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Cell-Free Mercury Volatilization Activity from Three Marine *Caulobacter* Strains

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Three mercury-resistant marine *Caulobacter* strains showed an inducible mercury volatilization activity. Cell-free mercury volatilization (mercuric reductase) from these three marine *Caulobacter* strains was characterized and compared with enzyme activities determined by plasmids of *Escherichia coli* and *Staphylococcus aureus*. The temperature sensitivity of the *Caulobacter* mercuric reductase was greater than that of mercuric reductase from other gram-negative sources. Cell-free enzyme activity required NADH or NADPH, with NADPH functioning much better at lower concentrations than NADH. The K_m for the *Caulobacter* enzyme was 4 μM Hg^{2+}. Ag^+ was a competitive inhibitor of *Caulobacter* mercuric reductase (K_i = 0.2 μM Ag^+), as with previously studied enzymes. Arsenite was a noncompetitive inhibitor of the *Caulobacter* enzyme with a K_i of 75 μM AsO_2^-.

Mercuric resistance has been the most thoroughly studied heavy metal resistance of plasmids and transposons (4, 12). It has been found in a wide range of procaryotes, both gram positive and gram negative, wherever mercury-resistant natural isolates have been sought (7–10). The mechanism of mercury resistance is reduction of Hg^{2+} to Hg^0 by the intracellular enzyme mercuric reductase, followed by volatilization of relatively nontoxic Hg^0. Among gram-negative mercuric resistance systems, three have been subject to DNA sequencing. They are identical at about 85% of their base pair positions (5, 12), sufficient similarity to assure positive Southern-blotting DNA-DNA hybridization results.

The DNA sequences of mercuric resistance determinants of two gram-positive microbes have also been determined (7, 13, 14). These systems also determine mercury volatilization and have genes for mercuric reductase, but the level of DNA homology is too low to be picked up by colony or gel blot hybridization.

Mercury volatilization assays were never made with newly characterized mercury-resistant marine *Caulobacter* strains, but Anast and Smit (1) used *mer* DNA from a gram-negative bacterium as a probe (in DNA-DNA colony-blotting analysis) and found no hybridization between the *mer* system of plasmid R100 and the DNA from the *Caulobacter* strains. Either the DNA sequences of the mercuric resistance determinants of the *Caulobacter* strains were sufficiently different from that of R100 as to give negative blotting results, or a different biochemical mechanism might determine mercury resistance in marine *Caulobacter* strains, or both. We now have demonstrated mercury volatilization activity with three marine *Caulobacter* strains (1), making the former hypothesis likely.

Growth inhibition by Hg^{2+} and induction of mercury resistance were measured. More than a 5 μM concentration of Hg^{2+} was required to inhibit growth of cells previously exposed to 1 μM Hg^{2+}. Thus, mercuric ion resistance in *Caulobacter* strain MCS-10 was inducible (Fig. 1), just as has been shown for all other mercuric resistance systems (8, 12) except that from a *Thiobacillus ferrooxidans* strain (9). Mercuric ion resistance in *Caulobacter* MCS-3 and MCS-22 (1) was similarly inducible (data not shown).

Volatilization of radioactive mercury by cells and cell-free enzyme was measured with the three marine *Caulobacter* strains MCS-3, MCS-10, and MCS-22 (1). When cells of the *Caulobacter* strains were suspended in assay buffer (8, 11), the cells rapidly lysed and thus whole-cell volatilization assays were complicated by the mixture of unlysed and lysed cells. Careful experiments were run on cell-free preparations following sonication of suspended cells and centrifugation to remove particulate debris (Fig. 2). Induced cells of *Caulobacter* strains volatilized mercury 15 times more rapidly than uninduced cells did (Fig. 2A and additional data not shown). This difference was of rate (V_{max}) rather than of affinity (K_m) (data not shown). For both uninduced and induced cells, the K_m was about 4 μM Hg^{2+}; Hg^{2+} concentrations above 10 μM showed inhibition of mercury volatilization (data not shown). Results similar to those in Fig. 2 were obtained with cell-free preparations from *Caulobacter* strains MCS-3 and MCS-22.

Mercury volatilization with cell-free enzyme from *Caulobacter* strain MCS-10 depended upon added reduced pyridine nucleotide, with NADPH functioning much more effectively than NADH (Fig. 2B). This result is similar to those with cell-free mercuric reductase from other bacterial sources (8, 9). This differentiation between NADPH and NADH is a property of the mercuric reductase enzyme (11) and not due to the use of crude enzyme preparations in the current studies. Mercury volatilization was most rapid when 0.5 to 5 mM β-mercaptoethanol was added. Dithiothreitol was not as effective as mercaptoethanol (data not shown).

