*THE BIOGENESIS OF MITOCHONDRIA, V. CYTOPLASMIC INHERITANCE OF ERYTHROMYCIN RESISTANCE IN SACCHAROMYCES CEREVISIAE**

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The recognition and study of respiratory-deficient mutants of yeast has been of fundamental importance in contributing to our knowledge of the genetic control of the formation of mitochondria. From these studies it has been recognized that cytoplasmic genetic determinants as well as chromosomal genes are involved in the biogenesis of yeast mitochondria.[1, 2] Following the recognition of the occurrence of mitochondrial DNA,[3, 4] attention has recently been focused on the relationship between mitochondrial DNA and the cytoplasmic determinant.[5] However, the information on this latter subject is limited and is derived from the study of a single class of mutant of this determinant, the respiratory-deficient cytoplasmic petite. This irreversible mutation is phenotypically characterized by the inability of the cell to form a number of components of the respiratory system, including cytochromes a, a_3, b, and c_1.[6] A clearer understanding of the role of cytoplasmic determinants in mitochondrial biogenesis could result from the characterization of new types of cytoplasmic mutations which do not result in such extensive biochemical changes. This would thus simplify the biochemical analyses as well as providing additional cytoplasmic markers to assist further genetic studies.

We have reported that the antibiotics chloramphenicol, lincomycin, and the macrolides erythromycin, carbomycin, spiramycin, and oleandomycin selectively inhibit *in vitro* amino acid incorporation by yeast mitochondria, while not affecting the yeast cytoplasmic ribosomal system.[7, 8] Further, these antibiotics do not affect the growth of *S. cerevisiae* on fermentable substrates but reversibly inhibit the *in vivo* synthesis of cytochromes a, a_3, b, and c_1; the inhibited cells appear to be phenocopies of the cytoplasmic petite mutant.[9, 10] This laboratory has also described the isolation of yeast mutants with varying degrees of sensitivity to the aforementioned antibiotics.[11] The mutants selected for resistance to high levels of erythromycin have been shown to contain mitochondria having an amino acid-incorporating activity which is completely resistant to the drug.[12] It was also shown that the inheritance of resistance to erythromycin did not segregate as a single chromosomal gene.[7]

This paper establishes that erythromycin resistance is inherited extrachromosomally. In addition, it is shown that the cytoplasmic determinant for erythromycin resistance and the factor associated with the cytoplasmic petite mutation, while closely related, do not appear to be identical.

Materials and Methods.—Nomenclature: The following symbols have been adopted to represent the various mutants used in this paper. The symbol ρ (rho) represents the cytoplasmic factor as described by Sherman,[2] where ρ^+ and ρ^- designate the ability or inability, respectively, to synthesize the respiratory enzymes cytochromes a, a_3, b, and c_1.

ER^s and ER^r designate, respectively, the determinants for sensitivity or resistance to erythromycin; ER^s strains were unable to grow on glycerol in the presence of 0.1 mg/ml erythromycin, and ER^s strains showed almost no inhibition of growth on glycerol in the presence of 8 mg/ml erythromycin and could still grow even in the presence of 20 mg/ml of the drug. Independent isolates of erythromycin-resistant strains are designated ER^{r-1}, ER^{r-2}, etc.

Media: YEP-glucose: 1% Difco yeast extract, 2% peptone, 2% glucose; 2% agar was added for solid media. YEP-glycerol: same as YEP glucose except 4% glycerol replaced the glucose. YEP-glycerol-erythromycin medium: YEP-glycerol medium supplemented with varying amounts of a sterile solution of erythromycin glucoheptonate. Synthetic medium: As described by Wickerham[13] with appropriate extra additions of amino acids and nucleotide bases as specified by Roman[14] to test the nutritional requirements of the strains. Sporulation medium: 0.25% tryptone, 0.06% glucose, 0.062% NaCl, 0.5% sodium acetate, 2% agar.

Strains: The yeast strains used are listed in Table 1. Spontaneous mutants resistant to erythromycin were isolated by plating cultures of sensitive strains onto YEP-glycerol

TABLE 1. *Yeast strains used.*

Strain no.	Genetic characters	Source
L410	α ur hi ER^s	Wilkie (formerly strain 41)
L411	α ur hi ER^{r-1}	From L410
L412	α ur hi ER^{r-2}	From L410
L413	α ur hi ER^{r-3}	From L410
L300	a ad_2 ER^s	Wilkie (formerly strain 40)
D243-2BR1	a ad_1 ER^s	Slonimski
D243-12A	a ad_1 tr_2 ly_2 ER^s	D243 of Sherman
LD32-10C	a hi tr_1 ER^{r-1}	Derived from crosses involving L411
LD32-1B	a hi tr_1 ER^{r-1}	
LD54-10C	a tr_1 ly_2 ER^{r-1}	

