GENETIC ANALYSIS OF MITOCHONDRIAL RESISTANCE TO TETRACYCLINE IN *SACCHAROMYCES CEREVISIAE*

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1. Introduction

It is now well established that mitochondria possess the machinery for synthesising proteins, and that antibacterial antibiotics which react with bacterial ribosomes selectively block mitochondrial synthesis, presumably by reacting with organelle ribosomes in the same way (see review of Roodyn and Wilkie, 1968). Antibiotics showing antimitochondrial activity in the yeast, *Saccharomyces cerevisiae*, include tetracycline (Clark-Walker and Linnane, 1966) and the strain-dependence of degree of inhibition by this antibiotic (*i.e.* genetic control of tolerance level) has been demonstrated (Wilkie, Saunders and Linnane, 1967). The work described here deals with the analysis of spontaneous resistant mutants isolated from sensitive strains of *S. cerevisiae* and provides more detailed information on the genetic control of mitochondrial resistance to tetracycline.

2. Materials and methods

Haploid strains of *S. cerevisiae* of this laboratory were used. Tests of tolerance levels of the mitochondrial system to tetracycline (TC) were essentially as described previously (Thomas and Wilkie, 1968) using a yeast extract (1 per cent.), peptone (1 per cent.), glycerol (4 per cent.) agar (YEPG) as non-fermentable medium and replacing glycerol with 2 per cent. glucose for fermentable medium (YEPS). Sensitivity is defined as the concentration of antibiotic required to inhibit grown completely, of a spot inoculum from a cell suspension of the strain under test on solid YEPG medium. Except where otherwise indicated, the series of concentrations used in tests were 0·025, 0·05, 0·1, 0·25, 0·5, 0·75, 1·0, 2·0, 3·0, 4·0 and 5·0 mg./ml. TC. Where a particular concentration is given as maximum tolerance or resistance level, the strain involved is totally inhibited at the next higher concentration in the series. Slowing down of growth rate was usually seen at maximum tolerance concentrations. When required, more accurate information on cell populations was obtained by plating cells on the drug.

Absorption spectra were obtained from YEPS-grown cells (approximately 10^9/ml.) in the Unicam SP800 spectrophotometer (Thomas and Wilkie, 1968). TC as the hydrochloride (Pfizer) in aqueous solution was added to sterilised liquid and solid medium in the latter just before pouring. Crosses were set up by mixing cells of differently auxotrophic strains of opposite mating type on solid minimal medium. All colonies (prototrophs) that came up were assumed to have arisen from individual zygotes formed by fusion of pairs of cells. Sporulation tests proved the diploid nature of all colonies

sampled in these cases. Anaerobic culture was carried out under nitrogen in a Fildes cylinder.

3. Results

Strains used, together with their respective drug-tolerance levels on YEPG medium are listed in table 1. In all of these strains, growth rate was generally unaffected by the presence of 5 mg./ml. TC in YEPS medium, testifying to the selective action of the drug on mitochondrial synthesis. (Some other strains have been found which are inhibited on a sugar medium containing 4 mg./ml. TC.) This was verified by growing strains to stationary phase in YEPS liquid medium containing 1 mg./ml. TC and recording absorption spectra. Peaks characteristic of cytochromes *a* and *b* seen in

Table 1

Tetracycline tolerance of haploid strains and spontaneous resistant mutants of Saccharomyces cerevisiae *on a non-fermentable agar medium*

Strain	Nuclear markers*	Resistant mutant	Tetracycline tolerance (mg./ml.)
A7	α; his-4; trp-5; ade-6	—	0·1
		7-521	1·0
A8	α; trp	—	0·05
		8-314	2·0
		8-317	1·5
A10	a; leu-1; his-7	—	0·025
		10-414	2·0
		10-435	1·0
		10-509	1·0
A32	a; leu	—	0·1
		32-344	1·0
A33	α; his	—	0·1
		33-321	3·0
D22	a; ade-2	—	0·25

* a, α, mating type; ade, his, leu, trp, requirement for adenine, histidine, leucine and tryptophane respectively.

untreated cultures, were missing in treated cells (fig. 1). The appearance of these mitochondrially-bound enzymes is believed to depend on protein synthesis in mitochondria. The inhibitory effect was reversible after four serial transfers in YEPS medium containing 3 mg./ml. TC.

