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Induction and selection of formaldehyde-based resistance in *Pseudomonas aeruginosa*

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SUMMARY

A formaldehyde resistant (R) phenotype of *Pseudomonas aeruginosa* was isolated from a formaldehyde-sensitive (S) parent by sequential treatment with 1,3,5-tris-(ethyl)hexahydro-*s*-triazine (ET). The resistance of the (R) strain to treatment with ET was approximately 3-fold higher than the parental (S) strain. Two modes of resistance to ET, and simultaneous resistance to formaldehyde, are demonstrated: (1) transient or induced resistance is expressed during short-term exposure to ET, and this resistance is gradually lost during subsequent growth in the absence of ET, and (2) resistance that results from a stable phenotypic change in the (S) strain following sequential treatment with ET ((R) strain phenotype). The observed activities of three forms of the formaldehyde oxidizing enzyme, formaldehyde dehydrogenase, are strongly correlated with the relative response of the (S) and (R) strains to treatment with ET. The observed resistance of the (R) strain appears to be due to high levels of an NAD^+-linked, glutathione-dependent form of formaldehyde dehydrogenase as well as a dye-linked formaldehyde dehydrogenase. The transient or induced response of the (R) strain involves an increase in activity of the dye-linked formaldehyde dehydrogenase. The induced response of the (S) strain and an ATCC strain of *P. aeruginosa*, however, is correlated with the two forms of the NAD^+-linked enzyme (glutathione-dependent (EC 1.2.1.1) and independent (EC 1.2.1.46)) with no contribution from the dye-linked enzyme.

INTRODUCTION

The development of resistance of *Pseudomonas* spp., especially *Pseudomonas aeruginosa*, to antibiotics and chemical biocides is well documented [6]. Microorganisms differ greatly in their intrinsic or inherent resistance to toxic chemical agents, and they may also acquire resistance in a number of different ways, including modification of the target of the chemical agent or developing a system for altering or detoxifying the chemical agent [2,3,5,6].

Previous reports on the phenomenon of regrowth of *P. aeruginosa* following treatment with 1,3,5-tris-(ethyl)hexahydro-*s*-triazine (ET) showed

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that the regrown populations were more resistant to the same levels of ET used in the initial testing [12,13]. Preliminary studies of the basis for resistance to ET or cross-resistance to formaldehyde suggested that formaldehyde dehydrogenase (glutathione-dependent, NAD^+-linked) was involved [13].

Other studies have described resistance development of microorganisms, mainly *Pseudomonas* species, in industrial systems to biocides, including [4,8]. These studies provided evidence for plasmid-mediated resistance, although the mechanism of resistance was not determined.

The experiments reported here describe the selection of a formaldehyde-resistant phenotype of *P. aeruginosa* from a sensitive parent using ET as the selective agent. Comparisons of the formaldehyde dehydrogenase activity of these two strains suggest a basis for the observed phenotypes.

MATERIALS AND METHODS

Media and culture conditions

All cultures were maintained on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI), grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) for 12 h, and transferred to fresh medium for 3–5 h prior to use to ensure that inocula were in exponential phase. Population levels of inocula were between 1 and $5 \cdot 10^7$ cfu/ml. Mineral salt base was prepared [11] containing 0.2% glucose as the carbon source (MSB-G).

All experiments were carried out in 250 ml flasks with a total suspension volume of 100 ml. Flasks were incubated at 30°C with rotary shaking at 200 rpm. Agar plates were incubated at 30°C for 48 h.

Biocide treatment

A 10% stock solution of ET in distilled water was used (10% active ingredient, w/v). A stock solution of formaldehyde was prepared from reagent grade (37.7%) formalin to give an equal molar concentration of formaldehyde as compared to the calculated formaldehyde content in the stock solution of ET. Since the synthesis of ET involves 3 mol of formaldehyde, and it is expected to release 3 mol upon chemical hydrolysis, 1 mM of ET equals 3 mM of formaldehyde.

Organisms

An isolate of *P. aeruginosa* obtained from contaminated metalworking fluid [12] was used in this study. The minimal inhibitory concentration (MIC) of formaldehyde by tube dilution was 5 mM in TSB for this strain (formaldehyde sensitive (S) strain). *P. aeruginosa* (ATCC 27853) strain was also used for studies of formaldehyde dehydrogenase.

