Oligomycin Resistance in Normal and Mutant Yeast*

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Yeast mutants with genetically altered mitochondria are extremely useful in the study of oxidative phosphorylation mechanisms (Beck et al., 1968). Such mutants are usually detected by their inability to grow on non-fermentable carbon sources, their spectral characteristics, and by selective staining. Drug resistance has also been employed in obtaining mitochondrial mutants (Thomas and Wilkie, 1968; Butow and Zeydel, 1968), but as yet this method has not been extended to include specific inhibitors of oxidative phosphorylation. This report describes the isolation of 50 oligomycin-resistant Saccharomyces cerevisiae mutants and the genetic characterization of two of these mutants and a naturally resistant strain. At least two Mendelian genes control the resistance in these strains. Resistance displays partial dominance and additivity in heterozygous diploids. Oligomycin partially inhibits cytochrome synthesis, and increases the frequency of mutation to cytoplasmic respiratory deficiency (ρ^-). Oligomycin resistance is accompanied by resistance to venturicidin.

Experimental

Yeast strains: Table I lists the strains used in the study along with some of their properties. Media: Cells were cultured on either YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose ± 2% Agar) or glycerol medium (same as YPD, except dextrose was replaced by 3% v/v glycerol). Liquid sporulation medium contained 0.98% potassium acetate, 0.05% dextrose and 0.1% Bacto-yeast extract. Nutritional requirements were scored on synthetic medium containing 0.67% Bacto-yeast nitrogen base without amino acids, 2% dextrose and 2% agar, which was supplemented with various amino acids or adenine sulfate (10-40 mg/l), as required. Antibiotic resistance was determined on glycerol medium containing from 0.05 to 10 μg antibiotic per ml. Screening for oligomycin-resistant mutants: Approximately 10^6 cells of strain D311-3A were spread on plates of glycerol medium (20 ml/plate) and

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Table I: Properties of Yeast Strains

Strain	Genotype or cross	Oligomycin concentration tolerated (μg/ml)
*D311-3A	a $tr_2ly_2hi_1$	< 0.25
G-2	a $tr_2ly_2hi_1or_n$	1.0-1.5
G-31	a $tr_2ly_2hi_1or_1$	2.0
*D501-2C	α ad_6	< 0.25
*1268-2B	α $ad_6hi_2or_2$	1.0-1.5
RJ-1	G-2 x 1268-2B	> 2.0
RJ-2	G-2 x D501-2C	1.0
RJ-3	G-31 x 1268-2B	> 2.0
RJ-4	G-31 x D501-2C	0.5-1.0
RJ-5	D311-3A x 1268-2B	1.0-1.5
RJ-7	D311-3A x D501-2C	< 0.25

Oligomycin tolerance was determined by observing growth of clones replicated on plates of glycerol medium containing one of the following concentrations of antibiotic: 0.25, 0.50, 1.0, 1.5, 2.0 μg/ml. Key to symbols: a, α - mating types; or - oligomycin resistance. Other symbols refer to nutritional requirements: tr - tryptophan, ly - lysine, hi - histidine, ad - adenine. *Obtained from Dr. Fred Sherman.

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exposed to UV irradiation for 10 or 60 seconds (giving 75 and 4.8% survival, respectively). After irradiation, a hole cut in the center of the agar was filled with 0.2 ml of sterile oligomycin solution (in ethanol).

Oligomycin concentrations of 1, 10, 50 and 100 μg/ml were used. Plates were incubated at 30°, and cells from colonies growing within the distinct zone of inhibition were used to start resistant clones. Each strain was further purified by streaking on YPD and selecting single colonies.

Determination of oligomycin resistance: About 10^6 cells of each strain to be tested were suspended in 0.5 ml sterile 0.05 M phosphate buffer, pH 7.0, in one of the wells of a Pepper inocula replicator (Pentex). Replica plates of YPD and glycerol medium containing various oligomycin concentrations were then inoculated. Thirty two strains (8 tetrads) may be tested simultaneously in this way.

Antibiotics: The oligomycin used consisted of a mixture of oligomycins A, B and C. Venturicidin (Glaxo) was recrystallized twice from ethanol-water before use.

Genetic analysis: Analysis of antibiotic resistance and nutritional requirements was carried out according to the general procedure described by Hawthorne and Mortimer (1960).