Aspects of the mechanism of resistance

A number of spontaneous resistant mutants were isolated from drug plates and their origin and resistance levels are recorded in table 1. Mechanisms of resistance would be expected to include alteration in binding site (presumably mitochondrial ribosome) and permeability changes, both at the cell membrane and mitochondrial membrane levels. Evidence for the involvement of organelle membrane in resistance is provided by experiments with anaerobically grown cells. It was found from analysis of mitochondrial resistance to erythromycin (Thomas and Wilkie, 1968) that mutants can be divided into two categories, namely, those that lose resistance following anaerobic culture and those that retain resistance under these circumstances.

Since mitochondrial membranes of *S. cerevisiae* synthesised under anaerobic conditions show considerable alteration from normal, aerobic mitochondria, both from electron microscope studies and biochemically (Criddle and Schatz, 1969), those mutants that lose resistance in this promitochondrial condition are believed to have a resistance mechanism based on changes in organelle membrane permeability to TC. This may depend on the lipid component of the membrane which is affected by anaerobic conditions, since there are indications that qualitative and quantitative alteration of cell wall

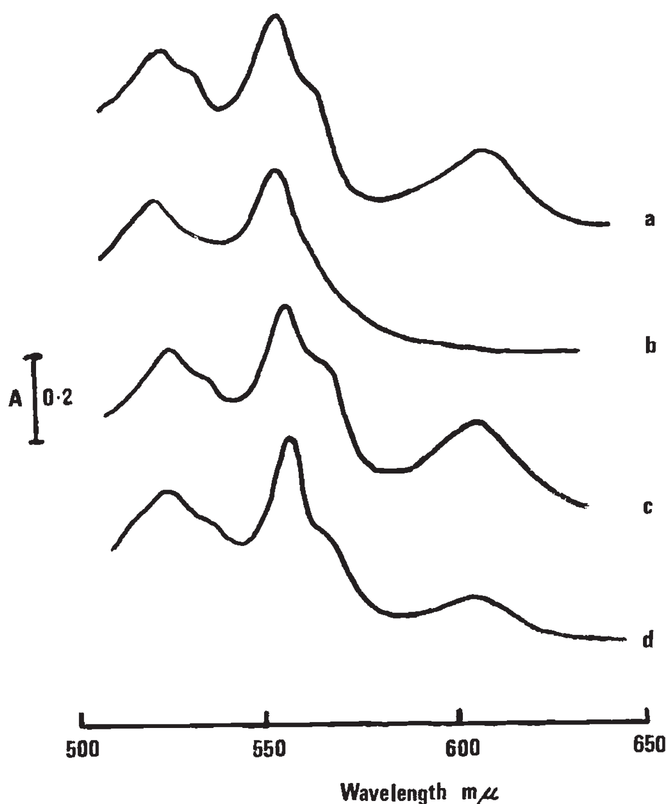

FIG. 1.—Absorption spectra of aerobic cultures of the TC-sensitive strain A10 (tracing *a*) and its resistant mutant 10-435 (*c*). Tracing *b*, strain A10 grown in the presence of 0·5 mg./ml. TC; tracing *d*, 10-435 grown in the presence of 0·75 mg./ml. TC. α peaks of cytochromes *a*, *b* and *c* occur at 605, 562 and 550 mμ respectively with β peaks of *b* and *c* at 530 and 520 mμ respectively.

lipids result in changes in TC tolerance in *Streptococcus pyogenes* (Norrington and James, 1970). A period of aerobic adaptation before testing, restores resistance in these cases.

