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Biogenesis of Mitochondria, XVIII. A New Class of Cytoplasmically Determined Antibiotic Resistant Mutants in *Saccharomyces cerevisiae*

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Abstract. New mutant yeasts resistant to the antibiotics chloramphenicol and mikamycin were isolated. They are mitochondrial mutants, characterized by several criteria as cytoplasmically determined. Biochemical studies show that amino acid incorporation into protein *in vitro* by mitochondria isolated from cells resistant or sensitive to mikamycin or chloramphenicol is inhibited by these antibiotics. Although aerobically-grown resistant strains of *Saccharomyces cerevisiae* are not affected by mikamycin or chloramphenicol, it is found that the mitochondrial protein-synthesizing system of anaerobically grown cells is inhibited *in vivo*. Cross resistance among the antibiotics chloramphenicol, mikamycin, erythromycin, lincomycin, carbomycin, and spiramycin is reported. All erythromycin resistant mutants, unlike the others, are resistant to erythromycin *in vivo* and *in vitro*. The results indicate that some of the cytoplasmic mutations (mikamycin and chloramphenicol resistance) are expressed at the mitochondrial membrane, whereas others (erythromycin resistance) possibly reflect changes in mitochondrial ribosomal proteins. We further suggest that conformational changes, either in the membranes or ribosomes, are likely to account for the observed antibiotic cross resistances.

The mitochondrial protein synthesizing system of *Saccharomyces cerevisiae* is specifically inhibited, *in vivo* and *in vitro*, by a number of antibacterial antibiotics: these include chloramphenicol, lincomycin, erythromycin, carbomycin, and spiramycin.[1,2] Yeast grown in the presence of these antibiotics lack the particulate mitochondrial cytochromes a, a_3, b, and c_1, thus are unable to grow on nonfermentable substrates. An erythromycin-resistant strain of *S. cerevisiae* showing cytoplasmic inheritance has been isolated and partially characterized in this laboratory.[3,4] *In vivo* and *in vitro*, its mitochondrial protein synthesizing system is unaffected by high levels of erythromycin. We have suggested, by analogy with bacterial mutants, that a change in the response of the mitochondrial protein-synthesizing system to erythromycin is a result of an alteration in a mitochondrial ribosomal protein.[3,4]

In this communication we report the isolation and some biochemical properties of two new cytoplasmically-determined mutant types of *S. cerevisiae* resistant to mikamycin and chloramphenicol. In contrast to the cytoplasmically-deter-

mined, erythromycin-resistant mutant reported from this laboratory, these mutants do not appear to have an altered mitochondrial protein-synthesizing system. It appears likely that the cytoplasmic determinants for mikamycin and chloramphenicol resistance are expressed through changes in proteins of the mitochondrial membrane. The new mutant strains isolated as resistant to a single antibiotic (chloramphenicol, mikamycin, or erythromycin) show extensive cross resistance to a number of other chemically unrelated antibiotics.

Materials and Methods. Media: The complex and synthetic media used have been described,[4] except that ethanol (2%) was used as a nonfermentable substrate instead of glycerol. Differential medium consists of 1% Difco yeast extract–2% peptone–0.1% glucose–2% ethanol. When this medium is supplemented with 0.3 mg/ml mikamycin or 4 mg/ml chloramphenicol, resistant yeast cells grow into large colonies by utilization of glucose and ethanol, whereas sensitive cells grow into pinpoint colonies, and utilize only glucose.

Isolation of mutants: Strains sensitive to an antibiotic are unable to grow on a nonfermentable substrate, such as ethanol, in the presence of that drug; in particular, at concentrations per milliliter of medium, of 0.01 mg mikamycin, 0.5 mg chloramphenicol, 0.1 mg erythromycin, 0.5 mg lincomycin, 0.3 mg carbomycin, 1.0 mg spiramycin, 0.5 mg tetracycline. Conversely, strains are resistant to these antibiotics by their ability to grow on such media in the presence, per milliliter of medium, of 0.3 mg mikamycin, 4 mg chloramphenicol, 4 mg erythromycin, 4 mg lincomycin, 0.6 mg carbomycin, 4 mg spiramycin, 2 mg tetracycline.