Resistance induction

Exponential cultures of the (S) strain in TSB at $1–5 \cdot 10^7$ cfu/ml were treated with 5 mM of formaldehyde or 1.67 mM (300 ppm) of ET (equimolar concentrations of available formaldehyde). When the populations had regrown ($5–7 \cdot 10^7$ cfu/ml), the cultures were collected by centrifugation and resuspended in fresh TSB at $1–5 \cdot 10^7$ cfu/ml. These populations were then treated with the same concentrations of formaldehyde or ET to determine the degree of resistance induction [13].

A stable resistant phenotype (R) strain was isolated following sequential treatment of the (S) strain survivors with increasing concentrations of ET. Induction of resistance was also tested in MSB-G medium with 0.1% methanol. Cultures of the (S) strain were grown in the presence or absence of methanol at $1–5 \cdot 10^7$ cfu/ml and challenged with 150 ppm of ET.

Preparation of cell-free extracts

A 2 l culture of each of three strains of *P. aeruginosa* ((S), (R) and ATCC 27853) were grown in TSB to a cell density of $1 \cdot 10^8$ cfu/ml and used to prepare cell-free extracts.

Duplicate cultures of the (S) strain and the ATCC strain were induced with 250 ppm ET and allowed to regrow, or the culture was grown in TSB containing 1% methanol.

Each of the control cultures or the ET-treated cultures were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer, pH 7.5. The cells were lysed by two passages through a French pressure cell at 4°C, and cell-free extracts were obtained by centrifugation.

Enzyme assays

Spectrophotometric enzyme assays were performed at 25°C in 1 cm quartz cuvettes (NAD^+-linked formaldehyde dehydrogenase) or 1 cm glass cuvettes (dye-linked formaldehyde dehydrogenase). Formaldehyde dehydrogenase activity was determined by measuring the formaldehyde-dependent reduction of NAD^+ to NADH (absorbance at 340 nm). The reaction mixture was prepared according to Ando, et al. [1]. For the glutathione-dependent activity, the reaction mixture contained 0.5 mM glutathione at pH 8.0.

Dye-linked formaldehyde dehydrogenase was assayed by measurement of the rate of reduction of DCPIP (2,6-dichlorophenolindophenol) at 600 nm. The complete reaction system in a volume of 2.0 ml contained 0.5 μmol DCPIP in 30 mM potassium phosphate buffer, 0.1 ml of cell-free extract, and 0.1 ml 50 mM formaldehyde at a pH of 7.5. The dependence of DCPIP reduction on the presence of PMS (phenazine methosulfate) was determined by conducting the reaction with or without 0.1 ml of 0.01% PMS. All reactions were initiated with formaldehyde and the absorbance change at 600 nm measured versus a reference cuvette containing all components except formaldehyde. The formaldehyde solution used for the enzyme assays was prepared by heating a 3% (w/v) aqueous suspension of paraformaldehyde to approximately 100°C overnight. The concentration of total protein in the cell-free extracts was measured by the Biuret method using bovine serum albumin as the standard [7]. One unit of NAD^+-linked formaldehyde dehydrogenase activity is expressed as the formation of 1.0 μmol of NADH per min. One unit of dye-linked formaldehyde dehydrogenase activity is expressed as 1.0 nmol of DCPIP reduced per min. The specific activity of the formaldehyde dehydrogenase activity is expressed as units per mg protein (Table 1).

