BIOCIDE RESISTANCE IN YEAST: ISOLATION AND GENERAL PROPERTIES OF TRIALKYL TIN RESISTANT MUTANTS

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1. Introduction

Organo-tin compounds are now widely used as biocides, e.g. trialkyl tin salts and triphenyl tin salts are in use in fungicides, algicides, molluscicides, wood preservatives and marine anti-fouling paints. The mode of action of these compounds appears to be the specific inhibition of energy conservation processes in mitochondrial and chloroplast systems [1, 2, 3], and the biocidal effects can be directly related to effects on oxidative phosphorylation and photosynthesis. The site of action of triethyl tin on mitochondrial energy conservation reactions is suggested to be a step in the energy transferring chain of oxidative phosphorylation [4, 5] or at the same site as oligomycin [2]. However, recent studies suggest that trialkyl tin compounds also mediate a rapid chloride–hydroxide exchange across the mitochondrial membrane [6].

Recent studies in this laboratory have been concerned with the genetics and biochemistry of a series of mutants in which mitochondrial function relating to oxidative phosphorylation has been altered. Studies on oligomycin resistant mutants of *Saccharomyces cerevisiae* have shown that oligomycin resistance is due to a modification of mitochondrial membrane systems involving a change in oligomycin sensitivity of mitochondrial adenosine triphosphatase (ATPase) [7]. This paper describes studies on the isolation and general properties of mutants of *S. cerevisiae* resistant to concentrations of triethyl tin (TET) 10–15-fold greater than that inhibiting the non-resistant strains, and their cross resistance to inhibitors and uncouplers of energy conservation reactions and mitochondrial protein synthesis inhibitors.

2. Materials and methods

2.1. *Strains*

The strains of *S. cerevisiae* used were as described in a previous communication [7]. All mutants discussed here were derived from haploid strain D22 (a, ad_2). Haploid strains D22 and D6 (α, arg, meth) were used as sensitive tester strains throughout.

2.2. *Media*

The complex and synthetic media used in the culturing of the haploid and diploid strains respectively were:

YEP Glu: 1% yeast extract, 2% peptone, 2% glucose, 2% agar;
YEP Gly: 1% yeast extract, 2% peptone, 4% glycerol, 2% agar;
MM Glu: Wickerham minimal medium [8], 2% glucose, 1.5% purified agar (Difco);
MM Gly: As MM Glu but glucose replaced by 4% glycerol.

Solid media containing drugs were made up as previously described [7]. Triethyl tin sulphate was a gift from Dr. W.N. Aldridge, 4,5,6,7-tetrachloro-2-(trifluoromethyl)-benzimidazole (TTFB) was a gift from Dr. R.B. Beechy and "1799" was a gift from Dr. P.G. Heytler. Carbonyl-cyanide, m-chlorophenyl-hydrazone (CCCP) and Mikamycin were purchased from Calbiochem.

2.3. *Isolation of mutants*

Mutants were isolated as resistant papillae appearing after spreading mutagenised (UV) yeast suspensions onto YEP Gly plates containing 5, 10 and 20 μM

triethyl tin sulphate. The mutants thus isolated are given prefixes EA, EB and EC respectively.

2.4. *Resistance to drugs*

Strains were assayed for maximum tolerance to drugs on solid media using the drop-out technique [9].

2.5. *Whole cell respiration*

Oxygen uptake by intact yeast cells was measured polarographically using an oxygen system. Triethyl tin sulphate (TET) was added as an aqueous solution and uncouplers were added as methanolic solutions.

2.6. *Genetic analyses*

The general techniques for mating of haploid strains and subsequent isolation of diploid colonies has been described [7]. Resistance of diploids was assayed either by replica plating isolated colonies onto YEP Gly plates containing triethyl tin sulphate or by aliquot plating of diluted cell suspensions onto MM Gly plates containing the drug.

3. Results and discussion

3.1. *General*

The properties of a series of yeast mutants resistant to TET are described in table 1. Mutants exhibiting 10–20-fold levels of resistance to inhibition of growth on an oxidisable substrate have been isolated and in all cases a 3–4-fold resistance on a fermentable substrate was also observed. The sensitivity of the wild type to low levels of TET when grown on an oxidisable substrate is consistent with its known mode of action on mitochondrial energy conservation reactions. However, the sensitivity to relatively high levels of TET when grown in the presence of glucose is not readily explainable but could be related to an uncoupler type effect on cellular ion transport. The isolation of TET resistant mutants which reflect specific modifications of mitochondrial membrane systems is thus complicated by the presence of additional site(s) of action of TET and it may be difficult to differentiate mitochondrial specific mutants from others involving cytoplasmic membrane systems such as plasma membrane mutants. The mutants described in table 1 have been selected on the basis of high increases in resistance on glycerol and low increases in resistance on glucose in which case the primary change would be associated with the mitochondrial energy conservation system. In contrast, other mutants have been isolated in which the degree of resistance on glucose parallels the degree of resistance on glycerol which would suggest that we are dealing with plasma membrane mutants or permeability mutants [10].

