CYTOPLASMIC MUTANTS OF YEAST:

The Isolation and Genetic Characterization of Cytoplasmic Mutants of *Saccharomyces cerevisiae* Defective in Oxidative Phosphorylation.

by

Mark Geoffrey Darlison. B.Sc. (Birm.).
M.Sc. (Warwick).

A Thesis submitted to the University of Warwick for the Degree of Doctor of Philosophy.

Department of Chemistry and
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June 1979.

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ABSTRACT

This thesis presents a method for the induction and selection of cytoplasmic respiratory deficient mutants of Saccharomyces cerevisiae, distinct from the pleiotropic rho^- petites. The procedure utilizes a parental stock carrying the nuclear recessive pet 9 (op_1) mutation. Mutagenesis is performed under growing conditions in the presence of manganese.

As a result of this treatment twenty one mutants, putatively designated mit^-, have been isolated. Among these are strains that are defective in the biosynthesis of either cytochrome b or cytochrome oxidase.

Two mutants, D603-3B/8 and D603-3B/9, have been intensively studied following the observation that they possess spectrophotmetrically detectable levels of cytochromes c, b and $a + a_3$. The mit^- mutations carried by these strains have been further genetically characterized and mapped with respect to known mitochondrial markers. These lesions have been located close to, and are possibly allelic with, the pho 1 segment of the genome. In addition, a very tight linkage to the $oli^r 2$ antibiotic resistance locus has been established.

Attempts to determine whether the mit^- mutations of D603-3B/8 and D603-3B/9 are allelic, have proved unsuccessful.

The effect of these lesions, in constructed mit^- diploids, on growth in glucose and glycerol liquid media has also been investigated.

It is suggested that D603-3B/8 and D603-3B/9 are defective in the biosynthesis of the CS-ATPase complex. Their potential use in studies of the biogenesis of this multisubunit enzyme is also discussed.

This Thesis is dedicated to those who cared about its completion.

ABSTRACT

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The effect of these lesions, in constructed mit^- diploids, on growth in glucose and glycerol liquid media has also been investigated.

It is suggested that D603-3B/8 and D603-3B/9 are defective in the biosynthesis of the OS-ATPase complex. Their potential use in studies of the biogenesis of this multisubunit enzyme is also discussed.

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ABBREVIATIONS

ADP	:	Adenosine-5'-Diphosphate
ATP	:	Adenosine-5'-Triphosphate
ATPase	:	Adenosine-5'-Triphosphatase
A + T	:	Adenine plus Thymine
CAP	:	Chloramphenicol
CsCl	:	Caesium chloride
DNA	:	Deoxyribonucleic Acid
EtBr	:	Ethidium Bromide
G + C	:	Guanine plus Cytosine
mRNA	:	Messenger Ribonucleic Acid
mtDNA	:	Mitochondrial Deoxyribonucleic Acid
NADH	:	Reduced Nicotinamide Adenine Dinucleotide
OS-ATPase	:	Oligomycin-Sensitive Adenosine-5'-Triphosphatase
P_i	:	Inorganic Phosphate
RNA	:	Ribonucleic Acid
r.p.m.	:	Revolutions per Minute
rRNA	:	Ribosomal Ribonucleic Acid
SDS	:	Sodium Dodecyl Sulphate
S.E.	:	Standard Error
T_m	:	Temperature of Melting (midpoint of melting curve)
tRNA	:	Transfer Ribonucleic Acid
TTC	:	2,3,5-Triphenyl Tetrazolium Chloride

NOMENCLATURE

The nomenclature for mitochondrial loci used throughout this thesis was that proposed at the Interdisciplinary Conference on the Genetics and Biogenesis of Chloroplasts and Mitochondria, München 1976 (Birky *et al.*, in press). In addition, ant^r and ant^s have been employed to denote general mitochondrial antibiotic-resistant and sensitive alleles respectively.

The symbols op_1 and OP_1 have also been used to represent the nuclear respiratory deficient mutation, also known as pet 9 (Lachowicz, 1968; Beck *et al.*, 1968), and the corresponding wild type allele.

DEFINITIONS

Grande yeast a grande yeast is defined as being respiratory competent, possessing the ability to grow on both fermentable and non-fermentable media.

Petite yeast a petite yeast is defined as being respiratory incompetent, lacking the ability to grow on non-fermentable media. Petites may arise through mutation of either the nuclear or mitochondrial DNA of a grande yeast.

Rho^+ strain a rho^+ strain is defined as possessing a fully functional mitochondrial genome.

Rho^- strain a rho^- strain is defined as possessing an irreversible cytoplasmically-inherited mutation that results in respiratory incompetence and the loss of mitochondrial protein synthesis. Crosses between established rho^- strains are never observed to result in restoration of the rho^+ state.

Rho^o strain a rho^o strain is defined as lacking detectable mitochondrial DNA.

Mit^- strain a mit^- strain is defined as possessing a cytoplasmically-inherited mutation that results in respiratory incompetence. Such strains however retain the capacity for mitochondrial protein synthesis and when crossed to suitable rho^- strains are capable of restoring the rho^+ state.

Syn^- strain a syn^- strain is defined as possessing a cytoplasmically-inherited mutation that results in the loss of mitochondrial protein synthesis. Such

strains are distinct from rho^- ones, since syn^- mutants are observed to revert to rho^+ and/or are capable of restoring the rho^+ state in crosses with suitable rho^- strains.

Mit^+ allele — a mit^+ allele is defined as the corresponding functional copy of a mit^- allele.

Mit^- allele — a mit^- allele is defined as the defective segment of mitochondrial DNA present in a mit^- strain.

CHAPTER ONE INTRODUCTION

1:1 General Introduction

The value of genetics as a tool in the unravelling of complex biochemical pathways and processes has long been realized. One phenomenon that has to date refused to reveal its secrets to biochemists and geneticists alike is that of oxidative phosphorylation. This is the mechanism by which the reduction of molecular oxygen is coupled to the conservation of energy in the form of adenosine-5'-triphosphate (ATP). The reducing equivalents necessary for this are generated by the oxidation of substrates of the tricarboxylic acid cycle. This energy conserving process is catalysed by macromolecular complexes located in the plasma membrane in the case of prokaryotes and the inner mitochondrial membrane of eukaryotes.

Essentially three hypotheses have been proposed in an attempt to describe, in molecular terms, the events that occur during oxidative phosphorylation.

1) The chemical hypothesis put forward by Slater (1953) argues that one or more high energy intermediates, generated by a series of chemical reactions involving components of the respiratory chain, drive ATP synthesis directly.

2) The chemiosmotic hypothesis postulated by Mitchell (1961, 1966) suggests that ATP synthesis is driven by the dissipation of a pH and electrochemical gradient established across a proton-impermeable membrane by passage of electrons along the respiratory chain.

3) The third model, the conformational hypothesis of Boyer (1965) proposes that energy derived from electron transport

may be conserved by a change in conformation of a protein and later utilized in the synthesis of ATP.

For a full account of these hypotheses, and for the evidence in favour and against each, the reader is recommended to consult the reviews by Greville (1969) and by Boyer and others (Boyer et al., 1977).

The terminal step in oxidative phosphorylation, and obviously in all of the models outlined above, is the synthesis of ATP from adenosine-5'-diphosphate (ADP) and inorganic phosphate (P_i). The enzyme responsible for this catalytic reaction is the ATP synthetase complex. This is also known as the adenosine-5'-triphosphatase (ATPase) complex, since the destruction of ATP with concomitant release of P_i is usually monitored in preference to the synthesis of ATP. Although the isolation and characterization of this and other complexes from various sources have resulted in a large increase in our knowledge of the reactions catalysed by these individual components, the overall mechanism remains to be elucidated.

A genetic approach to the problem was therefore considered; the isolation of mutants with defects in various subunits of the component enzyme complexes would possibly shed more light on their assembley, their functional role, their inter-relationships and on the process of oxidative phosphorylation itself. Because of its extensive exploitation in the early studies on the biogenesis of mitochondrial enzymes, later on during the first combined biochemical-genetic approaches and for the reasons outlined below, yeast appeared the most suitable organism for this investigation. The aim of this research was to isolate and characterize, as fully as possible, yeast mutants possessing

lesions in regions of mitochondrial deoxyribonucleic acid (mtDNA) responsible for the biosynthesis of the individual components of the oxidative phosphorylation machinery. More specifically the isolation of mitochondrial mutants defective in one or more subunits of the membrane bound oligomycin-sensitive adenosine-5'-triphosphatase (OS-ATPase) complex was attempted. Such mutants would also be of enormous value in the construction of a detailed genetic map of mtDNA.

There exist numerous books and reviews on yeast mitochondrial genetics and mtDNA (Sager, 1972; Linnane et al., 1975; Michaelis and Somlo, 1976; Nagley et al., 1977; Beale and Knowles, 1978; Borst and Grivell, 1978; Gillham, 1978; Linnane and Nagley, 1978) and the reader is recommended to consult these for further clarification and/or amplification.

1:2 Yeast as a Suitable Organism

The suitability of yeast for a combined genetic and biochemical study of the enzymes that participate in, and the mechanism of, oxidative phosphorylation has been discussed by Kováč (1974) amongst others and will only briefly be outlined here. Yeast is a eukaryotic organism possessing the capacity for growth on a variety of substrates and under a number of environmental conditions. It is well known that the mitochondria of yeast have properties very similar to mammalian mitochondria. An advantage of yeast however is the ability to substantially modify their mitochondrial phenotype by manipulation of the growth conditions or by genetic lesions. This property is a consequence of the capacity of this organism to generate sufficient energy for growth from the glycolytic

pathway alone. A further benefit, limited to the so-called "petite-positive" yeasts, derives from their efficiency to withstand both small and large modifications, or total deletion, of their mtDNA. This ability renders "petite-positive" yeasts ideal candidates for the isolation and characterization of specific mitochondrial mutations.

It should be pointed out however, that yeasts classified under the heading "petite-negative" (Bulder, 1964; DeDeken, 1966) are unable to resist mitochondrial damage and do not form viable petites. Bulder (1963) suggested that although the "petite-negative" yeasts underwent the petite mutagenic process, the loss of a functional mitochondrial genome proved lethal. It was later shown by Luha (Luha, 1972) that treatment of the "petite-negative" yeast Kluyveromyces lactis with the petite-inducing agent ethidium bromide (EtBr), resulted in the production of micro-colonies which ultimately autolysed. This difference between "petite-positive"and "petite-negative" yeasts may in some way be related to the difference in size of their mitochondrial genomes; the latter being smaller (Nagley et al., 1977) and resembling more closely the mtDNA of mammalian cells (Borst, 1972).

In biochemical-genetic investigations, the facultative anaerobe Saccharomyces cerevisiae has been the species of choice to date. From the genetic point of view there is a considerable bulk of information relating to both its nuclear and mitochondrial genomes. There also exist numerous genetically defined auxotrophic strains of S. cerevisiae and well characterized methods for their experimental manipulation. Biochemically yeasts, and in particular S. cerevisiae, have several important benefits.

The organism is easily maintained, cultured, capable of rapid growth in a chemically defined medium and is non-pathogenic. Further there exists a wealth of biochemical data on yeast as a whole and on the mitochondrion in particular. This is a consequence of the relative ease of isolation, fractionation and purification of the individual complexes of this organelle.

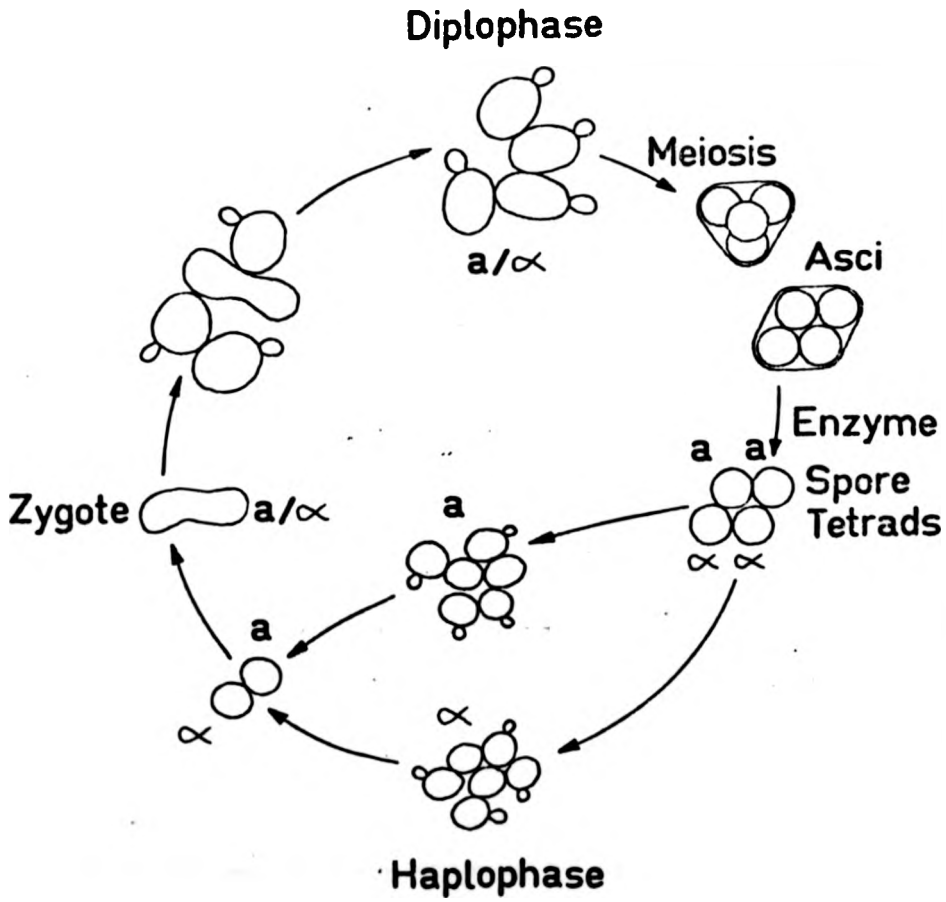
For these reasons S. cerevisiae was chosen as the organism most suited to fulfil the objectives outlined.

1:3 Characteristics of Saccharomyces cerevisiae.

S. cerevisiae is a unicellular eukaryote whose life cycle is depicted in Figure 1:1. When two haploid strains of opposite mating type are mixed under appropriate conditions fusion between two cells, carrying the a and α chromosomal alleles respectively, results in the formation of a zygote. Subsequent mitotic division of the zygote results in the formation of diploid cells. Both the haploid and diploid phase are stable and may be propagated by asexual budding. Under adverse conditions however, for example the presence of a poor substrate (Fowell, 1969), diploids may be induced to sporulate. Through meiosis these may give rise to four unordered haploid spores within an ascus, the mating-type alleles segregating 2:2 (a:α). Enzymic digestion of the ascus wall by a suitable preparation releases the spores which, following germination, may regenerate the haploid state.

Being a facultative anaerobe, S. cerevisiae possesses the ability to grow either anaerobically on fermentable sugars such as glucose or aerobically on a variety of different substrates. Anaerobic growth necessitates supplementation of the culture

Figure 1:1. Life Cycle of Saccharomyces cerevisiae[a]



a. Adapted from Mortimer and Hawthorne (1969).

medium with ergosterol and oleic acid (Linnane et al., 1975), usually in the form of Tween 80, since the biosynthesis of these two compounds requires the presence of oxygen. Clearly, oxygen is essential for growth on non-fermentable substrates.

