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With 1 *plate and* 1 *text-figure*
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Inhibition of mitochondrial synthesis in yeast by erythromycin: cytoplasmic and nuclear factors controlling resistance

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1. INTRODUCTION

The term 'rho (ρ) factor' is used to designate the cytoplasmic genetic information that controls the synthesis of some, if not all, bound enzymes of the inner membrane of the mitochondrion in *Saccharomyces cerevisiae*. These enzymes include cytochromes *a* and *b* and certain dehydrogenases (see Slonimski, 1953). The cytoplasmic mutant known as 'petite' lacks these enzymes (Sherman & Slonimski, 1964; Roodyn & Wilkie, 1967*a*) and has no inner mitochondrial membrane (Yotsuyanagi, 1962). The mutational change to petite is apparently irreversible and the notation $\rho-$ is generally applied to this class of mutant inferring loss of genetic information. A loss of mitochondrial DNA (MDNA) is in fact reported in these mutants by Tewari, Votch, Mahler & Mackler (1966), Corneo *et al.* (1966) and Moustacchi & Williamson (1966), whilst Mounolou, Jakob & Slonimski (1966) claim MDNA is still present in petite cells although grossly altered and presumed to be nonsense DNA. It may be concluded there is effective loss of MDNA in these cases and that this genetic material is the ρ factor. It may be concluded further that the synthesis of the outer membrane of the organelle is under nuclear control and proceeds independently of the ρ factor.

The antibacterial antibiotic erythromycin, in common with other inhibitors of protein synthesis in bacteria such as chloramphenicol, specifically inhibits the synthesis of the inner membrane assembly, resulting in a petite phenotype (Clark-Walker & Linnane, 1966, 1967) (Text-fig. 1). Apparently the inhibition is of the protein-synthesizing system of the mitochondrion at the level of translation of genetic information into polypeptide (Wilkie, 1967). The inhibitory effect is reversible.

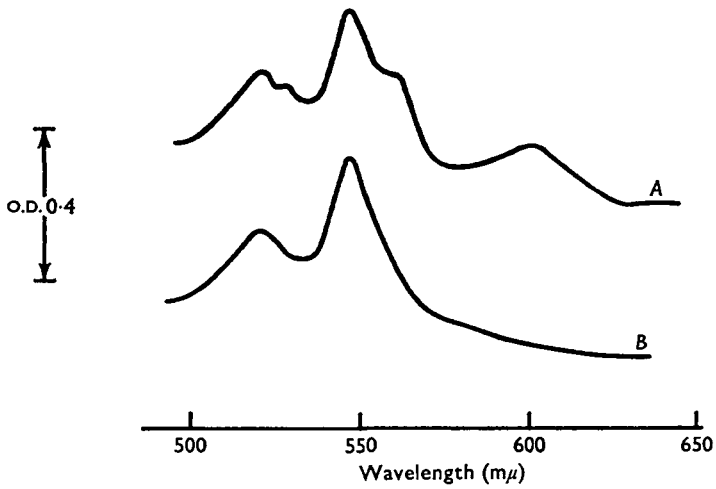
Yeast mitochondria *in vitro* incorporate amino acids into protein and this process is inhibited by chloramphenicol (Wintersberger, 1965) and erythromycin (Wilkie, Saunders & Linnane, 1967; L. A. Grivell, unpublished results).

A system for the isolation of spontaneous mutants resistant to these antibiotics has been described and cross-resistance relationships analysed (Wilkie *et al.* 1967). Genetic analysis has been concentrated on mutants resistant to erythromycin and the present paper reports on the results obtained. Attempts to work out the mechanism of drug resistance in these mutants have also been made in providing information on the biogenesis of the mitochondrion.

2. MATERIALS AND METHODS

Yeast strains are listed in Table 1.

Media used were as follows: YEPS: 1 % Difco yeast extract, 2 % Difco bacto-peptone, 2 % analar glucose, 2 % agar. YEPG: as for YEPS with the substitution of 4 % glycerol (v/v) as carbon source. MMS: minimal medium of Wickerham (1955), containing mineral salts, vitamins and glucose only. SPM: sporulating medium of 0·3 % raffinose, 0·2 % sodium acetate, 2 % agar. Incubation temperature was 30 °C.



Text-fig. 1. Cytochrome spectra of cells of a sensitive strain of *Saccharomyces cerevisiae* grown to stationary phase in the absence of erythromycin (curve *A*) and in the presence of 0·1 mg/ml erythromycin (curve *B*). α peaks of cytochromes *a*, *b* and *c* occur at the respective wavelengths 603, 557, and 550 mμ with β peaks of *b* and *c* at 532 and 521 mμ respectively.