Genetics of resistance

Resistant mutants 10-509 and 7-521 came into this class of aerobic-conditional resistance. Crosses were set up to determine the genetic basis of resistance in these mutants, the first cross being 10-509 × A7. A number of diploid colonies from this cross were isolated and tested for drug tolerance

by the spot-inoculum test. All showed the resistance level of the mutant (1 mg./ml.) indicating a dominant factor controlling resistance. One of the diploid clones was sporulated, ascospore tetrads microdissected and ascospore clones scored for antibiotic tolerance. All 10 tetrads analysed were found to segregate 2 : 2 resistant to sensitive (parental phenotypes), indicating a single nuclear gene change as the basis of resistance. In the cross 7-521 × A10, similar results were obtained, again indicating a dominant nuclear gene controlling resistance. It was not determined whether the same gene was mutant in each case. There was no evidence of modifying factors since all progeny of resistant ascospores inherited the tolerance level of the resistant parent, as determined by plating cell samples on medium containing TC.

Table 2

Inheritance of tetracycline resistance in crosses between aerobic-conditional resistant mutants

		Tetracycline tolerance (mg./ml.)* Tetrads					
Cross	Diploid	Type 1	No.	Type 2	No.	Type 3	No.
10-414 × 8-317	—	4·0	1	2·0	3	4·0	4
		4·0		2·0		4·0	
		<0·25		1·5		1·5	
		<0·25		1·5		<0·25	
8-317 × 32-344	1·5	1·5	2	1·5	2	1·5	3
		1·5		1·5		1·5	
		0·25		1·0		1·0	
		0·25		1·0		0·25	
10-509 × 8-317	5·0	5·0	2	1·5	3	5·0	7
		5·0		1·5		1·5	
		<0·25		1·0		1·0	
		<0·25		1·0		<0·25	

* Range of concentrations from 0·25 to 5·0 mg./ml. (see methods).

Resistant mutants which were not aerobic-conditional were then analysed in appropriate crosses with sensitive strains. Good 2 : 2 segregations of resistance and sensitivity were obtained in the case of mutants 10-414 and 8-317 (dominant genes), 10-435 and 8-314 (semi-dominant genes) and 32-344 (recessive gene). In these crosses there was no evidence of modifying factors affecting expression of resistance. Further crosses between resistant mutants were set up and analysed with results as shown in table 2. It can be seen from crosses 1 and 3 that there is positive interaction between resistance genes, while in cross 2 the dominant gene of 8-317 is apparently epistatic to the recessive resistance of 32-344. The frequency with which the various tetrad types arose suggests free recombination of the resistance genes of 10-414, 32-344 and 10-509 with that of 8-317. It was concluded that there are several if not many nuclear genes controlling tolerance level and that more than one mechanism of resistance may operate.

In the case of the unconditional resistance, a possible mechanism is alteration in the binding site of the antibiotic (presumably the mitochondrial ribosome). In cases of mitochondrial resistance in yeast to other antibiotics such as erythromycin and paromomycin, in which the mechanism was based

on alteration of the protein synthesising capacity of the organelle in the presence of the drug, genetic control was cytoplasmic (see Wilkie, 1970). Evidence of cytoplasmic inheritance of TC resistance is presented in the section that follows.

Cytoplasmic factors in TC resistance

Strains D22 and A33 have tolerance levels of 0·25 and 0·1 mg./ml. respectively. As in the strains and mutants discussed so far, tolerance level is a stable feature of these strains, that is, the character is transmitted to all daughter cells as deduced from plating experiments. From a cross between D22 and A33, 23 individual diploids (zygote clones) were isolated and scored for TC resistance, using the drop-inoculum method. Of these, one was resistant to 0·05 mg./ml., one to 0·1 mg./ml., fourteen to 0·25 mg./ml., five to 0·5 mg./ml. and two had a tolerance level of 0·75 mg./ml. Although the nuclear genetic complement of individual diploids is the same in any given cross, it is possible for cytoplasmic factors to recombine or reassort either in the zygotes or during growth of diploid colonies from zygotes (Thomas and Wilkie, 1968*a*). Thus analysis of diploids for variation is a first step in establishing cytoplasmic control. This is apparent from these results which contrast with those obtained in previous crosses, including those involving A33.