Under conditions of anaerobiosis or catabolite repression (i.e. the presence of a high concentration of glucose) mitochondria exhibiting gross structural and morphological changes are typical (Linnane et al., 1975). Such organelles have been shown to possess very low levels, and often an absence, of respiratory enzymes and other mitochondrial components. Drastic modification of the mitochondrial phenotype is also observed when yeast is grown in the presence of inhibitors of mitochondrial protein synthesis (Huang et al., 1966; Clark-Walker and Linnane, 1967) or respiration (Kovác̆ et al., 1970).

One further interesting characteristic of S. cerevisiae is the diauxic growth pattern displayed when glucose or other fermentable substrates are utilized as an energy source. This feature is a consequence of repression of respiration (the so-called Crabtree effect) by glucose and to a lesser extent other sugars (Matile et al., 1969). Yeast will exclusively utilize glucose for growth and will only metabolise the ethanol produced when the sugar concentration is sufficiently low for induction of the respiratory enzymes to occur.

1:4 Structure and Properties of Yeast Mitochondrial DNA

The association of DNA with yeast mitochondria was first demonstrated by Schatz and co-workers (Schatz et al., 1964). Differences in the melting point (T_m) and buoyant density in a caesium chloride (CsCl) gradient (Tewari et al., 1965) indicated

that this DNA species was distinct from nuclear DNA. These preliminary results provided a possible structural explanation for the observation that mitochondrial function is, at least in part, controlled by extrachromosomal genetic factors (Ephrussi and Hottinguer, 1951).

The mtDNA of S. cerevisiae is believed to exist in the form of highly twisted (supercoiled) circular molecules of approximately 25 μm in circumference (Hollenberg et al., 1970; Petes et al., 1973). This data was deduced from electron microscopic examination of lysed preparations of mitochondria and sphaeroplasts respectively. Although to date isolation of closed circular DNA molecules from yeast have proved relatively unsuccessful (Hollenberg et al., 1970; Clark-Walker, 1972) considerable circumstantial evidence in support of this hypothesis prevails. Mapping of mitochondrial drug-resistant loci by petite deletion analysis (Molloy et al., 1975) and complementation amongst newly arisen cytoplasmic petite mutants leading to respiratory competent diploids (Clark-Walker and Miklos, 1975) have both been interpreted in terms of a circular DNA genome. In addition restriction endonuclease cleavage maps of mtDNA are explained in this mode. The size of this mtDNA species, some 25 μm, indicates a molecular weight of approximately 50 x 10^6 daltons (Hollenberg et al., 1970). This value, based on a linear, double stranded configuration, has been established independently by sedimentation behaviour in a sucrose gradient (Blamire et al., 1972).

The buoyant density of mtDNA of S. cerevisiae in CsCl is now regarded to be 1.683 gm.ml^{-1} (Bernardi et al., 1970; Goldring et al., 1970). Although this figure infers, using the

established formula of Schildkraut and colleagues (Schildkraut et al., 1962), a guanine plus cytosine (G + C) base composition of 23 to 24%, chemical analysis indicates a value of approximately 17% (Mehrotra and Mahler, 1968; Bernardi et al., 1970; Grossman et al., 1971). This discrepancy was assumed to be due to some unusual feature of the structure of mtDNA. Later work by Bernardi and collaborators (Bernardi et al., 1972; Prunell and Bernardi, 1974) indicated that the observed ambiguity resulted from regions of mtDNA rich in adenine plus thymine (A + T). It is now generally recognized that the A + T rich domains are "spacer" regions separating the G + C rich, information-containing, regions (Prunell and Bernardi, 1974).

Early studies on mtDNA from cytoplasmic petites by a variety of workers (Mounolou et al., 1966; Mehrotra and Mahler, 1968; Bernardi et al., 1968) indicated, in general, a decrease in buoyant density compared to the corresponding wild-types. This reduction in buoyant density was correlated with a change in base composition of the DNA, G + C contents having been found as low as 3 to 4% (Bernardi et al., 1968; Mol et al., 1974). Increases in buoyant density have also been observed (see Nagley et al., 1977) and it seems likely that petites may exhibit a range of buoyant densities.

Two other points concerning the quantity of mtDNA per cell are relevant here. Firstly it has been demonstrated that petites usually possess a similar total mtDNA content to the corresponding grandes (Hall et al., 1976). Secondly Grimes and coworkers (Grimes et al., 1974) showed that the number of mtDNA molecules per diploid cell was approximately twice that of an isogenic haploid cell, estimated at 83 and 47 copies per cell

respectively. This doubling of the number of copies of mtDNA was further correlated with a doubling of the number of mitochondria per cell, an estimate of ten per haploid and twenty per diploid being reported. One may therefore conclude the presence of approximately four copies of mtDNA per mitochondrion. This figure has been established independently by Bleeg and colleagues (Bleeg et al., 1972) in a strain of S. carlsbergensis by correlating the amount of mtDNA detected with the number of mitochondrial particles stained by Janus Green B. The results of Hoffman and Avers (1973) are in sharp contrast to those discussed above. After thin sectioning and subsequent electron microscopy of a yeast cell these authors suggest the possible presence of a single giant mitochondrion.

1:5 The Petite Mutation: An Extrachromosomally-Inherited Character

The first extrachromosomally-inherited mutation, the petite mutation, was described by Ephrussi and coworkers (Ephrussi et al., 1949a). They observed that treatment of a population of yeast cells with acriflavine resulted in a rapid transformation; the resultant culture being composed solely of smaller cells that possessed a slower growth rate and which gave rise to colonies with a reduced diameter. In addition, this transformation was found to be stable, to occur only during budding and was observed in both haploid and diploid cultures. Ephrussi and his colleagues (Ephrussi et al., 1949a; Ephrussi and Hottinguer, 1950) also reported the presence of a similar cell type, at a frequency of approximately 1%, in normal cultures of several species of yeast. It was soon

established (Ephrussi and Hottinguer, 1950) that this cell type occurred at a very high frequency in the presence of acriflavine through induction of the mutation by this dye and not by selection of spontaneously arising petites.

Genetic analysis of the petite mutation (Ephrussi *et al.*, 1949*b*) indicated a non-Mendelian pattern of inheritance. When the mutant was crossed to a grande tester strain only grande diploids were observed. However, sporulation of these diploids resulted, with very rare exceptions, in a 4:0 (grande:petite) segregation. Backcrossing of the grande ascospores to the original petite mutant gave rise, again with only very rare exceptions, to grande diploid progeny. This same result was found with four successive backcrosses. The unlikely possibility that the infrequently occuring petites (5 out of a total of 596 ascospores examined) were a result of a number of independently segregating recessive nuclear genes, rather than new spontaneous mutants, was considered by L'Heritier (1949). He concluded that more than twenty genes, induced simultaneously by the action of acriflavine, would be required to account for the low frequency of petites detected.

Phenotypically the cytoplasmic petite mutant is characterized by its inability to utilize aerobic substrates (Tavlitzki, 1949), requiring a fermentable sugar such as glucose, for growth. Petite cells are respiratory deficient (Slonimski, 1949), they lack cytochromes *a* + a_3, *b* and c_1 (Slonimski and Ephrussi, 1949) and have lost the capacity for mitochondrial protein synthesis (Kužela *et al.*, 1969; Schatz and Saltzgaber, 1969).

The spontaneous frequency of petite mutation varies from strain to strain and has been shown to be controlled, at least

partially, by the nucleus. There have been several reports (Sherman, 1963; Negrotti and Wilkie, 1968; Weislogel and Butow, 1970) of nuclear mutants, that, either by virtue of the mutation itself, by growth in the presence of glucose or growth at non-permissive temperatures, exhibit greatly increased petite frequencies. Recently an extrachromosomal mutant possessing similar properties has been isolated (Handwerker et al., 1973). The petite phenotype may also be induced by a variety of agents, for example: acriflavine (Ephrussi et al., 1949a; Marcovich, 1951), ethidium bromide (Slonimski et al., 1968), berenil (Mahler and Perlman, 1973), ultraviolet light (Raut, 1954; Raut and Simpson, 1955), manganese (Putrament et al., 1973) and heat (Ycas, 1956; Sherman, 1959). The precise mode of action of many petite-inducing agents is not understood.

Although, as previously discussed, a haploid strain of yeast possesses some 40 to 50 copies of mtDNA (Grimes et al., 1974) it has been calculated that there are a much smaller number of targets per cell (Sugimura et al., 1966; Slonimski et al., 1968). Further, this value has been shown to depend upon the physiological state of the cells prior to the mutagenic treatment (Wilkie, 1963; Maroudas and Wilkie, 1968). Several authors have attempted to explain this discrepancy. It has been suggested (c.f. Roodyn and Wilkie, 1968; Slonimski et al., 1968) that not all copies of mtDNA are genetically competent. Rank and Person (1969) have proposed that this disparity, between the number of targets and molecules of mtDNA per cell, results from an increased rate of replication of defective mtDNA over normal mtDNA. A third model, in which there exists a regulatory mechanism controlling the expression of grande mtDNA, has been

put forward by Williamson (1970). Inactivation of only a small number of mtDNA molecules is suggested to be sufficient to destabilize the expression of the wild-type phenotype. More recently Clark-Walker and Miklos (1974) have postulated that petite mutants arise as a consequence of structural rearrangements between and within mtDNA molecules. Excision and insertion events are suggested to result in the generation of circular mitochondrial genomes, smaller than the complete genome. This model is also capable of explaining two other important properties of mtDNA. These are suppressivity and polarity of recombination, both of which will be discussed, in greater detail, later.

1:6 Suppressivity

One feature of the petites originally isolated by Ephrussi and coworkers (Ephrussi *et al.*, 1949a) was that upon crossing to a grande tester strain only grande diploids were observed (Ephrussi *et al.*, 1949b). Sporulation of these diploids resulted in the ascospores segregating 4:0 (grande:petite). Ephrussi and his collaborators (Ephrussi *et al.*, 1955) later reported the existence of a second type of cytoplasmic petite phenotypically identical but genetically distinct from the first. These two types were referred to as "neutral" petites and "suppressive" petites respectively. When these so-called "suppressive" petites were mated to a grande tester strain, subsequent sporulation of the zygotic progeny resulted in a segregation that was predominantly 0:4 (grande:petite). Further, if the zygotes were plated out directly after mating then the majority gave rise to petite clones. However, if the zygotes

were inoculated into culture media and allowed to multiply before transfer to sporulation medium, then the ascospores were found to segregate 4:0 (grande:petite). These results were interpreted as follows: the 4:0 (grande:petite) segregation, observed after several generations growth of the zygotes, is due to the sporulation of grande diploids. Since respiratory competence is a prerequisite of sporulation, petite diploids whether "neutral" or "suppressive" will fail to sporulate. If however the mating mixture is transferred to sporulation medium directly then the petite zygotes will sporulate and give rise to a 0:4 (grande:petite) segregation. That petite zygotes do sporulate whilst petite diploids do not is presumably a consequence of the mixed nature of the zygotic cytoplasm; respiratory enzymes having been contributed to the zygote from the grande tester strain.

Further experiments indicated that the degree of suppressivity of a particular petite could be transmitted to the vegetative progeny of that petite (Ephrussi and Grandchamp, 1965). Alternatively, petites could give rise to progeny that possessed either a higher or lower degree of suppressivity (Ephrussi et al., 1966). The degree of suppressivity may also be modified by the action of various mutagenic agents (Uchida, 1972; Nagley et al., 1973).

The suppressivity of a particular petite is usually established by crossing to a grande tester strain, plating out and subsequently analysing the resulting zygotic clones. Three types of clone are usually observed: one that is entirely respiratory deficient, one that is predominantly respiratory competent (a minority of the cells being spontaneous respiratory

deficient petites) and a mixed clone possessing both types of cell. The degree of suppressivity is defined as the probability that a zygote will produce an entirely petite clone. The suppressivity of a petite may be calculated from the equation:

$$S = \frac{X - Y}{100 - Y} \times 100$$ (Ephrussi and Grandchamp, 1965)

where S is the degree of suppressivity (%)

X is the percentage of entirely petite clones and

Y is the spontaneous frequency of petites observed in the grande tester strain.

Several authors (Carnevali et al., 1969; Coen et al., 1970; Williamson, 1970; Clark-Walker and Miklos, 1974) have attempted to explain the phenomenon of suppressiveness. To date however, strong evidence in favour of any one of these models is lacking. The mechanism of suppressiveness is not understood.

1:7 Extrachromosomal Antibiotic-Resistant Mutants of Saccharomyces cerevisiae

The isolation of cytoplasmic petite mutants, although important in establishing the involvement of mtDNA in the biogenesis of mitochondria, did not contribute significantly to our knowledge of mitochondrial genetics. Despite being genetically different these mutants were phenotypically indistinguishable. Further, the crossing of any two cytoplasmic petites, of opposite mating-type, was never observed to result in the production of grande diploid progeny (Wright and Lederberg, 1957; Roodyn and Wilkie, 1967; Slonimski et al., 1968). No

information concerning the organization of the mitochondrial genome could therefore be deduced. More recently it has been reported (Clark-Walker and Miklos, 1975) that respiratory competent diploids may be generated if newly arisen spontaneous cytoplasmic petites are crossed with one another.

A major advance in our understanding of mitochondrial genetics followed the observation that several antibacterial antibiotics had a profound effect on yeast mitochondrial protein synthesis (Clark-Walker and Linnane, 1966; Lamb et al., 1968). Cells grown in the presence of such drugs, for example chloramphenicol, appeared phenotypically identical to cytoplasmic petite mutants (Huang et al., 1966; Clark-Walker and Linnane, 1966, 1967). It was therefore postulated that mutants resistant to any one of these antibiotics might display extrachromosomal inheritance. In the late 1960's mutants resistant to erythromycin (Linnane et al., 1968a; Roodyn and Wilkie, 1968), spiromycin and paromomycin (Thomas and Wilkie, 1968) and exhibiting a non-Mendelian pattern of inheritance were isolated. Since that time many laboratories have been successful in isolating extrachromosomal yeast mutants, resistant to inhibitors of mitochondrial protein synthesis. In addition, cytoplasmically-inherited mutants resistant to respiratory chain inhibitors such as antimycin A (Bunn et al., 1970; Groot Obbink et al., 1977; Pratje and Michaelis, 1977) and mucidin (Šubík, 1975; Šubík et al., 1977) and inhibitors of the ATP synthetase complex, such as oligomycin (Avner and Griffiths, 1970, 1973a, b), venturicidin (Griffiths et al., 1975; Lancashire and Griffiths, 1975a) and triethyl tin (Lancashire, 1974; Lancashire and Griffiths, 1975b) have been described. As a direct result of

the isolation of these drug resistant mutants criteria of mitochondrial inheritance were established (Linnane et al., 1968a). The three main criteria are presented in Table 1:1 in the form of a comparison between the behaviour displayed by mitochondrial and nuclear genes.

Allelism tests, to distinguish whether two phenotypically identical drug resistance mutations are also genotypically identical, are performed by crossing the two mutants together. The appearance of sensitive diploid cells, arising through recombination, indicates that the two drug resistance mutations are conferred by separate loci. If only resistant diploid cells are observed, then the mutations may be regarded as allelic.