RESULTS

Sequential treatment of the (S) strain of *P. aeruginosa* with 5 mM formaldehyde or 1.67 mM ET (equimolar formaldehyde resulted in a gradual increase in resistance of the population) (Fig. 1). An isolate from a similar sequential treatment using increasing concentrations of ET had developed a stable resistance to ET or formaldehyde ((R) strain). This resistance was not lost after sequential transfers over a period of more than 3 months in TSB. The degree of resistance to ET of the (R) strain is shown in Fig. 2. Additional treatment of this strain with 1000 ppm ET (16.69 mM formaldehyde equivalents) did not result in any further increase in resistance. The absence of further increase of resistance of the (R) strain may indicate a threshold level

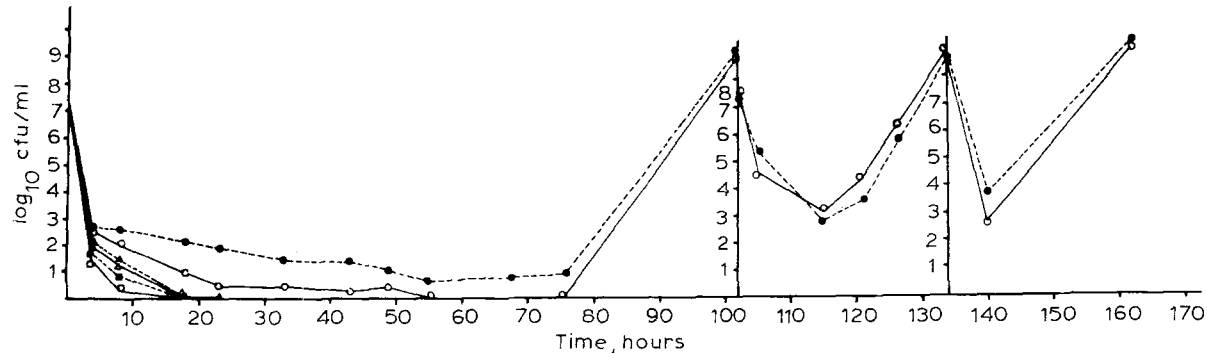

Fig. 1. Exponential cultures of the sensitive (S) strain of *Pseudomonas aeruginosa* were exposed to various levels of formaldehyde ET in tryptic soy broth (TSB). The survivors of the lowest concentration of biocide (5 mM formaldehyde or 1.67 mM ET) were resuspended in fresh TSB after approx. 100 h and treated with the same type and amount of biocide. The survivors of the second treatment were again resuspended in fresh TSB and treated with the same type and amount of biocide. ●-----●, 5 mM formaldehyde; ○——○, 1.67 mM ET (300 ppm: 5 mM formaldehyde equivalents); ▲-----▲, 6.66 mM formaldehyde; △——△, 2.22 mM ET (400 ppm); ■-----■, 8.31 mM formaldehyde; □——□, 2.77 mM ET (500 ppm).

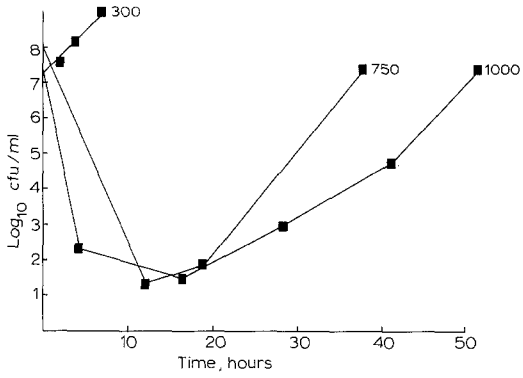

Fig. 2. The resistant (R) strain of *Pseudomonas aeruginosa* was treated with various concentrations of ET in tryptic soy broth (TSB). 300 ppm, 1.67 mM ET or 5 mM formaldehyde equivalents; 750 ppm, 4.17 mM ET or 12.5 mM formaldehyde equivalents; 1000 ppm, 5.56 mM ET or 16.69 mM formaldehyde equivalents.

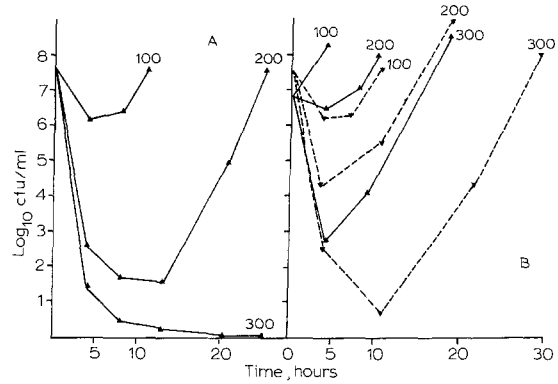

Fig. 3. (A) The sensitive (S) strain was treated with three levels of ET (100, 200, and 300 ppm). After the survivors of the 100 ppm treatment had recovered to approximately 1 · 10^8 cfu/ml, they were harvested and resuspended in fresh tryptic soy broth (TSB). (B) The survivors from the 100 ppm ET treatment (above) were divided into two parts. One part was immediately exposed to 100, 200, or 300 ppm ET (▲——▲), and the other part was subcultured in tryptic soy broth (TSB) for 50 h with several transfers to fresh media prior to exposure to ET (▼----▼).

