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# Mercuric reductase activity in a mercury-resistant strain of *Yersinia enterocolitica*

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## 1. INTRODUCTION

History of the discovery and elucidation of the mechanism of mercury resistance in bacteria has strikingly paralleled those of antibiotic resistances. For reasons which still remain unclear, these resistances are frequently linked, and molecular analysis has shown that heavy metals as well as antibiotic resistances are, in most cases, plasmid-determined [1–3]. Moreover, in some instances, these characters have been demonstrated to be encoded by translocatable elements [4–6].

The mercury-resistant Gram-negative bacterial strains isolated to date belong to one of the two following subclasses: those which display a 'narrow spectrum' of resistance, resistant to mercuric ion, fluorescein mercuric acetate and its brominated derivative (merbromin), and those showing a broad spectrum, comprising in addition organomercurial compounds such as merthiolate, phenylmercuric borate, methylmercuric and ethylmercuric salts.

Mercury and phenylmercury resistances are also commonly encountered in clinical isolates of *Staphylococcus aureus* with penicillinase plasmids [2].

The mechanism of mercury resistance in Gram-negative bacteria and *S. aureus* has been related to Hg° volatilization from $Hg^{2+}$ ion involving an NADPH-dependent mercuric reductase. Moreover, resistant strains of the broad spectrum subclass produce an organomercurial hydrolase which catalyzes the hydrolytic cleavage of the compound to liberate the $Hg^{2+}$ ion from the organic moiety [7].

We recently described the isolation of a mercury-resistant strain of *Yersinia enterocolitica* [8]. This strain, called 138A14, is resistant to mercuric chloride and merbromin, but sensitive to organomercurial compounds merthiolate and phenylmercuric borate. We assumed that the mechanism of resistance in 138A14 was identical to the one known in other mercury resistant gram negative bacteria. In this paper we report the volatilization of Hg° from mercuric chloride by 138A14, and we compare some characteristics of the crude enzyme with those of analogous preparations of *Escherichia coli* containing the R-100 plasmid, and of *Pseudomonas aeruginosa* harbouring the pVS1 plasmid with the Tn*501* transposon.

## 2. MATERIALS AND METHODS

*Y. enterocolitica* 138A14 has been described previously [8]. A mercury-sensitive isolate of *Y. enterocolitica* (136A21) was used as a control [8]. 138A14 and 136A21 belonged to the same biotype 1, serotype 13, 7, 8, 19, and bacteriophage type Xo. *P. aeruginosa* PAO9501 (pVS1) was obtained from V. Stanisich [4], and *E. coli* K-12 J53 (R100) from N. Datta (Hammersmith Hospital, London).

Bacterial strains were grown at 36°C with shaking in tryptone broth (Difco). Mercuric reductase activity was induced by adding 12 $\mu$M $HgCl_2$ after 8 h of pregrowth.

At stationary growth phase (after about 24 h of cultivation), cells were harvested by centrifugation at 4500 rev./min for 20 min, then washed twice with 0.05 M phosphate buffer, pH 7.0. The pellet was resuspended in 5 ml of 0.05 M Tris buffer, pH 7.5, and cells were disrupted by sonication for 10 min. The suspension of disrupted cells was clarified by centrifugation at 9000 rev./min for 25 min, then supernatant was heated at 80°C for 15 min before to be used as a crude enzyme extract.

Enzyme activity was tested by two methods. First, the vaporization of Hg° from the assay medium was followed by atomic absorption using a Coleman MAS-50 analyzer (Perkin-Elmer). Assays were run at 28°C or 37°C in 250 ml BOD bottle containing 100 ml of 0.05 M phosphate or Tris buffer pH 7.0, 100 $\mu$M $HgCl_2$, 50 $\mu$M NADPH, 2 mM or 10 mM 2-mercaptoethanol, and 200 $\mu$l crude enzyme extract or 2 ml intact cells suspension. Air was bubbled through the reaction mixture and vaporized Hg° was monitored as indicated in the manual.