1:8 Recombination and Polarity.

Evidence of recombination, between strains possessing different mitochondrial genotypes, was first presented by Thomas and Wilkie (1968). Crosses between yeast mutants resistant to erythromycin, spiramycin and paromomycin indicated that recombinant cell types could be generated. For example, when an ery^s par^r strain was crossed to an ery^r par^s strain, both ery^s par^s and ery^r par^r recombinants were found among the diploid progeny. The data of Thomas and Wilkie suggested that the ery^r and spi^r loci were closely linked to each other but were relatively distant to the par^r locus.

An early observation, in crosses between erythromycin-resistant and sensitive strains, was the frequent unequal proportions of erythromycin-resistant and sensitive diploids within the mating mixture (Coen et al., 1970). A similar phenomenon

Table 1:1 Criteria of Mitochondrial Inheritance in *Saccharomyces cerevisiae*[a]

Mitochondrial genes	Nuclear genes
1. Mitotic (vegetative) segregation of alleles after zygote formation, i.e. heteroplasmic state.	1. No mitotic segregation of alleles.
2. No segregation of alleles on meiosis of a homoplasmic diploid (4:0 or 0:4 tetrads)	2. Segregation of alleles on meiosis (2:2 tetrads)
3. i Loss of gene associated with rho^0 state [b].	3. No loss of nuclear genes associated with extranuclear petite mutation.
ii Retention or loss of gene in different rho^- isolates [c].	

a. Reproduced from Nagley *et al.* (1977).

b. A rho^0 petite is defined as lacking detectable mtDNA.

c. A rho^- petite is defined as possessing an irreversible cytoplasmically-inherited mutation that results in the loss of mitochondrial protein synthesis.

was also noted by these workers in crosses between chloramphenicol-resistant and sensitive strains. These and other results from Slonimski's laboratory helped to establish the rules of segregation and recombination of mitochondrial genomes.

This phenomenon of asymmetrical proportions of drug-resistant and drug-sensitive diploid clones in monofactorial crosses has been termed polarity of transmission (Coen et al., 1970). The existance of a polarity or sex factor, ω (omega), to explain these observations, has been postulated (Bolotin et al., 1971). Yeast strains have been subsequently designated ω^+ or ω^-. In heterosexual crosses (i.e. ω^+ x ω^- or ω^- x ω^+) a polarity of transmission will be detected for loci close to omega. As this distance from omega increases, the polarity of transmission decreases.

The greater the deviation from one, of the ratio of resistant to sensitive diploids, the more polar the cross is said to be. In homosexual crosses (i.e. ω^+ x ω^+ and ω^- x ω^-) no polarity is observed (Bolotin et al., 1971). Omega is believed to be located between, and very tightly linked to, the loci determining resistance to erythromycin and chloramphenicol (Howell et al., 1974). No polarity of transmission is observed for loci situated elsewhere on the mitochondrial genome, for example, for loci conferring resistance to paromomycin (Wolf et al., 1973) and oligomycin (Avner et al., 1973).

In heterosexual multifactorial crosses of the type cap^r ery^s x cap^s ery^r, unequal proportions of recombinant types (i.e. cap^r ery^r and cap^s ery^s) were also observed (Coen et al., 1970). This effect has been termed polarity of recombination and is defined as "the deviation from the 1:1 ratio of the two types

of reciprocal recombinants". Once again the greater the deviation, the more polar the cross. Polarity is not observed in equivalent homosexual crosses, although the frequency of recombinant types is usually lower (Bolotin et al., 1971).

Dujon and his coworkers (Dujon et al., 1974) have proposed a model to explain recombination of mitochondrial genomes. They propose that all regions of mtDNA undergo a process of non-reciprocal recombination, each recombinational event resulting in the production of one parental molecule and one recombinant molecule. In homosexual crosses and in heterosexual crosses involving non-polar regions of the genome, these recombinational events may occur in opposite directions and with equal frequencies. As a consequence, equal proportions of recombinant types are generated. In heterosexual crosses involving the polar region of the genome, these recombinational events are suggested to occur always in the same direction and beginning at omega. This asymmetrical gene conversion process, in which ω^- strands of mtDNA are converted to ω^+, is proposed to occur as follows. Pairing between ω^+ and ω^- strands results in an obligatory excision of the ω^- strand at the ω^- locus. This is followed by degradation of the ω^- sequence and resynthesis using the ω^+ strand as a template. As the distance from omega increases, the probability of this gene conversion process being halted also increases. Thus, loci that appear "loosely linked" to omega will exhibit polarity but to a much lesser extent.

Recently, a 1050 base pair insert has been detected and located within the 21S rRNA gene (Bos et al., 1978), a region of mtDNA that overlaps the erythromycin and chloramphenicol resistance loci and the polarity locus omega. Further, this

insert has been shown to be present in ω^+ strains but lacking in ω^- and ω^n (omega neutral) strains (Borst and Grivell, 1978); ω^n strains being derived from ω^- ones by mutation at the omega locus (Dujon et al., 1976). However, the absence of polarity in $\omega^+ \times \omega^n$ crosses indicates that this insert is not sufficient to determine polarity by itself (Borst and Grivell, 1978). This phenomenon is now believed to be governed by a small insertion, present in ω^- strains, adjacent to the 1050 base pair insert. It is further proposed that ω^n strains have only part of this second insertion, whilst in ω^+ strains it is absent. The mismatch at this second insertion, during recombination, is thought to be essential for the initiation of gene conversion. Evidence in support of this model derives from the work of Lukins and his associates (Lukins et al., 1977), who were able to generate an ω^+ cytoplasmic petite by deletion of an ω^- strain.

The observed maximum frequency of recombination between two "unlinked" mitochondrial drug resistance loci, uninfluenced by omega, is 20 to 25% (Wolf et al., 1973). This upper limit is explained if it is assumed that mating occurs repeatedly between both genetically different and genetically identical mtDNA molecules (Dujon et al., 1974). Mating and subsequent recombination between identical mtDNA molecules would not be experimentally detected.

Recombination between the mitochondrial genomes of two different cytoplasmic petites has also been demonstrated (Michaelis et al., 1973). Two petites possessing different drug resistance markers were crossed and, by a "marker rescue" technique involving the construction of triploids, recombinants were detected.

Further, the mtDNA of the petite diploids was found to have a buoyant density intermediate of the two parental mtDNAs thus lending support to the notion that recombination had occurred. In addition, these results suggested that the enzymes responsible for mitochondrial recombination were synthesized on the cytoplasmic ribosomes.

1:9 Mapping of Mitochondrial Genes

Three main methods exist for mapping the mitochondrial genome:

(i) recombination analysis

(ii) petite deletion analysis and

(iii) physical mapping by DNA/DNA hybridization.

Genetic mapping of mitochondrial markers by recombination analysis is based upon the assumption that the frequency of recombinant progeny decreases as the distance between two loci decreases. This method has a serious limitation however, being ineffective in determining the relative map positions of "unlinked" loci. As previously mentioned, "unlinked" mitochondrial drug resistance loci exhibit a maximum recombination frequency of 20 to 25% (Wolf *et al.*, 1973). Therefore, this method may only be used to order loci that are clustered closely together. Examples, in which recombination analysis has been employed successfully, include the region of the mitochondrial genome influenced by omega (Avner *et al.*, 1973; Wolf *et al.*, 1973; Netter *et al.*, 1974) and several mit^- regions (Kotylak and Slonimski, 1976, 1977; Trembath *et al.*, 1977).

It should be noted that absence of recombination between two

markers may be regarded as an indication of allelism.

Mapping by petite deletion analysis is based upon the premise that, since deletions may occur at random and in any region of the genome (Nagley et al., 1977), as the distance between two loci diminishes the probability of a deletion event taking place between them correspondingly decreases. Thus two "tightly linked" markers will usually be either deleted together or retained together upon petite induction. Further, since petites are unable to grow on non-fermentable substrates they must be crossed to a sensitive grande tester strain in order to assess loss or retention of drug resistance alleles (Molloy et al., 1975). Mit^+ alleles (i.e. the corresponding functional copies of mit^- alleles) may also be mapped by this method, but the petites must now be crossed to suitable mit^- tester strains instead of a grande tester (Schweyen et al., 1976). Approximate distances between markers have been estimated from the frequencies with which two markers are separated (Molloy et al., 1975; Schweyen et al., 1976; Trembath et al., 1976).

The third method for mapping mitochondrial genes was developed, and has been extensively used, in the laboratory of Linnane. Physical mapping involves the isolation of stable petites that retain various regions of the mitochondrial genome and the determination, by DNA/DNA hybridization analysis, of the physical length and location of the segment of grande mtDNA possessed by such petites (Sriprakash et al., 1976; Linnane et al., 1976; Nagley et al., 1976). The precise location of any new mitochondrial mutation may now be determined by crossing to these characterised petites. Appearance of the wild-type allele amongst the diploid progeny indicates the presence of the new

locus on the mtDNA of the petite tester. This method is applicable to drug-resistant mutants, mit$^-$ mutants and conditional mutants (Nagley et al., 1977). Genes for ribosomal ribonucleic acid (rRNA) and transfer ribonucleic acid (tRNA) may also be located by direct hybridization to the mtDNA of genetically and/or physically defined petites (Linnane et al., 1976). Two factors that might influence the correct location of mitochondrial markers by this method are: the possible presence of amplified DNA sequences in the reference petites and "new" mtDNA sequences not present in the grande mitochondrial genome (Sriprakash et al., 1976). However, using a library of genetically and physically defined petites any ambiguity should be eliminated.

A physical map of yeast mtDNA may also be constructed utilizing restriction endonucleases (Sanders et al., 1976).

1:10 Nuclear Oxidative Phosphorylation Mutants of Yeast

Although the cytoplasmic petites may be regarded as the first examples of yeast to exhibit a deficiency in oxidative phosphorylation (Slonimski, 1949; Tavlitzki, 1949; Ephrussi, 1956), their grossly modified biochemical properties rendered them unsuitable for investigations of the individual components of that process. More specific mutants, possessing lesions in the constituent complexes, were therefore sought. In the early 1960's Sherman isolated two classes of single-gene nuclear mutant designated p or pet (Sherman, 1963) and cy or cyc (Sherman, 1964). The pet mutants, of which there are now more than fifty documented (Fasman, 1976), are nuclear petites i.e. they lack the ability to grow on non-fermentable substrates. Biochemically they are

characterized by a complete absence of, or abnormally low levels of, either cytochromes $\underline{c}_1$, $\underline{b}$ and/or $\underline{a} + \underline{a}_3$ (Sherman and Slonimski, 1964; Mackler et al., 1965; Reilly and Sherman, 1965). One extensively studied mutant, pet 9 (also referred to in the literature as op_1), exhibits a normal cytochrome absorption spectrum (Kovác̆ et al., 1967). A modified mitochondrial adenine nucleotide translocase activity is believed to be responsible for the deficiency in oxidative phosphorylation of this mutant (Kolarov et al., 1972). Although the pet mutants are single-gene nuclear mutants it should be emphasized that many display more than one enzymic deficiency, indicating a strong interaction in the biosynthesis of the respiratory chain. Finally, it should be noted that certain pet mutations cause instability of the mitochondrial genome, resulting in high spontaneous rho^- frequencies (Sherman, 1963). In fact, the cytoplasmic petite mutation is lethal in strains possessing the pet 9 mutation (Kováčová et al., 1968). Such mutants therefore resemble the so-called "petite-negative" yeasts (Bulder, 1964; DeDeken, 1966).

The cyc mutants isolated by Sherman (1964) were found to possess lowered levels of cytochrome $\underline{c}$. Unlike the pet mutants, these strains retain the ability to grow on non-fermentable substrates, although often with a reduced efficiency.

Many other nuclear mutations, that directly or indirectly affect the ability of a cell to grow aerobically, have been described. A few of the more relevant ones will now be briefly discussed. For a more detailed account the reader is urged to consult the appropriate reviews (Kovác̆, 1974; Lloyd, 1974).

Parker and Mattoon (1969) have attempted to distinguish specific oxidative phosphorylation mutants from the much more

frequent rho$^-$ petites using the tetrazolium salt, 2,3,5-triphenyl tetrazolium chloride (TTC). The rationale of this approach being that petites retaining an intact respiratory chain might still be able to reduce TTC and thus be differentiated from the pleiotropic rho$^-$ petites which are unable to do so (Ogur *et al.*, 1957). Many nuclear petites designated aem (abnormal energy metabolism) and glt (glutamate requiring) were isolated. These were later shown to be either unable to assimilate glycerol and/or to be glutamate auxotrophs (Beck *et al.*, 1971).

Ebner, Schatz and colleagues (Ebner *et al.*, 1973; Ebner and Schatz, 1973) isolated a series of nuclear respiratory deficient mutants displaying a range of cytochrome absorption spectra. In addition one mutant, having greatly modified levels of cytochromes c_1, b and $a + a_3$, was found to lack both structurally and enzymically detectable mitochondrial F_1 ATPase (Ebner and Schatz, 1973). The F_1 ATPase is the name given to the nuclear coded, catalytically active component of the OS-ATPase complex (Tzagoloff *et al.*, 1973).

An ingenious method for the detection of oxidative phosphorylation mutants of yeast was devised by Tzagoloff and coworkers (Tzagoloff *et al.*, 1975a). A large number of mutants, isolated following a variety of mutagenic treatments, unable to grow on the non-fermentable substrates glycerol and ethanol were assayed for *in vivo* mitochondrial protein synthesis. Since cytoplasmic rho$^-$ petites lack this function (Kužela and Grečná, 1969; Kužela *et al.*, 1969; Schatz and Saltzgaber, 1969) Tzagoloff was able to select for both nuclear (Tzagoloff *et al.*, 1975b) and cytoplasmic (Tzagoloff *et al.*, 1975c) mutants possessing specific lesions in the process of oxidative phosphorylation.

Among the nuclear mutants isolated deficiencies in cytochrome oxidase, coenzyme QH_2-cytochrome c reductase, coenzyme Q and the OS-ATPase complex were described. Of three OS-ATPase deficient mutants, two lacked enzymically detectable F_1 ATPase whilst the other failed to integrate the F_1 component into the mitochondrial membrane. This latter mutant was found to lack subunit 9, a mitochondrially synthesized 45,000 dalton component, of the OS-ATPase complex (Tzagoloff and Akai, 1972).

Other nuclear mutations of note, resulting in an impaired ability to grow on non-fermentable media, include the pop mutations (Pretlow and Sherman, 1967) which affect the cellular levels of free and zinc porphyrins and protoporphyrins, and the unsaturated fatty acid auxotrophic mutation ole 1-1 (Resnick and Mortimer, 1966). Depletion of the normal levels of unsaturated fatty acids, in a strain possessing the ole 1-1 mutation, leads to a diminished capability for oxidative phosphorylation (Proudlock et al., 1969). Nuclear mutations conferring resistance to protein synthesis inhibitors (Wilkie et al., 1967; Roodyn and Wilkie, 1968) and ATPase inhibitors (Parker et al., 1968; Stewart, 1970; Douglas, 1977) have also been reported.

