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Mercury-Resistance and Mercuric Reductase Activity in *Chromobacterium, Erwinia,* and *Bacillus* Species

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Mercury resistant bacteria have been the most extensively studied of all the metal-tolerant bacteria (Barkay et al. 1985; Foster et al. 1979; Guenzi and Beard 1967; Hall 1970; Izaki et al. 1974; Izaki 1981; Kelly and Reanney 1984; Meissner and Falkinham 1984; Nelson and Colwell 1975; Olson et al. 1981; Olson et al. 1982; Silver and Misra 1984; Trevors et al. 1985; Weiss et al. 1977). Mercury resistance is usually mediated by two distinctly different enzymes encoded by plasmids (Izaki 1981). Mercuric reductase reduces Hg^{2+} to metallic mercury (Hg^{o}). The Hg^{o} then diffuses out of the cell and volatilizes from the growth medium (Izaki 1981). Organomercurial lyases have a molecular weight of 20,000 to 40,000, are composed of 1 or 2 subunits and require the presence of thiol (Silver and Misra 1984). They are responsible for catalyzing several reactions: phenylmercury to benzene and Hg^{2+}; methylmercury to methane and Hg^{2+}; and ethylmercury to ethane and Hg^{2+}.

Numerous mercury resistant bacterial strains have been isolated and studied in the past 20 years (Trevors et al. 1985). Most of these organisms belong to the genera Pseudomonas, Mycobacterium, Bacillus, Acinetobacter, Staphylococcus, Escherichia, Thiobacillus, Arthrobacter, Citrobacter, Enterobacter, Flavobacterium and Vibrio (Trevors et al. 1985). A recent study by Meissner and Falkinham (1984) described a 115 Mdal plasmid (pVT1) in Mycobacterium scrofulaceum, responsible for Hg^{2+} resistance. A plasmid-cured derivative lacking the pVT1 plasmid was unable to grow in the presence of 100 uM $HgCl_2$, and possessed no mercuric reductase activity. Plasmid-encoded Hg^{2+} resistance and mercuric reductase activity have not been detected in many species of bacteria.

A Chromobacterium, Erwinia and Bacillus species isolated from environmental samples were capable of growth in the presence of 50 μM $HgCl_2$. Cell-free extracts of the 3 organisms exhibited mercuric reductase activity that oxidized NADPH in the presence of $HgCl_2$. Negligible oxidation of NADPH was observed in the

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absence of $HgCl_2$. The Chromobacterium sp. did not contain any plasmid DNA. This would suggest that Hg^{2+} resistance was carried on the chromosome in Chromobacterium. A single 3 Mdal plasmid in the Bacillus sp. was refractory to curing. The Erwinia sp. contained 3 plasmids which were also refractory to curing. The location of the resistance genes is unknown in the Bacillus and Erwinia isolates.

MATERIALS AND METHODS

The Chromobacterium and Bacillus strains were isolated from freshwater sediment (Speed River) collected at Guelph, Ontario, Canada. The Erwinia sp. was isolated from an activated waste reactor. All organisms were isolated by plating serial dilutions of the sediment on LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 1000 ml H_2O) amended with 100 uM filter-sterilized mercuric chloride. The organisms were purified by restreaking on the same medium, and tentatively identified using standard morphological and biochemical tests. Identification was based on descriptions by Cowan (1979) and Kreig and Holt (1984).

For plasmid isolation and growth studies, cultures were grown in LB or nutrient broth for 16 h at 30°C with shaking at 100 rpm. Initial inocula were about 10 cells/ml. Mercuric chloride was filter-sterilized and added separately to the culture medium.

A 1.5 ml volume of a 16 h culture grown in the presence of 10 µmol $HgCl_2$ (to induce mercuric reductase activity) was harvested by centrifugation at 15,500 x g for 5 min at 4°C. The supernatant fluid was decanted and the cells resuspended in 1 ml of 150 mM phosphate buffer (pH 7) containing 4 mM 2-mercaptoethanol, and disrupted by sonication at 100 watts for 1 min at 2°C. The lysate was centrifuged at 15,500 x g for 15 min at 4°C and stored at 2°C.

The reaction mixture contained 0.5 ml of 150 mM phosphate buffer (pH 7), 0.5 ml of 100 µM $HgCl_2$, 0.5 ml of 2 mM 2-mercaptoethanol, 0.5 ml of 100 µM nicotinamide adenine dinucleotide phosphate (NADPH) dissolved in pH 7 phosphate buffer and 0.5 ml of the cell-free extract. The reaction was initiated by adding the cell-free extract to the remainder of the reaction mixture preincubated for 3 min at 20°C. Mercuric chloride dependent oxidation of NADPH (Izaki 1981) was measured by recording the decrease in absorbance at 340 nm using a LKB spectrophotometer. One unit of Hg^{2+} reducing activity is equivalent to a decrease in absorbance of 0.01/min at 20°C. This corresponds to 1.6 nmol/min if a one-to-one stoichiometry is assumed for the reduction of Hg^{2+} by NADPH (Izaki 1981).