(i) *Crossing procedure.* (*a*) Solid MMS was sown with cells of one strain and overlaid with cells of the crossing strain. Since in all crosses parental strains were differently auxotrophic, only the heterozygous zygotes were able to develop to give individual diploid colonies. (*b*) The two strains to be crossed were inoculated into liquid YEPS and incubated overnight. Cell samples are then seen to contain zygotes which are clearly distinguishable from haploid parental cells by their large size and odd shape. For further details of crossing techniques see Hawthorne & Mortimer (1960).

(ii) *Micromanipulation of zygotes.* This was carried out on agar block with the De Fonbrune micromanipulator. This instrument was also used to dissect ascospore tetrads (sexual progeny) which were induced to form on SPM. Random ascospore technique was that described by Beavan & Woods (1964).

(iii) *Anaerobic culture.* Strains were inoculated into liquid MMS containing 4 % glucose and appropriate growth factors where necessary. Growth of cells proceeded in a Fildes cylinder under a nitrogen atmosphere and cells were harvested after 7 days.

(iv) *Erythromycin.* A soluble salt, the lactobionate, was used and millipore-filtered stock solutions in water or ethanol were stored at 0 °C. Appropriate amounts were added to molten media at 45 °C prior to pouring of plates. The standard test series of plates contained the drug in the range of concentrations 0, 0·01, 0·05, 0·1, 0·5, 1 and 3 mg/ml. Higher levels were used to obtain maximum tolerance values in certain cases. All tests for erythromycin effects are necessarily carried out with glycerol as carbon source since inhibition of the synthesis of the respiratory system is a specific effect of the drug and is undetectable in the presence of fermentable substrate. *S. cerevisiae* being a facultative anaerobe can dispense with the mitochondrial system when sugar is available.

Table 1. *Erythromycin tolerance of sensitive and resistant haploid yeast strains and resistant mutants*

Strain	Resistant mutant	Nuclear markers*	Tolerance (mg/ml)	Genetic basis of resistance
74	—	*ar, me, α*	< 0·01	—
	741		> 3	ρ factor
	742		> 3	ρ factor
76	—	*hi, ur, α*	—	—
	761			ρ factor
26	—	ad_2, *α*	< 0·01	—
	261		3	ρ factor
	262		1	Dominant gene
16	—	ad_2, *ur, me, α*	< 0·01	—
	162		0·1	—
	163		0·1	—
	164		0·1	—
	166		0·1	—
36	—	*tr, ur,* ad_6, *ly, α*	< 0·01	—
	367		0·5	Dominant gene
21		*tr, me, α*	3	Dominant gene + ρ factor
10		*le, hi, a*	< 0·01	—
68		—	0·1	—
69		*tr, me, α*	0·1	Dominant gene

* For symbols, see Hawthorne & Mortimer (1960).

(v) *Resistant mutants.* Spontaneous mutants were isolated as individual colonies that came up following plating of cells of sensitive strains on drug medium. Resistance levels of all strains were determined by dropping out from cell suspensions onto the drug series of plates. Strains listed as resistant to a particular concentration usually show some degree of inhibition at that level of the drug but are completely inhibited at the next higher level in the series. It will be appreciated that inhibition of synthesis of the respiratory system where the substrate is non-fermentable leads to inhibition of growth.

(vi) *Petite induction.* The $\rho-$ mutation was induced by adding 5 μg/ml acriflavin to YEPS medium. At this concentration of the dye there was 100 % induction

among daughter cells in all strains used in these studies but no other mutagenic effect was apparent.

Petite mutants having no functional respiratory system cannot be tested directly for erythromycin defects—they cannot utilize glycerol.

(vii) *Cytochrome spectra.* Spectra of whole cells were run in the Unicam SP 800 split-beam recording spectrophotometer. The blank used was constructed essentially of cell walls of *S. cerevisiae* obtained by acetone extraction of freeze-dried cells followed by TCA treatment and a second extraction. This blank had no absorption peaks in the 700–300 mμ range.