1:11 Cytoplasmic Oxidative Phosphorylation Mutants of Yeast

In order to elucidate the function of mtDNA, and the role played in the biogenesis of its host organelle, mutants possessing specific lesions in this unique DNA species were sought. If defective proteins could be shown to be the product of unique mutational events occurring in mtDNA, this would serve as unequivocal evidence for the mitochondrial location of the gene(s)

responsible for the biogenesis of that protein. The cytoplasmic petites first described by Ephrussi and coworkers (Ephrussi et al., 1949a) were of little use in this respect due to their gross biochemical changes. Mitochondrial antibiotic-resistant mutants, although having their limitations, were of greater value. Increased resistance to erythromycin and paromomycin has been correlated with a mitochondrial protein synthetic function that is resistant to these drugs both in vivo and in vitro (Linnane et al., 1968b; Bunn et al., 1970; Kutzleb et al., 1973). However, cytoplasmic chloramphenicol-resistant mutants were found, by Bunn and coworkers (Bunn et al., 1970), to display a mitochondrial amino acid incorporation activity that was sensitive to this antibiotic. These authors proposed that erythromycin resistance was a consequence of an alteration in a mitochondrial ribosomal protein. Chloramphenicol resistance was thought to result from a change in the permeability of the mitochondrial membrane. This explanation does not ring true however, in the light of our recent knowledge of the organization of the mitochondrial genome.

Some of the mutants resistant to inhibitors of the mitochondrial OS-ATPase complex have also been studied biochemically (Griffiths et al., 1972; Mitchell et al., 1973; Shannon et al., 1973; Griffiths and Houghton, 1974; Somlo et al., 1974; Griffiths et al., 1975). Dependent upon the antibiotic resistance mutation being studied, the OS-ATPase complex may or may not exhibit an increased in vitro resistance. In general however, the resistance observed in vitro is considerably less than that observed in vivo. Although such mutants indicate the involvement of mtDNA in the biogenesis of the OS-ATPase complex, until

recently no direct evidence had been presented. In 1976 however, Tzagoloff and coworkers (Tzagoloff et al., 1976a) demonstrated that an oligomycin resistance mutation, at the oli^r1 locus (Avner et al., 1973), resulted in a modification of one of the mitochondrially synthesized OS-ATPase subunits. Subunit 9 of the complex, a proteolipid (Sierra and Tzagoloff, 1973), has an apparent molecular weight of 45,000 daltons in sodium dodecyl sulphate (SDS)-polyacrylamide gels (Tzagoloff and Akai, 1972). If pretreated with strong base or chloroform-methanol however, this component dissociates and runs with an apparent molecular weight of approximately 8,000 daltons. After radioactively labelling the mitochondrially synthesized proteins of oli^r1 resistant mutants and subsequent SDS-polyacrylamide gel electrophoresis, only the 8,000 molecular weight component was detected (Tzagoloff et al., 1976a). The corresponding wild type strains predominantly exhibited the 45,000 dalton molecular weight species. These results suggested that the oli^r1 mutation, in some way, influences the association-dissociation behaviour of the proteolipid. More recently, the amino acid sequence of subunit 9 from a wild type and an oli^r1 resistant mutant has been determined, and the oli^r1 mutation shown to result in a single amino acid substitution (A. Tzagoloff, personal communication).

A temperature sensitive mutant, L748, isolated in the laboratory of Linnane (Trembath et al., 1975), shows a greatly reduced ability to grow on non-fermentable substrates at 19°C as compared to 28°C. The extrachromosomal mutation, carried by this strain, confers resistance to oligomycin at both temperatures and maps at oli^r3 (Lancashire and Griffiths, 1975a). Biochemical analysis (Groot Obbink et al., 1976) has indicated that at the

restrictive temperature the synthesis of subunit 6, a 20,000 dalton component of the OS-ATPase complex (Tzagoloff and Meagher, 1971), is greatly diminished. Thus, the mitochondrial loci oli^r1 and oli^r3 are believed to be located in two separate genes that code for subunits 9 and 6 respectively (Groot Obbink et al., 1976). Labelling experiments have so far failed to reveal any structural difference between strains carrying the oli^r2 mutation (Avner et al., 1973) and the corresponding wild types.

Several research groups have, in an effort to isolate specific mitochondrial oxidative phosphorylation deficient yeast mutants, employed numerous and often laborious selection procedures. Flury and colleagues (Flury et al., 1974) attempted to isolate such mutants by testing respiratory deficient mutants for reversion to wild type; pleiotropic cytoplasmic mutants never having been observed to revert (Ephrussi, 1953). These workers describe an extrachromosomally-inherited mutant that lacks cytochrome oxidase, reduced nicotinamide adenine dinucleotide (NADH)- and succinate-cytochrome c reductase activities yet possesses significant levels of cytochromes b and c_1 and the capacity for mitochondrial protein synthesis. The mitochondrial ATPase activity of this strain is found to be resistant to oligomycin.

The isolation of extrachromosomal temperature sensitive mutants has also been reported (Handwerker et al., 1973; Storm et al., 1974; Storm and Marmur, 1975; Lancashire, 1976; Bolotin-Fukuhara et al., 1977). These authors believed that conditional mutants would be of enormous value in determining the gene products specified by mtDNA. Indeed, the extranuclear origin of components of the OS-ATPase complex (Groot Obbink et

al., 1976) and of certain tRNA and rRNA genes (Bolotin-Fukuhara et al., 1977; Monnerot et al., 1977) has been established as a consequence of this approach.

A dramatic advance in the elucidation of the mitochondrial genome has resulted from the recent work of Tzagoloff and his colleagues. With the knowledge that cytoplasmic rho$^-$ petites are characterized by the absence of mitochondrial protein synthesis (Kuželа and Grečná, 1969; Kuželа et al., 1969; Schatz and Saltzgaber, 1969) Tzagoloff decided to search for mutants that retained this function, yet still failed to grow on non-fermentable substrates. Following a range of mutagenic treatments, cells that failed to grow in the presence of glycerol and ethanol were incubated in a medium containing tritiated leucine and cycloheximide, an inhibitor of cytoplasmic protein synthesis (Kerridge, 1958; Siegel and Sisler, 1963, 1964; Lamb et al., 1968). Cells incorporating a significantly higher amount of radiolabel, as compared to a rho^o tester strain, were retained for further biochemical and genetic analysis. This procedure (Tzagoloff et al., 1975a) permitted the isolation of hundreds of potentially useful mutants. Cytoplasmic mutants were distinguished from nuclear ones by crossing to a rho^o tester strain and analysing the resultant diploids for respiratory competence and also by tetrad analysis (Tzagoloff et al., 1975a, c).

Among the nuclear mutants, deficiencies in coenzyme QH_2-cytochrome c reductase, cytochrome oxidase, coenzyme Q and the OS-ATPase complex were observed (Tzagoloff et al., 1975a, b). Preliminary studies (Tzagoloff et al 1975c) indicated that the cytoplasmic mutants possessed lesions in the biosynthesis of

coenzyme QH_2-cytochrome *c* reductase, cytochrome oxidase and the OS-ATPase complex. The extrachromosomally-inherited coenzyme QH_2-cytochrome *c* reductase deficient mutants were spectrophotometrically observed to lack cytochrome *b*, whilst the majority of the cytochrome oxidase mutants lacked cytochromes *a* + a_3. Further, when the mitochondrial products of some of the cytochrome oxidase mutants were radioactively labelled with [^{35}S] methionine or [^{3}H] leucine and subsequently analysed, the highest molecular weight subunit of the enzyme was found to be missing (Tzagoloff *et al.*, 1975*c*, *d*).

These mutants, designated mit$^-$, have been further genetically characterized and the lesions located on mtDNA (Slonimski and Tzagoloff, 1976). Essentially three methods have been employed to map these mutations:

(i) restoration of respiratory function in mit$^-$ x rho$^-$ crosses

(ii) restoration of respiratory function in mit$^-$ x mit$^-$ crosses and

(iii) analysis of recombination frequencies in crosses of antibiotic-sensitive mit$^-$ mutants with antibiotic-resistant rho$^+$ strains.

In this way three regions responsible for the biosynthesis of cytochrome oxidase (designated oxi 1, oxi 2 and oxi 3) and one responsible for coenzyme QH_2-cytochrome *c* reductase (designated cob) were delineated. Following the isolation of additional mit$^-$ mutants, the existence of two distinct regions controlling cytochrome *b* biosynthesis was demonstrated (Tzagoloff *et al.*, 1976*b*). These two regions, cob 1 and cob 2 were discriminated by mit$^-$ x mit$^-$ and mit$^-$ x rho$^-$ crosses. Biochemical analysis has

indicated that mutants possessing lesions in the region of cob 1 lack a major mitochondrial product that is identical to the apoprotein of cytochrome b (Katan et al., 1976). Mutants with lesions in cob 2 are either similar to those having mutations in cob 1 (i.e. they lack the cytochrome b apoprotein) or they lack the apoprotein but in addition exhibit a new, lower molecular weight, mitochondrial translation product (Tzagoloff et al., 1976b).

Mutants that appear to be defective in the biosynthesis of both cytochrome b and cytochrome oxidase have also been described (Kotylak and Slonimski, 1976). The mutations present in these strains were initially grouped into four regions, designated box 1 to 4, and found to map adjacent to the region controlling the biosynthesis of cytochrome b alone. The possibility that these box mutants were double mutants, similar to those described by Foury and Tzagoloff (1976a), was dismissed since they were observed to spontaneously revert to rho^+ with frequencies characteristic of single mit^- mutations (Kotylak and Slonimski, 1976; P. Slonimski, personal communication). This discrepancy, between the loss of two mitochondrial functions and a single mit^- mutation, has been resolved by Pajot and coworkers (Pajot et al., 1976; P. Pajot, personal communication). Mutations in either the box 1, box 2 or box 4 regions have been found to result in an increase in the sensitivity of cytochrome oxidase formation to catabolite repression by glucose. The synthesis and consequent spectrophotometric and enzymic detection of cytochrome oxidase was achieved when such mutants were grown in galactose. The synthesis of this enzyme could not be derepressed in strains possessing lesions in the box 3 region. The box mutations described above

and the cob mutations of Tzagoloff (Tzagoloff et al., 1976b) have now been correlated and ordered together on a map of mtDNA (Kotylak and Slonimski, 1977). There are now believed to be seven distinct regions (box 1 - 7) on mtDNA responsible for the biosynthesis of coenzyme QH_2-cytochrome c reductase (Borst and Grivell, 1978). Four of these (box 1, 4, 5 and 6) are thought to have a structural role, the others may have some regulatory function. However, mutation in any of these regions results in the defective biosynthesis of cytochrome b.

Recent studies (Cabral et al., 1977, 1978) have demonstrated that the oxi 1 segment contains the structural gene for subunit II of cytochrome oxidase. Two mutants possessing lesions in oxi 1 lack this subunit, but exhibit smaller mitochondrially synthesized polypeptides that are identifiable as fragments of subunit II.

Two further classes of mit^- mutants have also been isolated. These have been designated pho 1 (Foury and Tzagoloff, 1976b) and pho 2 (Tzagoloff et al., 1976c). Of four pho 1 mutants described (Foury and Tzagoloff, 1976b), two are capable of slow growth on glycerol. This author believes that these two mutants are "leaky" i.e. they possess only a modified, rather than a completely defective, oxidative phosphorylative capacity. Foury and Tzagoloff (1976b) have shown that the pho 1 region is closely linked to the oli^r2 drug resistance locus (Avner et al., 1973). Further, by crossing these mutants to a triple antibiotic-resistant rho^+ strain and a series of rho^- testers, the pho 1 segment has been located between the par^r1 (Wolf et al., 1973) and oli^r2 drug resistance loci. More detailed mapping by Somlo and colleagues (Somlo et al., 1977) places the pho 1 mutation of

M28-81 between oli^{r}2 and oli^{r}4 (Clavilier, 1976), a fourth locus conferring resistance to oligomycin. The results of preliminary biochemical studies have also been published. Foury and Tzagoloff (1976b) reported that pho 1 mutants exhibit an ATPase activity that is oligomycin insensitive. This activity is found in the post-ribosomal supernatant, indicating the presence of an F_1 ATPase component only. Further, this activity may be inhibited by antiserum to F_1 ATPase. Somlo and coworkers (Somlo et al., 1977) however suggest that the pho 1 ATPase is integrated into the mitochondrial membrane, but possibly in an abnormal fashion. No gross differences in mitochondrially synthesized proteins have so far been detected in pho 1 mutants (A. Tzagoloff, personal communication).

To date, only one mutant representing the mit$^-$ region designated pho 2 (Tzagoloff et al., 1976c) has been described and this also possesses a second mutation in the oxi 3 region. This pho 2 mutation is reported to be linked to oli^{r}1 and located between oli^{r}1 and oli^{r}2. Recent data (A. Tzagoloff, personal communication), indicates that this mutant has a deletion at the amino terminal end of subunit 9 of the OS-ATPase complex.

Tzagoloff has also reported the isolation of cytoplasmic pleiotropic petites, designated syn$^-$, that possess the ability to revert back to wild-type and/or give rise to respiratory competent diploids when crossed to a series of rho$^-$ testers (Tzagoloff et al., 1976c). In contrast, cytoplasmic rho$^-$ petites do not exhibit either capacity. Two of these syn$^-$ mutants, defective in the genes specifying aspartyl-tRNA and threonyl-tRNA, have been genetically mapped (Trembath et al., 1977). Both mutations lie in the cap-par segment of the mitochondrial genome. The

aspartyl-tRNA mutation maps between the cap^r locus and oxi 1 and the threonyl-tRNA lesion between oxi 1 and oxi 2.

Other groups of researchers have also isolated mit^- mutants of S. cerevisiae similar to those described by Tzagoloff and by Slonimski (Rytka et al., 1976; Cobon et al., 1976; Bolotin-Fukuhara et al., 1977).

1:12 Location of Genes on Mitochondrial DNA

In summary, regions responsible for the biosynthesis of coenzyme QH_2-cytochrome c reductase, cytochrome oxidase and the OS-ATPase complex have been located on mtDNA. A circular map, indicating the positions of some of the more important loci, is presented in Figure 1:2. Mapping of the two regions specifying the large (21S) and small (15S) rRNA molecules of the mitoribosome has been achieved by RNA/DNA hybridization. The 21S rRNA gene has been located within the segment containing the chloramphenicol and erythromycin drug resistance loci (Faye et al., 1974; Nagley et al., 1974) and the 15S rRNA gene has been found to map in the vicinity of the paromomycin drug resistance locus (Faye et al., 1975, 1976a). In addition, some twenty mitochondrial tRNA genes have been identified and mapped (Cohen and Rabinowitz, 1972; Casey et al., 1972; Cohen et al., 1972; Faye et al., 1973; Casey et al., 1974a, b; Fukuhara et al., 1976; Faye et al., 1976b; Martin et al., 1977).

It has proved considerably more difficult to assign mit^- mutations to specific structural or regulatory genes on mtDNA than to establish the location of mitochondrial RNA genes. Regions determining the rRNA and tRNA species are readily delineated.

