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The mechanism of microbial resistance to hexahydro-1,3,5-triethyl-*s*-triazine

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SUMMARY

Four strains of *Pseudomonas putida* and two unidentified *Pseudomonas* species that were resistant to hexahydro-1,3,5-triethyl-*s*-triazine (HHTT) were shown to be resistant to formaldehyde as well. Conjugation experiments revealed that: (a) HHTT and formaldehyde resistance was cotransferred in every case where exconjugants were recovered; (b) in every case HHTT resistance and formaldehyde resistance were expressed to the same level in the exconjugant as in the donor; (c) resistance to either HHTT or formaldehyde alone was never observed; and (d) in instances where HHTT and formaldehyde resistance in the exconjugants was unstable, the exconjugants lost resistance to both agents simultaneously and never to one agent alone. Resistant organisms (e.g. *P. putida* 3-T-15[2]) had high levels of formaldehyde dehydrogenase and this enzyme appeared to be constitutively expressed. It was concluded that resistance to HHTT was due to resistance to its degradation product, formaldehyde, via detoxification of formaldehyde by formaldehyde dehydrogenase. HHTT- and formaldehyde-sensitive organisms had barely detectable levels (most likely repressed levels) of formaldehyde dehydrogenase. Although speculative, it is possible that formaldehyde resistance may be due to a mutation resulting in derepression of the gene coding for formaldehyde dehydrogenase. While it could not be discerned whether HHTT resistance and formaldehyde resistance were carried on two separate but closely linked genes or if only one gene was involved, the evidence suggested that only one gene was involved. Similarly, it could not be determined whether HHTT and formaldehyde resistance was encoded by chromosomal or plasmid genes.

INTRODUCTION

We have shown that a major mechanism of antimicrobial action of hexahydro-1,3,5-triethyl-*s*-triazine (HHTT) was through its degradation product, formaldehyde [3]. Thus, the HHTT-resistant isolate, *Pseudomonas putida* 3-T-15[2] was resistant to formaldehyde as well. This organism could also use either HHTT or ethylamine, a component of HHTT, as a nitrogen source for growth. Neither HHTT, ethylamine or formaldehyde served as a carbon and energy source for growth. Nevertheless,

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by manometric experiments, *P. putida* 3-T -15[2] was shown to metabolize HHTT as evidenced by oxygen utilization. We speculated, therefore, that the oxygen uptake was due to oxidation of formaldehyde by formaldehyde dehydrogenase. Other investigators similarly have cited examples of formaldehyde-resistant yeasts and bacteria that exhibited high levels of formaldehyde dehydrogenase activity [8–10].

With the foregoing in mind, we initiated studies to determine: (a) that the mechanism of resistance to HHTT was actually due to resistance to formaldehyde via the organism's ability to oxidize formaldehyde; (b) whether the resistance to HHTT and to formaldehyde was genetically related; and (c) if HHTT resistance and formaldehyde resistance were coded by chromosomal or plasmid gene(s).

MATERIALS AND METHODS

Organisms. In addition to the HHTT- and formaldehyde-resistant *Pseudomonas putida* 3-T-15[2] and its HHTT- and formaldehyde-sensitive, novobiocin-cured derivative, *P. putida* 3-T-15[2] 11:21 [3,6], five other HHTT- and formaldehyde-resistant organisms isolated from contaminated kaolin slurries were examined. All contained from one to five plasmids each. Three of these isolates were identified as *P. putida* and they were assigned the strain designations: *P. putida* 3-T-15[2] B18, *P. putida* ACFX Ch. sm., and *P. putida* 3-T-15[2] R1-34. The remaining two isolates were shown to belong to the genus *Pseudomonas* but the species to which they belonged was not clearly determined, although they appeared not to be *P. aeruginosa*. They were given the designations: *Pseudomonas* sp. ACFX-TCX and *Pseudomonas* sp. 3-T-14. For comparative and control purposes, *P. putida* ATCC 12633 and *P. aeruginosa* PA01 were also used. *Escherichia coli* MC4100 and *E. coli* ML 308-225 were used in conjugation experiments as potential recipients.