3. RESULTS

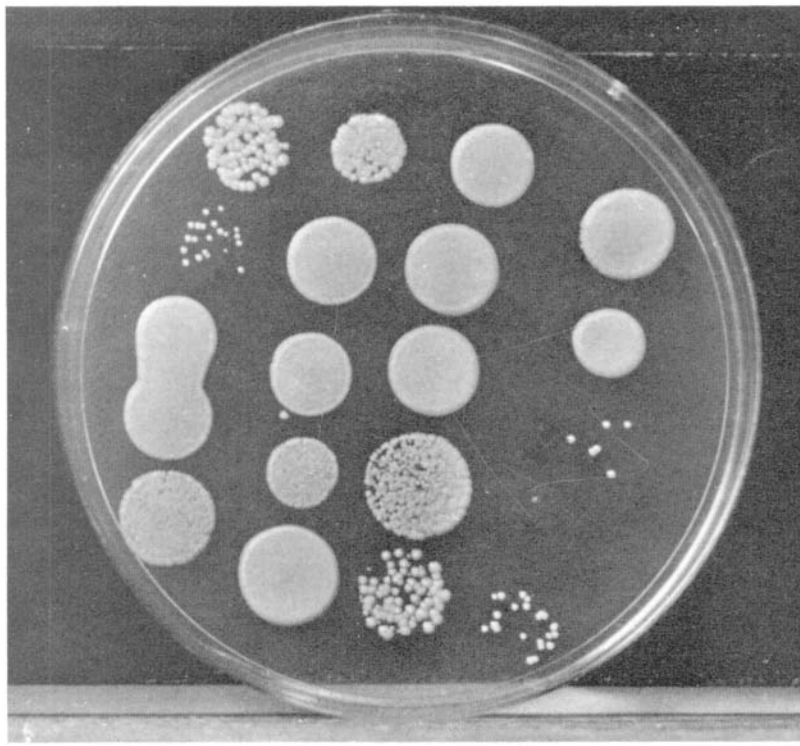
Spontaneous resistant mutants are listed in Table 1 with their respective resistance levels. An important feature of these mutants is the stability of resistance levels through many subcultures in the absence of the antibiotic. The frequency of resistant mutants picked up by these methods is of the order of 10^{-4}.

In several cases, genetic analysis of resistance in crosses to sensitive strains gave Mendelian ratios indicating gene control of resistance (Table 1). In the case of strains 741, 761 and 21 non-Mendelian segregation patterns were obtained, suggesting cytoplasmic control, and these cases were investigated in detail.

(i) *Analysis of crosses.* The cross between 741 and the sensitive strain 10 was made on solid medium. Cells were sampled from individual colonies that developed (each colony was assumed to have arisen from a single zygote) and tested for resistance. Results are shown in Plate 1 from which it can be seen that the percentage of resistant cells in individual drops which contained about 10^4 cells in each case varies from 0 to about 100. These patterns were similar on all drug plates of the test series. In other words, the diploid cells were either sensitive to 0·01 mg/ml (the level of sensitivity of strain 10) or capable of developing the full resistance of 741 ($>$ 3 mg/ml).

Cells from one of the wholly sensitive colonies were sporulated and ascospores analysed. Six complete tetrads each segregated 4:0 sensitive to resistant. Random spore suspensions were also tested and aliquots containing about 400 spores put down in each of the test plates. Approximately this number of colonies came up on the control medium but no colonies developed in the presence of the drug. Colonies on the control plates were then shown to be comprised of sensitive cells. Thus the progeny, both vegetative (diploid) and sexual (haploid), of this particular zygote had largely or wholly failed to inherit the factor for resistance.

The cross 741×10 was then set up in liquid medium and resulting zygotes micromanipulated. Eight zygotes were isolated, four onto medium containing 1 mg/ml erythromycin and four onto medium without the antibiotic. All zygotes gave rise to colonies. When cell samples were taken from the colonies on the non-selective medium, again wide variation in the percentage of resistant cells was observed between colonies. Sporulation and tetrad analysis of one of the mixed colonies gave 3 tetrads segregating 4:0 sensitive to resistant, 1 tetrad 3:1, 1



Samples of diploid vegetative progeny of twenty individual zygotes from a cross between a cytoplasmic resistant mutant and a sensitive strain dropped out on erythromycin medium (1 mg/ml). Each drop contained approximately 10^4 cells. The number of resistant cells can be seen to vary in each sample from none (col. 4, sample 4) to many or all.

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tetrad 1:3 and 1 tetrad 0:4 sensitive to resistant. On the other hand, all cells sampled from the four colonies on the drug medium were resistant and when one of the colonies was sporulated, random spores and spore tetrads were all found to have inherited resistance.

In all the above spore analyses nuclear markers generally segregated 2:2 in the complete tetrads and 1:1 in random platings.

Strain 761 was analysed precisely as for 741 with similar results.