Figure 1:2. Map of the Mitochondrial Genome of *Saccharomyces cerevisiae*.[a]

a. Adapted from Coruzzi *et al.* (1978)

cap^r indicates resistance to chloramphenicol.

ery^r indicates resistance to erythromycin.

par^r1 indicates resistance to paromomycin.

ana^r1 indicates resistance to antimycin A.

oli^r1 and oli^r2 indicate resistance to oligomycin.

oxi 1, oxi 2 and oxi 3 represent regions controlling the biosynthesis of cytochrome oxidase.

cob 1 and cob 2 represent regions controlling the biosynthesis of cytochrome b.

pho 1 and pho 2 represent regions controlling the biosynthesis of the OS-ATPase complex.

Note that the map distances are not representative of the physical distances between markers.

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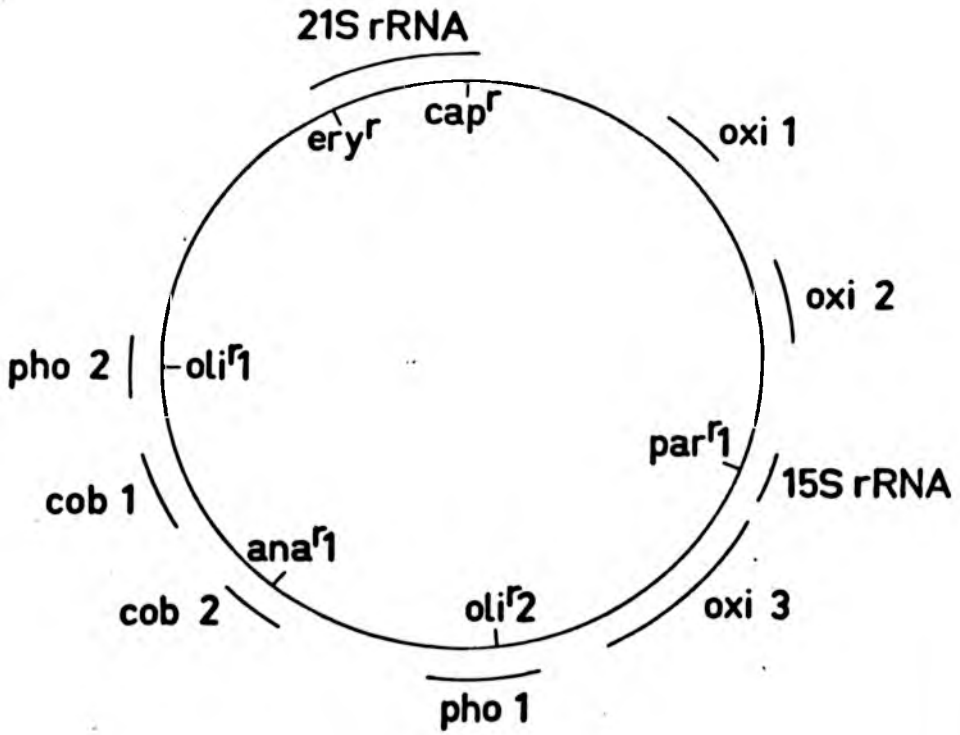

Deletion of such genes may be correlated with a loss of hybridization of the radioactively labelled mitochondrial RNA to the mtDNA of the petite possessing the lesion (Cohen and Rabinowitz, 1972; Cohen et al, 1972; Faye et al., 1973; Casey et al., 1974a). To date, only one mit$^-$ region has been positively assigned a structural or regulatory function. As previously discussed, mutations in the oxi 1 segment have been shown to result in the loss of subunit II of cytochrome oxidase (Cabral et al., 1977, 1978). Thus oxi 1 mutations are believed to be located within the structural gene for this protein.

In addition, one mutant possessing a lesion that maps in the region designated pho 2 (Tzagoloff et al., 1976c) is reported to contain a deletion of the amino terminal end of subunit 9 of the OS-ATPase complex (A. Tzagoloff, personal communication). Since this lesion has been positioned close to the oli^{r}1 drug resistance locus (Tzagoloff et al., 1976c), a mutation which has been shown to affect the association/dissociation behaviour of subunit 9 (Tzagoloff et al., 1976a), it is plausible that this pho 2 mutation is located within the structural gene for this polypeptide.

1:13 Aims of this Research

The primary goal of this research was the isolation of mitochondrially-inherited yeast mutants, specifically defective in one or more components of the OS-ATPase complex. When this work was instigated only one such mutant, designated M6-239 (Tzagoloff et al., 1975c), had been described. Today there still exists a scarcity of ATPase mutants. More recently,

Foury and Tzagoloff (1976b) have reported the isolation of three other mutants that map, along with M6-239, in the region designated pho 1. However, as already pointed out, two of these appear only partially defective in oxidative phosphorylation; retaining the ability to grow on non-fermentable media, albeit with a reduced efficiency. These mutants might be expected to be of only limited value in future biochemical investigations. Additionallly, a mutant lacking an oligomycin-sensitive ATPase and mapping close to the $oli^{r}1$ drug resistance locus has been reported (Tzagoloff et al., 1976c). A small number of less well characterized mit^{-} mutants mapping near the $oli^{r}1$ and $oli^{r}2$ loci have also been isolated (Bolotin-Fukuhara et al., 1977; P. Slonimski, personal communication).

This author believes that mitochondrially inherited ATPase mutants would be invaluable in:

i) categorically determining the number and identity of the component subunits of the complex specified by mtDNA. Biogenesis experiments (Tzagoloff and Meagher, 1972) have indicated that four such polypeptides are synthesized on mitoribosomes and therefore, tenably, mitochondrially coded.

ii) Such mutants might, in addition, yield information concerning the assembly and integration of the OS-ATPase complex into the mitochondrial inner membrane. A more complete understanding of the catalytic reaction(s) carried out by this enzyme might also shed light on the intricate process of oxidative phosphorylation itself.

iii) A collection of ATPase mutants might disclose the presence, if any exist, of regulatory genes responsible for the biosynthesis of this complex.

iv) Finally, they would be of great value in further elucidating the role of the mitochondrial genome and contribute significantly to the compilation of a complete map of mtDNA.

Such a project was also felt in line with, and a logical extension of, previous biochemical-genetic approaches employed successfully in this laboratory (Griffiths *et al.*, 1972; 1974, 1975; Griffiths and Houghton, 1974).

CHAPTER TWO THE ISOLATION AND PRELIMINARY GENETIC CHARACTERIZATION OF MIT$^-$ MUTANTS OF SACCHAROMYCES CEREVISIAE

2:1 Introduction

The first selection procedure to be successfully employed, in the isolation of specific respiratory-deficient mitochondrial mutants of yeast, was that of screening for *in vivo* mitochondrial protein synthesis (Tzagoloff *et al.*, 1975a). As previously discussed, this method permitted the isolation and subsequent genetic and biochemical characterization of a unique range of mutants, now referred to as mit$^-$ (Slonimski and Tzagoloff, 1976). Mit$^-$ mutants defective in the biosynthesis of coenzyme QH_2-cytochrome *c* reductase, cytochrome oxidase and the OS-ATPase complex have been obtained (Tzagoloff *et al.*, 1975c). A second series of specific mitochondrial mutants, designated syn$^-$ (Tzagoloff *et al.*, 1976c), has also been described. In contrast to mit$^-$ mutants, syn$^-$ mutants lack mitochondrial protein synthesis. However, their capacity to either revert to wild-type and/or restore the rho$^+$ state in crosses to suitable rho$^-$ tester strains distinguishes them from pleiotropic rho$^-$ petites. Syn$^-$ mutants possess lesions in the mitochondrial rRNA and tRNA genes (Tzagoloff *et al.*, 1976c; Trembath *et al.*, 1977).

A number of other methods have been utilized in the isolation of mit$^-$ and syn$^-$ mutants and these are outlined below.

Some workers (Rytka *et al.*, 1976; Bolotin-Fukuhara *et al.*, 1977) have looked for the appearance of grande diploid progeny following crosses between induced respiratory deficient strains

and a series of rho$^-$ testers. Since established rho$^-$ strains are unable to restore the rho$^+$ state when crossed to other rho$^-$ strains (Wright and Lederberg, 1957; Roodyn and Wilkie, 1967; Slonimski et al., 1968), this method has permitted the isolation of both mit$^-$ and syn$^-$ mutants. Both of these classes of specific mitochondrial mutant give rise to grande diploid progeny when crossed to rho$^-$ petites that retain the sequence that is defective in the mit$^-$ or syn$^-$ mutant (Slonimski and Tzagoloff, 1976; Tzagoloff et al., 1976c). In addition, by selecting several overlapping rho$^-$ testers, the approximate location of the newly-induced mutation may be immediately established on a circular map of mtDNA (Bolotin-Fukuhara et al., 1977).

In Linnane's laboratory the ability of some, but not all, strains to utilize galactose for growth has been exploited in an attempt to enrich for mit$^-$ mutants at the expense of rho$^-$ mutants (Rytka et al., 1976). This procedure was conceived following a report that mitochondrial protein synthesis was necessary for the induction of the enzymes responsible for the metabolism of galactose (Puglisi and Algeri, 1971). After mutagenesis of a parental stock, respiratory deficient strains that were capable of growth on galactose were crossed to a series of rho$^-$ tester strains to ensure that the mutants were indeed mit$^-$ and to facilitate their mapping. It is important to point out however that this method is not rigorous, since a large number of mit$^-$ mutants are found to be unable to grow on galactose (Rytka et al., 1976).

A further method employed by some workers (Kotylak and Slonimski, 1976; Schweyen et al., 1978) and adopted in this thesis utilizes a parental strain that carries the nuclear

mutation pet 9 (op_1); the rho^- state being lethal in such stocks (Kováčová et al., 1968). An assumption that is implicit in this approach is that small modifications of mtDNA, for example mit^- mutations, do not result in cell death. Induced $op_1 mit^-$ mutants may then be selected for by crossing the mutagenized $op_1 rho^+$ stock to an $OP_1 rho^o$ tester strain; the appearance of respiratory incompetent diploids indicating the presence of a mitochondrial or, improbably, a dominant nuclear mutation.

This section deals with the isolation and subsequent preliminary genetic characterization of a series of $op_1 mit^-$ mutants.

2:2 Materials and Methods

a) Materials

Strains The genotypes and origins of strains used in this section is presented in Table 2:1.

Chemicals Adenine sulphate was obtained from B.D.H. Chemicals Ltd., Poole, England.

Media NO, N3 and WO (minimal media) are essentially as described by Lancashire (1974). The recipes for these are presented below.

NO 1% (w/v) Difco yeast extract
1% (w/v) Oxoid bacteriological peptone.
2% (w/v) glucose
0.05M Na^+ K^+ phosphate buffer pH 6.25 (10 mM Na_2HPO_4 and 40mM KH_2PO_4)

N3 As for NO media but substituting 3% (w/v) glycerol for glucose.

Table 2:1 List of Strains Employed in Chapter Two

Name	Genotype		Origin
	Nuclear	Mitochondrial	
D6	∝, arg, met	ω^+, rho^+	Dr. D. Wilkie
*D6/ETH1	∝, arg, met	rho^o	This Thesis
D603-3B	a, ade 2, his 2, pet 9 (op_1)	ω^+, rho^+	Dr. J. Haslam
M6-239	∝	ω^+, mit^- (pho 1)	Dr. A. Tzagoloff (Tzagoloff et al., 1975c; Foury and Tzagoloff, 1976b)
M7-40	∝	ω^+, mit^- (cob 1)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976; Tzagoloff et al., 1976b)
M9-3	∝	ω^+, mit^- (oxi 2)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976; Tzagoloff et al., 1975c)
M9-94	∝	ω^+, mit^- (oxi 1)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976)
M10-150	∝	ω^+, mit^- (oxi 3)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976)
M11-82	∝	ω^+, mit^- (oxi 3, pho 2)	Dr. A. Tzagoloff (Tzagoloff et al., 1976c)
M17-162	∝, met	ω^+, mit^- (cob 2)	Dr. A. Tzagoloff (Tzagoloff et al., 1976b)
M17-231	∝, met	ω^+, mit^- (cob 1)	Dr. A. Tzagoloff (Tzagoloff et al., 1976b)
M28-81	∝, met	ω^+, mit^- (pho 1)	Dr. A. Tzagoloff (Foury and Tzagoloff, 1976b)

* The mitochondrial genotype of D6/ETH1 is putatively designated rho^o. The method of petite induction and a determination of the suppressivity of this strain is presented in the Appendix.

WO 0.67% (w/v) Difco yeast nitrogen base (without amino acids)

2% (w/v) glucose

Other media employed in this chapter:

N1 As for NO media but substituting 1% (v/v) ethanol for glucose.

YEPD (2%) 1% (w/v) Difco yeast extract
1% (w/v) Oxoid bacteriological peptone.
2% (w/v) glucose

YEPD (5%) As for YEPD (2%) but containing 5% (w/v) glucose.

YEPD (1%) plus adenine
1% (w/v) Difco yeast extract
1% (w/v) Oxoid bacteriological peptone
1% (w/v) glucose
0.01% (w/v) adenine sulphate

Where appropriate media was solidified by the addition of 2.3% (w/v) Oxoid agar No. 3 prior to autoclaving.

b) General Methods

Sterilization: Sterilization was normally performed by autoclaving at 15 pounds per square inch pressure for 15 to 20 minutes. Pipettes were sterilized by heating, to 160°C, overnight.

Preparation of agar slopes: YEPD (2%) media containing 0.01% (w/v) adenine sulphate and 2.3% (w/v) Oxoid agar No. 3 was heated over a bunsen to dissolve the agar. Bijou bottles or 2 dram silica glass vials were then approximately half-filled, capped and sterilized. The bottles or vials were then tilted at a suitable angle and the media allowed to cool. Slopes were

normally dried at room-temperature for apprximately 7 days.

Subcloning and subculturing: Newly isolated strains were subcloned twice on NO or N3 solid media before storage on agar slopes at approximately 4°C. Strains were routinely subcultured every six to twelve months.

Starter cultures: Starter cultures were normally grown for 24 to 48 hours in 50 ml NO liquid media in 250 ml conical flasks on a rotary shaker.

Lawns for mating: Cells, grown as described above, were harvested and washed twice in sterile distilled water. Approximately 2×10^7 cells were then plated onto the appropriate media.

Temperature: Unless otherwise stated, all incubations were performed at $29 \pm 1^{\circ}C$.

c) Special Methods

Manganese mutagenesis of D603-3B: Approximately 5×10^5 cells of a late logarithmic phase culture of D603-3B, grown in YEPD (5%) liquid media, were inoculated into 50 ml YEPD (5%) containing 8 mM manganese chloride in a 250 ml conical flask. After 7 days incubation on a rotary shaker, the cells were harvested, washed once in sterile YEPD (5%) and plated on YEPD (2%) solid media to give approximately 100 cells per plate.

Selection of $op_1 mit^-$ strains: After 4 to 5 days growth, the resultant colonies were velveteen replica plated (Lederberg and Lederberg, 1952) onto WO solid media, overlayed with a lawn of D6/ETH1. Diploid patches, arising after a further 3 days, were then replica plated onto N1 solid media to determine respiratory competence.

op_1mit^- mutants were scored after 2 to 3 days growth, picked from the YEPD (2%) master plates, subcloned twice and stored on agar slopes. Finally, op_1mit^- mutants were retested for the presence of the mitochondrial mutation.