Growth curves in liquid culture of wild type and mutant yeast strains also provide evidence for a specific effect on mitochondrial metabolism (fig. 1).

Growth on ethanol and the aerobic phase of growth on glucose are inhibited in the case of the parental strain D22 in the presence of 10 μM triethyl tin whereas growth of the resistant mutant D22-EC1 is virtually unaffected under the same conditions.

Table 1
TET resistance levels with glucose and glycerol as carbon sources.

Strain	Tolerance of strains to TET (μM)*	
	Glycerol	Glucose
D22	2	20–40
D6	4	40
D22-EB7	40	80
D22-EB8	40	160
D22-EB16	20	120
D22-EC1	20	80
D22-EC2	20	120
D22-EC7	40	120
D22-EC9	20	120
D22-EC10	20	120
D22-EC11	40	120
D22-EC12	40	120
D22-EC16	40	120
D22-EC17	40	120
D22-EC19	40	120
D22-EC23	20	80
D22-EC24	40	80

The tolerance level to TET is defined as the maximum concentration in the growth medium permitting normal growth of the strain. The concentrations of TET used were 2.0, 4.0, 10.0, 20.0, 40.0 μM on YEP Gly medium and 20, 40, 80, 120, 160, 320 μM on YEP Glu medium.

* Molarities are expressed in terms of the triethyl tin moiety of triethyl tin sulphate.

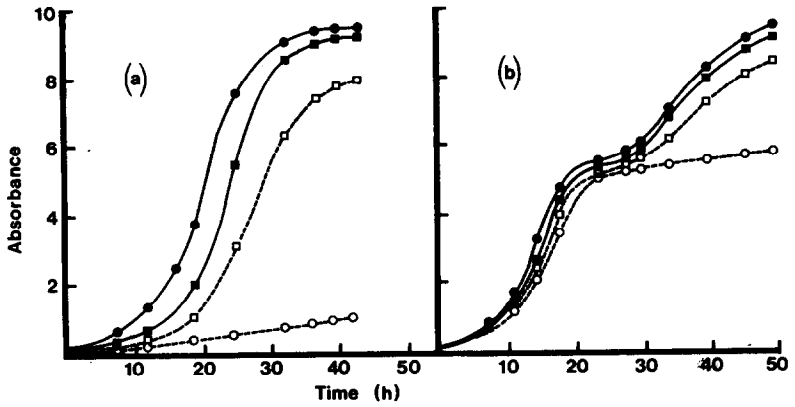

Fig. 1. Growth of wild type strain D22 and resistant mutant strain D22-EC1 using ethanol (a) and glucose (b) in the absence and presence of triethyl tin sulphate. A 1% inoculum of logarithmic phase cells was used in each case and the strains were cultured at 30° in shaking flasks, growth being followed turbidometrically (absorbance reading on EEL colorimeter). ●—●: strain D22 without drug; ○- - -○: strain D22 with 10 μM TET; ■—■: strain D22-EC1 without drug; □—□: D22-EC1 with 10 μM TET.

3.2. *Cross resistance to other alkyl tin compounds*

Cross resistance to other trialkyl tin biocides (trimethyl, tripropyl and tributyl) and to triphenyl tin has been investigated. TET resistant mutants were cross resistant to all trialkyl tins tested and to triphenyl tin (table 2). The differential sensitivity on glucose and glycerol was 8–10-fold with trimethyl tin, and 4–5-fold with tripropyl and tributyl tin but only 2–3-fold with triphenyl tin, suggesting that trimethyl and triethyl tin affect the oxidative metabolism in growing yeast more specifically than do the other tin compounds tested. No cross resistance to diethyl tin was detected.

3.3. *Cross resistance to oligomycin, TTFB, "1799" and mikamycin*

The cross resistance of TET mutants to inhibitors and uncouplers of oxidative phosphorylation and to a mitochondrial protein synthesis inhibitor are summarised in table 3. The mutants fall into three general classes: class 1, specific for TET with little cross resistance to other inhibitors; class 2, marked cross resistance to "1799" but not TTFB, and some cross resistance to mikamycin; class 3, cross resistance to oligomycin, TTFB, "1979" and mikamycin.