These results were interpreted as demonstrating cytoplasmic inheritance of resistance in 741 and 761 with the hypothesis that the mitochondrial DNA carried the genetic determinant. It could then be reasoned that fusion of sensitive and resistant cells in crosses gave zygotes with a mitochondrial complement consisting of the two parental types. Random inheritance of mitochondria among daughter cells during vegetative growth could explain the variation in the relative proportions of resistant and sensitive cells in resulting colonies on non-selective medium. On a selective medium containing erythromycin, only mitochondria of resistant type would be reproducible so vegetative progeny would all show resistance. Mitochondrial type of the ascospores would be determined by the mitochondrial complement of the parental diploid cells undergoing sporulation.

(ii) *Tests for correlation between functional ρ factor and resistance.* The hypothesis of resistance being due to a mutational change in MDNA was investigated making use of the fact that the mutation to $\rho-$ effectively destroys MDNA.

It is well known that acriflavin differentially induces the petite mutation at concentrations of a few μg/ml (Ephrussi, 1953). Petite mutants were isolated from 741, 761 and 10 following treatment with the dye, and the reciprocal crosses $741 \times 10\rho-$, $741\rho- \times 10$; $761 \times 10\rho-$, $761\rho- \times 10$ set up. As expected in crosses between $\rho+$ and $\rho-$ strains, all zygotes and their vegetative and sexual progeny were respiratory competent, $\rho+$. The accepted explanation of this classical example of cytoplasmic inheritance is that the $\rho+$ parent provides the functional ρ factor lacking in the petite parent to ensure respiratory competence in the zygote and its progeny.

In the cross $741 \times 10\rho-$ diploid colonies from individual zygotes were analysed. Twelve colonies on non-selective medium were sampled and found to be comprised of 100% resistant cells with no evidence of mixed colony type. Ascospores from a sporulation of one of these colonies were all resistant in random platings. Micromanipulated zygotes on selective and non-selective media likewise transmitted resistance to all progeny, vegetative and sexual. In the reciprocal cross $741\rho- \times 10$ the opposite was the case and no resistant progeny were found: on non-selective medium isolated zygotes gave colonies the cells of which were all sensitive and gave sensitive sexual progeny, while zygotes isolated onto drug medium were unable to form colonies.

These tests were also applied to the crosses involving 761 and once again the results were similar to those described for 741.

In yet another series of reciprocal, petite by normal, crosses between the resistant strains 261 and 742 and 10, a positive correlation between the ρ factor

and drug resistance was clearly seen. In direct contrast, the petite mutation in the gene-resistant strain 262 had no effect on the transmission of resistance (to 1 mg/ml): diploids from the cross $262\rho- \times 10$ all showed full resistance and random ascospores segregated 1:1 resistant to sensitive as expected for the dominant gene.

These findings provide good evidence that resistance to the inhibitory effects of erythromycin on mitochondrial synthesis can come about by a heritable change in MDNA. This DNA, although mutant in this respect, is apparently normal in all other respects. Thus resistant cells of 741 and 761 are fully respiratory competent and have a normal cytochrome spectrum (see Text-fig. 1A) not detectably different from the sensitive strains from which they were isolated. Furthermore, 741 and 761 show no inhibition of cytochrome synthesis in YEPS culture in the presence of erythromycin at stationary phase while in sensitive strains synthesis of cytochromes *a* and *b* is blocked (Text-fig. 1B).

(iii) *Analysis of strain 21.* This strain was naturally resistant to 3 mg/ml erythromycin and when crossed to a sensitive strain gave non-Mendelian inheritance of resistance. The petite of 21 was then isolated and crossed again to the sensitive strain. Cells were sampled from twelve individual colonies from zygotes on the crossing medium and all cells were resistant to 0·2 mg/ml. Random ascospores from a sporulation of one of the colonies segregated 1:1 sensitive to resistant to 0·2 mg/ml, indicating a dominant gene controlling resistance to 0·2 mg/ml of the drug. When the reciprocal cross was made with the sensitive strain as petite parent, all twenty diploids tested were found to be 100 % resistant to 3 mg/ml and random spores all developed into colonies on high levels of the drug. It was concluded that the resistance of strain 21 was due to a combination of mutant gene and mutant ρ factor and that most of the resistance was conferred by the latter.

(iv) *Aspects of the mechanism of resistance.* It is reported that mitochondria *in vitro* incorporate amino acids into protein but only into the insoluble fraction (Wintersberger, 1965). It was therefore adopted as a working hypothesis that mutational change in MDNA results in altered membrane protein (Woodward & Munkres, 1966) thereby introducing a permeability barrier to the drug at the level of the inner membrane. Similarly, since the synthesis of the outer membrane appears to be under the control of the nucleus, the mechanism of resistance in gene mutants could also be permeability effects but at the level of the outer membrane.