Restoration of the rho^+ state in $op_1mit^- \times OP_1mit^-$ crosses: op_1mit^- mutants were patched out from agar slopes onto NO solid media in gridded patterns. After 2 days incubation the mutants were velveteen replica plated onto separate lawns of OP_1mit^- tester strains and D6/ETH1 on NO solid media. The resultant diploids were then replica plated, after a further two days, onto both N1 and N3 solid media. Respiratory competence was usually scored after 2 days incubation.

Cytochrome absorption spectra of op_1mit^- mutants: Cells were inoculated from agar slopes into 100 ml YEPD (1%) plus adenine in 500 ml conical flasks and incubated on a rotary shaker. After 3 days the cells were harvested and washed twice in sterile distilled water. The cells were finally suspended at a concentration of 10^9 cells per ml in sterile 50% (v/v) glycerol. The spectra of samples, reduced by the addition of a few grains of sodium dithionite, were recorded on an SP1800 spectrophotometer equiped with a chart recorder against a Kleenex tissue blank.

Estimation of reversion frequencies of op_1mit^- mutants to op_1rho^+: Washed cells were spread onto NO solid media to give approximately 1000 - 2000 cells per plate. After 4 days growth colonies were velveteen replica plated onto WO solid media overlayed with a lawn of D6/ETH1. The resultant diploid patches were then replica plated, after a further 4 days, onto N3 solid media. Revertants were scored after 3 days.

2:3 Results and Discussion

A mutagenic protocol for the induction of mit^- mutants is presented along with a method for their detection and isolation. A haploid strain of S. cerevisiae possessing the nuclear mutation pet 9 (op_1) has been employed as the parental from which $op_1 mit^-$ mutants have been obtained. The rationale of this procedure is as follows. In strains carrying the op_1 mutation, the rho^- state is lethal (Kováčová et al., 1968). However, mit^- mutants are quite distinct from rho^- mutants, they retain the capacity for mitochondrial protein synthesis and are generally thought to result from very small modifications (in some cases point mutations) of mtDNA. It was therefore postulated that mit^- mutations might prove viable in strains also carrying the op_1 mutation. This being correct, detection of $op_1 mit^-$ mutants could be achieved by crossing a mutagenized $op_1 rho^+$ culture to an $OP_1 rho^o$ tester strain and subsequently analysing the resultant diploids for respiratory competence. Growth on non-fermentable media would indicate that the mitochondrial genotype of the haploid was rho^+. The production of respiratory incompetent diploids would suggest that a mitochondrial mutation of, conceivably, a nuclear dominant mutation, affecting mitochondrial function, had been induced. These two possibilities could then be readily distinguished by further genetic analysis. Syn^- mutants, since they phenotypically resemble rho^- mutants, were thought unlikely to prove viable in strains also possessing the op_1 mutation.

Manganese, in the form of manganese chloride, was chosen as mutagen since this metal had been recently shown to be extremely effective in inducing mitochondrial antibiotic-resistant

and rho^- mutations (Putrament *et al.*, 1973). Further, this chemical had been used with great success in the generation of mit^- mutants in grande strains by Tzagoloff and coworkers (Tzagoloff *et al.*, 1975a, c). This mutagen is suggested to act by interfering with mtDNA replication, through an interaction with the mitochondrial DNA polymerase (Putrament *et al.*, 1973). In addition, since several generations growth is necessary for the expression of newly-induced mitochondrial mutations, it was decided to mutagenize the $op_1 rho^+$ parental strain under growing conditions. The treated culture would, as a result, consist of $op_1 rho^+$ parental cells (possibly possessing additional nuclear mutations), non-viable $op_1 rho^-$ cells and, hopefully, $op_1 mit^-$ cells. The protocol outlined in section 2:2 is based upon a suggestion by Schweyen (personal communication).

The treatment described has been used successfully on three separate occasions. In all cases $op_1 mit^-$ mutants were found and with approximately equal frequencies. In Table 2:2 the number of independently isolated mutants and the total number of strains screened is presented. This frequency is considerably lower than that reported by Tzagoloff and coworkers (Tzagoloff *et al.*, 1975c) for cytoplasmically-inherited mutants that retain the capacity for mitochondrial protein synthesis. Their results indicate a frequency of 1 in 20. This disparity may reflect differences in the inherent characteristics of the parental strains or in the mutagenic protocols employed.

Occasionally when selecting for $op_1 mit^-$ mutants, sectored colonies were encountered in which some cells appeared $op_1 mit^-$ whilst others $op_1 rho^+$. In such cases the haploid colonies were picked from the master plates, subcloned once and usually four

Table 2:2 Frequency of Isolation of Putative op_1mit^- mutants

Approximate number of colonies screened	6,400
Number of independently isolated putative op_1mit^- mutants	21
Approximate frequency of isolation of putative op_1mit^- mutants	1 in 300

D603-3B was mutagenized and op_1mit^- mutants selected as described in Section 2:2.

clones retested by crossing again to D6/ETH1. Only one, out of two or more op_1mit^- clones was regarded as an independently isolated op_1mit^- mutant. Subsequent to their isolation, all op_1mit^- mutants were subcloned twice on NO solid media and retested to ensure the presence of the mit^- mutation.

In order to preliminarily characterize the mit^- mutations, newly-isolated op_1mit^- mutants were crossed to a series of OP_1mit^- tester strains possessing lesions in either oxi 1, oxi 2, oxi 3, cob 1, cob 2, pho 1 or pho 2. The resultant diploids were then analysed for restoration of the rho^+ state. The results are presented in Table 2:3. As may be observed, the majority of op_1mit^- mutants were found to possess lesions in the region of oxi 3. Thirteen strains, out of a total of twenty-one, failed to produce respiratory competent diploids when crossed to M10-150 and, in one instance, to M11-82. These strains did however give rise to respiratory competent progeny when mated to mit^- mutants possessing lesions in regions other than oxi 3. In addition, two mutants have been labelled "leaky oxi 3", since poor to moderate growth was detected on non-fermentable media when D603-3B/12 was crossed to M10-150 and M11-82 and D603-3B/17 was crossed to M10-150. A similar degree of growth was also observed when these two op_1mit^- mutants were mated to D6/ETH1, a putative rho^0 tester. It is important to point out that the amount of growth detected was substantially less than that normally found when op_1rho^+ strains are crossed to D6/ETH1. Fifteen mutants are thus proposed to be defective in the biosynthesis of cytochrome oxidase. Such mutants may be unable to synthesize one, or possibly more than one, subunit of this enzyme complex. Alternatively, synthesis of a defective subunit might result in the impaired integration of cytochrome oxidase into the inner mitochondrial membrane. oxi 3 mutations might also modify

Table 2:3 **Restoration of the Rho$^+$ State in Crosses Between Putative op$_1$mit$^-$ Mutants and OP$_1$mit$^-$ Tester Strains.**

Strains*	M6-239 (pho 1)	M7-40$^+$ (cob 1)	M9-3 (oxi 2)	M9-94 (oxi 1)	M10-150 (oxi 3)	M11-82 (oxi 3 + pho 2)	M17-162 (cob 2)	M17-231$^+$ (cob 1)	D6/ETH1	Mitochondrial Genotype
D603-3B	++	++	++	++	++	++	++	++	++	rho$^+$
D603-3B/1	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/2	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/3	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/4	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/5	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/6	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/7	++	++	++	++	-	-	++	++	-	oxi 3
D603-3B/8	++	++	++	++	++	++	++	++	-	unknown
D603-3B/9	++	++	++	++	++	++	++	++	-	unknown
D603-3B/10	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/11	++	++	++	++	++	++	++	++	-	unknown
D603-3B/12	++	++	++	++	+	+	++	++	+	"leaky oxi 3"
D603-3B/13	-	-	-	-	-	-	-	+	-	unknown
D603-3B/14	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/15	++	++	++	++	-	++	++	++	-	oxi 3

Continued.....

Table 2:3 Continued.

Strains*	M6-239 (pho 1)	M7-40+ (cob 1)	M9-3 (oxi 2)	M9-94 (oxi 1)	M10-150 (oxi 3)	M11-82 (oxi 3 + pho 2)	M17-162 (cob 2)	M17-231+ (cob 1)	D6/ETH1	Mitochondrial Genotype
D603-3B/16	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/17	++	++	++	++	+	++	++	++	+	"leaky oxi 3"
D603-3B/18	++	++	++	++	++	++	-	++	-	cob 2
D603-3B/19	+	+	++	+	+	+	+	+	+	unknown "leaky" mutation
D603-3B/20	++	++	++	++	-	++	++	++	-	oxi 3
D603-3B/21	++	++	++	++	+	N.T.	N.T.	N.T.	-	oxi 3

The experiment was performed as described in Section 2:2. Growth of the resultant diploids was usually scored on N1 and N3 solid media after 2 days. ++ indicates good growth, + indicates poor to moderate growth, - indicates no growth and N.T. indicates not tested. No significant growth of any $OP_1 mit^-$ lawn was observed on non-fermentable media.

* The parental strain D603-3B and all derived $op_1 mit^-$ mutants failed to exhibit growth on non-fermentable media.

+ M7-40 and M17-231 possess two independent mit^- mutations that map in the region designated cob 1. These two mutations may be discriminated by a series of rho^- tester clones (Tzagoloff et al., 1976b).

either directly or indirectly, possibly by a change in the conformation of the protein, the activity of this enzyme. Extensive biochemical analysis of oxi 3 strains is necessary to establish the effect of this type of lesion.

One op_1mit^- mutant, D603-3B/18, failed to produce respiratory competent diploids when crossed to M17-162. This strain was therefore assumed to possess a lesion in the cob 2 region of the mitochondrial genome. Two putative op_1mit^- mutants, D603-3B/13 and D603-3B/19, when crossed to OP_1mit^- testers and a presumptive rho^o strain, gave rise to progeny displaying an unusual pattern of growth. D603-3B/13 failed to produce respiratory competent diploids when crossed to D6/ETH1, a feature of op_1mit^- mutants. However, this strain also failed to produce respiratory competent diploids when crossed to seven out of the eight OP_1mit^- testers. When mated to the remaining OP_1mit^- mutant M17-231, which possesses a lesion in the region of cob 1, poor to moderate growth on N1 and N3 solid media was observed. D603-3B/19 is believed to carry a similar, but "leaky", mutation to that of D603-3B/13. When crossed to seven OP_1mit^- testers and the putative rho^o, D603-3B/19 exhibits poor to moderate growth. When crossed to M9-3, a mutant possessing a lesion in oxi 2, good growth on non-fermentable substrates is observed. It is not thought likely that these two strains carry lesions in six or so mit^- regions, since such mutants would closely resemble the lethal op_1rho^- mutants. However, this possibility cannot at present be excluded. It is conceivable that D603-3B/13 and D603-3B/19 are defective in their capacity for mtDNA recombination and/or expression. A third, and possibly more attractive, explanation is that these strains are defective in their ability to mate with other strains. Indeed, these mutants may prove to possess

additional nuclear, and not mit^-, mutations.

The three remaining mutants D603-3B/8, D603-3B/9 and D603-3B/11 were labelled mit^- because of their inability to restore the rho^+ state when mated to D6/ETH1. However respiratory competent diploids were produced when they were crossed to all of the $OP_1 mit^-$ tester strains. This test does not rule out the possibility that any of the above mutants possess lesions in one or more of the established regions (i.e. oxi 1, oxi 2, oxi 3, cob 1, cob 2, pho 1 or pho 2). On the other hand one, two or all of these $op_1 mit^-$ mutants may have lesions in, as yet, undiscovered regions of the mitochondrial genome.

In an attempt to eliminate the possibility that either D603-3B/8, D603-3B/9 or D603-3B/11 possess a mutation in one or more of the mit^- regions responsible for the biosynthesis of cytochrome *b* and/or *a* + a_3, the room temperature cytochrome absorption spectra of these and other mutants were recorded. The spectra of D603-3B, D603-3B/2 (mutated in the oxi 3 region), D603-3B/8, D603-3B/9 , D603-3B/11 and D603-3B/18 (mutated in the cob 2 region) are presented in Figure 2:1. The cell concentrations in each case were approximately 10^9 cells per ml. It should be emphasized that these traces do not represent quantitative estimates of the cytochrome contents. That is to say, the absorbance of one sample at one particular wavelength should not be compared to the absorbance of a second sample at the same wavelength. The reason for this is that many $op_1 mit^-$ mutants are unstable and give rise to $op_1 rho^-$ cells (W.E. Lancashire, personal communication). Although, as previously discussed, the rho^- condition is lethal in cells possessing the nuclear op_1 mutation, $op_1 rho^-$ cells may divide for a limited number of

Figure 2:1. Room-Temperature Cytochrome Absorption Spectra of Selected $op_1 mit^-$ Mutants and the $op_1 rho^+$ Parental Strain.

The experiment was performed as described in section 2:2. The cell concentration of each sample was approximately 10^9 cells per ml.

Absorbance

generations. If the frequency of conversion of op_1mit^- to op_1rho^- is high, then the number of op_1rho^- cells in the sample may be significant. Further, since op_1rho^- cells will presumably lack spectrally detectable cytochromes b and $a + a_3$, these will have a direct effect on the resultant absorption spectra.

As may be observed in Figure 2:1 the parental strain used in this study, D603-3B, displays peaks at 551 and 603 nm and a shoulder at 562nm. These maxima correspond to the α bands of cytochromes c, $a + a_3$ and b respectively. D603-3B/2, which possesses a mutation in the region of oxi 3, clearly lacks a peak at 603 nm, confirming a defect in the biosynthesis of cytochrome oxidase. D603-3B/8 and D603-3B/9 both possess spectrally detectable levels of cytochromes c, b and $a + a_3$, whilst D603-3B/11 lacks a cytochrome oxidase absorbance at 603 nm. Therefore, of the three op_1mit^- mutants that were able to restore growth, on non-fermentable substrates, in crosses with all OP_1mit^- tester strains, one, D603-3B/11, appears to have a deficiency in the biosynthesis of cytochrome oxidase. Thus it seems likely that this strain possesses a mutation in either oxi 1, oxi 2 or oxi 3. This lesion must however by non-allelic with the ones carried by M9-3, M9-94, M10-150 and M11-82. The possibility that D603-3B/11 possesses a mutation in another region of the mitochondrial genome, which directly or indirectly results in the loss of the absorbance peak at 603 nm, cannot be excluded.

D603-3B/8 and D603-3B/9 appear competent in the ability to synthesize cytochromes c, b and $a + a_3$. These mutants may possess lesions in regions of mtDNA responsible for biosynthesis of the oligomycin-sensitive ATPase complex. However, an alternative explanation, that D603-3B/8 and D603-3B/9 synthesize spectrally

generations. If the frequency of conversion of $op_1 mit^-$ to $op_1 rho^-$ is high, then the number of $op_1 rho^-$ cells in the sample may be significant. Further, since $op_1 rho^-$ cells will presumably lack spectrally detectable cytochromes b and a + a_3, these will have a direct effect on the resultant absorption spectra.