Media. All HHTT-resistant organisms were maintained on Luria (L) agar slants (10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar and deionized water to 1 liter) containing in final concentration 500 ppm HHTT (v/v). The other organisms used were maintained on slants or plates of L-agar. All cultures were kept at room temperature and transferred every 2–3 weeks.

For conjugation experiments, L-agar plates supplemented with the appropriate selective agents were used to recover the exconjugants.

Cultivation of organisms. For the formaldehyde dehydrogenase assays, each organism tested was cultivated aerobically on a rotary shaker at 30°C in 1 liter of the chemically defined basal salts medium previously described [5] and supplemented with 11 mM glucose (BSG). The organisms were harvested in late exponential phase by centrifuging (4°C) at 8000 × *g* and were washed with 0.01 M potassium phosphate buffer, pH 7.5. The washed cells were centriuged again and suspended to 20 ml with the same buffer containing 1 mM dithiothreitol (DTT) (Sigma Chemical Co., St. Louis, MO).

Cell extracts were obtained by passing the cells twice through a French press at 15000 lb/in[2]. Cell debris and intact cells were removed by centrifuging the suspension (4°C) at 20000 × *g* for 30 min. The cell extracts were dialyzed (molecular weight exclusion limit, 6000–8000) overnight at 4°C against 0.01 M potassium phosphate buffer, pH 7.5, containing 1 mM DTT.

Measurement of formaldehyde dehydrogenase activity. The cell extracts were assayed for formaldehyde dehydrogenase activity according to the procedure of Kato et al. [8]. Enzyme activity was expressed as nmol NADH formed/min per mg protein.

Minimal inhibitory concentration (MIC). MIC determinations were made in Antibiotic Medium 3 (Difco Laboratories, Detroit, MI) according to the serial dilution test-tube method of Anderson [1]. The results were recorded after 72 h of incubation at 32°C.

Curing experiments. Hall and Eagon [6] reported that *P. putida* 3-T-15[2] was cured of a 32.5 Md plasmid carrying HHTT resistance through treatment with novobiocin. In order to gain evidence whether HHTT and formaldehyde resistance were plasmid-mediated in the other HHTT- and formaldehyde-resistant strains, these isolates were subjected to

curing procedures with novobiocin (Sigma Chemical Co., St. Louis, MO) as previously described [6]. The organisms tested were: *P. putida* 3-T-15² B18, *P. putida* ACFX Ch. sm., *P. putida* 3-T-15² R1-34, *Pseudomonas* sp. ACFX-TCX, and *Pseudomonas* sp. 3-T-14.

Isolation and characterization of plasmid DNA. Plasmid DNA was extracted by the method of Kado and Liu [7] modified as previously described [4] but with a further minor modification in that samples were loaded onto a 0.65% agarose gel using a horizontal gel apparatus and electrophoresed in the TAE buffer at 25 V overnight.

Conjugation experiments. To determine whether HHTT resistance and formaldehyde resistance were genetically linked, mating experiments were carried out as previously described [6] using the various HHTT-resistant strains as donors. *P. putida* ATCC 12633 served as the recipient.

In order to evaluate the transfer compatibility between *P. putida* and other bacterial species, *P. aeruginosa* PA01, *E. coli* MC4100 and *E. coli* ML 308-225 were also tested as recipients.

Spontaneous mutants (i.e., resistant to different antibiotics) of each of these organisms were selected (see footnotes a and b to Table 3). Exconjugants that appeared on the selective medium (L-agar containing 250 μg/ml streptomycin and 500 ppm HHTT) were screened for resistance to formaldehyde by replica plating on L-agar containing 250 and 500 ppm formaldehyde.