Mutants falling into class 1 may be postulated to involve a change in the actual inhibitory or binding site of TET: preliminary studies on isolated mitochondria from resistant mutant D22-EC1 show that the ATPase system is less sensitive to TET than that of sensitive strain D22 [10]. The mutations in class 2 and 3 giving rise to cross resistance to other inhibitors affecting mitochondrial function are more difficult to

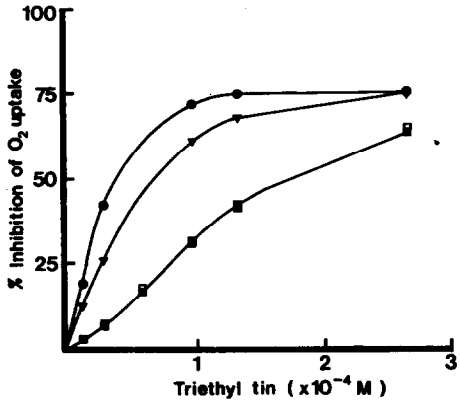

Fig. 2. Effect of TET on whole cell respiration in strains D22 (●—●), D22-EC1 (■—■), D22-EC2 (▼—▼) and D22-EC16 (□—□). Cells were grown up to late logarithmic phase in yeast extract-peptone-ethanol medium in the absence of TET, harvested by centrifugation, washed in sterile distilled water and resuspended to a concentration of about 10^9 cells/ml. The reaction mixture (3 ml) in the oxygen electrode consisted of 5 mM HEPES pH 7.0, 0.1 ml of yeast suspension, and glucose (1%) to initiate respiration. TET was added in various quantities as an aqueous solution.

Table 2
Cross resistance to different trialkyl tin compounds.

Strain	Tolerance* of strain to alkyl tin compounds (μM)				
	Trimethyl tin chloride	Tripropyl tin chloride	Tributyl tin chloride	Triphenyl tin chloride	Diethyl tin chloride
D22	10	2	2–4	4	400
D6	20	4	4	10	400
D22-EB7	80	10	10	10	400
D22-EB8	80	10	10	10	400
D22-E16	80	10	4–10	10	400
D22-EC1	40	4	10	4–10	400
D22-EC2	80	10	10	10	400
D22-EC7	80	10–20	10	10	400
D22-EC9	20	10	10	10	400
D22-EC10	80	10	10	10	400
D22-EC11	80	10	10	10	400
D22-EC12	40	10	10	10	400
D22-EC16	80	10–20	10	10	400
D22-EC17	80	10	4–10	10	400
D22-EC19	80	10	10	10	400
D22-EC23	20	5–10	4–10	10	400
D22-EC24	80	10	10	10	400

* The tolerance of the strain to the trialkyl tin compound is defined as the maximum concentration used which allowed growth of the strain on solid YEP Gly medium. The actual concentrations used in the plates were 2, 4, 10, 20, 40, 80 and 160 μM for the trialkyl tin compounds, and 200, 400 and 800 μM for diethyl tin chloride.

explain especially since these mutants show cross resistance to an inhibitor of mitochondrial protein synthesis. A series of mutants showing similar multiple cross resistance to mitochondrial protein synthesis inhibitors has been reported [11] where it is suggested that the primary site of mutation may have caused a general conformational change to be transmitted throughout the mitochondrial membrane so causing the sites of action of other inhibitors to be modified. A similar explanation may apply in the case of class 2 and 3 mutants or alternatively we suggest that a basic structural unit of the mitochondrial membrane e.g. a "structural protein" or a lipid component, may have been modified in the mutants. These and other alternative explanations are currently being investigated in this laboratory.

3.4. *Inhibition of whole cell respiration by triethyl tin sulphate*

Oxygen uptake by whole cells was studied in sensitive strain D22 and resistant mutants D22-EC1, D22-EC2 and D22-EC16. Late logarithmic phase cells were used and glucose was used as the substrate in the oxygen electrode. In the case of the parental strain respiration was found to be quite sensitive to inhibition by TET, 40 μM giving 50% inhibition of oxygen uptake (fig. 2). The resistant mutants D22-EC1 and D22-EC16 were found to be significantly more resistant to the inhibitory effects of TET, a 160 μM level being required to give 50% inhibition of respiration in these strains. Strain D22-EC2 also showed some resistance to TET in the oxygen electrode but not to such an extent as the other two mutants. The phenotypic character of the mutant cells which have been cultured in the absence of TET suggests that the resistance mutations we are dealing with are not explained in terms of inducible detoxification mechanisms.