A diploid from this cross with resistance to 0·25 mg./ml. was investigated further. Constituent cells all showed this resistance level as determined by plating on drug plates. Cells were sporulated for tetrad analysis and from a total of 13 complete tetrads analysed, seven were of a type in which all four products of meiosis inherited resistance to 0·25 mg./ml. and the remaining six tetrads segregated 3 : 1 for tolerance to 0·25 mg./ml. to tolerance to 0·05 mg./ml. TC. It was tentatively concluded that these segregation patterns, together with the variation seen among zygote clones, indicated the presence of cytoplasmic factors for resistance in strain D22.

The spontaneous resistant mutant 33-321, isolated from A33, had a TC tolerance of 3 mg./ml. This resistance was not lost under anaerobiosis and cells when plated, were able to grow into colonies in the presence of 3 mg./ml. in the medium. In these plating experiments, more than 1000 cells were tested at each concentration of the drug used.

A cross was made between 33-321 and D22 and a number of individual diploids were isolated and tested for resistance level. In this analysis, a total of 46 diploids were tested by the drop inoculum method on medium containing respectively 0·5 and 1·0 mg./ml. TC. Four of the diploids were completely inhibited at 0·5 mg./ml., six were resistant to 1·0 mg./ml. and the remaining 36 were resistant to 0·5 mg./ml. One diploid from each of the three classes was analysed further by plating on a drug series and the results are recorded in table 3. It can be seen that cells sampled from a particular clone all showed the same tolerance level. This homogeneity within zygote clones indicated that tolerance level was a characteristic of the zygote, while variation between zygotes in this respect, indicated cytoplasmic control of TC tolerance.

The diploid resistant to 1·5 mg./ml. listed in table 3 was sporulated and ascospore tetrads scored for tolerance levels. The tetrad types and their frequencies are given in table 4. If tetrads are scored on the basis of the proportion inheriting resistance to 3 mg./ml., that is, inheriting the resistance

factor of 33-321, then 27 tetrads show 2 : 2 segregation and one shows 1 : 3 segregation in this respect. Taken at their face value, these results for the resistant diploid indicate that a mendelising gene controls resistance and that it is semi-dominant in the heterozygote. The range in tolerance levels among the " sensitive " ascospore progeny in these tetrads (0·05 to 1·0 mg./ml.) is not readily deduced in terms of nuclear genes. Tetrad analysis of the sensitive diploid (table 3, type 3) gave F_1 progeny which varied in TC tolerance between 0·05 and 0·75 mg./ml., with most tetrads having all four

TABLE 3

Analysis of different diploid clones from the cross 33-321 × D22 by cell plating

Clone type (see text)	Number of colonies from platings* Tetracycline concentration (mg./ml.)					
	0	0·25	0·5	0·75	1·0	1·5
1. Resistant to 1·0 mg./ml. TC	1280	1262	1240	1256	1268	1248
2. Resistant to 0·5 mg./ml. TC	1096	1112	1081	1075	0	0
3. Sensitive to 0·5 mg./ml. TC	802	772	0	0	0	0

* Similar aliquots plated at each concentration of TC in each series.

TABLE 4

Tetrad analysis of a resistant diploid from the cross 33-321 × D22 (table 3, type 1)

Tetracycline tolerance of tetrads in mg./ml.