Chloramphenicol-resistant mutants are commonly due to nuclear mutations affecting whole cell permeability;[3,4] these mutations are frequently recessive in diploids.[5] Hence, to minimize the selection of these whole-cell permeability mutants, we have utilized sensitive diploid strains as suggested by Slonimski[5] for the isolation of cytoplasmically-determined antibiotic resistant mutants. Cultures of the sensitive, prototrophic, diploid strain, N-1300, were irradiated with ultraviolet light, and after overnight growth on yeast extract–peptone–ethanol to allow for phenotypic lag, the cells were plated on this medium, supplemented with either 0.3 mg/ml mikamycin or 4 mg/ml chloramphenicol. The resistant, prototrophic, diploid mutants, N-1301 and N-1302, selected on mikamycin- and chloramphenicol-containing media respectively, were then sporulated to obtain resistant haploids (L-3000 and L-3100 from N-1301; L-3200 and L-3300 from N-1302). Spontaneous cytoplasmic erythromycin-resistant mutants, L-3001 and L-3301, were isolated by plating cultures of erythromycin sensitive strains L-3000 and L-3300 respectively onto medium containing 4 mg/ml erythromycin. All the mutant strains isolated were stable with respect to their antibiotic resistance.

Mating procedures, the method of tetrad analysis, and the determination of the proportion of sensitive and resistant cells that arise from single diploid zygotes (denoted by us as mixedness), were essentially as described.[4,6,7] Cells were crossed for 4 hr, then plated on minimal medium to select the prototrophic diploid cells arising from the cross. The auxotrophic haploids that have been used in the present study are characterized by the following growth requirements, all of which derive from mutations of nuclear genes: L-3000, L-3100, L-3200, L-3300, L-3001, and L-3301 require uracil and histidine; L-2200 and L-2300 require adenine, tryptophan, and lysine; L-2265 requires leucine, arginine, tryptophan, and threonine; L-5628 requires only adenine.

Effect of antibiotics on anaerobically grown cells: Cells were grown anaerobically for 22 hr on a 5% glucose–1% Difco yeast extract–salts medium.[8] Antibiotics, when present, were 0.1 mg/ml mikamycin or 1 mg/ml erythromycin; these concentrations are about ten times greater than the amounts required to prevent growth of sensitive strains on ethanol medium. Cells were harvested, washed free of antibiotic, and resuspended in a 0.2% glucose–1% yeast extract–salts medium plus or minus antibiotic. After 6 hr aeration, whole-cell respiration was measured with an oxygen electrode.

Protein synthesis: Assays of mitochondrial protein synthesis *in vitro* were as described.[2]

Antibiotics: Antibiotics were gifts from: chloramphenicol, Parke Davis, Sydney, Australia; erythromycin glucoheptonate, Eli Lilly and Co., Sydney, Australia; lincomycin hydrochloride, The Upjohn Co., Kalamazoo, U.S.A.

Results. Genetic analysis of mikamycin and chloramphenicol resistance: Cytoplasmic determinants in yeast behave differently from nuclear genes in at least three ways. (*a*) The cytoplasmic determinants show 4:0 segregation as a result of meiosis; that is, they segregate, all or none, into the four haploid spores that arise from the sporulated diploid cells. This property has been demonstrated for the cytoplasmic-petite and the erythromycin-resistance determinant mutations,[4,9] and, more recently, a chloramphenicol-resistant determinant.[5] (*b*) Single uninuclear-diploid zygotes resulting from crosses between antibiotic sensitive and resistant cells contain a mixture of both determinants which subsequently segregate and eventually give rise to both resistant and sensitive diploid progeny.[4-7] (*c*) The proportion of sensitive and resistant cells which arise from the diploid zygotes is directly influenced by the nuclear alleles determining mating type. Thus, as first shown by Slonimski and colleagues, there is a "polarity" or directional transfer of genetic information from the "*a*" (donor) parent to the "*α*" (recipient) parent in crosses involving cytoplasmic determinants.[5,6]

