet transilluminator and the camera.

An attempt was made to isolate mercury-sensitive derivatives of each isolate by repeatedly culturing and transferring (10 transfers) the isolates through LB broth without $HgCl_2$, followed by plating on non-selective LB agar. Colonies which formed on this medium were plated on LB agar amended with 100 µM $HgCl_2$. All the colonies tested still retained their $HgCl_2$ resistance. Since the spontaneous loss of plasmids did not occur, the isolates were grown in LB broth amended with different sublethal concentrations of filter-sterilized ethidium bromide as a curing agent. Serial decimal dilutions were made in 0.85% (w/v) sterile saline and spread on LB agar. Isolated colonies were then replica plated to LB agar containing 100 µM $HgCl_2$, in an attempt to obtain mercury-sensitive derivatives which could be examined for the loss of one or more plasmids.

Growth of the bacterial isolates in LB broth alone and LB broth amended with 100 µM $HgCl_2$ was determined by measuring the absorbance at 550 nm with a Spectronic 20 spectophotometer. A 100 µl aliquot of log-phase cells was used to inoculate a 500 ml sidearm Bellco flask containing 50 ml of appropriate media. All flasks were incubated at 25°C in the dark with shaking at 120 rpm.

RESULTS AND DISCUSSION

The four mercury resistant bacteria were tentatively identified as Aeromonas hydrophila, Pseudomonas sp., Pseudomonas mendocina and Citrobacter freundii). Brief summaries of the bacterial genera are presented according to the descriptions given by Cowan (1979). Aeromonas species are Gram-negative motile rods, aerobic and facultatively anaerobic, displaying catalase and oxidase positive reactions. Sugars are degraded fermentatively and the amino acid arginine is broken down.

Pseudomonas species are Gram-negative rods, with aerobic metabolism. They display catalase and oxidase positive reactions. Sugars are degraded by oxidative metabolism and fluorescent diffusible yellow pigment may be produced.

Citrobacter species are Gram-negative mobile rods, aerobic and facultatively anaerobic. They exhibit oxidase negative and catalase positive reactions, and degrade sugars fermentatively with gas being produced. Citrate can be utilized as a sole carbon source.

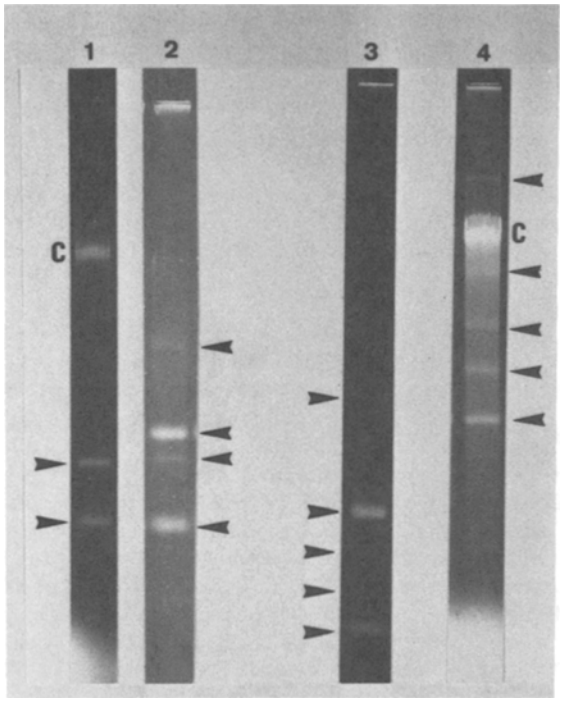

Figure 1. Agarose gel electrophoresis of plasmids isolated from mercury resistant bacteria. Lane 1, Pseudomonas sp., Lane 2, Pseudomonas mendocina, Lane 3, Citrobacter freundii, Lane 4, Aeromonas hydrophila. Arrows indicate the positions of the plasmid bands. C is chromosomal DNA.

Aeromonas hydrophila (lane 4) contained five plasmids, Pseudomonas mendocina (lane 2), four plasmids, Citrobacter freundii (lane 3), five plasmids and the Pseudomonas sp. harbored two plasmids (lane 1) (Fig. 1). No common plasmids (in size or numbers) were present in the four mercury- resistant isolates. Agarose gel electrophoresis of the precipitated plasmid DNA was repeated at least five times over a period of five months to ensure the reproducibility of isolating the plasmid DNA. The plasmid isolation procedure proved suitable for the isolates belonging to different genera. This is important because plasmid isolation procedures that are satisfactory for one organism may be unsuitable for other organisms.

At concentrations of 100 μM, $HgCl_2$ did not significantly reduce the growth rates and final cell yields in cultures of Aeromonas hydrophila, Pseudomonas mendocina, and Citrobacter freundii (Figs. 2-4). However, the onset of logarithmic growth was delayed about 8 hours in the Pseudomonas sp. (Fig. 5). An attempt to isolate spontaneous derivatives sensitive to $HgCl_2$ by repeated growth and transfers through LB broth (without $HgCl_2$) proved unsuccessful.