The inhibition of whole cell respiration by TET (150 μM) was found to be relieved by CCCP and other classical uncoupling agents of oxidative phosphorylation. In the control experiment TET had no effect on the respiration when maximally stimulated by CCCP (15 μM). This observation would suggest

Table 3
Cross resistance of TET mutants to oligomycin, TTFB, "1799" and mikamycin.

	Tolerance* of haploid strains to inhibitors (μg/ml)					
	Strain	TET (μM)	Oligomycin	TTFB	"1799"	Mikamycin
Class 1	D22-EB7	40	< 1.25	10	20	< 5
	D22-EC1	20	< 1.25	10	10–20	< 5
	D22-EC9	40	< 1.25	10	5	< 5
	D22-EC23	20	< 1.25	10	5	< 5
	D22-EC10	20	< 1.25	10	20	< 5
Class 2	D22-EB8	40	< 1.25	10	40	10
	D22-EB16	20	< 1.25	10	40	5
	D22-EC2	20	< 1.25	10	40	5
	D22-EC17	40	< 1.25	10	40	5
Class 3	D22-EC7	40	> 10	30	60	10
	D22-EC16	40	> 10	> 40	> 60	10
	D22-EC24	40	> 10	> 40	40	10
	D22-EC11	40	1.25	20	40	10
	D22-EC19	40	1.25	20	40	10
	D22	2	< 1.25	10	5	< 5

Strains were tested for resistance to inhibition of growth on solid YEP Gly medium containing various concentrations of the drugs.

* Ranges of concentrations used: Triethyl tin sulphate, 2, 4, 10, 20 and 40 μM; oligomycin, 1.25, 2.5, 5.0 and 10.0 μg/ml; TTFB, 2.5, 5.0, 10.0, 20.0, 30.0 and 40.0 μg/ml; "1799", 5, 10, 20, 40 and 60 μg/ml; mikamycin, 5, 10 and 100 μg/ml.

that the inhibition of respiration in whole yeast cells is due to TET blocking a step in the energy conservation system on the ATPase side of the site of action of uncouplers, a fact in agreement with the postulated mode of action of trialkyl tin compounds [1–3]. Having established this fact it seems reasonable to state that in isolating mutants resistant to TET when growing on an oxidisable substrate we are dealing with mutations in the oxidative phosphorylation complex of the mitochondrion or with mutations preventing TET interacting with this system.

3.5. *Genetic studies*

The diploid strains obtained by mating the resistant mutants to sensitive strain D6 were all found to be more resistant to TET than the control strain D22 X D6 obtained by crossing the two sensitive strains [10]. When diploid cells from a mass mating were grown up to give single colonies and subsequently tested for resistance to TET by replica plating it was found that most strains give rise to either mixed

Table 4
Resistance of diploids derived from TET resistant X sensitive crosses.

Strain	No. of colonies	No. of resistant colonies*	No. of sensitive colonies	No. of mixed colonies
D22 X D6	68	0	68	0
D22-EC1 X D6	56	5	10	41
D22-EC2 X D6	61	19	15	27
D22-EC11 X D6	66	0	0	66
D22-EC12 X D6	77	1	19	57
D22-EC16 X D6	75	3	25	47
D22-EC19 X D6	63	4	0	59
D22-EC23 X D6	80	0	0	80

Diploid colonies were obtained by prototrophic mass mating on MMGlu solid medium followed by spreading a suspension of diploids onto the same medium. The resulting colonies were replicated onto YEP Gly solid medium containing 20 μM TET to test for reistance.

* Resistant colonies on replating and checking for reistance were found to contain a maximum of 2% sensitive cells.

colonies consisting of resistant and non-resistant cells or mixtures of resistant and non-resistant colonies, whereas the diploid D22 X D6 derived from the two sensitive haploids yielded only sensitive cells (table 4). Diploid cells from the primary mass mating were also assayed for resistance by aliquot plating of cells onto MM Gly plates containing TET, and ratios of resistant to non-resistant cells obtained agreed fairly well with those obtained as described in table 4, the only exception being in the case of mutants belonging to class 3, which gave a very low percentage of resistant cells.

The genetic behaviour of all three classes of TET resistant mutant suggests that the mutational events is inherited in a non-Mendelian fashion and further genetic analyses suggest that the TET resistance in mutants of class 1 and 2 is inherited in an extra chromosomal fashion [10].

Extrachromosomal inheritance of resistance to oligomycin and triethyl tin has now been demonstrated in strains of *S. cerevisiae* and cross resistance to uncoupling agents has been demonstrated in both classes of mutants. Studies of these mutants together with studies of specific uncoupler resistant mutants also isolated in this laboratory should provide a useful tool for the studies on organisation and function of mitochondrial membranes.

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