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Mutants of Yeast with Altered Oxidative Energy Metabolism: Selection and Genetic Characterization

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Isolation of a series of mutants, characterized by decreased ability to utilize nonfermentable carbon sources for growth and presence of all cytochromes, is reported. A total of 161 mutants, showing deficient growth on glycerol but able to reduce 2,3,5-triphenyltetrazolium chloride, were isolated, purified, and characterized by ability to grow on various carbon sources. Mutants showing decreased growth were examined by low-temperature spectroscopy, and the 35 strains shown to possess all cytochromes were retained for further studies. These strains were characterized by growth on various nonfermentable carbon sources, relative yield on glucose medium, and respiration (Q_{O_2}) of glucose and ethyl alcohol. Genetic studies revealed that at least 19 of the 35 mutants are the result of mutation in single nuclear genes. Furthermore, at least 11 complementation groups are represented among these 19 mutants. Mutants within two complementation groups were shown to be very similar in various properties. These studies demonstrate that a large number of nuclear genes control oxidative energy metabolism and that the characteristics of mutants of the general class are extremely diverse.

In the study of the mechanisms of respiration, and particularly oxidative phosphorylation, the usual approach involves fractionation of isolated mitochondria by chemical or mechanical means. The resulting fractions are then examined separately or in artificially reconstituted systems produced by mixing fractions. Mattoon and Sherman (13) first introduced the use of mutant yeast mitochondria in the study of oxidative energy metabolism in a mutant strain deficient in iso-1-cytochrome *c*. This approach has the unique advantage of deleting or altering a single component of the mitochondrial system in vivo, thus providing a means of studying these altered components in either the context of the whole cell or of the intact, isolated mitochondrion. Subsequently, several reports have appeared describing the use of mutants in the study of oxidative phosphorylation (3, 8, 9).

The yeast *Saccharomyces cerevisiae* has a special advantage in screening for mutants with altered respiration or oxidative phosphorylation because it is a facultative organism, capable of deriving sufficient energy for growth from glycolytic metabolism. Thus, mutants defective in oxidative energy metabolism will grow on a fermentable carbon source such as glucose, whereas they will not grow on a nonfermentable substrate such as glycerol or ethyl alcohol. Known yeast mutants lacking the ability to grow on nonfermentable carbon sources include the cytoplasmic "petite" mutant (ρ^-; 5, 20) and a number of nuclear-gene mutants with similar phenotype. Some of these nuclear mutants, designated "*p*" mutants, have been described in detail by Sherman and Slonimski (22). Also, a number of other mutants affecting aspects of the mitochondrion, such as its sensitivity to various inhibitors of mitochondrial protein synthesis or mitochondrial function, have recently been reported (10, 19, 26–28).

Of prime interest in the present study are mutants which, though they contain an intact electron transport chain, exhibit decreased ability to grow on nonfermentable carbon sources. This class of mutants is expected to contain members with altered oxidative phosphorylation as well as mutants with other metabolic aberrations useful in studying both mitochondrial origin and function. The present report describes 35 such mu-

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tants and includes methods of selection, isolation procedures, genetic characterization, and preliminary biochemical characterization.

MATERIALS AND METHODS

Strains and nomenclature. Heterothallic strains of *Saccharomyces cerevisiae* obtained from F. Sherman were used. All mutants were derived from haploid strain D311-3A ($a\ ly_2\ hi_1\ tr_2$). Strain D213-1B ($\alpha\ tr_1\ ad_1$) served as a normal tester strain in genetic studies. Two series of mutants were selected. The first, the "M" series, consisted of mutants induced by ultraviolet (UV) light (M-1 through M-20) or ethylmethanesulfonate (EMS), and the second, the "P" series, consisted of mutants induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NMG). The mutants were numbered consecutively within these series in the order isolated.

Media and growth conditions. The YPDG medium used for selection of mutants contained 1% yeast extract (Difco), 2% peptone (Difco), 0.1% dextrose, 3% glycerol, and 1% agar (Ionagar no. 2, Colab Laboratories, Chicago Heights, Ill.). The medium used to assay for reversion and to determine ability to grow on glycerol, YPG, consisted of 1% yeast extract (Difco), 2% peptone (Difco), 3% glycerol, and 1% agar. Liquid media used consisted of 1% yeast extract (Difco) 2% peptone (Difco), and either 2% dextrose (YPD), 3% glycerol (YPG), or 3% 3% ethyl alcohol (YPE). Cells were cultured at 30 C unless otherwise noted.

The media used for genetic studies included presporulation medium [0.8%-yeast extract (Difco), 0.3% peptone (Difco), 10% dextrose, and 1% agar], sporulation medium [0.98% potassium acetate, 0.5% dextrose, and 0.1% yeast extract (Difco)], synthetic minimal medium [SD; 0.67% yeast nitrogen base (Difco) without amino acids, 2% dextrose, and 1% agar], synthetic complete medium (SD + 5; SD medium with 40 mg of L-lysine hydrochloride per liter, 20 mg of L-histidine hydrochloride per liter, 10 mg of L-tryptophan per liter, 10 mg of adenine sulfate per liter, and 20 mg of methionine per liter), and deletion media (SD 5 − X; SD + 5 minus one of the above amino acids or adenine).