On the other hand, mercuric reductase activity was assayed by following the oxidation of NADPH. Routine enzyme assays were carried out at 37°C in 0.05 M Tris buffer, pH 8.1, added with 100 $\mu$M NADPH, 10 mM 2-mercaptoethanol, 250 $\mu$M $HgCl_2$, and 10 $\mu$l crude enzyme extract. The oxidation of NADPH was followed spectrophotometrically at 340 nm. One unit of enzyme activity was defined as the amount of enzyme causing an initial decrease of absorbance of 0.01/min in the *A* at 340 nm.

Specific activity was inferred from catalytic activity per mg of protein, whose concentration was determined by the Coomassie blue method of Bradford [9] with bovine serum albumin as a standard.

## 3. RESULTS

As expected, both crude enzyme extract as well as intact cells of 138A14 volatilized Hg° from $HgCl_2$. Vaporization of Hg° occurred quickly after the introduction of the enzyme preparation. Maximal production of Hg° was reached after 3 min, then the rate of metal volatilization decreased progressively (Fig. 1). 138A14 crude extracts displayed an NADPH-dependent mercuric reductase activity which required the presence of 2-mercaptoethanol. While NADPH was rapidly oxidized by crude enzyme extract in the presence of $HgCl_2$, NADH was only slowly oxidized. Enzyme activity was optimal with an NADPH concentration near 50 $\mu$M.

Figs. 2 and 3 show that the rate of NADPH oxidation increases both with $Hg^{2+}$ and 2-mercaptoethanol concentrations. In addition, as the concentration of 2-mercaptoethanol rises, increasingly more $Hg^{2+}$ is required to obtain maximal activity.

The effect of pH on enzyme activity was investigated. Optimal activity was found at pH 7.3, which was the same value observed with crude extracts of R100, whereas PAO extracts showed optimal activity at pH 7.5.

Study of heat stability revealed that 85% of the activity remained after treatment at 80°C for 15 min, whereas the enzyme was completely inactivated after heating at 100°C for 15 min.

We examined the effect of temperature on enzyme activity of crude extract of 138A14 in comparison with that of PAO and R100. Maximal

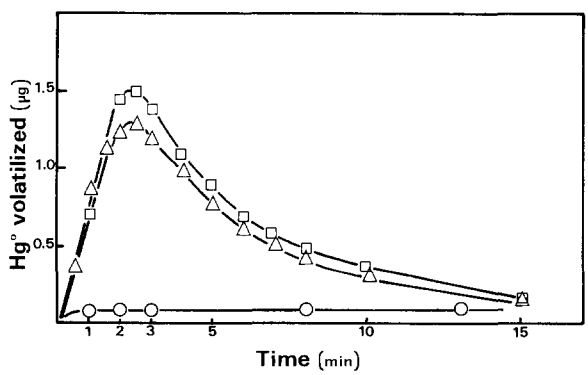


Fig. 1. Mercury volatilization from $HgCl_2$ by sonicated cells ($4 \cdot 10^{10}$ cell equivalents) at 28°C in phosphate buffer 0.05 M pH 7.0, □, *Y. enterocolitica* 136A14; △, *E. coli* (R100); ○, *Y. enterocolitica* 136A21 (control). Experimental details are given in the text.

