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Plasmid-Encoded Mercuric Reductase in *Mycobacterium scrofulaceum*

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A Chesapeake Bay water isolate of *Mycobacterium scrofulaceum* containing a 115-megadalton plasmid (pVT1) grew in the presence of 100 μM HgCl₂ and converted soluble ²⁰³Hg²⁺ to volatile mercury at a rate of 50 pmol/10⁶ cells per min. Cell extracts contained a soluble mercuric reductase whose activity was not dependent on exogenously supplied thiol compounds. The enzyme displayed nearly identical activity when either NADH or NADPH served as the electron donor. A spontaneously cured derivative lacking pVT1 failed to grow in the presence of 100 μM HgCl₂ and possessed no detectable mercuric reductase activity.

Previous studies have demonstrated that the increased frequency of recovery of representatives of the *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* (MAIS) group from water samples collected in the southeastern United States (6) correlates with the high frequency of persons showing evidence of prior infection (4) and the high frequency of recovery of mycobacteria of this group from specimens submitted to state tuberculosis laboratories (9, 10). The wide geographic distribution of members of this group of slow-growing, human pathogens throughout the southeastern United States and their ability to grow in natural waters (8) suggested that they should demonstrate phenotypic and genotypic changes which permitted survival in the diverse aquatic environments from which they were recovered. Accordingly, we examined the heavy-metal resistance of a large number of environmental and clinical MAIS isolates and identified a number of strains which were resistant to 100 μM HgCl₂ (5). Since mercury resistance in both gram-positive (22) and gram-negative (19, 21) bacteria is due to the presence of plasmids which encode for synthesis of a mercury-volatilizing mercuric reductase, we sought to determine whether the mercury resistance in one environmental MAIS isolate was also due to plasmid-encoded mercuric reductase. The results of experiments reported in this paper provide evidence that the resistance to 100 μM HgCl₂ of an isolate of *M. scrofulaceum* is due to the presence of a mercuric reductase whose appearance correlates with the presence of a plasmid. The identification of this selectable marker on plasmid DNA will allow the development of techniques for genetic analysis of this important group of human and animal pathogens.

*M. scrofulaceum* W262 was isolated from a brackish surface water sample of the Cheser River at Chesapeake, Md., near the Chesapeake Bay. Experiments were conducted with cells grown at 30°C in Middlebrook 7H9 medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.4% (vol/vol) glycerol (MG broth). The mercury-volatilizing activity was induced by growing strain W262 in MG broth containing 100 μM HgCl₂. The mercury-sensitive strain used in these experiments was obtained after several generations of growth of strain W262 in MG broth, followed by plating on nonselective Middlebrook 7H10 (BBL) agar medium containing 0.4% (vol/vol) glycerol. This spontaneous mercury-sensitive strain (W262C) was obtained from a colony which appeared on this medium and failed to grow in the presence of 100 μM HgCl₂.

Whole cell mercury volatilization assays were conducted with log-phase cultures of strain W262 grown in MG broth plus 100 μM HgCl₂. Cells were harvested and suspended in 1/50 volume of MG broth containing 10 μM HgCl₂ and 2 μM ²⁰³Hg(NO₃)₂ (total 12 μM Hg²⁺). All experiments were conducted at 30°C in test tubes (16 by 120 mm). Air was gently bubbled through the solution to maintain homogeneity and to ensure rapid volatilization of the mercury.

Cell extracts were prepared by suspending log-phase cells from 500 ml of MG broth plus 100 μM HgCl₂ in 10 ml of 50 mM sodium phosphate buffer (pH 7.0). Cells were disrupted by two passages through a French Pressure Cell (American Instruments Co., Silver Spring, Md.) at 20,000 lb/in². After disruption, the suspension was cleared by centrifugation at 10,000 × g for 10 min. The cell-free supernatant was tested for mercuric reductase activity in a minimal assay mixture (17) containing 1 ml of extract, 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.2 mM MgSO₄, 0.5 mg of bovine serum albumin per ml, and 2 μM ²⁰³Hg(NO₃)₂. Thiols compounds (final concentration, 1 mM) or NADPH or NADH (final concentrations, 200 μM) were added as indicated in individual experiments.

Plasmid isolation was by the method of Kado and Liu (11). Mycobacterial strains were grown in 30 ml of MG broth containing 10% OADC (BBL). Upon onset of late log phase, cultures were exposed to D-cycloserine (1 mg/ml) and ampicillin (100 μg/ml) for 18 hr as described by Crawford and Bates (1). Cells were then harvested and suspended in 1 ml of E buffer (40 mM Tris-acetate, 2 mM EDTA, pH 7.9), to which was added 2 ml of lyzing solution (3% sodium dodecyl sulfate, 50 mM Tris, pH 12.5) (11). The lysate was incubated at 60°C for 20 min, followed by extraction with 2 volumes of phenol saturated with 0.5 M sodium chloride. Extractions were carried out mechanically at 4 rpm on a New Brunswick roller-drum apparatus (New Brunswick Scientific Co., New Brunswick, N.J.). Extracted lysates were cleared by centrifugation at 10,000 × g for 60 min. Fifty microliters of the aqueous supernatant was mixed with 10 μl of tracking dye (0.25% brom cresol purple in 50% glycerol-0.05 M Tris-acetate, pH 8.2), followed by electrophoresis with a vertical slab apparatus (Hoefer Instruments, San Francisco, Calif.). Slab dimensions were 16 cm by 18 cm by 3 mm. DNA-grade agarose (0.7%; Bio-Rad Laboratories, Richmond, Calif.) was dissolved in TEB buffer (89 mM Tris, 89 mM boric acid,
paring their weights (in megadaltons) of plasmids.