All liquid cultures, except those used in experiments reported in Table 4, were carried out in Erlenmeyer flasks containing an amount of medium equal to 10% or less of the total flask capacity. Flasks were shaken on a rotary shaker operated at 200 rev/min at 30 C.

Mutagenesis procedures. A suspension of yeast containing approximately 1.5×10^4 cells per ml was spread on YPDG plates, 0.2 ml per plate, and allowed to dry. The plates were then irradiated with UV light from two, 8-w Westinghouse germicidal lamps, at a distance of approximately 32 cm, for various times up to 60 sec.

For chemical mutagenesis, cells were suspended in sterile water to a concentration of approximately 2.5×10^8 cells/ml. For NMG treatments, a fresh solution containing 20 μg of NMG (Aldrich Chemical Co., Cedar Knolls, N.J.) per ml was prepared for each experiment in a buffer consisting of 0.1 M citric acid and 0.2 M Na_2HPO_4, *p*H 7.7 (12). An 0.5-ml sample of the cell suspension was diluted with 5 ml of the NMG solution and incubated in a shaking water bath at 30 C for various times up to 120 min. The treatment was terminated by dilution in 50 mM phosphate buffer, *p*H 7.0.

For EMS treatments, a solution was prepared by adding 0.15 ml of EMS (K & K Laboratories, Inc., Plainview, N.Y.) to 4.85 ml of 0.2 M PO_4 buffer (*p*H 8.0) and shaking well with a vortex mixer. To this solution we added 0.5 ml of the above cell suspension, and the resulting mixture was incubated with shaking for various times up to 180 min. The reaction was terminated by 1:10 dilution of a sample in 1% $Na_2S_2O_3$ in 50 mM phosphate buffer, *p*H 7.0 (2).

After treatment with either EMS or NMG, the cells were plated on YPDG medium, after appropriate dilution, to give approximately 100 viable (colony-forming) cells per plate.

2,3,5-Triphenyltetrazolium chloride (TTC) staining procedure. YPDG plates containing mutagen-treated cells were incubated for about 48 hr until the larger colonies reached a diameter of approximately 2 to 2.5 mm. Fisher TTC Overlay Agar (Fisher catalogue no. J-1144-C) was prepared, cooled to 45 to 50 C, and poured over the plates to a thickness of approximately 1 mm. The plates were incubated in the dark for various periods up to 24 hr to allow full development of the red color. Colonies of interest were removed from these plates by puncturing the agar above a selected colony with a sterile Pasteur pipette. The colony, within a plug of agar, was then transferred from the pipette to a slant of YPD medium. The TTC staining procedure has previously been used to detect the presence of ρ^- mutants (16) and other respiration-deficient mutants (9, 22, 24).

Spectroscopic technique. The mutant strains were examined spectroscopically after freezing in liquid nitrogen, with a Zeiss hand spectroscope (Zeiss catalogue no. 50-20-00) mounted on a monocular microscope body, by the method of Sherman (21).

Genetic analyses. Standard techniques of mating, sporulation, ascus dissection, and tetrad analysis were employed (6, 14). The media used for determining auxotrophic requirements of segregants are listed above. YPG medium was routinely used to determine the ability of the segregants to utilize a nonfermentable carbon source. A Pepper inoculator (Pentex, Inc., Kankakee, Ill.) was used in place of velveteen for replica plating.

Q_{O_2} determination. Respiration (Q_{O_2}) of intact cells was determined by using a Clark oxygen electrode in a 3-ml, semi-closed system at 25 C (1). The medium used consisted of 16 mM KH_2PO_4 buffer (*p*H 6.5) containing either 100 mM glucose or 100 mM ethyl alcohol, or this buffer with no added substrate. Q_{O_2} was expressed as microliters of oxygen consumed per hour per milligram (dry weight) of cells. Dry weight was determined by filtration of the cell suspension through a preweighed filter (0.45 μm pore size) and drying the filter containing the cells at 80 C for at least 24 hr.

RESULTS

Selection of mutants. Examination of TTC-stained YPDG plates containing colonies derived from mutagen-treated cells revealed colonies of various sizes and colors. Degree of staining ranged from none (white) to very dark red. Many very small colonies (diameter less than 0.5 mm) were observed. Colonies which were smaller than the average population (2 to 2.5 mm) and showed at least some pink coloration were classified as mutants in this study and were isolated. Table 1 shows the per cent survival after mutagen treatment, as determined by plating untreated and treated cells on YPD medium. The mutation frequency refers to the number of mutants (i.e., small, pink colonies) per 100 surviving cells. As will be seen, UV gives rise to the highest frequency of mutation, but at the same time it induces the highest percentage of ρ^- mutants. It should be noted that the frequency of spontaneous occurrence of ρ^- in D311-3A is 1% or less. The induction of mutants with the other mutagens, EMS and NMG, was dose-dependent (i.e., relatively linear dose-response curve), and seven relatively low doses, with respect to survival, gave rise to a significant number of mutants. A total of 161 mutants were isolated and further characterized.