The symbols, a, α, represent mating type; ur, hi, tr_1, ly_2, and ad_1, ad_2, represent chromosomal genes which result in a growth requirement for uracil, histidine, tryptophan, lysine, and adenine, respectively. ER^s, ER^{r-1}, etc., as described in the section on nomenclature.

medium containing 8 mg of erythromycin/ml of medium; only resistant cells grow under these conditions. The erythromycin-resistant cells so selected are stable with respect to resistance. Cytoplasmic petites were induced by culturing respiratory-competent cells on YEP-glucose liquid media containing 2.5 μg/ml euflavine. Individual petite colonies were then isolated from the culture by plating on YEP-glucose medium.

Determination of erythromycin resistance: Preliminary tests for erythromycin resistance were made by dropping out suspensions of 10^5 to 10^6 cells onto YEP-glycerol agar plates and onto YEP-glycerol-erythromycin agar plates containing erythromycin in the concentrations 0.1 and 8 mg/ml. Glycerol was used as the energy source for growth, since growth on a nonfermentable substrate in the presence of the drug is dependent on normal mitochondrial respiratory activity. The ability of a strain to grow on glycerol in the presence of the antibiotic concentration is the measure of resistance of the organism.

The dropping-out method is unsuitable for distinguishing between suspensions of cells containing mixtures of resistant and sensitive cells and suspensions composed entirely of resistant cells. Suspensions scored as resistant on the basis of confluent growth on erythromycin plates could still contain many nongrowing sensitive cells as the spot becomes confluent if as few as 10% of the cells are resistant. The method used to determine the proportion of resistant and sensitive cells was to plate dilutions of the cultures on YEP-glycerol and YEP-glycerol-erythromycin medium to give 100–300 colonies per plate.

Mating procedures: The crossing of auxotrophic haploid strains was performed by the mass-mating technique of Lindegren and Lindegren,[15] followed by prototrophic selection of single diploid colonies on minimal medium.[16] Crosses were also performed by mixing freshly grown suspensions of the parental strains on solid minimal medium; diploid

papillae appeared after 3–4 days. Analyses of diploids isolated by the two methods yielded similar results.

Tetrad analysis: Diploids were grown on YEP-glucose, washed, and a thick suspension placed on the sporulation medium. Asci usually formed after 3–4 days, but were allowed to mature for 7 days before dissection. The ascus wall was digested by snail gut contents to expose the ascospores which were then separated by microdissection.[17] Spores were germinated and grown into small colonies, then tested for erythromycin resistance and for nutritional requirements determined by chromosomal genes.

Results.—Genetic behavior of erythromycin resistance in crosses: Crosses were performed between erythromycin-resistant and erythromycin-sensitive strains. Preliminary experiments appeared to yield erythromycin-resistant diploid colonies, but on closer examination of some of these colonies they were found to be composed of a mixture of resistant and sensitive diploid cells. Such mixed diploid clones, presumably the vegetative progeny of a single zygotic event, were obtained from all $ER^r \times ER^s$ crosses performed (Table 2). In a

TABLE 2. *Resistance of diploids resulting from crosses of erythromycin-resistant and -sensitive strains.*

Cross no.	Strains crossed				No. of diploids tested	Erythromycin Resistance S	M	R
54*	L411	(ER^{r-1}) ×	D243-12A	(ER^s)	54	—	54	—
60*	LD54-10C	(ER^{r-1}) ×	L410	(ER^s)	64	—	64	—
52	L411	(ER^{r-1}) ×	D243-2BR1	(ER^s)	51	—	3	(48)†
25	L411	(ER^{r-1}) ×	L300	(ER^s)	24	1	9	(14)
38	LD32-1B	(ER^{r-1}) ×	L410	(ER^s)	2	—	1	(1)
39	LD32-10C	(ER^{r-1}) ×	L410	(ER^s)	2	1	1	—
71	L412	(ER^{r-2}) ×	D243-12A	(ER^s)	19	—	4	(15)
55	L413	(ER^{r-3}) ×	D243-12A	(ER^s)	19	—	1	(18)
53	L410	(ER^s) ×	D243-12A	(ER^s)	10	10	—	—
16	L410	(ER^s) ×	L300	(ER^s)	10	10	—	—
61*	L411	(ER^{r-1}) ×	LD54-10C	(ER^{r-1})	18	—	—	18

S, sensitive (inhibited by 0.1 mg/ml erythromycin); R, resistant (not inhibited by 8 mg/ml erythromycin); M, a mixture of resistant and sensitive cells.

