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OBSERVATIONS OF RESISTANCE AND CROSS-RESISTANCE TO FORMALDEHYDE AND A FORMALDEHYDE CONDENSATE BIOCIDE IN *PSEUDOMONAS AERUGINOSA*

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Abstract: An isolate of *Pseudomonas aeruginosa* from metalworking fluid was treated with sublethal concentrations of formaldehyde (FA) or hexahydro-1,3,5-tris-ethyl-s-triazine (ET). Following 100 hours of treatment, the cells were significantly more resistant to both FA and the FA condensate biocide regardless of the compound used in the initial treatment. This development of resistance and cross-resistance suggests that the ET does indeed release FA. The specific activity of FA dehydrogenase increased about 18 fold as a result of the induction of resistance with the ET. The mode of resistance to FA or the ET appears to be an increased rate of detoxification of FA (NAD^+ linked oxidation).

Introduction

Triazine biocides are produced via the condensation of formaldehyde (FA) and substituted amines. The most common end products are derivatives of hexahydro-1,3,5-triazine.

Holtzman and Rossmoore (1977) demonstrated that the biocidal activity of hexahydro-1,3,5-tris (2 hydroxyethyl)-s-triazine was blocked by homocysteine, suggesting that the antimicrobial activity of this compound is a result of FA release. Although those results were not in agreement with measurements of FA (by the dimethone complex method), the data presented here as well as other results (DeMare *et al.* 1972) suggests that FA is the toxic component of the triazine biocides.

Isolates of FA-resistant bacteria and yeast have been shown to contain enzymatic activity that results in the detoxification of FA (Kato *et al.* 1982, 1983). This report demonstrates that the induction of resistance in *Pseudomonas aeruginosa* to FA or hexahydro-1,3,5-tris ethyl-s-triazine (ET) results in cross-resistance as well as increased levels of FA dehydrogenase.

Materials and Methods

An isolate of *Ps. aeruginosa* was obtained from contaminated metalworking fluid and maintained on trypticase soy agar (TSA) or trypticase soy broth (TSB). Exponential cultures in TSB at 1–5 × 10^7 cfu/ml were treated with 5.25 mM FA or 1.75 mM ET (equimolar concentrations of available FA) for 100 hours. When the populations had regrown and reached a density of 5–7 × 10^7 cfu/ml (Fig. 1a), the cultures were collected by centrifugation and resuspended in fresh TSB at 1–5 × 10^7 cfu/ml. These populations were then treated with the same concentrations of FA or ET to determine the degree of resistance induction or treated with the alternate biocide to determine cross-resistance.

A 2 liter culture of *Ps. aeruginosa* was treated with ET as described above for approximately 100 hours, and the regrown population was harvested by centrifugation. The cells were resuspended in 50 mM K phosphate buffer, pH 7.5, lysed by two passages through a French Pressure Cell, and a cell-free extract was obtained by centrifugation. Formaldehyde dehydrogenase activity was determined by measuring the FA-dependent reduction of NAD^+ (absorbance of NADH, 340 nm). The reaction mixture was prepared according to Ando *et al.* (1979) with 0.5 mM glutathione at a pH of 8.0.

Results and Discussion

If the biocidal activity of FA condensate compounds is a result of FA release, then microorganisms capable of detoxification of FA could develop resistance in industrial systems treated with such biocide. A strain of *Ps. aeruginosa* isolated from metalworking fluid was exposed to sublethal concentrations of FA or ET. Treatment with ET has been previously shown to result in an initial kill followed by regrowth (Sondossi *et al.* 1984). The result of treatment of cultures with FA or ET is shown in Fig. 1a. The regrown populations were then exposed to the same biocide as in the initial treatment (Fig. 1b) or to the opposite biocide (Fig. 1c). Clearly, a degree of resistance has developed to the biocide used in the initial treatment as well as cross-resistance to the alternate biocide. One intrepretation of these results is that the toxic moiety of the FA condensate biocide is indeed FA. A test of this interpretation was done with purified FA dehydrogenase from *Ps. pudita* (Sigma Chemical Co.). If FA is released by ET, then ET should serve as a substrate for FA dehydrogenase. There was no detectable difference in the rate of NADH formation, in excess of substrate, by the purified enzyme using either FA or ET (unpublished results).

Some microorganisms have been reported to oxidize FA but not to assimilate it. Kato *et al.* (1982, 1983) reported isolation of FA-resistant, non-methylothrophic bacteria and yeasts from soil. Resistance was due to detoxification of FA by enzymatic oxidation to formic acid or dismutation to methanol and formic acid.