Purification of mutant strains. After isolation of the mutants, the strains were purified by streaking cells from the isolated culture on YPDG medium at a cell density sufficiently low to give formation of single colonies. In most cases, at least two distinct colony sizes were observed on this medium, indicating that the original mutant clone consisted of a mixture of cell types. In these cases, a small clone and a large clone were isolated and designated "S" and "L," respectively (except in the cases of M-1 through M-20 where more than two subclones were isolated). A total of 300 subclones were thus collected and studied in more detail with respect to their growth on a medium containing glycerol as the sole carbon source. Some of these were also characterized with respect to their absorption spectra.

Growth of mutants on glycerol. The subclones were plated on various media, using a Pepper inoculator, to determine growth on glycerol and also to confirm the presence of the original auxotrophic markers of the parent strain D311-3A. Growth on glycerol (Table 2) was estimated as follows: normal = greater than 50% of the control strain, D311-3A, after 48 hr of incubation; slow = less than 50% of the control; none = no apparent growth after 48 hr of incubation. Table 2 lists the results for all subclones tested and also indicates the ability of the subclones to

TABLE 1. *Derivation of mutant strains*

Mutagen	Per cent survival	Per cent ρ^{-a}	Mutation[b] frequency	No. of mutants isolated[c]
UV	0.1	35	10	20
EMS	58	5	0.4	26
	33	5	1.4	39
NMG	80	6	1.7	8
	62	8	3.0	15
	34	16	5.2	7
	7	17	5.2	29
	0.3	20	4.0	17

[a] The per cent ρ^- was determined by counting the number of white colonies on TTC-stained YPDG plates. The normal strain, D311-3A, usually shows about 1% ρ^- under the same conditions. Also included in this figure are any p ρ^+ mutants unable to reduce TTC.

[b] Mutants are defined as small colonies on YPDG medium which show a positive staining reaction (red) with TTC. Mutation frequency is the number of colonies, thus defined, per 100 colonies formed.

[c] The total number of mutants was 161.

grow on SD + 5 medium. It was noted that seven of the strains growing poorly on glycerol showed little or no growth on SD + 5. The finding will be discussed in more detail in a future publication.

Spectroscopic examination. Only those strains showing slow or no growth on glycerol (Table 2) were carried further in the screening procedure. A total of 100 strains were examined spectroscopically. These were plated for confluent growth on YPD medium and incubated at 30 C for 3 days; the cytochrome spectra of the intact cells were then observed by the method of Sherman (21). Table 3 lists the distribution of strains in six general spectroscopic categories. Forty strains showed the presence of cytochrome c only, indicating that these cultures were composed largely or entirely of ρ^- cells or were one of the p mutants of similar phenotype to ρ^- (22). Most of the strains were the "S" subclones of the original cultures. A few were pink because of the presence of a mutant gene causing a requirement for adenine (25). The remaining 60 strains showed the presence of all cytochromes in various ratios. The spectra observed were very generally categorized as follows: (1) only cytochrome c present, (2) all cytochromes present in low concentrations, (3) normal spectrum, (4) all cytochromes present in high concentrations but apparently normal ratios, (5) low cytochrome a concentrations with respect to the other cytochromes, and (6) low

TABLE 2. *Growth of mutant subclones on glycerol medium*

Growth on YPG[a]	No. of subclones[b]	No. of subclones[c] not growing on SD + 5
Normal	197	3
Slow	43	4
None	60	3

[a] Growth was estimated after 48 hr of incubation on solid YPG medium. "Normal" represents an estimated value of 50% or greater of the normal strain, D311-3A. "Slow" represents growth less than 50% normal.

[b] Subclones were derived from the 161 mutant strains listed in Table 1 by plating on YPDG medium and selecting clones representative of the population. Thus, for most strains, at least two subclones were isolated, one large and one small. The total number of subclones was 300.

[c] It was noted that some of the subclones isolated showed no detectable growth on synthetic complete medium (SD + 5) after 48 hr. The numbers of subclones of this type are tabulated according to their growth on glycerol. The total number of subclones not growing on SD + 5 was 10.

TABLE 3. *Cytochrome spectra of mutant subclones as a function of growth on glycerol*

Spectral type[a]	Growth on YPG medium[b]	
	None	Slow
(1) Only *c*	40[c]	0
(2) All low	5	4
(3) Normal	6	31
(4) All high	6	0
(5) Low *a*	2	4
(6) Low *c*	0	4

[a] Whole-cell spectra were examined after freezing the cells in liquid nitrogen. Six basic categories were observed: (1) typical ρ^- spectrum in which only cytochrome *c* was present; (2) all cytochromes present in markedly decreased amounts; (3) spectrum normal as compared with control, D311-3A; (4) all cytochromes increased in amounts but ratios normal; (5) low cytochrome *a* content relative to the other cytochromes present; (6) low cytochrome *c* content relative to the other cytochromes present.