Type 1	No.	Type 2	No.	Type 3	No.	Type 4	No.
3·0	4	3·0	5	3·0	2	3·0	2
3·0		3·0		3·0		3·0	
0·25		0·25		0·5		0·5	
0·05		0·25		0·5		0·1	
Type 5		Type 6		Type 7		Type 8	
3·0	7	3·0	1	3·0	1	3·0	1
3·0		3·0		3·0		3·0	
0·5		0·5		0·75		0·75	
0·25		0·5		0·1		0·25	
Type 9		Type 10		Type 11			
3·0	3	3·0	1	3·0	1	—	—
3·0		3·0		1·5		—	
0·75		1·5		1·0		—	
0·5		0·1		0·25		—	

products showing resistance to 0·25 mg./ml., but no ascospore inherited resistance to 3 mg./ml. indicating loss of the resistance factor in the sensitive diploid. The analysis of sensitive zygote clones was taken to the F_2 generation following arbitrary selection of first generation segregants, designated 4b, 5b and 3c with respective TC tolerances of 0·05, 0·05 and 0·75 mg./ml. The crosses 4b × 5b, 4b × 3c and the back-cross 4b × D22 were made and analysed by isolating one diploid in each cross and sporulating it to obtain tetrad data. In each case, tetrads again showed a range of tolerance levels from 0·05 mg./ml. (the level inherited by most ascospores in the cross

4b × 5b) to 0·75 mg./ml., the latter being shown by the majority of the F_2 progeny in the crosses 4b × 3c and 3c × D22. In many of these tetrads, all four products of meiosis showed the same level of tolerance to TC. The inheritance of tolerance levels is obviously complex and the tentative conclusion is reached that reassortment of cytoplasmic factors takes place in determining tolerance in these cases, and that the factors originate from strain D22. The complexity is underlined by the uniformity of phenotype both of zygote clones and sensitive segregants (in relative terms) seen in the previous crosses involving strains A7, A8, A10 and A33.

Transmission of the resistance factor of strain 33-321 was also followed to the second generation in crosses between F_1s each of which had inherited resistance to 3 mg./ml. (see table 4). The results of these crosses are given in table 5. In each cross, a diploid taken at random was found to express the

TABLE 5

Inheritance of TC resistance in the cross 33-321 × D22; F_2 tetrads from resistant × resistant F_1 crosses

	Tetracycline tolerance in mg./ml.								
		Tetrads							
F1 crosses	Diploid	Type 1	No.	Type 2	No.	Type 3	No.	Type 4	No.
1a × 5a	3·0	3·0	10	3·0	2	3·0	1	3·0	1
		3·0		3·0		3·0		3·0	
		0·25		0·25		1·0		0·75	
		0·25		0·1		0·25		0·1	
1a × 2a	3·0	Type 1		Type 2		Type 5			
		—	6	—	2	3·0	2		
		—		—		3·0			
		—		—		0·1			
		—		—		0·1			
4d × 6b	3·0	Type 1		Type 2		Type 4		Type 5	
		—	8	—	2	—	1	—	1

high level of resistance to 3 mg./ml. This would be expected on the assumption that the diploids are homozygous for a resistance gene inherited originally from the resistant mutant 33-321. Tetrad analysis however, gave the unexpected results recorded in table 5. In each of the crosses there is a strict 2 : 2 segregation of resistance to (relative) sensitivity in the tetrads, with the variation of tolerance levels among the sensitive progeny characteristic of this series of crosses.

The results of the analysis of the spontaneous resistance in strain 33-321 taken together, lead to the hypothesis that a cytoplasmic genetic factor controls the resistance. In all the above crosses, nuclear marker genes generally segregated 2:2

The cytoplasmic petite mutation and TC resistance

The majority of the respiratory deficient mutants in *S. cerevisiae* known as *petite* or *rho*-minus ($\rho-$) have severe aberration of their mitochondrial DNA such as to render it largely non-sensical. Other *petites* have no detectable mitochondrial DNA (Williamson, 1970). The resulting loss of genetic information appears to be irreversible in all *petites*. This situation was

exploited in locating mitochondrial antibiotic-resistance factors in mitochondrial DNA by inducing the $\rho-$ mutation and demonstrating concomitant loss of the resistance factor (Thomas and Wilkie, 1968). The cytoplasmic factors postulated in the present investigation, in resistance to TC, were tested for ρ factor association.