Table 1 shows the results obtained from crosses between mikamycin-sensitive and resistant strains in which the resistance determinant is present in "*a*" and

TABLE 1. *Characteristics of cytoplasmic inheritance shown by mikamycin- and chloramphenicol-resistant mutants.*

	Diploid progeny			
	Mikamycin		Chloramphenicol	
Cross	% resis.	% sens.	% resis.	% sens.
L-3000 (aMC^r) × L-2300 (αMC^s)	38	62	—	—
L-2200 (aMC^s) × L-3100 (αMC^r)	63	37	—	—
L-3000 (aMC^r) × L-2265 (αMC^s)	14	86	16	84
L-5628 (aMC^s) × L-3100 (αMC^r)	78	22	75	25
L-3200 (aCL^r) × L-2265 (αCL^s)	—	—	3	97
L-5628 (aCL^s) × L-3300 (αCL^r)	—	—	98	2

MC^r and MC^s, CL^r and CL^s denote the determinants for resistance and sensitivity to mikamycin and chloramphenicol, respectively; the symbols "*a*" and "*α*" denote cell mating types. A resistant and a sensitive diploid colony were selected from each of the six crosses, sporulated, and the resultant asci dissected; the segregation in individual asci derived from resistant diploids was 4:0 for resistance and sensitivity, while the converse was true for sensitive diploids. The nuclear alleles segregated 2:2 in all asci.

"*α*" mating type strains. All three criteria for a cytoplasmic gene are satisfied by both the mikamycin and chloramphenicol determinants (Table 1). The influence of cell mating type on the polarity of the cross is particularly apparent in the case of the chloramphenicol determinant; the transfer of the determinant from the "*a*" type cell to the subsequent diploid progeny is very low, only about 3%. On the other hand, the mikamycin determinant that originates from the "*a*" type cell is transferred to the diploid progeny about 40% in some crosses and about 20% in others. It may be considered that some form of maternal inheritance is being observed, in which the "*a*" type cell is acting as a male

donor, and the "α" is the female recipient. As suggested by Slonimski,[5] the observed differences in polarity may also reflect different gene loci within a cytoplasmic chromosome (mitochondrial DNA?).

Ethidium bromide, which does not affect nuclear genes, quantitatively induces the cytoplasmic petite mutation, and also in most instances induces a loss of the erythromycin determinant.[6,7] We have similarly found that the chloramphenicol and mikamycin determinants are on occasion lost as a result of ethidium bromide-induced petite mutation.

Antibiotic cross resistance: We have reported[3,10] that yeast strains isolated as resistant to erythromycin are cross resistant to lincomycin, carbomycin, spiramycin, and oleandomycin (strain L-411). Similarly, strains L-3000 and N-1301, originally selected for resistance to mikamycin, were found to be cross resistant to chloramphenicol, lincomycin, carbomycin, and tetracycline. The chloramphenicol-resistant mutants, N-1302 and L-3300, were found to be cross-resistant to lincomycin and tetracycline.

Although the mutants were ostensibly selected as single step mutations, the observed cross resistances make it difficult at this stage of our investigations to be certain of their precise genotype and they may represent multiple gene mutations. However, our current studies suggest that most likely we are examining single gene mutations which confer multiple antibiotic resistances. In reciprocal genetic crosses between strains, the observed cross resistances appear to segregate together; pending further work we assume they are single step mutations. We have elected to use the following system of nomenclature. The symbols MC^r and MC^s denote resistance or sensitivity to the five antibiotics mikamycin, chloramphenicol, lincomycin, carbomycin, and tetracycline, conferred presumably by a single cytoplasmic genetic determinant. Similarly CL^r and CL^s designate a cytoplasmic determinant conferring resistance or sensitivity to chloramphenicol, lincomycin, and tetracycline. In the subsequent sections of the paper ER^r and ER^s designate, respectively, a cytoplasmic determinant conferring resistance or sensitivity to erythromycin, lincomycin, carbomycin, and spiramycin simultaneously.