[b] Growth of mutant strains on glycerol (YPG) was determined in the same manner as in Table 2. A total of 59 strains showed no growth, and a total of 43 showed slow growth.

[c] Number of strains.

cytochrome *c* concentration with respect to the other cytochromes. Although subclasses of spectral types could also be distinguished within certain categories, the general description was deemed adequate for the present study. Table 3 also affords comparison of the ability of mutant strains to utilize glycerol with their respective spectral type. Mutants exhibiting the presence of all cytochromes were selected for further study. In cases where both subclones, "S" and "L," of a given original strain showed similar or identical spectral and growth properties, only the "S" strain was chosen. In the case of strain M-17, the "S" and "L" clones exhibited somewhat different spectral types and growth patterns, and both were retained.

In all, 35 mutants which exhibited decreased growth on glycerol and presence of all cytochromes were selected for further detailed study.

Determination of growth on various carbon sources. All of the mutant strains were grown in liquid cultures at room temperature (approximately 25 C) in media containing one of the following: no added carbon source (YP), dextrose (YPD), glycerol (YPG), or ethyl alcohol (YPE). Total yield of cells was determined by filtering the entire culture through a filter of predetermined weight (pore size, 0.45 μm), followed by washing, drying and weighing. Table 4 compares the growth yield of the mutant strains on these media. Growth on a given medium is expressed as a percentage of the normal control strain yield for that medium. Our P-26S stock had apparently reverted in these experiments, possibly by the spontaneous occurrence of a suppressor mutation at an unlinked locus, as suggested by the segregation pattern observed in the heterozygous diploid (Table 6).

It should be noted that even when glucose was the substrate most mutants grew less efficiently than the normal strain. The decrease in ability to grow on ethyl alcohol is particularly striking in all cases. Normal strain (D311-3A) did not grow well on liquid YPG medium in these experiments, yielding only about 10% the quantity of cells on YPG as on YPD. Therefore, the samples of cells were very small, so that values for growth on glycerol were subject to great error. On YPE, on the other hand, the yield of D311-3A was approximately 115% of the YPD yield. Growth of D311-3A on YP yielded less than 3% the number of cells compared to YPD, and few of the mutant strains showed any detectable growth on YP medium. It should be noted that the growth patterns on glycerol and ethyl alcohol did not appear to be correlated with spectral type, except that the four strains which showed decreased cytochrome *a* content (M-23, M-69, M-84, P-33S) did not grow at all on either ethyl alcohol or glycerol (17).

Respiration of intact cells. Table 5 lists endogenous respiration (Q_{O_2}) without added sub-

strate and with added glucose or ethyl alcohol for normal and mutant strains. It should be noted that most of the strains tested respired in medium containing ethyl alcohol, but in some cases the rate was not significantly higher than the endogenous respiration rate. This poses the question of whether these strains oxidize ethyl alcohol. The values presented in this table represent maximum Q_{O_2} values determined polarographically. Samples were taken for most strains at 24 and 48 hr of growth, and Q_{O_2} varied with time of harvesting in nearly every case. The cells were all grown in liquid YPD medium.

The major findings presented in Table 5 are as follows: (i) all strains respire; (ii) some strains respire very poorly, with Q_{O_2} values of less than 5% of normal; (iii) some strains show an increased Q_{O_2} at 48 hr, which is in contrast with the decrease in the normal strain; (iv) some strains with low growth yield on glucose (Table 4) show a high Q_{O_2} value, indicating possible decreased efficiency of oxidative energy metabolism.

Genetic analysis. All mutant strains were crossed with a normal strain (normal with respect to growth on glycerol), D213-1B. Prototrophic diploid clones were selected on SD medium, and cultures were allowed to sporulate. Analyses of at least five tetrads from each of these heterozygous diploids were carried out, giving the results presented in Tables 6 and 7. Table 6 shows that, although 13 strains showed a clear 2:2 segregation of growth to nongrowth of the spore isolates on glycerol, many crosses deviated from this pattern. A number of segregations, although suggestive of a 2:2 pattern, showed a great deal of variability in growth on glycerol among the segregants. In many cases, one segregant was negative on glycerol (no growth), whereas the other three showed various degrees of growth. Often, as in the case of JP-276, of the three glycerol-positive segregants, one showed considerably less growth than the other two, indicating that the expression of the original mutant phenotype may have been enhanced by the presence of a modifying gene or genes segregating independently (see below). Still other diploids, such as JP-274, showed less variability among segregants, with nearly all of them growing to some extent on glycerol. From 3 of the heterozygous diploids, only a very small percentage of germinating spores was obtained, and, from 6 others, few or no tetrads with 4 germinating spores were obtained out of at least 15 tetrads dissected. One diploid (JP-277) showed very poor sporulation. These 10 strains have not yet been analyzed in sufficient detail to draw conclusions regarding the genetic nature of the lesions, but it is expected that all are subject to the application of standard tetrad analysis procedures if sufficient tetrads are dissected and analyzed.