Previous experiments (8, 9) had shown that the mercuric reductases from gram-negative bacteria were very heat resistant and survived incubation at temperatures 20°C higher than those needed to inactivate mercuric reductases from gram-positive bacteria. The mercuric reductase from *Caulobacter* strain MCS-10 was intermediate in heat resistance between the resistances of the enzymes from *Escherichia coli* and *Staphylococcus aureus* plasmid pI258. With a 10-min heating period, 70°C was required to reduce the *Caulobacter* strain MCS-10 enzyme activity to 10% of the initial value, whereas 82°C was required to comparably inactivate enzyme activity from previously studied gram-

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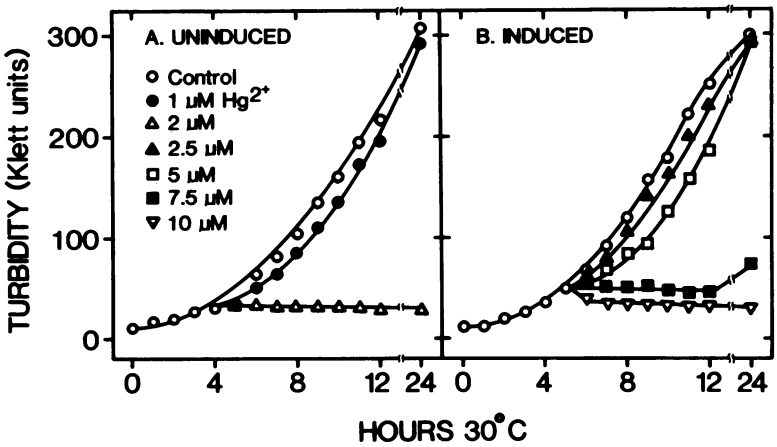

FIG. 1. Mercury inhibition of growth of mercury-resistant *Caulobacter* strains. Cells of *Caulobacter* strain MCS-10 were grown at 30°C in sea salts-peptone-yeast extract (1) overnight and then diluted (0.8 ml into 40 ml of fresh medium) and grown for 3 h. After 1 h of additional incubation without (A) or with (B) 1 μM Hg^{2+} as inducer, the cultures were divided and 0 to 10 μM Hg^{2+} was added. Growth was followed by turbidity in a Klett colorimeter (green no. 54 filter) during incubation with shaking at 30°C.

