OLIGOMYCIN RESISTANT MUTANTS IN YEAST

P.R.AVNER and D.E.GRIFFITHS
School of Molecular Sciences, University of Warwick, Coventry, CV4 7AL, England

Received 20 August 1970

1. Introduction

The increasing amount of information available relating to the structure and activity of yeast mitochondria has made feasible the use of a genetic approach to the problem of the mechanisms of respiration and oxidative phosphorylation by isolating mutants with lesions in mitochondrial metabolism [1]. Such mutants are usually detected by either their inability to grow on non-fermentable carbon sources, their staining reaction to dyes feeding into the electron transport chain, their spectral characteristics or their resistance to drugs which affect oxidative phosphorylation or electron transport [2, 3].

Some of the mutants so far isolated, such as the cytoplasmic petites and a number of nuclear gene mutants with a similar phenotype are unsuitable for biochemical analysis of energy coupling reactions or respiration due to the large extent of the biochemical lesions. However, other more specific mutants [4–7] have been reported.

The purpose of the investigation was to attempt to define a series of mutants in which mitochondrial function relating to oxidative phosphorylation had been altered, and to carry out a systematic genetic and biochemical study on them. The results presented relate to a series of 50 mutants of *Saccharomyces cerevisiae* which were resistant to concentrations of oligomycin 4–20 fold greater than that inhibiting the non-resistant strain. The cross resistance to other inhibitors of oxidative phosphorylation such as *N*, N^1-dicyclohexylcarbodiimide (DCCD) and aurovertin was investigated.

2. Materials and methods

2.1. *Strains and nomenclature*

Two heterothallic strains of *S. cerevisiae* D22 (ad_2, α, ρ^+) and D6 (arg, met, ρ^+) obtained from Dr.D.Wilkie were used. All mutants were derived from either of these two haploids strains whose drug resistance is shown in table 1. All mutants derived from D6 have that number as a prefix and similary with D22. Series A, B, C, refer to whether the mutants were isolated from oligomycin plates containing 2.5, 5.0 or 10 μg/ml of the drug, respectively. The two parental strains D6 and D22 served as sensitive testers throughout the genetic studies.

2.2. *Media*

The normal growth media for the haploid strains were:

YEPG: 2% peptone, 1% yeast extract, 2% glucose, and 2% agar (oxoid No. 3);
YEPGly: 2% peptone, 1% yeast extract, 4% glycerol, 2% agar;
YEPEtOH: 2% peptone, 1% yeast extract, 0.5% ethanol (added after autoclaving), 2% agar.

The liquid growth media used normally were as above but minus the agar.

The synthetic media used for genetic studies on diploids were:

MMG: Wickerham minimal medium glucose [8];
MMGly: Wickerham minimal medium glycerol – as above but 4% glycerol instead of glucose as the carbon source.

North-Holland Publishing Company – Amsterdam

MMPDM: minimal medium for petite determination – as above but 4% glycerol and 0.5% glucose served as the carbon source. Agar was added when necessery at a concentration of 1.5% [Ionagar No.2 Oxoid]. Haploid strains were cultivated in the above media supplemented with either adenine (10 mg/l) or arginine and methionine (10 mg/l)

Drug plates were prepared by adding the drug as an ethanolic solution to autoclaved YEPGly agar cooled to 50°. All drug plates were used within 24 hr of pouring.

Oligomycin was purchased from Sigma Chemical Co., carbonyl cyanide phenyl hydrazone (CCCP) from Calbiochem, paromomycin was kindly donated by Parke Davis and Co. Ltd., erythromycin a kind gift from Eli Lilly, whilst spiramycin was generously donated by May and Baker. Aurovertin was a gift from Professor H.A. Lardy.

2.3. *Isolation of mutants and testing for oligomycin resistance*

Oligomycin resistant mutants were isolated by ultraviolet irradation for 80 sec (10% viability) of a cell suspension containing 2×10^7 cells/ml followed by plating out of 4×10^6 cells onto YEPGly plates containing 2.5, 5.0 or 10.0 μg/ml of oligomycin. Colonies that grew after incubation for 5 days at 30° were isolated, streaked out onto YEPGly plates containing 2.5 μg/ml oligomycin and single colonies isolated. These colonies were subsequently tested for their degree of resistance by replica plating and by plating out cells onto drug plates. Growth was examined after 3 days incubation at 30°.