Table 7 lists the specific mutant strains which are either known or strongly suspected to result from mutations in single nuclear genes.

Complementation studies. Segregants from the heterozygous diploids containing the mutant gene and appropriate complementary auxotrophic

TABLE 4. *Growth of mutants in various media*

Strain	Relative Growth on YPD (%)[a]	Relative growth on nonfermentable carbon sources (%)		
		YPG	YPE	YP
D311-3A (normal)	100	100	100	100
M-1B	27	0[b]	6	0
M-2A	29	0	6	0
M-4A	54	70	0	70
M-8	38	0	0	0
M-17S	44	0	0	0
M-17L	91	0	0	0
M-20A	43	60	4	70
M-23	64	0	0	0
M-25S	35	—[c]	—	—
M-26S	65	—	0	0
M-33S	68	—	—	—
M-44S	42	0	0	0
M-49S	83	0	0	0
M-50S	43	—	—	—
M-65S	72	0	10	0
M-69S	27	0	0	0
M-74S	36	0	3	0
M-82S	83	0	0	0
M-84	21	0	0	0
P-3S	95	0	0	0
P-5S	94	94	22	0
P-12S	86	0	0	0
P-16S	70	47	0	0
P-17S	41	0	0	0
P-20S	84	0	0	0
P-24S	32	0	0	0
P-26S	93	100	110	—
P-28	90	80	10	0
P-33S	77	0	0	0
P-37L	98	0	11	0
P-40S	105	0	24	0
P-44S	86	0	0	0
P-55S	69	70	15	0
P-64S	75	75	14	0
P-72S	81	50	0	0

[a] Relative growth refers to dry weight yield for a given mutant as a percentage of the dry weight yield for strain D311-3A on the same medium. Media used consisted of 1% yeast extract (Difco), 2% peptone (Difco), and one of the following carbon sources: none (YP); 2% dextrose (YPD); 3% glycerol (YPG); or 3% ethyl alcohol (YPE). Shake-tube cultures (3 ml) were employed.

[b] No growth was detectable.

[c] Values were not determined.

TABLE 5. *Respiration of mutant and normal strains with various substrates*

Strain	Oxygen uptake[a]					
	Glucose		Ethyl alcohol		Endogenous	
	24 hr[b]	48 hr	24 hr	48 hr	24 hr	48 hr
D311-3A[c]	65 ± 5	29 ± 4	58 ± 6	24 ± 7	31 ± 9	12 ± 4
D213-1B[c]	87 ± 6	41 ± 6	117 ± 8	34 ± 3	56 ± 2	27 ± 3
M-1B		6		5		3
M-2A		5		5		3
M-4A		24		19		19
M-8	3	6		5		2
M-17S	8	15				
M-17L	18	76		60		45
M-20A		31		24		24
M-23		67		67		28
M-25S	3	1				
M-26S	62	31				
M-33S	46	33				
M-42S	50	51	61	63	33	45
M-49S	32	30	41	21	17	18
M-50S	4	13		14		6
M-65S	39	33	33	34	29	22
M-69S	3	1				
M-74S	23	28	24	33	4	8
M-82S	56	41	52	32	26	19
M-84	9	1		1		
P-3S	76	29	75	24	34	13
P-5S	36	36		36		21
P-12S	16	48		39		26
P-16S	38	29		28		19
P-17S	47	21	48	23	28	17
P-20S	21	59		59		31
P-24S	20	19	35	35	6	4
P-26S	67	53	77	31	51	
P-28	56	33	56	24	64	
P-33S	12	21	10	19		
P-37L	66	43	63	25	33	
P-40S	62	33	61	29	40	
P-44S	61	55	53	47	32	
P-55S	53	33	72	65		
P-64S	42	64	36	55		
P-72S	78	42	78	35		

[a] Microliters of O_2 per mg (dry weight) per hour. O_2 uptake (Q_{O_2}) was determined polarographically as described.

[b] Cells were grown in liquid YPD medium at 30 C for 24 and 48 hr, respectively.

[c] Values for the normal control strains are the averages of several determinations. Standard deviations are included for these determinations.

markers were crossed to all of the mutants in the original collection. The data in Table 8 summarize the findings of the complementation studies with only the strains listed in Table 7 (i.e., those which are thought to represent mutation in a single nuclear gene). Strains were included within a complementation group only when reciprocal crosses gave rise to noncomplementing diploids (i.e., YPG-negative). Table 9 shows a comparison of growth yield, Q_{O_2}, and spectra for the two representatives of the respective complementation groups. The similarities are striking in these cases, indicating that the lesions involved in the noncomplementing strains are similar if not identical.