It is possible to bring about apparent complete disruption of the yeast mitochrondrion by growing cells anaerobically in the absence of fatty acids (see Lukins, Tham, Wallace & Linnane, 1966). Anaerobic cultures of a number of resistant strains were obtained, the cells washed and dropped out on the drug series of plates to test for retention of resistance in the absence of intact mitochondrial membranes. Two gene-resistant mutants, strains 367 and 69, resistant to 0·5 and 0·1 mg/ml respectively, were no longer resistant and were inhibited at the lowest level of 0·01 mg/ml. On the other hand, 741 and 761 retained full resistance. Of five other strains tested (162, 163, 164, 166 and 68) each resistant to 0·1 mg/ml, only strain 68 retained its resistance (the genetic basis of resistance in these five cases is not yet known). Where resistance is lost following anaerobic growth it is

a reasonable assumption that resistance depends on the presence of mitochondrial membrane, and that in the case of gene mutants we are dealing with mutations affecting the outer membrane, although inner membrane alteration could equally explain the results.

4. DISCUSSION

Erythromycin is known to bind to the bacterial ribosome (the 70 S type) in blocking protein synthesis in the bacterial cell (see Vazquez, 1966). In view of the recent findings of ribosomal-type units in yeast mitochondria (for review see Roodyn & Wilkie, 1967*b*) with RNA sedimentation values resembling more those of bacteria than of higher cells (Linnane, 1967), it is not unlikely that these units are the sites of erythromycin activity in the mitochondrion. Results with the ρ factor mutants give support to the idea that MDNA is involved in specifying these components of the mitochondrial system and that mutation results in altered 'ribosomes' leading to resistance. Resistance of this kind would not require the continued existence of the membranes. Alteration in the 80 S ribosome of yeast has been described in conferring resistance to cycloheximide (Cooper, Banthorpe & Wilkie, 1967) and the altered ribosomes seem in no way impaired in function. Sinclair, Stevens, Sanghavi & Rabinowitz (1967) find that yeast MDNA is basically a filament of 5–6 μ in length or roughly 15000 base pairs. An estimated 10000 nucleotide pairs would suffice to specify both the ribosomal and the transfer RNA of the organelle, leaving enough information to code for about ten proteins of average molecular weight of 20000. It is clear there is not enough information in MDNA to specify the complete protein-synthesizing system and all the proteins of the inner membrane. Other information, perhaps entering the developing mitochondrion as m-RNA of nuclear origin, is required. Gene mutants with resistance not associated with mitochondrial membrane may be involved in this aspect of organelle biogenesis. Other mechanisms of resistance that are unlikely to be affected by anaerobic treatment include permeability changes at the cell membrane and drug inactivation. However, it is difficult to see how mutant MDNA could be involved in either of these processes.

Before these problems can be adequately tackled, it will be necessary to isolate and identify the components of an *in vitro* protein-synthesizing system from mitochondria. Work with an *in vitro* system is proceeding independently in Linnane's laboratory in the analysis of erythromycin resistance in mutants from strains related to those described here and their results are awaited with interest. In the meantime the present work is being extended to the analysis of mutants resistant to other mitochondrial inhibitors, in particular the aminoglycoside antibiotics, in the hope that mitochondrial genetics will be possible. It is clear that these mutants are of importance in the more general problem of mitochondrial biogenesis.

SUMMARY

Erythromycin-resistant mutants were separated into two categories showing Mendelian and non-Mendelian inheritance of resistance respectively. The cytoplasmic factor was located in the mitochondrion based on the fact that the loss of the mitochondrial genetic unit associated with the petite mutation (the ρ factor, assumed to be the mitochondrial DNA) resulted in a corresponding permanent loss of resistance. Conversely, the petite mutation in gene resistant mutants had no effect on the inheritance of resistance.

Analysis of vegetative and sexual progeny of isolated zygotes from a number of crosses between resistant and sensitive strains indicated that different mitochondrial types, both sensitive and resistant, co-exist in the yeast cell and are distributed at random among daughter cells under non-selective conditions. Only resistant mitochondria are in evidence if zygotes are put down to proliferate on medium containing the antibiotic.

Resistant strains were tested for retention of resistance following anaerobic culture (known to cause disruption of mitochondria). The cytoplasmic mutants retained their resistance but two gene-resistant mutants tested both lost their resistance. It was suggested in the latter case that resistance is dependent on the existence of intact mitochondrial membrane (possibly inner membrane). In the former mutants the mechanism of resistance may be the alteration of one or other of the components of the protein-synthesizing system of the organelle.

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Note added in proof. Since the completion of this paper, Linnane and co-workers report the independent finding of a correlation between erythromycin resistance and the ρ factor (personal communication).