Other. Protein was determined by the Lowry procedure as modified by Markwell et al. [11]. Reagent grade formalin (37% formaldehyde, J. T. Baker Chemical Co., Phillipsburg, NJ) was the source of formaldehyde. All other chemicals and reagents in the highest state of purity were purchased from commercial sources.

RESULTS

Formaldehyde dehydrogenase activity

As shown in Table 1, cell extracts of HHTT- and formaldehyde-resistant *P. putida* 3-T-15² exhibited high levels of formaldehyde dehydrogenase activity, while cell extracts from HHTT- and formaldehyde-sensitive, novobiocin-cured *P. putida* 3-T-15² 11:21 had barely detectable levels of this enzyme. *P. putida* ATCC 12633 also had low formaldehyde dehydrogenase, but the activity was 2-fold higher than that exhibited by *P. putida* 3-T-15² 11:21. The formaldehyde dehydrogenase found in all three organisms was dependent upon NAD^+ and reduced glutathione (GSH) for activity.

MIC values

MIC determinations showed that each HHTT-resistant isolate was also equally resistant to formaldehyde, and that the HHTT-sensitive, novobiocin-cured strain, *P. putida* 3-T-15² 11:21, was susceptible to formaldehyde as well (Table 2). Moreover, exconjugants resulting from matings between the HHTT-resistant isolate, *P. putida* 3-T-15² R1-34, and *P. putida* ATCC 12633 and *P. aeruginosa* PA01 also showed high levels of resistance to formaldehyde as well as to HHTT.

Curing experiments

As evidenced by agarose gel electrophoresis, three classes of plasmid profiles were detected in the five additional HHTT-resistant isolates used in this study (data not shown). Both *Pseudomonas* sp. ACFX-TCX and *Pseudomonas* sp. 3-T-14 contained one large plasmid approximately 61 Md in size. *P. putida* ACFX Ch. sm. and *P. putida* 3-T-15² B18 contained five plasmids ranging in size from about 65 Md to less than 5 Md. Four plasmids were detected in *P. putida* 3-T-15² R1-34, three of which appeared to be shared with strains ACFX

Table 1

Formaldehyde dehydrogenase activity in dialyzed cell extracts

Organism	Enzyme activity[a]
P. putida 3-T-15²	500[b]
P. putida 3-T-15² 11:21	6.5
P. putida ATCC 12633	13

[a] Enzyme activity = nmol NADH formed/min per mg protein.
[b] This is an average value. Enzyme activities ranged from 400 to 600 for this organism.

Table 2

MIC determinations

Organism	MIC (ppm)	
	HHTT	formaldehyde
P. putida 3-T-15[2]	1000	1000
P. putida 3-T-15[2] 11:21	31	16
P. putida 3-T-15[2] R1-34	1000	1000
P. putida 3-T-15[2] B18	1000	1000
P. putida 3-T-15[2] ACFX Ch. sm.	1000	1000
Pseudomonas sp. 3-T-14	1000	1000
Pseudomonas sp. ACFX-TCX	1000	1000
P. putida ATCC 12633	125	31
P. aeruginosa PA01	125	31
P. putida ATCC 12633 (HHTT, HCHO)[a]	1000	1000
P. aeruginosa PA01 (HHTT, HCHO)[b]	1000	1000

[a] An exconjugant from the mating of *P. putida* ATCC 12633 with *P. putida* 3-T-15[2] R1-34.
[b] An exconjugant from the mating of *P. aeruginosa* PA01 with *P. putida* 3-T-15[2] R1-34.

Ch. sm. and 3-T-15[2] B18. Lacking in *P. putida* 3-T-15[2] R1-34, however, were two large plasmids (approximately 65 Md and 35–40 Md respectively) possessed by strains ACFX Ch. sm. and 3-T-15[2] B18. However, no one plasmid was detected that was shared by every HHTT- and formaldehyde-resistant isolate.