One mutant, M-82S, apparently did not complement with a majority of the other strains tested. This suggests that the mutation in M-82S is at least semidominant. However, the heterozygous cross of M-82S with D213-1B yielded a majority of segregants which grew well on YPG, indicating that this may be a relatively unstable mutation or, alternatively, that the expression of the mutation

TABLE 6. *Genetic analysis of heterozygous diploids*

Mutant parent strain	Spectral type[a]	Heterozygous diploid strain	Ratio of segregation on YPG growth to nongrowth[b]	Comments on analyses
M-1B	2	JP-260	2:2	
M-2A	2	JP-261	2:2	
M-4A	4	JP-262		Very poor spore germination
M-8	2	JP-256	Probably 2:2	Few analyzed, poor germination
M-17S	2	JP-258	Probably 2:2	Few analyzed, poor germination
M-17L	3	JP-257	2:2	
M-20A	4	JP-263		Very poor spore germination
M-23	5	JP-264	2:2	
M-25S	5	JP-265	2:2	
M-26S	3	JP-266	2:2	
M-33S	2	JP-267	2:2	
M-42S	4	JP-268	2:2	
M-49S	3	JP-269		Poor germination[c]
M-50S	2	JP-270		Poor germination[c]
M-65S	3	JP-271	Probably 2:2	Possibile 1:3 in 2 tetrads
M-69S	5	JP-272	2:2	
M-74S	2	JP-273	2:2	
M-82S	3	JP-274		Probably 3:1
M-84	5	JP-275		Very poor spore germination
P-3S	3	JP-276		Probably 3:1
P-5S	3	JP-277		Poor sporulation
P-12S	3	JP-278		Poor germination[c]
P-16S	3	JP-279		Poor germination[c]
P-17S	6	JP-280		Indication of 3:1
P-20S	3	JP-281	Probably 2:2	1 Nongrower and 1 poor grower[d]
P-24S	4	JP-282	2:2	
P-26S	3	JP-283	3:1	1-2:2; 2-3:1; 2-4:0
P-28	3	JP-284	2:2	
P-33S	5	JP-285		Poor germination
P-37L	3	JP-286	Probably 2:2	Great variability in growth on YPG
P-40S	3	JP-287	Probably 2:2	All grow, 2 grow less well
P-44S	3	JP-288		Indication of 3:1
P-55S	6	JP-289	2:2	
P-64S	3	JP-290		Poor germination
P-72S	6	JP-291	3:1	Mutant haploid grows well on YPG

[a] Spectra were determined as in Table 3. Designation of the type is the same.
[b] Standard genetic procedures were used in the analysis of tetrads. Growth of segregants on various media was determined after replica plating on these media by using a Pepper inoculator. In scoring growth on YPG, estimates of the growth of each segregant were made. In some cases, the term nongrowth can be replaced with "limited growth."
[c] In most tetrads dissected, three spores formed macrocolonies, whereas the fourth spore formed a microcolony. Isolation of these microcolonies before overgrowth by the macrocolonies has not been possible with the standard procedure.
[d] All tetrads from this cross showed two spores growing well on glycerol, whereas a third spore grew poorly and a fourth spore not at all.

may depend on a number of factors, in particular the nature of the background genome.

DISCUSSION

The purpose of the experiments reported here was to isolate mutants of yeast with lesions in oxidative phosphorylation for use in studying the underlying mechanisms of this process. It seemed likely that at least some mutants unable to use or inefficient in the use of nonfermentable carbon sources for growth, but which retain at least some respiratory capacity, might possess lesions in enzymes of oxidative phosphorylation.

The method used for selecting mutants has been extremely productive, and 35 mutants of the general phenotype sought have been isolated. Upon closer examination, it was found that some of the mutants collected can in fact utilize nonfermentable carbon sources for growth but with lower efficiency than normal. On the other hand, other mutants give no detectable growth on

TABLE 7. *Summary of mutants thought to contain single-gene mutations*[a]

Original mutant strain	Heterozygous diploid strain	2:2 Segregation on YPG medium
M-1B	JP-260	Yes
M-2A	JP-261	Yes
M-8	JP-256	Probably[b]
M-17S	JP-258	Probably[b]
M-17L	JP-257	Yes
M-23	JP-264	Yes
M-25S	JP-265	Yes
M-26S	JP-265	Yes
M-33S	JP-267	Yes
M-42S	JP-268	Yes
M-65S	JP-271	Probably
M-69S	JP-272	Yes
M-74S	JP-273	Yes
P-20S	JP-281	Probably
P-24S	JP-282	Yes
P-28	JP-284	Yes
P-37L	JP-286	Probably
P-40S	JP-287	Probably
P-55S	JP-289	Yes

[a] Data for this table are taken directly from Table 6.

[b] Few complete tetrads were examined but treatment of the data as a random spore analysis suggested that these strains do indeed segregate 2:2 on YPG.

nonfermentable carbon sources, and some of these exhibit a very low respiratory capacity. Of the 35 mutants studied, at least 23 have a respiratory capacity which is at least 50% as high as that of the normal strain when glucose is the substrate, and all of these show some loss in efficiency of growth on glycerol and ethyl alcohol. However, the extent to which this loss merely reflects decreased respiration is, at the moment, not entirely clear. In selecting mutants for further study, it is important to note that at least a few of them, such as M-42S, show decreased growth on all carbon sources but normal or nearly normal respiration. It might be suggested that these are mutants in which phosphorylation is uncoupled from respiration.