2.4. *Aurovertin resistance*

Assays for aurovertin resistance were carried out using YEPGly plates on which were spread approx. 1000 cells. 400 μg aurovertin were added as an ethanolic solution to a central well cut in the plates and inhibition zones were measured after 3–4 days. Controls were run using ethanol.

2.5. *DCCD resistance*

Resistance of the strains was tested by plating out approx. 1000 cells, onto DCCD containing plates of minimal medium supplemented with adenine, arginine and methionine and with either ethanol or glycerol as carbon source. The media were buffered at either pH 7.3 or pH 6.0 using 0.1 M phosphate buffer. Growth at 30° was scored after 5 days.

Table 1
Resistance to oligomycin and cross resistance to aurovertin.

Strain	Tolerance of haploid to oligomycin	Tolerance of diploids ($OL^r \times OL^s$) to oligomycin	Tolerance of haploid to aurovertin
D22A2	2.5	N.T.	Resistant
D22A3	10.0	2.5	N.T.
D22A4	> 10.0	2.5	Resistant
D22A5	> 10.0	2.5	Resistant
D22A7	> 10.0	1.0	N.T.
D22A9	5.0	2.5	N.T.
D22A12	> 10.0	1.0	N.T.
D22A18	2.5	1.0	Resistant
D22B9	> 10.0	2.5	Resistant
D22B15	> 10.0	1.0	Resistant
D22B16	> 10.0	1.0	Resistant
D22B21	> 10.0	> 10.0	N.T.
D6A1	2.5	1.0	N.T.
D6A4	> 10.0	> 10.0	Resistant
D6B2	> 10.0	0.5	N.T.
D22	0.5	–	Nil
		(D6 × D22) 1.0	N.T.
D6	0.5	–	Nil

N.T. = Not tested

The level of oligomycin tolerance is defined as the maximum concentration of oligomycin permitting growth of the strain. Levels of oligomycin used were 0.5, 1.0, 2.5, 5.0, 10.0 μg/ml. Aurovertin resistance is defined as a failure to form inhibition zones when 400 μg aurovertin were added to a centre well.

2.6. *Petite frequency*

The effect of oligomycin on petite frequency was tested by growth in MMG + supplements in the presence and absence of oligomycin. Growth tubes were shaken for 48 hr at 30°. Petite frequency was estimated using the tetrazolium overlay technique [10].

2.7. *Genetic analyses*

Crosses were performed by the mass mating technique and the resulting zygotes were selected on MMG. Synchronised zygotes were obtained following a modified procedure of Jakob [9]. As diploids obtained in the initial 4 hr after mating grew only poorly on MMGly, oligomycin resistance was

assayed using MMPDM plates containing oligomycin and colonies containing resistant cells identified by the presence of tetrazolium positive papillae. Diploids obtained after 4 hr were assayed using MMGly oligomycin plates.

3. Results and discussion

The 50 oligomycin resistant mutants isolated showed degrees of resistance (table 1) varying from 2.5 to >10 μg/ml oligomycin as compared to sensitivity to 0.5 μg/ml oligomycin exhibited by the parental strain. The mutants appeared perfectly stable for 9 months after isolation. All oligomycin resistant mutants tested showed cross resistance to aurovertin when assayed as described in table 1. The level of aurovertin necessary to inhibit the oligomycin sensitive strains D6 and D22 is at least 40 times greater than the level of oligomycin required for inhibition; 20 μg/ml of aurovertin failed to inhibit the growth of either strain.

None of the oligomycin resistant strains showed any cross resistance to erythromycin, paromomycin, spiramycin, CCCP or 2, 4-dinitrophenol. The resistant strains were tested using a range of concentrations of these compounds from 1–1000 times the minimal concentration necessary to inhibit the growth of the oligomycin sensitive strains, D6 and D22.

N, N'-Dicyclohexylcarbodiimide (DCCD) is a potent inhibitor of oxidative phosphorylation and energy transfer processes in animal mitochondria [11] and in yeast cells [12] and is readily available in contrast to aurovertin. Genetic studies are complicated by its instability in aqueous solution at acid pH but it is reasonably stable at neutral pH. Consequently studies of DCCD resistance were carried out at pH 7.3 rather than at pH 6.0. At pH 7.3 the oligomycin sensitive strains D6 and D22 are inhibited by 0.5 μM DCCD with glycerol as the carbon source (table 2) but required 125 μM DCCD when glucose was the carbon source.