of formaldehyde toxicity, but it is also possible that further treatment with 1000 ppm ET or with lower levels of ET or formaldehyde might result in increased levels of resistance.

The response of the (R) strain to either ET or formaldehyde was equivalent, as is true also for the (S) strain (Fig. 1), and this response represents a stable phenotypic change that is not lost upon repeated subculture in the absence of ET (data not shown).

A second type of resistance can be achieved by short-term treatment of the (S) strain with low levels of ET (100 ppm or 1.67 mM formaldehyde equivalents, Fig. 3A). Such a treatment resulted in a change in the response of the survivors to subsequent exposure to ET (compare Figs. 3A and 3B). This response is referred to as induction or transient resistance, since the sensitivity of the treated cultures gradually returns following growth in the absence of ET (Fig. 3B). Growth of the (S) strain in mineral salts base containing glucose and methanol also resulted in the induction of resistance to ET (Fig. 4).

The results shown in Table 1 reveal that the (S) and (R) strains as well as another strain of *P. aeruginosa* (ATCC 27853) are all capable of the oxidative use of formaldehyde via formaldehyde dehy-

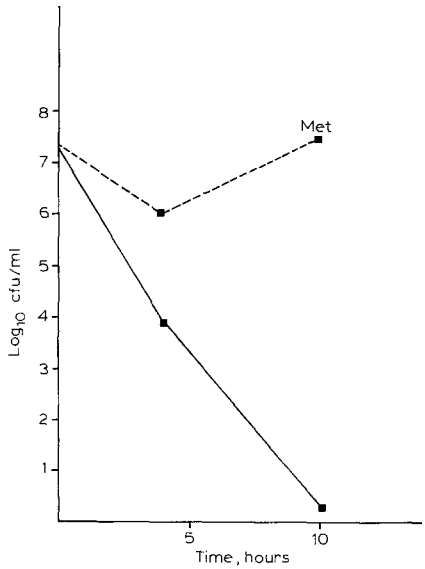

Fig. 4. The sensitive (S) strain was pregrown in mineral salt base containing 0.2% glucose as the carbon source (MSB-G) medium with or without added methanol (0.1%). The cultures were then harvested by centrifugation, resuspended in MSB-G and treated with 150 ppm ET. ■-----■ Met, pregrown in 0.1% methanol; ■——■, pregrown without methanol.

Table 1

Specific activity of formaldehyde dehydrogenase (FADH) in cell-free extracts of *Pseudomonas aeruginosa*

DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; ET, 1,3,5-tris-(ethyl)hexahydro-*s*-triazine; n.d., not detectable.

Strain	Inducer	NAD^+-linked FADH (μmol NADH/min per mg protein)		DCPIP-linked FADH (nmol DCPIP/min per mg protein)	
		glutathione-independent	glutathione-dependent	PMS-independent	PMS-dependent
ATCC 27853	none	n.d.	11.20	n.d.	1.047
ATCC 27853	ET	7.90	82.44	n.d.	0.71
Sensitive	none	n.d.	2.65	n.d.	n.d.
Sensitive	ET	4.08	47.19	n.d.	n.d.
Resistant	none	n.d.	219.59	2.26	9.86
Resistant	ET	11.79	200.23	5.94	26.49
Resistant	methanol	2.375	260.13	3.5	13.48

drogenase). The (S) strain and the ATCC strain (27853) contain detectable levels of the glutathione--dependent, NAD^+-linked formaldehyde dehydrogenase, and this enzyme is inducible by exposure to ET. In the absence of induction, the (R) strain contains from 20 to 100-times the activity of this glutathione-dependent formaldehyde dehydrogenase, as compared to either the ATCC strain (27853) or the (S) strain. However, this form of formaldehyde dehydrogenase is present in high levels in the (R) strain and apparently is not inducible to any significant degree by exposure to ET or growth in medium containing methanol.