Another consideration in selecting mutants for further biochemical analyses is the effect of various uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol (DNP). Preliminary work has shown that the responses of mutants M-8, M-17S, and M-17L to DNP are abnormal. In particular, respiration of the M-17 strains is not stimulated by DNP in either the whole cells or in isolated mitochondria (18). Such studies provide information about the state of oxidative phosphorylation coupling mechanisms in the mutants and will thus be useful in characterizing all of the mutants.

The present study concerns not only the molecular basis of mitochondrial function but also the genetic control of mitochondrial structure, function, and biogenesis. Studying mutants of the type described provides further answers to the question of the origin of mitochondrial components, that is, the question of whether a given mitochondrial structure is coded by nuclear or mitochondrial deoxyribonucleic acid (DNA). It is already clear that the structure of cytochrome *c* (23, 24) and that of malic dehydrogenase (4, 11) are determined by nuclear genes, and a number of other nuclear genes, including those reported here, are known to affect mitochondrial structure and function (9, 17, 19, 22). However, mitochondria are known to contain DNA possessing different properties from nuclear DNA (15). Furthermore, some mutant genes affecting mitochondrial composition (5) or resistance of the mitochondrial protein-synthesizing apparatus to inhibitors such as erythromycin (10, 26) are inherited cytoplasmically. These facts suggest that at least a portion of the genetic information necessary for the production of normal mito-

TABLE 8. *Summary of results of complementation tests*[a]

Complementation group	Tester strain[b]	Original mutant strain	Mutant strains not complemented by tester[c]
A	JP260-1C	M-1B	M-1B
B	JP256-6A	M-8	M-8
C	JP258-1B	M-17S	M-17S, M-17L
D	JP265-5D	M-25S	M-25S, M-69S
D	JP272-3D	M-69S	M-25S, M-69S
E	JP266-3D	M-26S	M-26S
F	JP267-5C	M-33S	M-33S, M-74S
F	JP273-3A	M-74S	M-33S, M-74S
G	JP264-2C	M-23	M-23
H	JP271-2C	M-65S	M-65S
I	JP282-3B	P-24S	P-24S
J	JP286-2B	P-37L	P-37L
K	JP261-4D	M-2A	M-2A
L	D360-5B[d]	p_9	D360-7D (p_9)[d]

[a] Complementation tests were carried out by crossing a haploid tester carrying the mutant gene with all of the original mutant strains and plating the resulting diploid cultures on YPG medium.

[b] Testers used are YPG-negative, meiotic segregants of the diploid strain constructed by crossing the original mutant strain with D213-1B.

[c] Strains are included in complementation groups only if both reciprocal crosses gave rise to YPG-negative diploids.

[d] Obtained from F. Sherman.

chondria resides in cytoplasmic genes, presumably located in mitochondrial DNA.

With this information in mind, genetic characterization of the 35 mutants isolated was undertaken to determine whether any of these mutants resulted from a cytoplasmic mutation. A second important objective was to determine which mutants resulted from mutation in single nuclear genes, since these would be the most likely source of mitochondria with lesions in single components. A third consideration was determination of the number of repeats of mutation in the same gene which had occurred (i.e., how many genes are represented among the mutants) in order to avoid duplication of biochemical analyses in different strains containing the same or allelic mutations.

A number of factors have complicated the genetic analyses, as pointed out above. Among the problems are poor germination of spores, apparent additional nutritional requirements, and variability of growth of segregants on glycerol (YPG) medium.

The problem of poor germination prevented, in some heterozygous crosses, the collection of enough complete tetrads for meaningful analysis from the total number dissected in the initial experiments. In two cases (M-8 and M-17), random spore analyses were carried out to obtain an indication of the nature of the segregation of the mutant genes. In other cases (M-49S, M-50S, P-12S, P-16S), analysis would require dissection of additional tetrads. Finally, in the three cases (M-4A, M-20A, M-84) in which germination was extremely low (less than 10%), new heterozygous crosses made with a different normal tester strain might result in improved germination.

It is probably significant that germination was decreased in some of the heterozygous crosses, since all heterozygous diploids were identical except for the selected mutation. The poor germination in most cases was probably the result of some chromosomal aberration, polyploidy, aneuploidy, or a chance combination of the mutant genes with certain other genes from the background genome, with the resulting combination preventing germination. Alternatively, the lesion may be a dominant cytoplasmic mutation, and most spores inheriting the mutant gene are incapable of germinating. Although this is an attractive possibility, there is no conclusive evidence that it is the case.