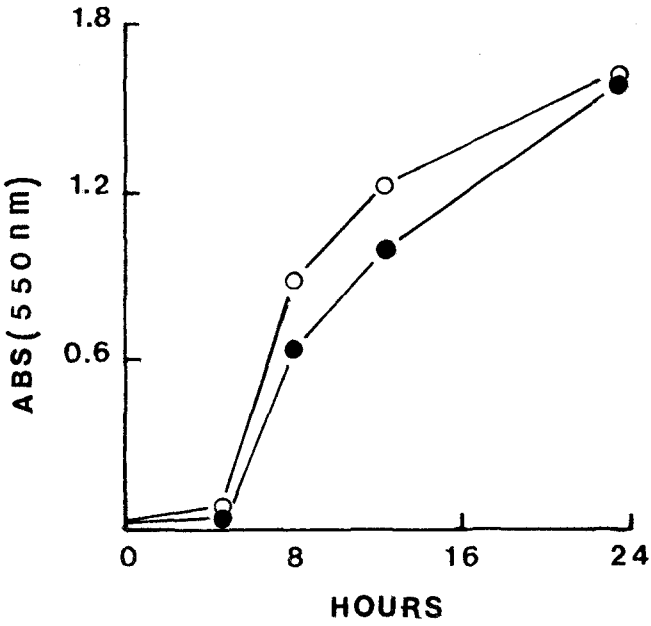

Figure 2. Growth of Aeromonas hydrophila in the absence (O) and presence (●) of 100 μM $HgCl_2$.

Also, plasmid curing experiments using sublethal concentrations of ethidium bromide did not yield any $HgCl_2$ sensitive derivatives. It is therefore unknown which of the plasmids controls mercury resistance, or if the resistance is indeed plasmid-encoded.

When determining if an organism is metal resistant, there is no standard acceptable concentration which designates the boundary between metal resistance and susceptibility in bacterial isolates (Trevors et al., 1985). In the present study, a $HgCl_2$ level of 100 μM was used. This was based on the findings of Meissner and Falkinham (1984), who investigated mercuric reductase activity in Mycobacterium scrofulaceum.

Recently, Duxbury and Bicknell (1983) suggested that bacteria found in soils containing relatively low concentrations of metals, could possibly function without the need for plasmid-encoded metal resistance. In the present study, the four isolates were resistant to 100 μM $HgCl_2$. This is a relatively high metal concentration and can be considered a level that designates true metal resistance. The presence of mercury resistant bacteria in sediment suggests that bacteria are present that can cope with high levels of mercury pollution. It may also be possible to use mercury resistant and volatilizing bacteria to detoxify mercury in activated reactors.

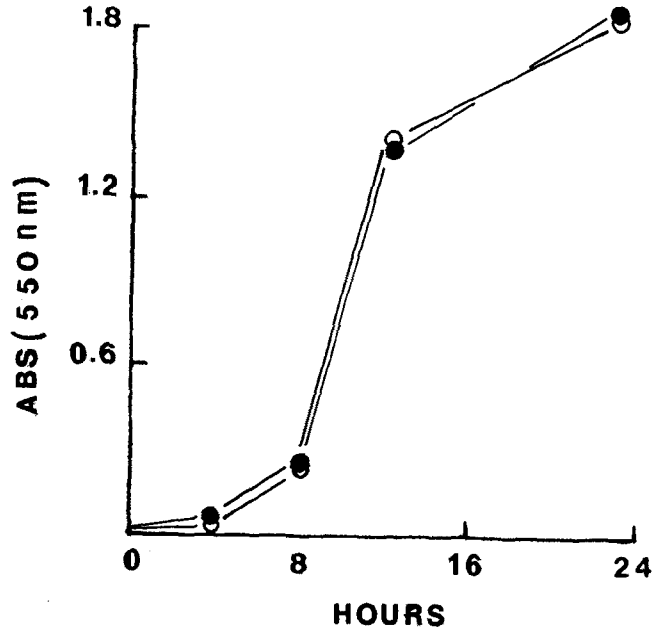

Figure 3. Growth of Pseudomonas mendocina in the absence (O) and presence (●) of 100 μM $HgCl_2$.

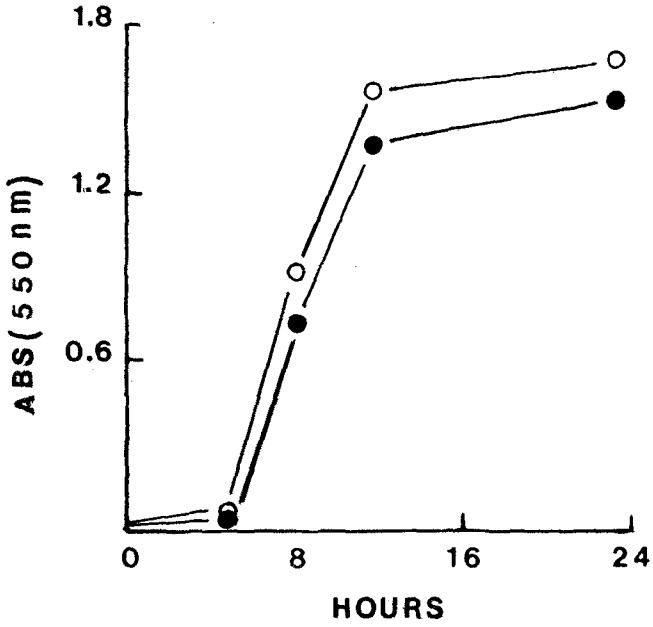

Figure 4. Growth of Citrobacter freundii in the absence (O) and presence (●) of 100 μM $HgCl_2$.

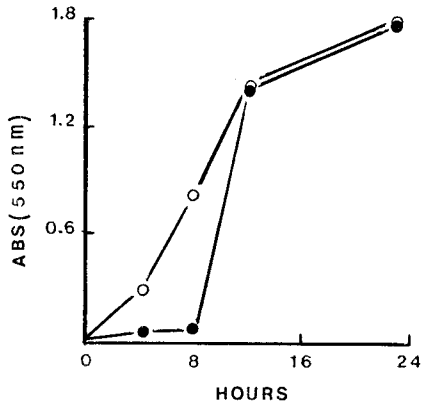

Figure 5. Growth of Pseudomonas sp. in the absence (O) and presence (●) of 100 μM ($HgCl_2$).

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