Mitochondrial protein synthesis: Studies in this laboratory have shown that mitochondrial protein synthesis in mutants selected for resistance to erythromycin is resistant to this antibiotic *in vitro*.[3,10] It was anticipated that the MC^r and CL^r strains would similarly contain mitochondria whose amino acid incorporating activity *in vitro* would be antibiotic resistant. However, as shown in Table 2, mitochondrial protein synthesis in strains L-3000 (MC^r) and L-3300 (CL^r) is sensitive to all the antibiotics tested. The question of whether the whole cells of the mutants are permeable to the antibiotics immediately arises. Determination of whole cell permeability of several strains resistant to mikamycin and chloramphenicol, by methods previously employed in this laboratory,[3] has shown that the cells are apparently permeable to the drugs and that therefore the mitochondria are exposed to these antibiotics *in vivo*.

To investigate any relationship between erythromycin resistance and chloramphenicol resistance, a spontaneous, cytoplasmic, erythromycin-resistant mutant, L-3301, was isolated from L-3300 (CL^r). The expression of chloram-

TABLE 2. *The effect of antibiotics on mitochondrial protein synthesis in antibiotic sensitive and resistant mutants.*

Yeast strain	Cytoplasmic resistance determinant	% Antibiotic inhibition of [^{14}C]leucine incorporation					
		Mikamycin	Chloramphenicol	Lincomycin	Carbomycin	Spiramycin	Erythromycin
L-3000	MC^r	81	82	67	80	86	84
L-3001	MC^r ES^r	82	75	78	66	65	0
L-3300	CL^r	77	88	84	88	88	90
L-3301	CL^r ER^r	80	83	20	16	7	7
L-411	ER^r	74	70	12	0	0	0

Mitochondria were isolated from the strains indicated and assayed for [^{14}C]leucine incorporation in the presence and absence of antibiotics. Specific activities in the absence of antibiotics were about 1–7 pmol of [^{14}C]leucine incorporated/mg mitochondrial protein per 20 min. Results are expressed as the average % inhibition of the mitochondrial activity in the presence of 0.1 mM antibiotic

phenicol resistance is not altered by the new mutation; L-3301 remains resistant *in vivo* and sensitive *in vitro* to chloramphenicol (Table 2). However, it becomes resistant both *in vivo* and *in vitro* to erythromycin, spiramycin, carbomycin, and lincomycin. Thus, this erythromycin mutation has the same phenotypic properties as we have reported in earlier papers for strain L-411,[3,10] included in Table 2 for comparison. It is apparent that in one strain, two different types of cytoplasmically determined antibiotic resistance coexist, one expressed *in vivo* and *in vitro*, the other only *in vivo*. Strain L-3301 would be denoted in our terminology as CL^r ER^r. However, it has now emerged that not all mutants selected for erythromycin resistance behave identically with regard to cross resistance. A spontaneous, cytoplasmic, erythromycin-resistant mutant, L-3001, derived from L-3000 (MC^r), was found to be resistant *in vivo* to erythromycin and spiramycin, as well as to mikamycin, lincomycin, carbomycin, and chloramphenicol; however, protein synthesis by its mitochondria *in vitro* was resistant only to erythromycin (Table 2). Thus, the erythromycin mutation introduced in strain L-3001 is different than the previously described erythromycin mutation and confers cross resistance to spiramycin *in vivo*, but not *in vitro*. We denote this second erythromycin mutation as ES^r/ES^s to distinguish it from the previously described ER determinant; strain L-3001 would be denoted MC^r ES^r in our nomenclature.

A summary of the rather complicated effects of antibiotics on the strains discussed, *in vivo* and *in vitro*, is shown in Table 3. The mutants fall into two classes; one type with mitochondrial protein synthesis resistant to the antibiotics *in vivo* and *in vitro* (Table 3 italics), the other type resistant only *in vivo*. The involvement of two different systems was considered to account for these observations, one type of mutation, as suggested earlier, being a probable mitochondrial-ribosomal mutation, the other a mitochondrial-membrane mutation.