Many of the oligomycin resistant strains showed a 10–25 fold increased resistance to DCCD when grown on glycerol, compared to the parental strains D6 and D22. It is not possible at this stage to state that all oligomycin resistant strains show cross resistance to DCCD because of poor growth of some strains at pH 7.3.

When grown on glucose as carbon source at both pH 6.0 and pH 7.3 there was no correlation between oligomycin resistance and DCCD resistance. At pH 6.0 with glycerol there was again no correlation between oligomycin and DCCD resistance. This may be due to: (a) DCCD having a specific effect on energy conservation reactions at neutral pH only; (b) hydrolysis of DCCD at pH 6.0 leading to variable concentrations of DCCD within the yeast cell; (c) variable hydrolysis of DCCD at pH 6.0 leading to reaction products which affect energy conservation reactions but at a different locus to DCCD and oligomycin.

Table 2
Sensitivity of yeast strains to DCCD.

Strain	Tolerance of haploids to oligomycin (μg/ml)	Tolerance of haploids to DCCD (μM)			
		Carbon source			
		Glycerol		Glucose	
	pH 5.8	pH 7.3	pH 6.0	pH 7.3	
D6	0.5	<0.5	20.0	>125.0	
D22	0.5	<0.5	20.0	>125.0	
D22B10	5.0	++++			
D22B14	2.5	++			
D22B21	>10.0	+			
D22B8	>10.0	++			
D22B16	>10.0	+++			

++++ Resistant to >10 μM DCCD
+++ Resistant to 5.0–10 μM DCCD
++ Resistant to 2.5–5.0 μM DCCD
+ Resistant to 1.0–2.5 μM DCCD

Media used were MMG and MMGly and adenine, arginine, and methionine. All media were made up in 0.1 M phosphate buffer. DCCD was added as a freshly made ethanolic solution to autoclaved media cooled to 50°.

The results on cross resistance to aurovertin and DCCD taken together with evidence that other oligomycin resistant mutants show cross resistance to venturicidin [2a, b] – all inhibitors affecting oxidative phosphorylation despite their different molecular structures – strongly suggest that the resistance to these mutants is not due to changes in cell permeablility but to a change at the locus of action of these inhibitors, the mitochondrial ATP synthetase complex or the interaction of this complex with the mitochondrial membrane. Supporting evidence for this view has come from studies in this laboratory on the oligomycin sensitivity of ATPase in yeast submito-

Table 3
Effect of oligomycin on petite frequency resistant and sensitive strains

Strains	Level of oligomycin Tolerance	Oligomycin concentration used in induction (μg/ml)	Increase in % petite frequency relativ to control
D6	1.0	5	+300
D22	0.5	10	+500
D22A4	>10.0	5,10	+ 30
D22A5	>10.0	10	+380
D22A7	>10.0	10	± 30
D22A15	>10.0	10	± 30
D22B10	5.0	5	± 30
D22B16	>10.0	10	± 30
D22OC410	>10.0	10	± 30
D6B2	>10.0	10	+170

A 1% inoculum of mid log phase glucose grown cells was inoculated into minimal medium + supplements containing oligomycin and grown with shaking for 48 hr, at 30°. Control tubes contained no oligomycin but equivalent amounts of EtOH to that added to the oligomycin growth tubes. Cells were plated onto YEPG and incubated for 5 days at 30°. Petites were indentified using a tetrazolium overlay [10].

chondrial particles.

ATPase in submitochondrial particles from the parental strain D22 is inhibited 50% by 8 μg oligomycin/mg/protein whereas the ATPase activity in submitochondrial particles derived from strains D22A4 and D22B9 are inhibited 50% by 50 μg oligomycin/mg/protein and 10% by 750 μg oligomycin/mg/protein respectively (J.R.Turner, personal communication).

Similarly, studies on mitochondrial membrane proteins from oligomycin sensitive and resistant strains show marked differences when examined by gel-electrophoresis (Broughall and Avner, to be published). The significance of these observations and their relation to changes in the components of the ATP synthetase complex and/or its interaction with the energy conservation system of the mitochondrial membrane are being investigated.