As may be observed in Figure 2:1 the parental strain used in this study, D603-3B, displays peaks at 551 and 603 nm and a shoulder at 562nm. These maxima correspond to the α bands of cytochromes c, a + a_3 and b respectively. D603-3B/2, which possesses a mutation in the region of oxi 3, clearly lacks a peak at 603 nm, confirming a defect in the biosynthesis of cytochrome oxidase. D603-3B/8 and D603-3B/9 both possess spectrally detectable levels of cytochromes c, b and a + a_3, whilst D603-3B/11 lacks a cytochrome oxidase absorbance at 603 nm. Therefore, of the three $op_1 mit^-$ mutants that were able to restore growth, on non-fermentable substrates, in crosses with all $OP_1 mit^-$ tester strains, one, D603-3B/11, appears to have a deficiency in the biosynthesis of cytochrome oxidase. Thus it seems likely that this strain possesses a mutation in either oxi 1, oxi 2 or oxi 3. This lesion must however by non-allelic with the ones carried by M9-3, M9-94, M10-150 and M11-82. The possibility that D603-3B/11 possesses a mutation in another region of the mitochondrial genome, which directly or indirectly results in the loss of the absorbance peak at 603 nm, cannot be excluded.

D603-3B/8 and D603-3B/9 appear competent in the ability to synthesize cytochromes c, b and a + a_3. These mutants may possess lesions in regions of mtDNA responsible for biosynthesis of the oligomycin-sensitive ATPase complex. However, an alternative explanation, that D603-3B/8 and D603-3B/9 synthesize spectrally

detectable yet enzymically inactive cytochromes, cannot be ruled out.

D603-3B/18 lacks both a peak at 603 nm and a shoulder at 562 nm. The spectrum clearly resembles that of a rho^- petite. This mutant is known to possess a lesion in the region of cob 2; how then do we account for the loss of absorbance at 603 nm? By analogy with the so-called box mutants, the synthesis of cytochrome oxidase may be highly sensitive to repression by glucose in this mutant (Pajot et al., 1976). Alternatively, D603-3B/18 may possess two or more mit^- mutations, one (a lesion in cob 2) affecting the biosynthesis of cytochrome b and another affecting the biosynthesis of cytochrome oxidase. Double mutants of this type have been described (Foury and Tzagoloff, 1976a). However, one observation is thought to be of prime importance to this discussion. If a suspension of D603-3B/18 is counted, diluted and plated on NO solid media, only 2 to 3% of the cells give rise to colonies (data not presented). The implication of this finding is that D603-3B/18 cultures contain a vast majority of $op_1 rho^-$ cells. If this is indeed the case, then the cytochrome spectrum of this $op_1 mit^-$ mutant would be obscured by the high frequency of $op_1 rho^-$ cells. Thus it is not possible to state categorically whether D603-3B/18 possesses one or more mit^- mutations.

It is also necessary to comment upon the instability of other $op_1 mit^-$ mutants, and of the $op_1 rho^+$ parental to $op_1 rho^-$. D603-3B, D603-3B/2, D603-3B/8, D603-3B/9 and D603-3B/11 do not exhibit an exceptionally low viability when counted and plated on NO solid media, in contrast to D603-3B/18 (data not presented).

Finally it should be noted that the reversion frequency of

each $op_1 mit^-$ mutant, to $op_1 rho^+$, was determined on the same sample from which the cytochrome spectrum was obtained. This estimation was necessary in order to eliminate the possibility that the presence of $op_1 rho^+$ cells, within the sample, had contributed to the observed spectrum. In the case of D603-3B/2, D603-3B/11 and D603-3B/18 this is clearly not the case, since none of these $op_1 mit^-$ mutants exhibited a full complement of cytochromes. However, D603-3B/8 and D603-3B/9 were shown to possess absorbances corresponding to cytochromes *c*, *b* and *a* + a_3.

The estimation of the reversion frequency of $op_1 mit^-$ to $op_1 rho^+$ is hampered by the fact that $op_1 rho^+$ cells themselves are unable to multiply on non-fermentable media, due to the presence of the op_1 mutation. Thus, to detect $op_1 rho^+$ revertants, cells must be crossed to an $OP_1 rho^0$ tester strain and the resultant diploids analysed for respiratory competence. Obviously, this method limits the number of cells that may be tested. Of some 1500 to 3000 cells, of both D603-3B/8 and D603-3B/9, tested no obvious $op_1 rho^+$ revertants were detected. It is firmly believed that revertants occurring at a frequency lower than this would not significantly contribute to the observed absorption spectrum.

D603-3B/8 and D603-3B/9 were therefore credited with the presence of spectrally detectable, although arguably inactive, cytochromes *c*, *b* and *a* + a_3. These two $op_1 mit^-$ mutants were therefore thought worthy of further study.

2:4 Summary

1) A mutagenesis procedure, employing manganese, for the induction of mit^- mutants in an $op_1 rho^+$ strain of *S. cerevisiae*

is described, along with a method for their selection. Twenty one independently isolated mutants have been obtained as a result.

2) By a combination of mit^- x mit^- crosses and room-temperature cytochrome absorption spectra these mutants have been analysed. Sixteen have been found to be defective in the biosynthesis of cytochrome oxidase and one in the biosynthesis of cytochrome b. Two strains, possessing unidentified lesions, exhibit peculiar properties in mit^- x mit^- crosses. The remaining two mutants were capable of restoring respiratory competence in crosses with all $OP_1 mit^-$ tester strains.

3) These latter two mutants, D603-3B/8 and D603-3B/9 , possess spectrally detectable levels of cytochromes c, b and a + a_3 and have been retained for further genetic characterization.

CHAPTER THREE FURTHER GENETIC CHARACTERIZATION OF TWO MIT$^-$ MUTANTS, D603-3B/8 AND D603-3B/9.

3:1 Introduction

On the basis of the results obtained and presented in the previous chapter, two op$_1$mit$^-$ mutants (D603-3B/8 and D603-3B/9) were selected for intensive study. These mutants were found to be incapable of yielding respiratory competent diploids when crossed to a putative rho^o tester strain and, as a result, were tentatively classified as mit$^-$. D603-3B/8 and D603-3B/9 were capable of restoring the rho$^+$ state when mated to eight OP$_1$mit$^-$ mutants possessing lesions in either oxi 1, oxi 2, oxi 3, cob 1, cob 2, pho 1 or pho 2. However, as previously discussed, these results do not preclude the mutations present in D603-3B/8 and D603-3B/9 from being located within one of the above regions. In addition, both of these mutants exhibited spectrally detectable levels of cytochromes *c*, *b* and *a* + *a*$_3$. Mit$^-$ mutants deficient in coenzyme QH_2-cytochrome *c* reductase or cytochrome oxidase have been shown to lack absorption maxima corresponding to cytochromes *b* and *a* + *a*$_3$ respectively (Tzagoloff *et al.*, 1975*c*). This observation implies that the mutations carried by D603-3B/8 and D603-3B/9 are not located within regions of mtDNA specifying cytochrome biosynthesis. It is plausible that these mutants carry lesions in regions that code for components of the OS-ATPase complex or, as yet, undiscovered mitochondrial functions.

It is clear that D603-3B/8 and D603-3B/9 are not mutated in any of the mitochondrial rRNA or tRNA genes. Such mutants, designated syn$^-$ (Tzagoloff *et al.*, 1976*c*), would be incapable of

mitochondrial protein synthesis and would thus lack cytochromes b and a + a_3. In addition, since syn^- mutants are phenotypically rho^-, it is believed that combination with the nuclear op_1 mutation would prove lethal.

It was now felt important to further establish the mitochondrial nature of these two mutations and their relationship to one another i.e. to test for allelism.

3:2 Materials and Methods

a) Materials

Strains The genotypes and origins of strains used in this section are presented in Table 3:1.

Chemicals: Suc digestif d'Helix pomatia was obtained from Industrie Biologique Française, Gennevilliers, France.

Media: YP10 and WO (10%) are as described by Kotylak and Slonimski (1976). PSP (presporulation media) and SP (sporulation media) are as described by Lancashire (1974). YPD is as described by Tzagoloff and coworkers (Tzagoloff et al., 1975d). The recipes of these are presented below.

YP10 1% (w/v) Difco yeast extract
1% (w/v) Oxoid bacteriological peptone
10% (w/v) glucose

WO (10%) 0.67% (w/v) Difco yeast nitrogen base (without amino acids)
10% (w/v) glucose

YPD 1% (w/v) Difco yeast extract
2% (w/v) Oxoid bacteriological peptone

Table 3:1. **List of Strains Employed in Chapter Three.**

Name	Genotype		Origin
	Nuclear	Mitochondrial	
D603-3B	a, ade 2, his 2, pet 9 (op_1)	ω^+, rho^+	Dr. J. Haslam
D603-3B/2	a, ade 2, his 2, pet 9 (op_1)	ω^+, mit^- (oxi 3)	This Thesis
D603-3B/8	a, ade 2, his 2, pet 9 (op_1)	ω^+, mit^- (unknown)	This Thesis
D603-3B/9	a, ade 2, his 2, pet 9 (op_1)	ω^+, mit^- (unknown)	This Thesis
D603-3B/11	a, ade 2, his 2, pet 9 (op_1)	ω^+, mit^- (unknown)	This Thesis
D603-3B/18	a, ade 2, his 2, pet 9 (op_1)	ω^+, mit^- (cob 2)	This Thesis
D6	α, arg, met	ω^+, rho^+	Dr. D. Wilkie
*D6/ETH1	α, arg, met	rho^o	This Thesis
D22	a, ade 2	ω^+, rho^+	Dr. D. Wilkie
*D22/ETH1	a, ade 2	rho^o	This Thesis

Table 3:1 (continued). List of Strains Employed in Chapter Three.

Name	Genotype: Nuclear	Genotype: Mitochondria	Origin
D41	∝, ura, his	ω^+, rho^+	Dr. D. Wilkie
IL8-4D	∝, his 1	ω^+, rho^+, cap^r-321, ery^r-514	Prof. P.P. Slonimski (Avner et al., 1973)
DL211		mit^- (oxi 3)	This Thesis
(A diploid segregant obtained by crossing D603-3B/2 to D6)			
DL223		mit^- (unknown)	This Thesis
(A diploid segregant obtained by crossing D603-3B/8 to D6)			
DL224		mit^- (unknown)	This Thesis
(A diploid segregant obtained by crossing D603-3B/9 to D6)			
D273-10B	∝	ω^+, rho^+	Dr. A. Tzagoloff (Tzagoloff et al., 1975c, d)
M6-239	∝	ω^+, mit^- (pho 1)	Dr. A. Tzagoloff (Tzagoloff et al., 1975c; Foury and Tzagoloff, 1976b)
M10-150	∝	ω^+, mit^- (oxi 3)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976)

• The mitochondrial genotypes of D6/ETH1 and D22/ETH1 are putatively designated rho^0. The method of petite induction and a determination of the suppressivity of these strains is presented in the Appendix.

Table 3:1 (continued). List of Strains Employed in Chapter Three.

Name	Genotype: Nuclear	Genotype: Mitochondria	Origin
D41	α, ura, his	ω^+, rho$^+$	Dr. D. Wilkie
IL8-4D	α, his 1	ω^+, rho$^+$, cap^r-321, ery^r-514	Prof. P.P. Slonimski (Avner et al., 1973)
DL211 (A diploid segregant obtained by crossing D603-3B/2 to D6)		mit$^-$ (oxi 3)	This Thesis
DL223 (A diploid segregant obtained by crossing D603-3B/8 to D6)		mit$^-$ (unknown)	This Thesis
DL224 (A diploid segregant obtained by crossing D603-3B/9 to D6)		mit$^-$ (unknown)	This Thesis
D273-10B	α	ω^+, rho$^+$	Dr. A. Tzagoloff (Tzagoloff et al., 1975c, d)
M6-239	α	ω^+, mit$^-$ (pho 1)	Dr. A. Tzagoloff (Tzagoloff et al., 1975c; Foury and Tzagoloff, 1976b)
M10-150	α	ω^+, mit$^-$ (oxi 3)	Dr. A. Tzagoloff (Slonimski and Tzagoloff, 1976)

• The mitochondrial genotypes of D6/ETH1 and D22/ETH1 are putatively designated rho^o. The method of petite induction and a determination of the suppressivity of these strains is presented in the Appendix.

2% (w/v) glucose

PSP 0.5% (w/v) Difco yeast extract
0.2% (w/v) potassium dihydrogen orthophosphate
0.2% (w/v) ammonium sulphate
2% (w/v) glucose

SP 0.25% (w/v) Difco yeast extract
0.1% (w/v) glucose
0.1M potassium acetate

NO, N1, N3 and WO media are as described in section 2:2.

b) General Methods

These are as described in section 2:2.

c) Special Methods

Mitotic segregation of mit$^-$ mutations: The method employed was essentially that described by Kotylak and Slonimski (1976). Appropriate strains were inoculated, from agar slopes, into 10 ml YP10 liquid media in boiling tubes and incubated without shaking for approximately 40 hours. The cells were then harvested, washed once in sterile distilled water and 5 x 10^6 cells of the two parental strains inoculated into 5 ml YP10 liquid media in a preincubated 50 ml conical flask. After vigorous shaking on a water bath for 90 minutes, the cells were pelleted in a conical centrifuge tube using a BTL bench centrifuge, (swing out rotor) at 2,500 revolutions per minute (r.p.m.) for 5 minutes. The tube and contents were then incubated, without decanting the supernatant and without shaking, for 30 minutes. After discarding the supernatant, the cells were resuspended in 5 ml YP10. The suspension was then transferred to a sterile preincubated 50

ml conical flask and again shaken vigorously on a water bath. After 90 minutes, the cells were harvested as previously described, washed twice in sterile distilled water and finally resuspended in 5 ml distilled water. 0.05 ml of the cell suspension was then inoculated into 5 ml WO (10%) in a boiling tube and incubated without shaking. After approximately 70 hours the cells were collected, washed twice in sterile distilled water and plated on WO solid media to give approximately 50 to 100 cells per plate. After three days the resultant diploid colonies were velveteen replica plated onto NO and N1 or N3 solid media. The results were scored after an additional 2 days incubation.

Production of synchronous zygotes: Two methods were employed in the production of synchronous zygotes:

(i) the first is exactly the same method as described above for the demonstration of the mitotic segregation of mit^- mutations.

(ii) the second, essentially that outlined by Tzagoloff and colleagues (Tzagoloff et al., 1975d), is presented below. Appropriate strains were inoculated from agar slopes, into 10 ml YPD liquid media in boiling tubes and incubated without shaking for approximately 40 hours. After harvesting and washing once in distilled water, 5×10^6 cells of the two parental strains were inoculated into 5 ml YPD liquid media in a preincubated 50 ml conical flask. The flask and contents were then vigorously shaken on a water bath. After 90 minutes, the cells were pelleted in a conical centrifuge tube using a BTL bench centrifuge (swing-out rotor) at 2500 r.p.m. for 5 minutes and incubated, without decanting the supernatant and without shaking, for between 3½ and 5 hours. After this time, the supernatants were discarded and the pellets washed once in 5 ml sterile distilled water. The

cells were finally resuspended in a small volume (0.5 to 1 ml) of distilled water.

Sporulation of zygotes: The conditions employed, in an effort to sporulate zygotes produced by crossing $op_1 mit^-$ and $OP_1 mit^-$ mutants to an $OP_1 rho^+$ tester strain, are described below.

(i) For zygotes produced according to Kotylak and Slonimski (1976): Either 0.1 ml of the suspension of zygotes, in distilled water, was inoculated into 2 ml SP liquid media in a 100 ml conical flask or 0.2 ml was inoculated into 10 ml SP liquid media, in the same sized vessel. The zygotes were then incubated on a water bath, with vigorous shaking, for between 5 and 7 days.