The *petite* mutation was induced in strains D22, A33 and 33-321 by plating cells on YEPS medium containing 20 p.p.m. acriflavine (Ephrussi, 1953). Almost all colonies that came up on these plates were *petite*. One *petite* colony was selected from each strain and a series of crosses set up as shown in table 6. In each cross, 12 individual diploid clones all of which were respiratory competent, were analysed. It can be seen that reciprocal crosses gave the same results indicating no correlation of TC resistance factors with the ρ factor. This applies both to the low level of resistance of strain D22 and the high level resistance of 33-321. However, since the degree of aberration of mitochondrial DNA was not determined in these *petites*, it is possible that regions containing resistance factors remained functional. Since mitochondrial genomes can undergo recombination (Thomas and Wilkie, 1968*a*), the results could be interpreted as indicating

TABLE 6

Inheritance of TC resistance in crosses involving the petite ($\rho-$) *mutation*

Cross	No. of diploid clones	No. resistant to TC*	
		0·25 mg./ml.	1·0 mg./ml.
A33 × D22 $\rho-$	12	12	0
D22 × A33 $\rho-$	12	12	0
33-321 × D22 $\rho-$	12	12	12
D22 × 33-321 $\rho-$	12	12	12

* Concentrations of 0·25 and 1·0 mg./ml. TC only were used.

preferential transmission of recombinant $\rho+$ genomes carrying TC resistance factors. However, mitochondrially located genetic factors have not been seen to segregate in a 2 : 2 ratio as a general rule, so that altogether a mitochondrial location of resistance factors in these cases seems unlikely.

4. DISCUSSION

The stability of TC tolerance levels in all strains, resistant mutants and recombinants (where tested), is a notable feature and facilitated genetic analysis. In aerobic-conditional mutants, control of resistance was nuclear in that different zygote clones had similar tolerance levels in any one cross, and ascospore tetrads showed mendelian segregations. In the cross between D22 and A33 on the other hand, control of tolerance levels appeared to be cytoplasmic, since diploid clones from different zygotes varied widely in TC tolerance and ascospore tetrads tended to have all four products showing the same tolerance level. However, during vegetative growth no reassortment of hypothetical cytoplasmic factors was seen either in diploid clones from zygotes or in haploid cultures from ascospores. In other words, reassortment took place only in zygotes or during meiosis and a mechanism for stabilising the cytoplasmic complement during cell division must be postulated. Since strain A33, the low tolerance parent, did not show these

segregation patterns when crossed to other strains, it was concluded that the cytoplasmic factor was present in the cells of D22. Variation among zygotes and ascospores could have a simple quantitative basis in which higher tolerance levels correspond to more resistance units per cell than lower levels. This is supported to some extent by the fact that there is a correlation between the resistance of the strains in first-generation crosses and tolerance levels of their second-generation progeny.

The determinant for high-level resistance in the mutant 33-321 appeared also to be cytoplasmic in that zygote clones varied in tolerance from relatively sensitive to highly resistant in a cross with D22. Supporting evidence for this conclusion came from the analysis of the sensitive clones in which the resistance factor did not re-appear in the tetrads. However, segregations of the type described for the D22 × A33 cross, were seen in these tetrads, indicating that the factor controlling tolerance levels in D22 were independent of the high-level resistance factor. The zygote clones that inherited high level resistance, when sporulated, gave tetrads segregating 2 : 2 resistant to sensitive, an apparent contradiction of the hypothesis of a cytoplasmic location of the resistance factor. However, the strict 2 : 2 segregation of resistance to sensitivity in F_2 tetrads from resistant × resistant crosses is a striking departure from an expected homozygous-resistant condition of the diploids on the basis of nuclear control and strengthens the case for cytoplasmic inheritance. An extra-chromosomal resistance factor in this system would seem to have a correlation with chromosomal transmission, at least during meiosis, in manifesting the Mendelian-like inheritance pattern obtained.