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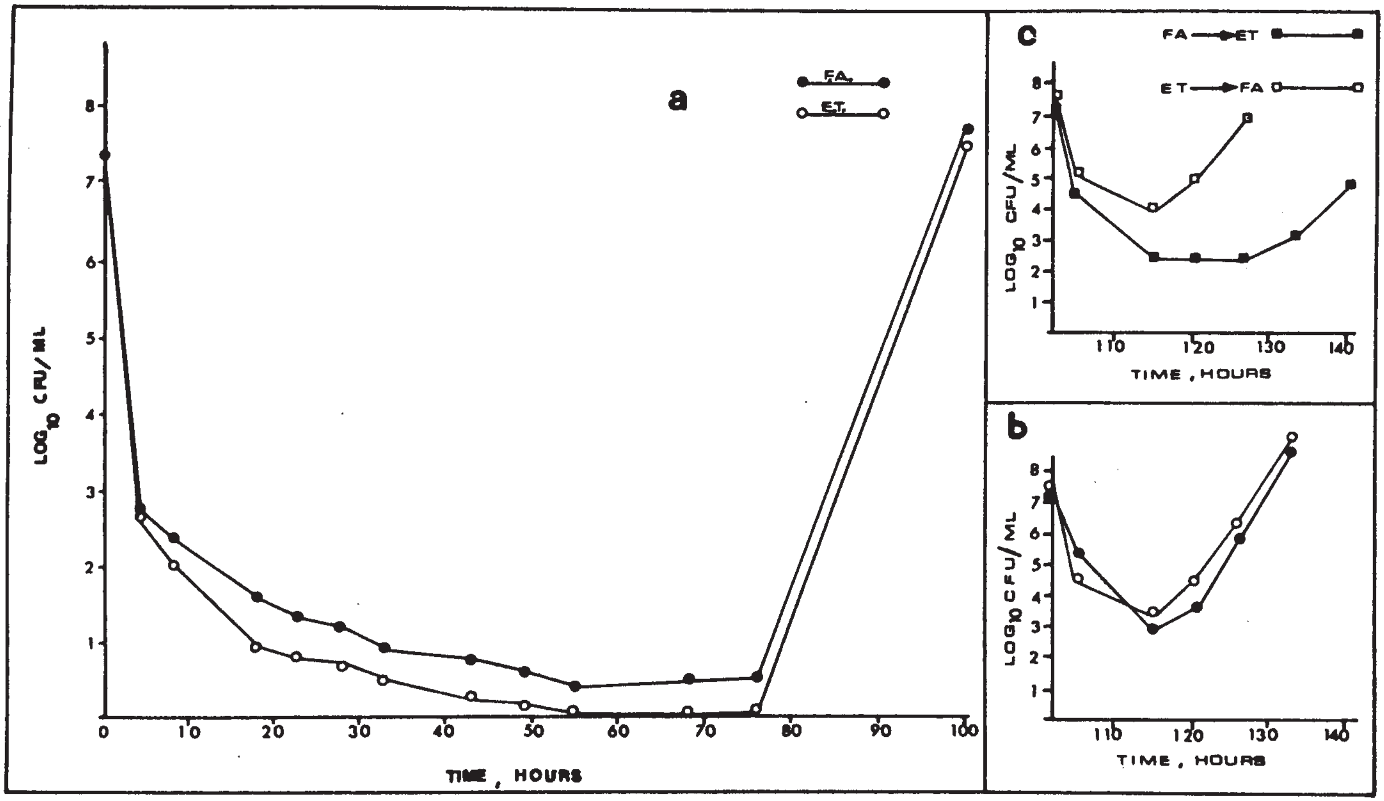

Fig 1. (a) Initial treatment of *Pseudomonas aeruginosa* with formaldehyde (FA), 5.25 mM, or hexahydro-1,3,5-tris ethyl-s-triazine (ET), 1.75 mM. (b) Subsequent treatment with same concentrations of FA and ET. (c) Treatment with alternate biocides.

Since treatment of *Ps. aeruginosa* with ET results in development of resistance to FA, the mechanism of resistance may involve FA detoxification. An assay for FA-oxidizing activity (FA dehydrogenase) was done on crude extracts of untreated cells and cells treated for 100 hours with ET. The specific activity of FA dehydrogenase increased from 0.16 to 2.83 (μ moles NADH formed/hr/mg protein) as a result of treatment with the FA condensate biocide.

These results are part of a series of experiments to demonstrate the development of resistance of microorganisms to FA and FA condensate biocides. In addition, we hope to demonstrate the release of FA from these compounds and thus determine the general mode of action as well as development of resistance or cross-resistance.

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