The glutathione-independent formaldehyde dehydrogenase was not detectable in any of the three strains tested unless the cultures had been previously exposed to the formaldehyde condensate biocide ET (Table 1). This enzyme was also induced to low levels in the (R) strain by pregrowth in MSB containing glucose and methanol.

The dye-linked form of formaldehyde dehydrogenase was present only in very low or undetectable levels in the ATCC strain (27853) or the (S) strain, and exposure to ET had no effect. The (R) strain, however, contained this enzyme in measurable amounts, and the enzyme was inducible by exposure to ET and, to a lesser extent, by growth in the presence of methanol. The activity of the DCPIP-linked formaldehyde dehydrogenase in the (R) strain was stimulated 4–5-fold by the addition of PMS.

DISCUSSION

Pseudomonas aeruginosa is not a methylotroph and is not thought to assimilate one-carbon compounds, such as methanol or formaldehyde, into cell material. The use of formaldehyde, however, as an electron source via oxidation of formaldehyde to formic acid can be accomplished by a wide range of microorganisms utilizing an NAD^+- or dye-linked formaldehyde dehydrogenase [1,9,10].

The experiments reported here provide evidence for two modes of resistance to the formaldehyde condensate biocide ET in *P. aeruginosa*. Both types of resistance to ET confer simultaneous resistance to formaldehyde. One type of resistance is inducible by short-term exposure to ET and is gradually lost during subsequent growth in the absence of ET. The basis for this transient resistance appears to be two forms of an NAD^+-linked formaldehyde dehydrogenase. A glutathione-independent formaldehyde dehydrogenase is induced by ET, and the observed activity of the glutathione-dependent enzyme is greatly stimulated by exposure to ET.

A second mode of resistance is represented by a stable phenotypic change in the cells following sequential treatment of the sensitive (S) strain with ET. This resistance allows survival of the (R) strain in higher concentrations of ET or formaldehyde, and the basis for this mode of resistance apparently also involves the oxidative detoxification of formaldehyde, as was observed for the transient mode of resistance.

The detoxification of formaldehyde by the (R) strain involved three forms of formaldehyde dehydrogenase. The first enzyme (glutathione-independent, NAD^+-linked) is a minor component of the resistance and requires induction by ET or growth in methanol. The second enzyme (glutathione-dependent, NAD^+-linked) is essentially independent of induction by ET or methanol and is present at high, constant levels of activity. The third enzyme (dye-linked formaldehyde dehydrogenase) is unique to the (R) strain (except for low levels detected in the ATCC strain 27853) and is inducible by ET or methanol.

Studies of mode of resistance do not directly confer information about the mode of action of biocides. Although we can state with certainty that the formaldehyde condensate biocide (ET) used for these studies is biocidal via the release of formaldehyde, this does not shed light on the mode of action of formaldehyde per se. Nevertheless, the methods used here allow studies of modes of resistance, and such studies may provide clues about the mode of action of a particular biocide by, for example, identifying the altered target that changes during resistance development.

Although the evidence presented here is circumstantial, the correlated changes in formaldehyde dehydrogenase activity and resistance strongly suggest that the observed resistance involves the oxidative detoxification of the biocide. Other studies [14] compare the response of the (R) and (S) strains to a number of biocides, some of which are thought to release formaldehyde as the toxic agent and others that probably do not. These studies of the comparison of the responses of an (R) and (S) strain to a wide range of biocides are attempts to study the mode of action of biocides via studies of mode of resistance. Theoretically, a strain resistant to any biocide could be selected; comparison of the response of a (R) strain and its (S) parent to various biocides would allow the characterization of biocides into classes or types based on the pattern of resistance and cross-resistance observed.

This approach to the modes of action of biocides offers great promise, in a practical as well as theoretical sense, in providing the information necessary for choosing an appropriate alternative biocide should resistance develop in an in-use system or in providing the clues necessary to develop new biocides with altered modes of resistance.

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