In an attempt to determine whether different

Table 3

Conjugal mating experiments

Donor[a]	Recipient[b]	Transfer frequency[c]
P. putida 3-T-15[2] B18	*P. putida* ATCC 12633	$1.3 \cdot 10^{-3}$[d]
P. putida 3-T-15[2] R1-34	*P. putida* ATCC 12633	$3.7 \cdot 10^{-6}$[d]
P. putida ACFX Ch. sm.	*P. putida* ATCC 12633	$2.0 \cdot 10^{-6}$[d]
Pseudomonas sp. ACFX-TCX	*P. putida* ATCC 12633	$1.0 \cdot 10^{-8}$[e]
Pseudomonas sp. 3-T-14	*P. putida* ATCC 12633	$5.0 \cdot 10^{-8}$[e]
P. putida 3-T-15[2] R1-34	*P. aeruginosa* PA01	$3.5 \cdot 10^{-8}$[d]
P. putida 3-T-15[2] R1-34	*E coli* ML 308-225	No exconjugants recovered
P. putida 3-T-15[2] R1-34	*E. coli* MC 4100	No exconjugants recovered

[a] All donor strains were rifampicin, HHTT and formaldehyde resistant and streptomycin sensitive. The rifampicin-resistant strains were isolated as spontaneous chromosomal mutations.
[b] All recipient strains were rifampicin, HHTT and formaldehyde sensitive and streptomycin resistant. The streptomycin-resistant strains were isolated as spontaneous chromosomal mutations.
[c] Frequency of transfer was calculated as the number of HHTT- and formaldehyde-resistant exconjugants recovered divided by the number of donor cells present at the commencement of mating.
[d] Exconjugants from these matings exhibited stable resistance to both HHTT and formaldehyde with MIC values of 1000 ppm for each agent.
[e] These exconjugants lost resistance to both HHTT and formaldehyde upon one transfer through L-agar containing no HHTT.

plasmids in these strains could carry the same genetic information responsible for HHTT- and formaldehyde resistance, we subjected each of these five strains to curing treatments using novobiocin. Of 100–200 novobiocin-treated subisolates per strain that were tested, we failed to observe any cured strains in that we were unable to demonstrate loss of resistance to either HHTT or formaldehyde.

Mating experiments

Our data suggested that HHTT resistance and formaldehyde resistance were inseparable. Thus, in an attempt to elucidate whether HHTT resistance and formaldehyde resistance were genetically linked, conjugational matings were carried out. Each of the five HHTT- and formaldehyde-resistant isolates (but not strain 3-T-15^2) were mated with *P. putida* ATCC 12633. Transfer frequencies for the matings are shown in Table 3. *P. putida* 3-T-15^2 B18 transferred HHTT resistance and formaldehyde resistance with a high frequency when mated with *P. putida* ATCC 12633. When *P. putida* 3-T-15^2 R1-34 and *P. putida* ACFX Ch. sm. served as donors to *P. putida* ATCC 12633 as recipient, lower transfer frequencies were noted. By agarose gel electrophoresis screens, we were unable to detect concomitant transfer of plasmid DNA, although the exconjugants appeared to be stable. More importantly, all exconjugants showed that cotransfer of HHTT resistance and of formaldehyde resistance had occurred.

Matings between *Pseudomonas* sp. ACFX-TCX and *Pseudomonas* sp. 3-T-14 and *P. putida* ATCC 12633 showed the lowest frequency of transfer (Table 3). The latter two exconjugants also exhibited unstable inheritance of HHTT and formaldehyde resistance in that they lost resistance to both agents upon one transfer through non-selective media (i.e., L-agar containing no HHTT).

Resistance to HHTT and to formaldehyde was transferable to *P. aeruginosa* PA01 although at a low transfer frequency (Table 3). Nevertheless, *P. aeruginosa* exconjugants were recovered from the matings and had MIC values of 1000 ppm to both HHTT and formaldehyde (Table 2). When plasmid screens were performed on the donor, *P. putida* 3-T-15^2 R1-34, on the recipient, *P. aeruginosa* PA01, and on the exconjugant, we were unable to detect a concomitant transfer of a plasmid.