3.1. *Effect of oligomycin of petite frequency*

Table 3 shows that the petite frequency of the oligomycin sensitive strains D6 and D22 increase at least three fold when these cells are grown in the presence of oligomycin. In contrast, only two of the resistant strains tested (D22A5 and B6B2) showed an increase in petite frequency when grown in the presence of oligomycin. This difference in responce of the resistant and sensitive strains does not seem to be due to inhibition of the sensitive strains at the end of the glycolytic growth phase in the presence of oligomycin [13]. The results show that strains D22A5 and D6B2 differ from the other strains tested and suggest that at least in these two resistant strains oligomycin resistance is not due to its altered permeability.

Table 4
Resistance of diploids derived from oligomycin resistant x oligomycin sensitive crosses

Strain	No. of colonies	No. of resistant colonies	No. of non-resistant colonies	No. of mixed colonies
D22A3 X D6	23	0	0	23
D22A4 X D6	78	0	0	78
D22A7 X D6	31	0	0	31
D22A12 X D6	120	0	0	120
D22B9 X D6	422	0	0	422
D22B1 X D6	234	0	60	174
D6A1 X D22	38	0	5	33
D6A4 X D22	248	11	3	234
D6B1 X D22	24	0	0	24
D6B2 X D22	67	0	23	44

Diploid colonies were obtained by mass mating followed by plating out and auxotrophic selection. Resistance to 2.5 μg and 5.0 μg/ml oligomycin was tested by replica plating

3.2. *Genetic studies*

Diploids obtained by mass mating resistant strains to a ρ^+ sensitive tester when analysed by plating out showed varying levels of resistance (table 1). Most of the diploids appeared to exhibit resistance intermediate between that of the parental strains, but some exhibited resistance no greater than obtained on crossing the two non resistant strains D6 and D22 and others–within the limits of the concentrations tested appeared to have levels of resistance as high as that of the haploid resistant strain from which they were derived. Single diploid colonies were obtained from some of the strains and examined by replica plating. All the strains so far tested gave either mixed colonies consisting of resistant and non resistant cells, or mixtures of resistant and non resistant colonies

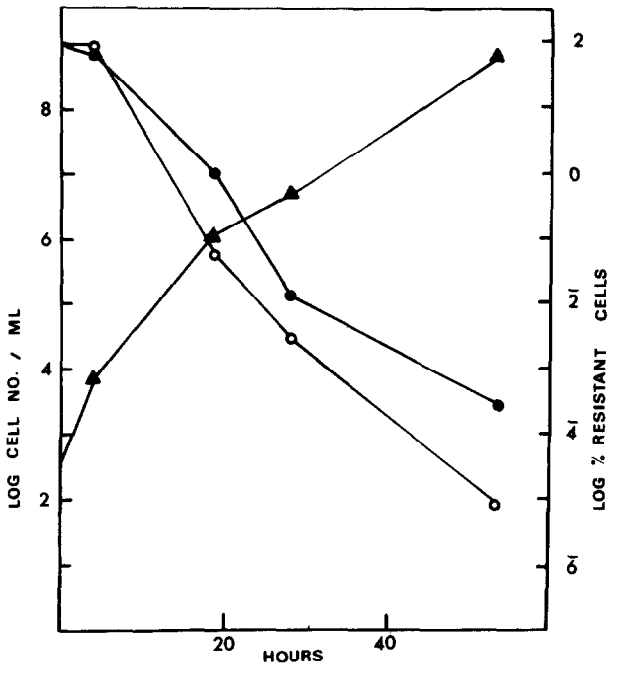

Fig. 1. Cell density and % resistant diploids plotted against time. Synchronised zygotes were isolated folowing the procedure of Jakob [9] A 1% inoculum of these zygotes was inoculated into MMG and grown at 30°. Aliquots were taken for estimation of cell density, and number of resistant cells. Early cell counts were estimated on MMG plates and resistance estimates made using oligomycin containing MMPDM plates which were overlayed with tetrazolium chloride and colonies derived from initially resistant cells identified by the presence of tetrazolium positive papillae. Later resistance estimates were made using MMGly and oligomycin plates, and cell counts made using MMGly plates. •—•: Log of % No. of resistant cells/ 2.5 mg/ml; ○—○: Log of % No. of resistant cells 5.0 mg/ml; ▲—▲: Log cell No./ml