A comparison can be made with episomal systems in bacteria, in which chromosomal association of a genetic factor alternates with a cytoplasmic location and a model for the inheritance of the high-level TC resistance along the following lines may be postulated. A replicating extra-chromosomal factor has been acquired by strain 33-321, either by infection or mutation of a pre-existing factor, and its presence confers a high level of resistance to TC. During vegetative growth, all daughter cells inherit the unit, but in a cross with a sensitive strain, some zygotes either fail to inherit the factor or, having done so, fail to transmit it and give rise to sensitive clones. To explain 2 : 2 segregation of the cytoplasmic factor in tetrads from resistant zygote clones (whether from resistant × sensitive or resistant × resistant crosses), it may be assumed that in vegetative and meiotic cells the factor is integrated at a particular site in a chromosome. The unit replicates with the chromosome resulting in its chromosome-linked transmission to two of the four products of meiosis and to each daughter cell in mitosis. At zygote formation, the factor assumes a cytoplasmic location and may fail to replicate in some zygotes which would give rise to sensitive clones. This model does not explain the finding that resistant clones from resistant × sensitive crosses do not develop the full resistance of zygote clones from resistant × resistant crosses. Perhaps the distinction is due to a quantitative effect and in the latter clones there are two integration sites, one on each of the homologous chromosomes involved. Whatever the difference in vegetative diploid cells, the situation in meiotic cells appears to be the same giving rise to similar products.

There are obvious variations on this basic scheme of alternating sites, but a cytoplasmic location in vegetative cells would seem unlikely if a close

comparison with bacterial episomes is made. This follows from the finding that acriflavine treatment, which efficiently eliminates these units when not integrated in the bacterial chromosome, had no detectable effect on the resistance factor during *petite* induction.

Arguments in favour of cytoplasmic inheritance based on segregation data are not compelling, and to substantiate them it is necessary to establish the biochemical identity of hypothetical cytoplasmic factors. Although this has been achieved in recent years in the case of organelles including kinetoplasts and basal bodies, all of which have associated DNA, other autonomous systems are more difficult to identify. That they exist is not unlikely since there are DNA species of unidentified function in cells, including so-called satellite DNA (Moustacchi and Williamson, 1966), and DNA associated with microsomes (Bond, Cooper, Courington and Wood, 1969). These systems may be much more widespread than is realised and an extension of the studies on TC resistance reported here could be a useful starting point in their identification.

5. Summary

1. Six haploid strains of *Saccharomyces cerevisiae* were investigated and concentrations of tetracycline (TC) required to inhibit mitochondrial synthesis and arrest growth on non-fermentable, agar medium, determined.

2. Inhibitory concentrations ranged from 0·025 to 0·25 mg./ml. Growth was not arrested in any strain at a concentration of 5 mg./ml. TC when fermentable substrate was available. The inhibitory effect was reversible in these experiments.

3. Spontaneous TC-resistant mutants were of two types, showing respectively aerobic-conditional and -unconditional resistance.

4. Genetic analysis of aerobic-conditional mutants indicated control of resistance by a nuclear gene in each case. These were either recessive or dominant depending on the mutant, and showed various degrees of interaction including epistasis, in pairwise crosses.

5. In an aerobic-unconditional mutant with high-level resistance to TC, the resistance factor appeared to be cytoplasmic. There was no evidence of an association of the postulated cytoplasmic resistance factor with mitochondrial genetic material (the *rho* factor), but its transmission appeared to have some features in common with a bacterial episome.

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