Finally, we were unable to transfer HHTT resistance or formaldehyde resistance to either strain of *E. coli* through conjugational matings (Table 3).

DISCUSSION

Isolates that were resistant to HHTT were concomitantly resistant to formaldehyde as well. Through conjugation experiments, we showed that: (a) HHTT and formaldehyde resistance was cotransferred in every case where exconjugants were recovered; (b) by MIC determinations that were made on exconjugants from each mating, in every case HHTT resistance and formaldehyde resistance were expressed to the same level as in the donor organism; (c) HHTT resistance and formaldehyde resistance always cotransferred and resistance to either agent alone was never observed; and (d) in instances where HHTT and formaldehyde resistance in the exconjugants was unstable, the exconjugants lost resistance to both agents simultaneously, never to one agent alone.

Our data did not permit us to discern whether HHTT resistance and formaldehyde resistance were two separate entities carried on closely linked genes or if only one gene was involved. The evidence suggested, however, that only one gene was involved. Therefore, we conclude that resistance to HHTT exhibited by the isolates that were studied actually resulted from resistance to its degradation product, formaldehyde, and that resistance was due to detoxification of formaldehyde by formaldehyde dehydrogenase.

The formaldehyde dehydrogenase possessed by these HHTT isolates appeared to belong to a specific class of formaldehyde dehydrogenases. Of nine types of formaldehyde dehydrogenase that have been described [2], only one NAD^+-dependent formaldehyde dehydrogenase also required GSH. Thus, this enzyme appears to be formaldehyde: NAD^+ oxidoreductase (EC 1.2.1.1).

P. putida 3-T-15^2 expressed high levels of for-

maldehyde dehydrogenase activity with no prior exposure to HHTT or to formaldehyde (i.e., the cells were cultivated in BSG with no added HHTT or formaldehyde). Thus, it appears that the gene coding for this enzyme is constitutively expressed.

Barely detectable levels of formaldehyde dehydrogenase activity were noted in *P. putida* ATCC 12633 and *P. putida* 3-T-15[2] 11:21. Whether this was true formaldehyde dehydrogenase activity due to a repressed level of formaldehyde dehydrogenase or whether the activity was due to some other substance present in the cell extracts or reaction mixtures could not be discerned. A possible explanation is that formaldehyde resistance is due to a mutation in a regulator gene that functions in the wild-type organism to repress the gene coding for formaldehyde dehydrogenase and, in the resistant organism, the mutated regulator gene no longer functions to repress the gene coding for formaldehyde dehydrogenase, and this latter gene is then fully expressed or perhaps even amplified.

Evidence was previously reported strongly suggesting that HHTT resistance in *P. putida* 3-T-15[2] was plasmid mediated [6]. However, we failed to find evidence that HHTT or formaldehyde resistance was plasmid mediated in the five additional strains of isolates that were studied in this present work. Thus, we were unable to 'cure' the isolates of HHTT or formaldehyde resistance with novobiocin, and we were unable to detect the acquisition of plasmid DNA in exconjugants. Similarly, in a previous report (T. E. Hall, M.S. Thesis, University of Georgia, 1984) it was found that *P. putida* 3-T-14 was resistant to novobiocin curing. These data, however, do not rule out plasmid-mediated HHTT or formaldehyde resistance in these various isolates, since *Pseudomonas* is notoriously difficult to cure of plasmids. Those curing techniques that are successful with *E. coli,* for example, frequently fail with *Pseudomonas*. Thus, whether HHTT and formaldehyde resistance in the various strains studied in this work are encoded by chromosomal or by plasmid genes cannot be discerned at this time. The answer to this question will have to await further experimentation.

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