(table 4). This behaviour strongly suggests the presence of cytoplasmic determinants and this is currently being checked by tetrad analysis. Similar results have been reported by Stuart [7]. Mixed colonies were obtained in the case of strains resistant to > 10 μg/ml oligomycin whether or not resistance of the diploids was tested at the 2.5 or 5.0 μg/ml level. However, in the latter case, less resistant growth was observed and this has been comfirmed by qantitative aliquot plating of diploids obtained by mass mating in which far fewer resistant colonies grew after plating onto 5.0 μg/ml MMGly oligomycin plates than 2.5 μg/ml MMGly oligomycin plates. This decrease in the number of resistant cells as the oligomycin concentration was raised is not due to some zygotes giving rise to cells resistant to 5.0 μg/ml oligomycin and other zygotes giving rise to cells resistant to 2.5 μg/ml since, on replica plating diploid clones of D6 X D22B9, all the individual clones showed cells resistant to both 2.5 and 5.0 μg/ml oligomycin. The frequency of cells resistant to 2.5 and 5.0 μg/ml oligomycin however clearly varied from clone to clone.

The kinetics of the loss of resistance in diploids are shown in fig. 1. The number of resistant colonies in a cross of a resistant strain with a sensitive tester falls continuously with increasing number of generations; the lower transmission of resistance at the 5.0 μg/ml level than at the 2.5 μg/ml level is shown, even though during the first generations of growth after mating the number of cells showing resistance to both levels remains approximately the same. The size of colonies arising on the plates appears rather heterogeneous and this could be for three reasons: first, the active oligomycin concentration on the plates may fall, leading to an increasing number of diploids since more diploids appear resistant at lower levels of oligomycin; second, the number of resistant cytoplasmic particles, perhaps resistant mitochondria per cell, may influence growth rate even where there are sufficient to support cell growth; third, different clones seem to have different transmission rates of resistance and this will influence colony size on growth plates. A simple explanation for the results in fig. 1 is that a higher number of resistant "particles" are necessary for oligomycin resistance at the 5 μg/ml level than at the 2.5 μg/ml level. As the resistant strain used D22B9 is resistant to > 10 μg/ml oligomycin all the derived diploids would initially be resistant to greater than 5 μg/ml oligomycin but continued segregation of resistant and sensitive "particles" would lead to a drop in the number of resistant cells and a faster decline in the relative numbers of cells resistant to the higher levels of oligomycin would be expected.

If the behaviour of strain D22B9 is typical of other oligomycin resistant mutants then rigorously standardised growth conditions are necesarry when comparisons are made of the levels of resistance of, for example, petites derived from resistant strains [14] and in determining the level of resistance of diploids derived from resistant strains.

Acknowledgements

P.R.A. thanks the Medical Research Council for a Research Studentship.

References

[1] F.Sherman, in: Methods in Enzymology, Vol. 10, eds. R.W.Estabrook and M.E.Pullman (Academic Press, New York, 1967) p. 610.
[2a] J.H.Parker, I.R.Trimble Jnr. and J.R.Mattoon, Biochem. Biophys. Res. Commun. 33 (1968) 590;
[2b] K.D. Stuart, Biochem. Biophys. Res. Commun. 39 (1970) 1045.
[3] R.A.Butow and M.Zeydel, J. Biol. Chem. 243 (1968) 2545.
[4] J.Beck, J.R.Mattoon, D.C.Hawthorne and F.Sherman, Proc. Natl. Acad. Sci. U.S. 60 (1968) 186.
[5] J.H.Parker and J.R.Mattoon, J. Bacteriol 100 (1969) 647.
[6] L.Kovac, T.M.Lachowicz and P.P.Slonimski, Science 158 (1967) 1564.
[7] K.Wakabayashi and N.Gunge, FEBS Letters 6 (1970) 302.
[8] L. Wickerham, J. Bacteriol. 52 (1946) 293.
[9] H.Jakob, Genetics 52 (1965) 75.
[10] M.Ogur, R.St.John and S.Nagai, Science 125 (1957) 928.
[11] R.B.Beechey, A.M.Roberton, T.Holloway and I.G.Knight, Biochemistry 6 (1967) 3867.
[12] L.Kovac, T.Galeotti and B.Hess, Biochim. Biophys. Acta 153 (1968) 715.
[13] P.R.Avner and D.E.Griffiths, unpublished observations.
[14] E.B.Gingold, G.H.Saunders, H.B.Lukins and A.W.Linnane, Genetics 62 (1969) 735.