* Diploid clones tested for presence of sensitive cells by replating.

† Numbers within parentheses indicate primary diploid clones which were not tested for the presence of sensitive cells by replating. It is likely that most, if not all, of these colonies were mixed.

more detailed analysis of the two crosses 54 and 60 (Table 2), 54 and 64 diploid colonies, respectively, were selected and plated to determine the proportion of resistant cells (see *Methods*). All of these primary diploid colonies were shown to contain both resistant and sensitive cells, the percentage of resistant cells per colony ranging from as low as 0.01 per cent to values approaching 100 per cent. Figure 1 illustrates the frequency distribution of resistant and sensitive cells in the primary diploid colonies. It should be emphasized that the clones derived from the individual diploid cells of the primary mixed colonies are either completely sensitive or completely resistant, i.e., individual cells making up the primary mixed diploid colonies do not give rise to further mixed clones. It follows that resistance or sensitivity of the cells comprising the primary diploid colony arising from the zygote is established as a stable characteristic of each individual cell by the time a visible colony has formed.

The control crosses $ER^s \times ER^s$ and $ER^r \times ER^r$, as would be anticipated, gave completely sensitive and completely resistant diploid clones, respectively.

The mixed diploid clones obtained from the $ER^r \times ER^s$ crosses are difficult to explain on the basis of a chromosomal inheritance of erythromycin resistance. For chromosomal inheritance it would be expected that all diploids from a single cross, necessarily having the same genotype, would have identical resistance

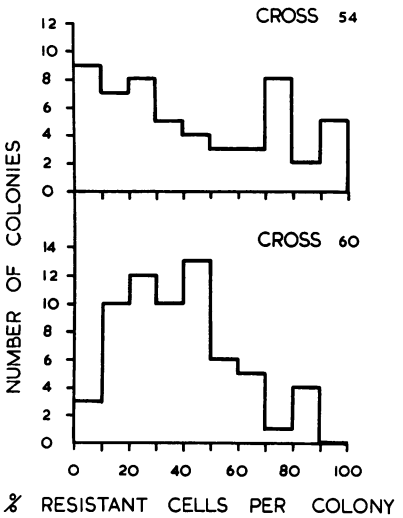

FIG. 1.—Frequency distributions of the percentage of resistant cells per primary diploid colony from cross 54 (L411 (ER^{r-1}) × D243-12A (ER^s)) and cross 60 (LD54-10C (ER^{r-1}) × L410 (ER^s)). The diploid colonies arose from the zygotes formed in the crosses.

characteristics, that is, all would be either resistant or sensitive. The mixed characteristics of the diploids arising from a single zygote thus immediately suggest that erythromycin resistance is inherited extrachromosomally.

Tetrad analysis: In yeast, extrachromosomal inheritance can be readily demonstrated by tetrad analysis, as was done in the case of the ρ factor.[1, 18] Accordingly, the individual sensitive and resistant diploids isolated from the mixed clones resulting from the $ER^r \times ER^s$ crosses were sporulated and the ascospores analyzed to determine the segregation of the erythromycin-resistance characteristic. The results, which are presented in Table 3, show tetrad ratios inconsistent with chromosomal inheritance. In the cases where a resistant diploid was selected the segregation was 4:0 for resistance:sensitivity, whereas sensitive diploids selected from the crosses yielded 0:4 segregation. In addition, several mixed diploid clones were transferred directly to sporulation medium, giving rise to asci which on dissection yielded spores showing either 4:0 or 0:4 segregation for erythromycin resistance. In all tetrads the known chromosomal markers in the crosses showed 2:2 segregation. Hence the results of these analyses establish an extrachromosomal mode of inheritance for erythromycin resistance.

Effect of petite induction on the inheritance of erythromycin resistance: Euflavine, in low concentrations, is known to induce cytoplasmic petite mutations, while having no effect on yeast chromosomal genes.[1] Erythromycin acts on sensitive yeast cells to inhibit the synthesis of cytochromes a, a_3, b, c_1 to produce

TABLE 3. *Tetrad analysis of diploids from crosses between erythromycin-resistant (ER^r) and erythromycin-sensitive (ER^s) strains.*

Cross no.	Resistance of diploid colony selected	No. of tetrads analyzed	Segregation in Spores ER^r:ER^s	
38	R	2	2 (4:0)	
25	R	8	8 (4:0)	
54	R	8	8 (4:0)	
25	S	2		2 (0:4)
39	M	14	2 (4:0)	12 (0:4)
54	M	4	1 (4:0)	3 (0:4)
55	M	7	3 (4:0)	4 (0:4)