Therefore, to determine the presence of curing by using plasmid pVT1. Both strains W262 and W262C were cured by using pVT1. Mercury-volatilizing activity was detected from whole cells and cell extracts of strain W262 grown in the presence of 10 or 100 μM HgCl₂ (Table 1). The sensitivity to 100 μM HgCl₂ and lack of mercuric reductase activity in strain W262C grown in the presence of 10 μM HgCl₂ (Table 1) is consistent with our belief that the enzyme is encoded by pVT1 in strain W262. The fact that strain W262C retained the ability to grow in 10 μM HgCl₂ is not surprising since a substantial number of clinical and environmental MAIS isolates also grow in the presence of 10 μM HgCl₂ (5). The nonspecific resistance of MAIS bacteria to high levels of antibacterial compounds is characteristic of these organisms and apparently results from an unusually high permeability barrier (14, 16).

As with other bacterial species (21, 22), the mercuric reductase of strain W262 is induced by growth in Hg²⁺-containing media. Maximum volatilizing activity by either whole cells or cell extracts is observed only when those assays are conducted with cells grown in the presence of 100 μM HgCl₂ (Table 1).

Bacterial mercuric reductases are cytoplasmic enzymes (17), and the enzyme from strain W262 is associated with the soluble fraction of cell extracts. Seventy percent of the original enzyme activity of crude cell extracts was present in the supernatant after centrifugation at 145,000 × g for 60 min (data not shown). The activity of the enzyme was completely dependent on NAD(P)H, and there was essentially no difference in activity when either NADH or NAPD was supplied as the electron donor (Table 1). Plasmid-encoded reductases...
Table 1. Characterization of mercury volatilization by cells and cell extracts of *M. scrofulaceum* W262 and W262C

<table>
<thead>
<tr>
<th>Strain</th>
<th>HgCl₂ concn (μM)</th>
<th>Nucleotide or thiol addition</th>
<th>Volatilization rate</th>
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</thead>
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<tr>
<td>Whole cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W262</td>
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<td>None</td>
<td>50</td>
</tr>
<tr>
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<td>10</td>
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<td>16</td>
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<td>None</td>
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<tr>
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<td>None</td>
<td>&lt;1</td>
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<tr>
<td>Cell extract</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>100</td>
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<td>&lt;1</td>
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<td>NADPH or NADH + β-ME</td>
<td>&lt;1</td>
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<tr>
<td>W262</td>
<td>100</td>
<td>NADPH + β-ME</td>
<td>42</td>
</tr>
</tbody>
</table>

" Concentration in MG broth in which cells of either strain were grown to late log phase.
" Cofactors were added to the reaction mixture at 200 μM NADPH and 1 mM β-mercaptoethanol (β-ME).
" pmol of ^{209}Hg volatilized per 10⁶ cells per min.

from other species often show activity in the presence of either cofactor, but activity is usually greater with NADPH than with NADH (20). However, there are the reports of mercury volatilization by a soil pseudomonad (7) and a *Streptomyces* sp. (18) whose mercuric reductases use either cofactor equally, as we have observed.

Mercuric reductases from gram-positive microorganisms (such as *Staphylococcus* spp. and *Bacillus* spp.) are inactivated by temperatures above 60°C (15). Incubation for 10 min at 63°C completely inactivated the mercuric reductase from strain W262 (data not shown). Additionally, the enzyme retained activity after several freeze-thaw cycles (data not shown), and in this respect it resembles the reductase from *Thiobacillus ferrooxidans* (15).

Unlike previously studied mercuric reductases (17, 20, 22), the activity of the enzyme in freshly prepared extracts of strain W262 was not dependent on exogenous thiol compounds. Addition of β-mercaptoethanol at concentrations necessary for the activity of reductases from other species (15) had little or no effect on the enzyme from *M. scrofulaceum* (Table 1). In other species, maximum reductase activity occurs when the reduced thiol is present at concentrations nearly 100 times in excess of that of the substrate Hg₂⁺ (17, 21). This large molar excess of sulfhydryl compound implies that the actual substrate for the reductase may be a thiol (or dithiol) adduct of mercury and not ionic mercury (20). The fact that the reductase from *M. scrofulaceum* W262 is active in the absence of exogenous thiol compounds may indicate that other compounds present in cell extracts from this strain may react with Hg₂⁺ and thereby render it a suitable substrate for the enzyme.

*M. scrofulaceum* is closely related to two other mycobacterial species: *M. avium* and *M. intracellulare* (23). Although plasmids have been isolated from these two latter species (2, 13), this is the first report of plasmids in an environmental isolate of *M. scrofulaceum*. Although there is evidence that mycobacterial plasmids encode for restriction and modification (3) and are involved in colonial morphology variation (13), neither is a useful marker for genetic selection. The discovery of a plasmid-encoded mercuric reductase in *M. scrofulaceum* suggests that this trait will serve as an important marker in the genetic analysis of these species as well as epidemiological studies.

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LITERATURE CITED