RESULTS AND DISCUSSION

The Hg^{2+} resistant isolates were tentatively identified as a Chromobacterium, Erwinia and Bacillus sp. (Table 1). The organisms did not fit into an exact identification scheme.

Table 1. Morphological and biochemical characteristics of the mercury resistant isolate

Characteristic	Test results		
Morphology	rod	rod	large rod
Gram-stain	-	-	+
Motility	+	+	+
Catalase	+	+	+
Oxidase	+	-	-
Gelatin liquefaction	-	+	-
Indole	-	-	-
Simmons citrate	+	-	-
Urease	-	-	-
Voges-Proskauer	-	-	-
Nitrate reduction	+	-	+
ONPG hydrolysis	+	-	-
Arginine hydrolysis	-	-	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-
H_2S production	+	-	-
Tryptophane deaminase	-	-	-
Carbohydrates utilized			
Mannitol	+	-	+
Inositol	+	-	+
Sorbitol	+	-	+
Rhamnose	+	-	+
Sucrose	+	-	+
Melibiose	+	+	+
Amygdalin	+	-	+
Arabinose	+	+	+
Glucose	+	+	+
Tentative identification	*Chromobacterium* sp.	*Erwinia* sp.	*Bacillus* sp.

Table 2. Mercuric reductase activity; NADPH oxidation dependent on Hg^{2+} in cell-free extracts

Cell-free extract	Assay conditions	nmol Hg^{2+} reduced/min
Chromobacterium sp.	$HgCl_2$ present	6.4
Erwinia sp.		16.0
Bacillus sp.		20.8
Chromobacterium sp.	No $HgCl_2$	0.80
Erwinia sp.		0.48
Bacillus sp.		0.96

However, many environmental isolates rarely do. In addition, the Chromobacterium sp. did not produce purple pigmentation which is usually, but not always associated with this species. Pigment production is usually most distinct when Chromobacterium spp. are grown in media containing tryptophan (Kreig and Holt 1984). However, non-pigmented variants are also possible (Cowan 1979).

NADPH was oxidized by cell-free extracts of the 3 organisms in the presence of Hg^{2+} (Table 2). However, negligible oxidation of NADPH occurred in the absence of Hg^{2+}. For example, in the absence of $HgCl_2$, reductase activity was less than 1 nmol/min. This can be attributed to background oxidation of NADPH by other enzymes still functioning in the cell-free extracts. NADPH oxidation due to mercuric reductase activity has been demonstrated by Izaki (1981) in a mercury-resistant Bacillus cereus strain. Cell-free extracts of this organism were used as a positive control for mercuric reductase activity. In cell-free extracts of B. cereus, reductase activity was always observed, indicating that the assay technique of Izaki (1981) was suitable.

It is known that mercury-resistance can be located on plasmids in certain bacteria (Trevors et al. 1985). The 3 isolates were examined for the presence of plasmid DNA using the method previously described by Trevors and Oddie (1986) and Meissner and Falkinham (1984). The Chromobacterium isolate did not contain any plasmids. It would therefore appear that the Chromobacterium sp. carried the mer operon on the chromosome or possibly a transposon located on the chromosome. However, the Erwinia sp. contained a 3.7, 2.5 and 1.4 Mdal plasmid. The Bacillus sp. contained 1 small plasmid about 3 Mdal. The single plasmid harbored in the Bacillus sp. and the 3 Erwinia plasmids were refractory to curing with agents like sodium dodecyl sulfate, novobiocin, ethidium bromide and elevated growth temperature. Some plasmids are very stable and to date have not been cured (Trevors 1986). A plasmid-cured derivative may have provided evidence for plasmid-encoded Hg^{2+}-resistance.

Investigations on the ecology, physiology and genetics of mercury resistance have only been reported in a limited number of bacteria species (Foster et al. 1979; Nelson and Colwell 1975; Olson et al. 1982; Robinson and Tuovinen 1984; Schottel 1978; Trevors et al. 1985). It is known that inorganic mercuric ion and organomercurial resistance is determined by an inducible operon of closely linked genes (Silver and Misra 1984). A report by Silver and Misra (1984) summarized the present understanding of plasmid-determined resistance to mercury: (1) it is the best understood of all heavy metal resistances, (2) detoxification produces volatile Hg^{o} which is less toxic than the other forms of mercury, (3) the enzymes responsible are being further characterized and sequenced in order to gain a better understanding of mercury resistance.

The microbial transformations of mercury are probably one of the best examples of metal metabolism. As more and different mercury resistant bacteria are studied, additional information will be

gathered on the extent of mercuric reductase activity in both Gram-negative and -positive bacteria isolated from different environments.

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