It has been previously reported that diploids homozygous for some genes for respiration deficiency in both *Saccharomyces* (22) and *Schizosaccharomyces* (7) are incapable of undergoing sporulation. Also, ρ^- diploid strains do not sporulate. Preliminary experiments in this

TABLE 9. *Comparison of mutants in complementation groups D and F*

Strain	Spectrum[a]	Relative growth on glucose[b]	Q_{O_2} on glucose[c]
		%	
M-25S	Low *a*	35	1
M-69S	Low *a*	43	1
M-33S[d]	All low	68	33
M-74S	All low	41	28

[a] Spectral designation is the same as in Table 3.

[b] Relative growth yield expressed is as a percentage of the normal strain (D311-3A) after 48 hr of growth in liquid YPD medium.

[c] Q_{O_2} of a 48-hr culture in glucose as in Table 5 (footnote *a*).

[d] M-33S is the only strain of these four showing any growth on YPG plates.

laboratory have shown that, in some cases, the heterozygous diploid strains used for dissection have a significantly decreased respiration compared to the normal parent, suggestive of a gene-dosage effect. Perhaps this is related in some way to the abnormal germination observed in many strains, and it seems highly probable that it is the cause of the extremely poor sporulation in the case of strain JP-277.

The most difficult problem in analyzing the mutants with respect to the segregation of the inability to grow on glycerol is found in the variability of growth of the segregants on YPG. Since some of the mutants grow reasonably well on YPG plates, it is often difficult to differentiate between mutant and normal segregants, especially since segregation of other genes greatly affects the expression of the lesion. This variability, in many cases, has precluded determination of the number of genes affected in a given mutant. This difficulty may be corrected by using a different medium to score segregation of the mutant genes.

Although none of the data from tetrad analysis definitely indicated cytoplasmic inheritance of the mutant genes, one strain, M-82S, showed highly abnormal complementation patterns with most of the other mutant strains. All of the mutant tester strains, when crossed to M-82S, gave rise to a diploid which grew poorly on glycerol. Moreover, when a tester derived from JP-274 (M-82S × D213-1B) was crossed with the 35 original mutant strains, nearly all of the diploids grew poorly on glycerol, including the homozygous cross (JP274-3D × M-82S). This suggests that the mutant gene in M-82S is at least semidominant. Tetrad analysis of segregants from the heterozygous diploid JP-274 showed an aberrant segregation pattern on YPG medium. In five tetrads, over

half of the spores grew well on glycerol, only three spores showed no growth, and the remaining segregants showed variable growth on YPG. Although the pattern is difficult to analyze from a limited number of tetrads and could be consistent with a two-gene segregation, it could also be consistent with cytoplasmic inheritance in which only a limited number of segregants receive the mutant gene. For this to be true, it is necessary to postulate that the mutation is semidominant, since, as stated above, crosses of a YPG-negative segregant with the other mutant strains produce YPG-negative diploids. This hypothesis, though purely speculative, is subject to further test and offers attractive possibilities.

The complementation data are relatively clear, at least for crosses involving testers thought to contain a single mutant gene (i.e., segregants from heterozygous crosses showing 2:2 segregation on YPG; Table 7). There are 13 testers which appear to contain single-gene mutations and which complement with all or nearly all of the mutants. These testers, when back-crossed to the original mutant strains from which they were derived, give rise to a YPG-negative homozygous diploid. There exists in the collection of mutants one repeat each of two of the testers (Tables 6 and 7). The other testers are apparently single representatives of mutations at the site, although this does not necessarily imply that some other mutant strains are not allelic to them, since complementation can occur among allelic mutants. It should be noted that the noncomplementation of pairs was confirmed by making the appropriate reciprocal crosses in each case. On the strength of these complementation tests and the similarities of the strains as presented in Table 9, it seems safe to assert that the two members of each pair are allelic to each other, if indeed they are not actual repeats of the same mutation.

It was pointed out that M-17S and M-17L have sufficiently different properties to warrant the retention of both subclones of the original M-17 strain. Although these mutants will be the subject of a future publication, it is important, in the context of the genetic analyses presented here, to point out that the cross of M-17S × M-17L produced a diploid which did not grow on YPG medium. Since it is apparent that these two forms (S and L) are interconvertible (18), this finding is surprising and suggests an instability, possibly of cytoplasmic origin, superimposed on a single nuclear-gene mutation.

A few complementation tests were attempted with segregants from heterozygous diploids not showing a clear 2:2 segregation on YPG. In every case, these tests were ambiguous, since the "homozygous" diploid combination showed at least some growth on glycerol so that it was impossible to differentiate clearly this growth from growth of the other diploids tested.

In conclusion, this study represents a broad survey of the results of screening for mutants having altered oxidative energy metabolism and, in particular, altered oxidative phosphorylation. It is apparent that there are many mutants, in addition to the mutants previously reported (3, 8, 9, 22), showing decreased growth on nonfermentable carbon sources, but possessing a functional respiratory chain. Many of these new mutants represent mutation in single nuclear genes, providing further evidence that a large portion of the genetic regulation of mitochondria is encoded in the nucleus of the cell. In further characterizing these mutants biochemically, it is likely that a great deal of information may be acquired with regard to the mechanisms of oxidative energy metabolism and the control mechanisms governing mitochondrial biogenesis.

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