Aerobic and anaerobic growth of cells in the presence of antibiotics: Yeast cells cultured anaerobically on growth-limiting amounts of unsaturated fatty acids and ergosterol (5% glucose–1% yeast extract medium) contain poorly developed mitochondria, deficient in internal membrane and unable to respire due to the absence of the cytochromes.[8] On aeration of these lipid-depleted anaerobic cells, functional mitochondria develop with normal amounts of cytochromes and respiratory ability; this induction is dependent on an active mito-

TABLE 3. *The effects of antibiotics on antibiotic sensitive and resistant mutants in vivo and in vitro.*

Yeast strain	Selector antibiotic	Phenotype: *in vivo/in vitro* Mikamycin	Chloramphenicol	Lincomycin	Carbomycin	Spiramycin	Erythromycin
L-3000	Mikamycin	R / S	R / S	R / S	R / S	S / S	S / S
L-3001	Mikamycin (Erythromycin)	R / S	R / S	R / S	R / S	R / S	*R* / *R*
L-3300	Chloramphenicol	S / S	R / S	R / S	S / S	S / S	S / S
L-3301	Chloramphenicol (Erythromycin)	S / S	R / S	*R* / *R*	*R* / *R*	*R* / *R*	*R* / *R*
L-411	Erythromycin	S / S	S / S	*R* / *R*	*R* / *R*	*R* / *R*	*R* / *R*

In vivo phenotypes R and S denote the ability and inability respectively, of cells to grow on ethanol medium containing the particular antibiotic. *In vitro* phenotypes R and S denote resistance, defined as less than 25% inhibition of [^{14}C]leucine incorporated into protein by isolated mitochondria, and sensitivity, defined as greater than 60% inhibition by the antibiotic.

chondrial protein synthesizing system. To investigate the role of mitochondrial membranes in antibiotic resistance *in vivo*, mutant strains were grown anaerobically in the presence or absence of antibiotics, then aerated for an additional 6 hr in the presence or absence of antibiotics. The results are shown in Table 4. Strains resistant to erythromycin (L-411, L-3001) develop normal respiratory activity in the presence of this drug, whether it is present during anaerobic growth, aerobic induction, or both; erythromycin-sensitive strains (L-3000) do not develop respiration when aerated in the presence of the antibiotic.

TABLE 4. *The effect of antibiotics on the induction of respiration in anaerobically-grown cells.*

Yeast strain	Antibiotic present: Anaerobic growth	Aerobic induction	Whole cell respiration (pg-atoms O_2/min per mg dry wt cells)
L-411	None	None	80
	Mikamycin	Mikamycin	3
	Erythromycin	Erythromycin	25
L-3000	None	None	28
	Mikamycin	Mikamycin	0
	Erythromycin	Erythromycin	0
L-3001	None	None	36
	Erythromycin	Erythromycin	34
	None	Mikamycin	0
	Mikamycin	None	0

Cells were grown anaerobically and aerated both in the presence or absence of antibiotics as indicated.

The effect of antibiotics on the strains *in vivo* under *fully aerobic* conditions, and *in vitro*, on the amino acid incorporating activity of the isolated mitochondria, was as follows:

L-411: *in vivo* Erythromycin (Resis.) Mikamycin (Sens.); *in vitro* Erythromycin (Resis.) Mikamycin (Sens.).

L-3000: *in vivo* Erythromycin (Sens.) Mikamycin (Resis.); *in vitro* Erythromycin (Sens.) Mikamycin (Sens.).

L-3001: *in vivo* Erythromycin (Resis.) Mikamycin (Resis.); *in vitro*, Erythromycin (Resis.) Mikamycin (Sens.).

These results are consistent with the hypothesis that erythromycin resistance in these strains is due to an altered mitochondrial ribosome which is unaffected by erythromycin under all circumstances.