Results

Screening: Mutation to oligomycin resistance is relatively common. With 10 sec. irradiation, 24 resistant clones were isolated, representing a frequency of 3.9 mutants per 10^6 survivors. With 60 sec., 27 clones were obtained, and the frequency was 68 per 10^6 survivors. Fifty of the 51 clones selected showed resistance to at least 0.5 μg oligomycin per ml in a subsequent test. Two mutants, G-2 and G-31 were selected for detailed genetic analysis.

Oligomycin resistance: When either G-2 or G-31 was crossed with haploid tester strain, D501-2C, the resulting diploids, RJ-2 and RJ-4 exhibited resistance between that of the mutant and tester haploids, and greater than that of the diploid, RJ-7, made by crossing the tester strain with the parent of the two mutants, D311-3A (Table 1). Crosses between mutants and a second tester, 1268-2B, gave highly resistant diploids, suggesting that the tester itself might be resistant, as indeed it proved to be (Table 1).

Segregation of or genes: Resistance to oligomycin is designated by the symbol or; the corresponding sensitive allele by OR. Tetrad analysis of diploids, RJ-2 and RJ-4, in which strain D501-2C is the sensitive haploid parent, showed 2:2 segregation of or:OR, indicating that a single gene is responsible for resistance in either mutant (Table 2). Therefore, the mutant allele in G-31 is designated or_1, while the mutant allele in G-2 is tentatively designated or_n, since its genetic linkage to or_1 has not yet been determined. However, when diploids (RJ-1 and RJ-3) derived from strain 1268-2B and the two or mutants were analyzed, half of the tetrads showed either 3:1 or 4:0 segregation, indicating that two or more or genes were present. Therefore, a cross between strain 1268-2B and the sensitive parent strain, D311-3A, was made, and the resulting diploid, RJ-5, was subjected to tetrad analysis. Since 5 of the 6 tetrads displayed 2:2 segregation, it is likely that the resistance of 1268-2B is also determined by a single gene. The resistant

Table 2. Tetrad analyses of oligomycin resistant (or) strains.

Tetrad Type (Res:Sens)	Number of Tetrads				
	RJ-1	RJ-2	RJ-3	RJ-4	RJ-5
4:0	0	0	1	0	0
3:1	3	0	4	0	1
2:2	3	6	5	6	5

Analyses were performed as indicated in the text. Sensitive segregants were unable to grow on glycerol medium containing 0.25 μg oligomycin per ml. Resistant segregants grew on medium containing oligomycin concentrations of 0.5 μg/ml or higher.

allele in 1268-2B is designated $\underline{or}_2$ because it displays a low degree of linkage to either $\underline{or}_1$ or $\underline{or}_n$.

Cross-resistance studies: When venturicidin was used in place of oligomycin, resistance patterns were exactly parallel. Table 3 shows that strains bearing resistant alleles $\underline{or}_1$, $\underline{or}_2$ or $\underline{or}_n$ were all resistant to venturicidin. At least 5 times as much venturicidin as oligomycin could be tolerated. Diploid strains RJ-3, RJ-4, and RJ-5 also showed resistance to venturicidin concentrations at least as high as 10 μg/ml.

Table 3. Resistance of various strains to oligomycin and venturicidin

Strain	Inhibitor concentration tolerated	
	Oligomycin (μg/ml)	Venturicidin (μg/ml)
D311-3A	< 0.25	0.10
D501-2C	< 0.25	0.05
1268-2B	1.0	>10
G-31	2.0	>10
G-2	0.5	>10
RJ-7	< 0.25	0.10

Antibiotic tolerance was determined using the concentrations listed for Table 1. Venturicidin was also tested at concentrations of 0.05, 0.10, 5.0 and 10.0 μg/ml.

Table 4. Effects of oligomycin on properties of yeast cells.

Property	Control	+ Oligomycin
% inhibition on glycerol medium	0	100
% inhibition on YPD	0	~ 30
% ρ^-	0.5	20
Cytochrome a	+ +	~ 0
Cytochrome b	+ +	+
Cytochrome c	+ + +	+ +
Cytochrome c_1	+	±
Q_{O_2}	30-50	4-9

Inhibition of growth of strain D311-3A was determined as described in the screening procedure or by weighing cells after growth in liquid dextrose medium. The central well of the plates contained 0.2 ml of oligomycin solution, 100 μg/ml. The %ρ^- induced was determined with cells from the partially inhibited zone on plates of dextrose medium by observing differential growth on plates of glycerol medium containing 0.1% dextrose, followed by a tetrazolium overlay test [Ogur, M., St. John, R., Nagai, S., Science 125, 928 (1957)]. Cytochrome content was estimated spectroscopically after freezing cells in liquid nitrogen. Cells used for the cytochrome study and Q_{O_2} determination were grown in liquid YPD medium containing 2 μg oligomycin/ml. Q_{O_2} (glucose) was measured at 25° with a Clark oxygen electrode.