R, M, S, denote resistant, mixed, and sensitive clones as described in Table 1. The resistant and sensitive diploids were selected from mixed diploid clones. The cross numbers refer to the crosses as set out in Table 2.

a phenocopy of the cytoplasmic petite mutation.[9, 10] The cytoplasmic petite mutation and erythromycin resistance both involve some intrinsic changes in the mitochondrial system.[5, 10, 12] We have consequently investigated the effects of euflavine induction of cytoplasmic petites upon the cytoplasmic inheritance of erythromycin resistance so as to examine the relationship between the ρ factor and the resistance determinant.

Erythromycin resistance or sensitivity cannot be assayed with petite (ρ^-) cells, since being respiratory-deficient they are unable to grow on glycerol. It follows that it is not possible to test directly whether petites derived by euflavine induction from erythromycin-resistant strains retain their resistance characteristic. However, if such a petite is crossed with a respiratory-competent, erythromycin-sensitive strain, the inheritance of erythromycin resistance can be traced through the resulting respiratory-competent diploids.

Analyses of several crosses of the type $ER^r\ \rho^- \times ER^s\ \rho^+$ produced respiratory-competent diploids all of which were erythromycin-sensitive (Table 4). Tetrad

TABLE 4. *Analyses of crosses involving "petites" derived from erythromycin-resistant strains ($ER^r\ \rho^- \times ER^s\ \rho^+$).*

Cross $ER^r\ \rho^- \times ER^s\ \rho^+$	Diploid clones	No. of tetrads analyzed	Segregation in spores ER^r:ER^s
L411 $\rho^- \times$ L300 ρ^+	80-All ER^s	6	0:4
L411 $\rho^- \times$ D243-12A ρ^+	20-All ER^s	3	0:4
L412 $\rho^- \times$ D243-12A ρ^+	20-All ER^s	3	0:4

Single diploid clones were selected in each case for sporulation and dissection. All spores were respiratory-competent (ρ^+) and all chromosomal markers segregated 2:2.

The 80 resistant diploids from the cross L411 $\rho^- \times$ L300 ρ^+ represent the sum of results from 4 experiments using independently isolated L411 ρ^- clones.

analysis of these diploids revealed exclusively 0:4 (resistant:sensitive) segregation. These results suggest a close relationship between the ρ factor and the factor determining erythromycin resistance, since, following the petite mutation ($\rho^+ \rightarrow \rho^-$), resistance does not reappear in the diploids or spores.

It might therefore be anticipated that in reciprocal crosses of $ER^r\ \rho^+ \times ER^s\ \rho^-$ the petites in this cross being derived from erythromycin-sensitive strains, all diploids and spores would prove to be resistant to the antibiotic. In most

TABLE 5. *Analyses of crosses involving "petites" derived from erythromycin-sensitive strains (ER^r ρ^+ × ER^s ρ^-).*

Cross ER^r ρ^+ × ER^s ρ^-	Diploid clones	No. of tetrads analyzed	Segregation in spores ER^r:ER^s
L411 ρ^+ × L300 ρ^-	50-All ER^r	8	4:0
L411 ρ^+ × D243-12A ρ^-	17-All ER^r	5	4:0
L412 ρ^+ × D243-12A ρ^-	18-All ER^r	4	4:0
L411 ρ^+ × L300 ρ^-w	40 { 38-Mixed	9	6 (4:0), 3 (0:4)
	40 { 2-ER^s	3	0:4

The 50 resistant diploids from the cross L411 ρ^+ × L300 ρ^- represent the sum of results from 4 experiments using independently isolated red-pigmented L300 ρ^- clones. L300 ρ^-w denotes an unpigmented cytoplasmic petite isolated from strain L300. For other details see legend to Table 4.

cases this result was obtained, as shown in the upper part of Table 5. These results support the concept of a close relationship between the ρ factor and the determinant for erythromycin resistance. However, an interesting exception has been observed involving a single petite (L300 ρ^-w), shown to be cytoplasmically determined and derived from strain L300. Strain L300 produces a red pigment as a result of the ad_2 gene and, although nearly all of the petites induced by euflavine in this strain were also red, isolate L300 ρ^-w was unpigmented. This isolate still required adenine for growth and showed 2:2 segregation for adenine requirement in crosses with strains prototrophic for adenine. When L300 ρ^-w is crossed with a respiratory-competent erythromycin-resistant strain, respiratory-competent diploid clones comprised of sensitive as well as resistant cells are obtained (Table 5); tetrad analysis gives both 0:4 and 4:0 segregation for resistance and sensitivity (Table 5).