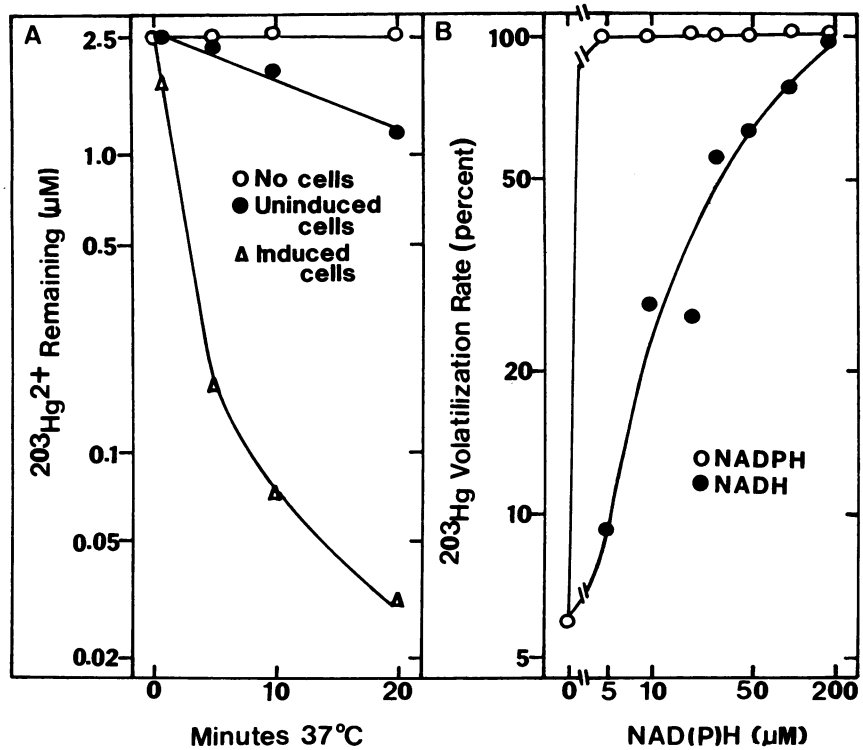

FIG. 2. Volatilization of radioactive ^{203}Hg by cell-free preparations from induced or uninduced cells. Cells of *Caulobacter* strain MCS-10 (1) were grown and induced as described in the legend to Fig. 1. After 1 h of induction, the cells were harvested by centrifugation at 9,000 × *g* for 10 min and suspended in 50 mM sodium phosphate buffer (pH 7.4) at a turbidity of 5,000 Klett units. The suspended cells were sonicated (W225 sonicator; Heat Systems-Ultrasonics, Plainview, N.Y.) for 5 min (intermittently) at full power with cooling on ice. After removal of cell debris by centrifugation for 5 min in an Eppendorf microcentrifuge, the supernatant fluids were used as crude cell-free enzyme. (A) Induction. Cell-free enzyme (6.25 μg of protein) from uninduced or induced cells was added to 0.2 ml of complete assay mixture (8, 11) containing 0.2 mM NADPH and 2.5 μM $^{203}Hg^{2+}$. Samples (25 μl) were removed for counting of radioactivity periodically during shaking at 200 rpm in a water bath at 37°C. Uninduced cell extract, induced cell extract, or buffer was added to start the reactions. (B) Dependence of mercury volatilization on NADH or NADPH. The cell-free extract from induced cells of strain MCS-10 was assayed in complete assay mixture with 6.25 μg of cell protein, 2.5 μM $^{203}Hg^{2+}$, and various concentrations of NADH or NADPH. The volatilization rates were determined from the exponential loss phase as described in the legend to Fig. 2A. The 100% value was 16 nmol/min per mg of protein.

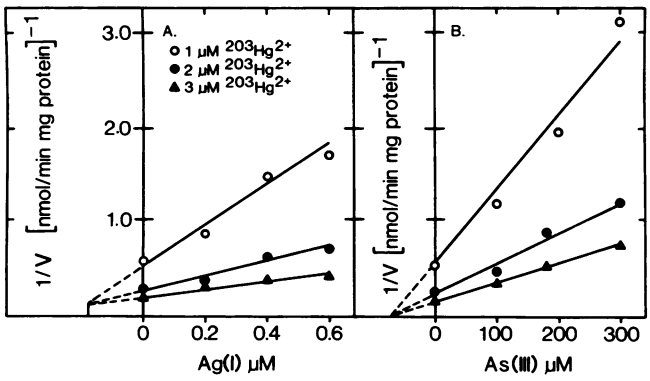

FIG. 3. Inhibition of mercuric reductase activity by Ag(I) and As(III) salts. Cell-free crude enzyme from *Caulobacter* strain MCS-10 was assayed as described in the legend to Fig. 2 in the presence of increasing concentrations of $AgNO_3$ or $NaAsO_2$ and 1, 2, or 3 μM $^{203}Hg^{2+}$. The initial rates of volatilization are plotted in reciprocal units in a Dixon plot.

negative systems (data not shown). Enzyme from a representative gram-positive system (pI258 [7]) was inactivated under these conditions at 62°C. The three *Caulobacter* enzymes were the most heat labile of those from gram-negative bacteria tested to date (9 and unpublished data).

Silver ions competitively inhibited the enzyme activity from *Caulobacter* strain MCS-10 with a K_i of 0.2 μM Ag^+ (Fig. 3A), or an affinity higher by at least 1 order of magnitude than the K_m of 4 μM Hg^{2+}. This was also the case in previous studies with cell-free mercuric reductases. As(III), an inhibitor of the related flavoprotein lipoamide dehydrogenase (6), also inhibited *Caulobacter* strain MCS-10 mercuric reductase activity (Fig. 3B), but unlike Ag^+, AsO_2^- functioned as a noncompetitive inhibitor of mercury volatilization with a K_i of 75 μM AsO_2^-. The *Caulobacter* mercuric reductase was approximately 10 times more sensitive to arsenite inhibition (Fig. 3B) than were the mercuric reductases from *E. coli* or *S. aureus* plasmids (data not shown).

The mercuric reductase system from the marine *Caulobacter* strains seems basically similar to that from previously studied bacteria and accounts for the mercuric ion resistance of these strains (8, 9, 12). However, Anast and Smit (1) reported that the DNA from these marine *Caulobacter* strains was not recognizable by colony blot hybridization using R100 *mer* operon DNA as a radioactive probe. It is clear that the mechanism remains basically the same, but it seems likely that the DNA sequences have diverged sufficiently during evolution so as not to be detected in DNA-DNA hybridization procedures. The sequence diversity is sufficient to explain the failure to detect colony blotting with the marine *Caulobacter* strains and an R100 probe (1) and also the frequent occurrence of mercury-resistant colonies that do not blot with R100 probes in environmental screening studies (2, 3; T. Barkay and B. H. Olson, personal communications). What appears needed for environmental screening using blotting procedures (1, 3) is a small number of *mer* operon probes that can reflect the range of DNA sequences encoding resistance to mercury.

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