Effects of oligomycin on mitochondrial biogenesis: Presumably oligomycin inhibits yeast growth on glycerol medium by inhibiting oxidative phosphorylation in vivo. Incomplete inhibition of growth on dextrose medium (YPD) is observed, suggesting that glycolytic ATP formation is normal in the presence of oligomycin (Table 4). However, oligomycin action in vivo is not limited to inhibition of mitochondrial function; the antibiotic also affects mitochondrial biogenesis. The Q_{O_2} and cytochrome content of cells grown on YPD are both diminished markedly when oligomycin is present. Furthermore, the frequency of mutation to cytoplasmic respiratory deficiency ($\rho^+ \rightarrow \rho^-$) is increased about 40-fold above the spontaneous level by oligomycin. However, it should be stressed that the increased level of ρ^- cells is not sufficient to account either for the decrease in Q_{O_2}, or for the complete absence of growth of cells on glycerol medium.

Discussion

Oligomycin resistance may be due to mutation-induced mitochondrial changes, to decreases in permeability of the plasma membrane or induction of a detoxication process. If a mitochondrial basis for oligomycin resistance is assumed, the present demonstration that or genes show Mendelian segregation represents another example of the dependence of mitochondrial inheritance upon chromosomal information. A series of Mendelian genes, designated p, which control various respiratory functions, have been characterized by Sherman (1963). Moreover, Sherman et al. (1966) have demonstrated that the structural gene for yeast-iso-1 cytochrome c is chromosomal, as are the structural genes for malate dehydrogenase in Neurospora (Munkres and Richards, (1965).

Yeast genes controlling resistance to chloramphenicol have been shown to be Mendelian, but the basis for this resistance appears to involve altered cell permeability. Erythromycin resistance, on the other hand, has a mitochondrial basis and displays extrachromosomal inheritance (Thomas and Wilkie, 1968; Linnane et al., 1968) In growing yeast, both chloramphenicol and erythromycin (a macrolide) inhibit synthesis of cytochromes a + a_3, b and c_1, but not cytochrome c (Huang et al., 1966). Since these drugs inhibit bacterial protein synthesis, it has been postulated that they inhibit yeast cytochrome synthesis by interacting with mitochondrial ribosomes. The demonstration that oligomycin also inhibits cytochrome synthesis to some extent, may be interpreted in different ways: (1) Mitochondrial ATP formation, which is inhibited by oligomycin, may play a significant role in synthesis of mitochondrial protein. Perhaps this "dependent" synthesis involves mitochondrial ribosomes. The primary product of such synthesis is not necessarily cytochrome, but may be a "lattice" protein whose presence is required before further steps

in the self-assembly of the mitochondrial membrane can proceed (including addition of cytochromes to the lattice). (2) Oligomycin may form a complex with a lattice protein (or lipid), thereby masking lattice sites essential for continued self-assembly. (3) Since oligomycin is itself a macrolide (Lardy, Witonsky, and Johnson, 1965) it may, like erythromycin, act directly upon mitochondrial ribosomes. This alternative seems less likely, since oligomycin has no antibiotic activity against a variety of bacteria (Smith, Peterson and McCoy, 1954).

Regardless of the mechanism by which oligomycin inhibits cytochrome synthesis, the resulting aberrant mitochondria appear to have increased susceptibility to the cytoplasmic mutation, $\rho^+ \rightarrow \rho^-$. Such susceptibility may reflect either a deficiency in mitochondrial ATP required for mitochondrial DNA synthesis, or may be a consequence of an incomplete lattice.

Cross-resistance to venturicidin suggests that the mechanism by which this antibiotic inhibits yeast growth is very similar, if not identical, to that of oligomycin. Walter, Lardy and Johnson (1967) have shown that the *in vitro* action of venturicidin on oxidative phosphorylation in rat liver mitochondria resembles that of oligomycin. The higher level of resistance to venturicidin found in *or* mutants correlates with their observation that complete inhibition of mitochondrial ATPase requires higher concentrations of venturicidin than of oligomycin.

The previous findings (Smith, Peterson, and McCoy, 1954) that oligomycin was inactive against *S. cerevisiae* may likely have resulted from the use of dextrose rather than glycerol as carbon source.

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