The results are similar to those obtained in the cross between L300 ρ^+ (ER^s) and L411 ρ^+ (ER^r) which yields mixed clones (Table 2). Both strains L300 ρ^-w and L300 ρ^+ are able to transfer the erythromycin-sensitive character to the diploid, whereas the red-pigmented L300 ρ^- strains have lost this ability. These observations suggest that the genetic site for erythromycin-resistance or -sensitivity, although cytoplasmic, is not identical to that determining respiratory competence.

Discussion.—The results presented in this paper strongly support the conclusion that erythromycin resistance is inherited in an extrachromosomal fashion. The conclusion is based upon the mixed nature of diploid clones obtained from crosses of resistant and sensitive cells, the tetrad analysis of the diploid cells, and the change in the inheritance of erythromycin resistance on petite induction by euflavine.

An outstanding characteristic of crosses involving erythromycin-resistant and -sensitive strains has been the isolation of diploid colonies arising from single zygotic events which, on replating, were shown to consist of a mixture of sensitive and resistant diploid cells. This situation suggests the occurrence of an initial unstable state in the zygote which subsequently leads to the segregation of resistant and sensitive organisms. Unstable states involving cytoplasmic factors have been previously postulated to occur in yeast.[19] It was observed that cells grown in the presence of low concentrations of euflavine gave rise on

plating to colonies which were composed of a mixture of petite and normal cells. The proportion of each cell type varied from colony to colony. The authors suggested that euflavine induced an unstable state in the cells which resulted in the cells giving rise to mixed clones. Ephrussi *et al.*[20] have more recently postulated that an unstable "premutational state" results from a cross between a suppressive petite and a normal strain. The present results suggest that an analogous unstable state, again involving cytoplasmic factors, exists in zygotes formed after the fusion of ER^r and ER^s cells.

The behavior of the erythromycin resistance characteristic after petite induction may be conveniently discussed in terms of two simple models.

(1) A cytoplasmic determinant for erythromycin resistance is part of the ρ factor. In this case the ρ^+ diploids selected from an ER^r ρ^- × ER^s ρ^+ cross would be expected to be all erythromycin-sensitive; conversely, the ρ^+ diploids selected from an ER^r ρ^+ × ER^s ρ^- cross would be expected to be all erythromycin-resistant. Exceptions to this proposition would require recombination or complementation between ρ factors, phenomena which have not been reported to date.

(2) The resistance "gene" is carried by a separate cytoplasmic factor with similar genetic behavior to the ρ factor, and which may be similarly affected by euflavine. Hence, loss of the resistance characteristic could still occur concomitantly with petite induction, as a result of euflavine acting on the two factors. In this case, however, petite induction might sometimes occur without loss of the resistance characteristic.

In general, the resistance characteristic of all diploids formed by crossing a petite strain with a respiratory-competent strain was that of the respiratory-competent parent, the result predicted on the basis of a single factor (model 1). However, the petite L300 ρ^-w, derived from a sensitive organism, strain L300, represents an exception to this pattern. This petite, when crossed with an erythromycin-resistant respiratory-competent strain, yielded diploids having either the erythromycin-sensitive characteristic of strain L300, or the erythromycin-resistant characteristic of the respiratory-competent parent. That is, L300 ρ^-w has not lost the ability to transfer the erythromycin-sensitive characteristic to diploids. This result tends to argue against model 1 and support the concept of two separate factors (model 2). However, it must be borne in mind that the two models are simple and that the situation may be more complicated than that suggested by either of them. The simplest conclusion which is consistent with all the data is that there appears to be two cytoplasmic factors which, although separate, are not entirely independent.

As shown in the next paper of this series, erythromycin resistance involves a change in the protein-synthesizing system of the mitochondria, and it appears most likely that the erythromycin resistance is associated with an alteration in the mitochondrial ribosome.[12] Thus the cytoplasmic location of the genetic factor for erythromycin resistance strongly suggests cytoplasmic coding for the formation of mitochondrial ribosomes. The question remains as to whether the mitochondrial DNA is the cytoplasmic determinant involved.

Summary.—Erythromycin resistance in *Saccharomyces cerevisiae* has been

shown to be inherited in an extrachromosomal fashion. An investigation of the relationship between the cytoplasmic factor for erythromycin resistance and the ρ factor has indicated that these two factors, although related, may not be identical. It is suggested that the cytoplasmic coding for erythromycin resistance indicates cytoplasmic coding for the mitochondrial ribosomes.

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