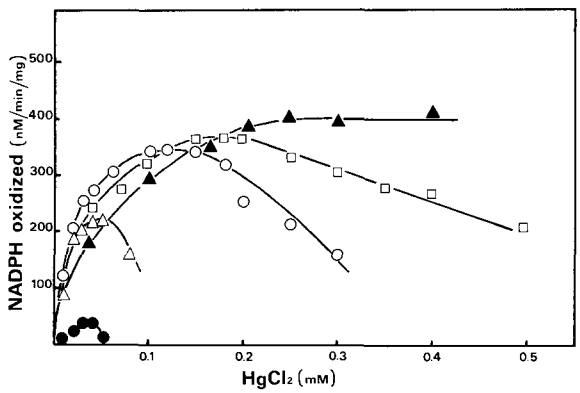


Fig. 2. Effect of 2-mercaptoethanol and $HgCl_2$ concentrations on oxidation of NADPH by crude enzyme extract of *Y. enterocolitica* 138A14 at 37°C in 0.05 M Tris buffer pH 8.1. Concentrations of 2-mercaptoethanol: ●, 0.2 mM; △, 0.5 mM; ○, 2 mM; □, 6 mM; ▲, 10 mM.

activity was observed between 37°C and 43°C, without significant difference from one strain extract to the other.

Finally, study of ionic strength effect pointed out the great sensitivity of enzyme activity of 138A14 to NaCl concentration.

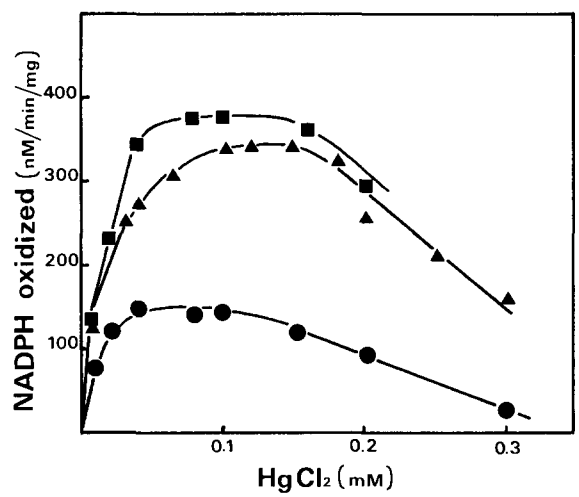


Fig. 3. Effect of $HgCl_2$ concentration on oxidation of NADPH by crude enzyme extract of *Y. enterocolitica* 138A14 (▲); *Ps. aeruginosa* PAO9501 ($pVS_1$) (■); *E. coli* J53 (R100) (●). Concentration of 2-mercaptoethanol: 2 mM.

## 4. DISCUSSION

The most interesting finding reported here is the first description of a mercuric reductase activity exhibited by bacteria of the genus *Yersinia*. This enzymatic activity does not seem to differ from that of other similar enzymes still characterized in *Ps. aeruginosa* harbouring the Tn*501* transposon, and in *E. coli* containing the R100 plasmid with MER operon. The mercuric reductase of 138A14 is inducible, NADPH-dependent, requires the thiol compound 2-mercaptoethanol, and is remarkably heat-resistant.

These results suggest that 2-mercaptoethanol is a substrate of the enzyme in the form of mercaptide. In addition, the mercury-free thiol compound seems to act as an activator of the enzyme.

When we began this study, we assumed that a mercury-resistant isolate of *Y. enterocolitica*, because of psychrotrophic property of this species, might produce a mercuric reductase able to work at low temperature with more efficiency than similar enzymes synthetized by mesophilic bacteria such as *E. coli*. Our results are not in agreement with this assumption. These data argue in favour of a common origin of mercuric reductases encountered in enteric bacteria and in *Pseudomonas*, whose spreading would have been facilitated by the transposability of their genetic determinants.

We are currently studying the genetic control of mercury resistance in 138A14, and a new transposon (Tn*3926*) localized on a 29 kpb non-conjugative plasmid associated with $Hg^{2+}$ resistance of that strain, has recently been characterized (manuscript in preparation). This transposon seems to have some sequences in common with Tn*501*, especially within the area of the reductase gene.

On the other hand, purification of the mercuric reductase of 138A14 is in progress to estimate its $M_r$ value, determine if it has a bound FAD moiety, and test if it immunologically cross-reacts with other known mercuric reductases.

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