However, in direct contrast to erythromycin, the effect of mikamycin on the mitochondrial protein synthesizing system *in vivo* is greatly influenced by the conditions under which the cells are grown. Thus, the mikamycin-resistant strains L-3000 and L-3001 are unaffected by up to 1 mg/ml mikamycin in aerobic culture, whereas synthesis of cytochromes *a*, a_3, *b*, and c_1 by the sensitive strain L-411 is inhibited by 20 μg/ml mikamycin. However, when these three strains are grown anaerobically, with or without 100 μg/ml mikamycin but aerated in the presence of 100 μg/ml mikamycin, they fail to develop respiration. These results suggest that the mitochondrial protein synthesizing system is inaccessible to the antibiotic when cells are grown under aerobic conditions and contain well developed mitochondrial membranes. On the contrary, when cells are grown anaerobically and contain grossly altered mitochondrial membranes, the mitochondrial protein synthesizing system in mikamycin-resistant strains is accessible and sensitive to mikamycin.

Discussion. Erythromycin-resistant mutants already characterized are resistant to erythromycin *in vivo* and *in vitro*[3,10] whereas the new cytoplasmically-determined mikamycin- and chloramphenicol-resistant mutants described in this paper are resistant *in vivo*, but sensitive *in vitro*, to the antibiotics. Not only is amino acid incorporation by isolated mitochondria inhibited by the antibiotic in these new strains, but also the oxygen-induced development of respiration by anaerobically-grown cells *in vivo* is inhibited by the antibiotic, a result which we interpret as due to an inhibition of mitochondrial protein synthesis under these conditions. However, it should be added that a high concentration of mikamycin does partially inhibit yeast cell respiration as, indeed, does chloramphenicol. Nonetheless, the primary effect of chloramphenicol and, we suggest, mikamycin is on mitochondrial protein synthesis; low concentrations of both antibiotics do not directly inhibit respiration. We have suggested that yeast cytoplasmically-determined erythromycin resistance is a consequence of a specific alteration in a mitochondrial ribosomal protein and this still appears likely. However, the mikamycin- and chloramphenicol-resistant mutants cannot be easily explained by this hypothesis. Membrane changes appear to be involved in the mutation to mikamycin resistance, thereby rendering the mitochondria impermeable to mikamycin, chloramphenicol, lincomycin, and carbomycin. Damage to the mitochondrial membrane during isolation and assay would then allow the antibiotics to penetrate the mitochondria *in vitro*. The anaerobic studies have indicated that mitochondrial membrane structure is an important factor in whole cell resistance or sensitivity to mikamycin *in vivo*.

Two possibilities to account for the overall results suggest themselves, both of which involve a mutation that results in a conformational change in a single protein. This protein may be part of the membrane or ribosome. As a result of the putative conformational change, multiple cross resistances to antibiotics are conferred on the system. The mutation could be considered analogous to

the situation in *Escherichia coli* recently reported by Apirion *et al.*[11] in which one mutation conferring antibiotic resistance, as a result of a change in a single ribosomal protein, leads to changes in the reaction of neighboring proteins to other antibiotics. Or, the change may be exclusively in a membrane protein that affects the accessibility of the mitochondrial ribosome to a number of antibiotics. We interpret the data to suggest that the erythromycin mutation is probably expressed as a change in a ribosomal protein and mikamycin resistance as a change in a membrane. Neither of these suggestions is mutually exclusive, and we favor the view that they coexist and that interactions between them may occur. Investigators in a number of laboratories have had only limited success in the preparation of mitochondrial ribosomes from different organisms. The yields of ribosomes obtained are small and, in our hands, the separation of ribosomes from yeast mitochondria is not a simple task. The ribosomes of yeast mitochondria are intimately associated with the organelle membranes; the difficulty of their separation by the use of detergents suggests a strong interaction. Indeed, it is not unlikely that the ribosomes are part of the membrane. This possibility is under active investigation.

Abbreviations: MC, resistance or sensitivity to five antibiotics; CL, cytoplasmic determinant conferring resistance or sensitivity to three antibiotics; ER, cytoplasmic determinant for four antibiotics [see text].

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