(ii) For zygotes produced according to Tzagoloff et al., (1975d): 0.025 ml or 0.05 ml of a suspension of zygotes, in distilled water, was inoculated into either 2 ml SP liquid media or 10 ml liquid media in a 100 ml flask. The zygotes were then incubated on a water bath with vigorous shaking, for up to 5 days.

In all cases, zygotes were transferred to sporulation medium within approximately 3 hours of formation.

The degree of sporulation of the zygotes was estimated by microscopic examination.

Sporulation of diploids: (Lancashire, 1974) A loop of cells was inoculated into 10 ml PSP liquid media in a 100 ml conical flask. After vigorous shaking on a water bath for approximately 24 hours, one drop of the suspension was inoculated into 10 ml SP liquid media in a 100 ml conical flask. After similar incubation for a further 4 to 5 days, the degree of sporulation was estimated by microscopic examination.

Tetrad digestion and Spore germination: To 0.9 ml of the sporulated suspension in a sterile bijou bottle was added 0.1 ml of a 1:1 dilution of snail enzyme (suc digestif d'Helix pomatia) in sterile distilled water. The suspension was incubated with occasional vortexing, until digestion of tetrads was observed microscopically. The suspension was then serially diluted in sterile distilled water and 0.1 ml of each dilution plated out onto NO solid media. After 4 days incubation colonies were picked off, patched out onto fresh NO solid media in gridded patterns and incubated for a further 2 to 3 days.

Selection of $\propto OP_1 mit^-$ clones: Colonies, patched out as described above, were velveteen replica-plated onto N3 solid media and lawns of D603-3B, D6/ETH1 and D22/ETH1 on NO solid media. After 3 to 4 days growth, diploid patches were replica plated onto N3 solid media and growth of the haploids scored. Diploids were scored for respiratory competence after a further 3 days. Table 3:2 presents the pattern of growth predicted for the various possible cell types.

Discrimination between $\propto OP_1 mit^-$ and $\propto OP_1 rho^-$ colonies: Colonies that gave rise to growth patterns similar to those predicted for $\propto OP_1 mit^-$ and $\propto OP_1 rho^-$ cell types were normally subcloned once on NO solid media. Putative $\propto OP_1 mit^-$ strains plus D603-3B, D603-3B/2, D603-3B/8, D603-3B/9, D603-3B/18, D6/ETH1 and D22/ETH1 were then inoculated into 10 ml NO liquid media in boiling tubes and incubated for approximately 48 hours, without shaking. A drop of the $op_1 rho^+$, $op_1 mit^-$ and presumptive rho^o cultures was then mixed with a drop of a putative $\propto OP_1 mit^-$ culture on the surface of an NO plate. After 2 to 3 days

Table 3:2 **Predicted Growth Patterns of the Various Cell Types Following Sporulation and Digestion of Tetrads in Crosses of the Type: $aop_1 mit^-$ x $\propto OP_1 rho^+$.**

Cell Type	Growth of haploid on N3	Growth of haploid and/or diploid on N3 when crossed to:		
		D603-3B ($aop_1 rho^+$)	D6/ETH1 ($\propto OP_1 rho^o$)	D22/ETH1 ($aOP_1 rho^o$)
$aop_1 rho^+$	-	-	+	-
$aop_1 mit^-$	-	-	-	-
$aop_1 rho^-$		.....NON-VIABLE.....		
$aOP_1 rho^+$	+	+	+	+
$aOP_1 mit^-$	-	-	-	-
$aOP_1 rho^-$	-	-	-	-
$\propto op_1 rho^+$	-	-	-	+
$\propto op_1 mit^-$	-	-	-	-
$\propto op_1 rho^-$		.....NON-VIABLE.....		
$\propto OP_1 rho^+$	+	+	+	+
$\propto OP_1 mit^-$	-	+	-	-
$\propto OP_1 rho^-$	-	+	-	-

\+ indicates the presence of growth; - indicates the absence of growth.

incubation, the resultant diploids were velveteen replica plated onto N3 solid media. Respiratory competence was scored after a further 3 to 4 days.

Estimation of reversion frequencies of constructed mit$^-$ diploids to rho$^+$: Washed cells were spread on N3 solid media to give approximately 0.5 or 1.0 x 10^8 cells per plate. Revertants were usually scored after 3 to 4 days.

Statistical treatment of results: Standard errors were calculated according to the equation of Mather (1951):

$$\text{Standard error (S.E.)} = \sqrt{\frac{p\,(1 - p)}{n}}$$

where, for the purpose of this study,
p = the proportion of petite diploids or recombinant types and
n = the number of diploids analysed.

The frequencies of petite diploids or recombinant types are presented as per cent $\pm$ 95% confidence limits, calculated as $\pm$ 2 S.E. (Coen et al., 1970).

3:3 Results and Discussion:

In order to further establish the cytoplasmic nature of the mutations present in D603-3B/8 and D603-3B/9, their behaviour in crosses to rho$^+$ tester strains was studied. Rather than employ the standard procedure for demonstrating mitotic segregation of mitochondrial drug resistance mutations, i.e. mass mating on prototrophic selection media followed by quantitative replica plating of random diploid cells on drugged and undrugged

media (Coen et al., 1970; Avner and Griffiths, 1973b), the method of Kotylak and Slonimski (1976) was used. In this method preculture of the parental strains, zygote formation and the vegetative multiplication of these cells occurs in 10% glucose. These conditions were thought necessary since rho^+ cells are respiratory competent, unlike mit^-, and are therefore able to utilize the ethanol produced by the fermentation of glucose. As mit^- cells are incapable of using ethanol for growth, rho^+ cells would, if unchecked outgrow mit^- cells under aerobic conditions. This selective advantage of rho^+ cells may be minimized however, by the use of strong repressive conditions (i.e. high concentration of glucose) and the absence of forced aeration (Kotylak and Slonimski, 1976). Tables 3:3 and 3:4 present the results of crosses between D603-3B, D603-3B/2, D603-3B/8, D603-3B/9 and D603-3B/11 and two rho^+ tester strains, D6 and IL8-4D respectively.

With the exception of D603-3B/11 x D6 no clear mitotic segregations, of mit^- mutations, were observed. However, a general trend did emerge. The frequencies of respiratory incompetent diploid segregants were found to be greater in crosses involving D603-3B/2, D603-3B/8, D603-3B/9 and D603-3B/11 than in the control crosses with D603-3B. This is more evident in crosses to D6 than to IL8-4D. An increase in the number of respiratory deficient diploid segregants may arise through either a dominant nuclear mutation, that influences the petite frequency, or a cytoplasmic mutation. On the basis of these experiments alone it is not possible to exclude the first explanation. However, when other results are taken into consideration, for example restoration of the rho^+ state in crosses with mitochondrial

Table 3:3 Mitotic Segregation of Mit^- Mutations in Crosses of the Type: $op_1 mit^- \times OP_1 rho^+$.

Cross	Colonies scored on:		Number of	Frequency of
	NO	N3 and/or N1	petites	petites (%)
D603-3B x D6*	668	654	14	2.10 ± 1.11
D603-3B/2 x D6*	606	536	70	11.55 ± 2.60
D603-3B/8 x D6*	674	624	50	7.42 ± 2.02
D603-3B/9 x D6*	615	552	63	10.24 ± 2.45

Cross	Colonies scored on:		Number of	Frequency of
	NO	N3	petites	petites (%)
D603-3B x D6+	506	496	10	1.98 ± 1.24
D603-3B/11 x D6+	562	431	131	23.31 ± 3.57

The experiments were performed as described in section 3:2. Growth of diploids was scored, on NO and either N1 and/or N3 solid media, after 2 days.

+ Results obtained from a single experiment. * Results obtained from a combination of four individual experiments.

The petite frequencies (%) are presented ± 95% confidence limits, calculated as described in section 3:2.

Table 3:4 Mitotic Segregation of Mit^- Mutations in Crosses of the Type: $op_1 mit^- \times OP_1 rho^+$.

Cross	Colonies scored on: NO	N3	Number of petites	Frequency of petites (%)
D603-3B x IL8-4D	280	259	21	7.50 ± 3.15
D603-3B/2 x IL8-4D	349	317	32	9.17 ± 3.09
D603-3B/8 x IL8-4D	243	214	29	11.93 ± 4.16
D603-3B/9 x IL8-4D	260	210	50	19.23 ± 4.89

The experiments were performed as described in section 3:2. Growth of diploids was scored, on NO and N3 solid media, after 2 days. The results were obtained from a single experiment.

The petite frequencies (%) are presented ± 95% confidence limits, calculated as described in section 3:2.

mit^- mutants but not with a putative rho^o tester strain, the latter interpretation seems more likely.

The conditions of strong glucose repression and anaerobiosis discussed earlier and also by Kotylak and Slonimski (1976) appear to have been insufficient to nullify the selective advantage of grande diploid segregants in glucose liquid media. The observed results are thought to have arisen as a direct consequence of this selective advantage; rho^+ segregants being capable of growth on glucose and ethanol whereas mit^- segregants may only utilise glucose.

One further point, clearly establishing that a mitotic segregation had occurred, was the successful isolation of mit^- diploid segregants. Inspection of the diploid replicas on NO solid media revealed the presence of at least three types. In addition to the large, cream-coloured replicas and the small, white replicas, an intermediate size could also be detected. These patches, which were observed to occur at a very low frequency, appeared pale cream in colour. Only the large, cream-coloured patches were capable of growth on non-fermentable media (i.e. N1 or N3 solid media). These three types of replica were believed to correspond to rho^+, rho^- and mit^- diploid segregants respectively. It is important to point out that scoring of mit^- segregants, in crosses of the type $op_1 mit^-$ x $OP_1 rho^+$, was not possible. Although some mit^- segregants could be clearly identified, many replicas could not be categorically classified as either mit^- or rho^-. Since the segregation of mit^+ and mit^- alleles could not be accurately determined, the numbers of respiratory competent and respiratory incompetent diploids were recorded.

In an attempt to prove that a mitotic segregation of mit^+ and mit^- alleles had occurred, presumptive mit^- diploid segregants were isolated, subcloned and tested for the ability to spontaneously revert to rho^+. Three putative mit^- diploids: DL221, DL223 and DL224 obtained by crossing D6 to D603-3B/2, D603-3B/8 and D603-3B/9 respectively, were studied. A low frequency of revertants, of the order of 10^{-8} per cell, was detected in each case. Since rho^- cells never revert, DL221, DL223 and DL224 must be mit^-; the ability of many mit^- mutants to spontaneously revert being well established.

One final point is worthy of comment and that is the difference in the frequencies of respiratory competent and respiratory incompetent diploid segregants observed with the two rho^+ tester strains. A more significant difference in the frequency of petite diploids was found, in crosses of the type $op_1 mit^- \times OP_1 rho^+$ as compared to the control cross, when D6 was the tester strain. This may reflect a variation in the input of mtDNA copies from the two grande testers; the input of mit^+ alleles being greater for IL8-4D. This would result in an increase in the frequency of rho^+ segregants, at the expense of mit^- segregants. Differences in the growth rates of the various diploid segregants would also explain this disparity.

To summarize, the manganese-induced mutations present in D603-3B/8 and D603-3B/9 have been shown to be non-Mendelian by three criteria:

(i) inability to produce respiratory competent diploid progeny when crossed to a putative rho^o tester strain,

(ii) the demonstration, although not clear cut, of a mitotic segregation of mit^+ and mit^- alleles and

(iii) the ability to restore the rho^+ state, presumably by recombination, in crosses to other well characterized mit^- mutants possessing lesions in oxi 1, oxi 2, oxi 3, cob 1, cob 2, pho 1 and pho 2.

Attempts were now made to establish whether the cytoplasmic mutations, carried by these two strains, were allelic. Isolation of $\propto OP_1 mit^-$ meiotic segregants was therefore necessary. Subsequent crosses of the type $aop_1 mit^- \times \propto OP_1 mit^-$ followed by an analysis of the diploid progeny for respiratory competence, would indicate whether the two mutants possessed a common lesion. Clearly, $aop_1 mit^- \times \propto op_1 mit^-$ matings would be futile; the presence of the homozygous op_1 mutation in the resultant diploids would obscure any recombination that might occur between the two mitochondrial genomes. Irrespective of the presence of functional rho^+ copies of mtDNA, such diploids would be respiratory incompetent. D603-3B/8 and D603-3B/9 were therefore crossed to a grande tester strain and sporulation induced.

It is well established that mit^- diploids are incapable of sporulation. Zygotes however, possessing both functional and non-functional mitochondria, are competent (Tzagoloff et al., 1975c). Thus sporulation of the latter was effected in the search for $\propto OP_1 mit^-$ segregants.

Initially zygotes were produced essentially by the method of Kotylak and Slonimski (1976). However, sporulation of zygotes, generated by mating the $op_1 mit^-$ mutants to D6, proved unsuccessful. Whilst zygotes from the cross D603-3B x D6 regularly gave rise to a high proportion of tetrads, those produced by mating D603-3B/8 and D603-3B/9 to the same tester strain only infrequently sporulated. Induction of sporulation was normally attempted in 10 ml

SP liquid media. However 2 ml SP liquid media was also employed following a report (B. Dujon, personal communication) that a reduced volume of medium resulted in an increase in the frequency of tetrad formation. This was suggested to be due to enhanced aeration of the cell suspension.

The possibility that the nucleus was interferring with the sporulation process was eliminated on the basis of two observations.

Firstly, as already pointed out, zygotes arising from the cross D603-3B x D6 regularly gave rise to a high frequency of tetrads. Secondly, a randomly selected rho^+ segregant, from the cross D603-3B/9 x D6, also gave rise to a high proportion of tetrads after 6 days incubation in 10 ml SP liquid media.

Zygotes arising from the cross D603-3B/2 (known to contain a lesion in the region of oxi 3) x D6 were also subjected to the same treatments, in order to ascertain whether the lack of sporulation was a characteristic of all the $op_1 mit^-$ mutants isolated. Although occasional tetrads could be microscopically observed, the frequency appeared considerably lower than for D603-3B x D6 zygotes, but slightly higher than for D603-3B/8 and D603-3B/9 derived zygotes. This inability to sporulate efficiently thus seemed to be a property of all $op_1 mit^-$ mutants. Further, these frequencies of sporulation were considered unacceptably low for the successful isolation of $\propto OP_1 mit^-$ meiotic segregants.

Similar results to those discussed above were also obtained when zygotes were generated by the method outlined by Tzagoloff and coworkers (Tzagoloff *et al.*, 1975*d*). Zygotes produced by mating D603-3B/2, D603-3B/8 and D603-3B/9 to D6 only occasionally yielded tetrads. However, zygotes from the control cross, D603-3B x D6, readily sporulated. In an effort to demonstrate

that this absence of sporulation was not a consequence of the experimental procedures employed, two well characterized OP_1mit^- mutants (obtained from Dr. A. Tzagoloff) were subjected to the method described by him (Tzagoloff *et al.*, 1975*d*). M6-239 (possessing a lesion in the region of pho 1), M10-150 (possessing a lesion in the region of oxi 3) and the parental strain D273-10B were mated to D22. Good sporulation of the resultant zygotes was observed, in all three instances, after 5 days incubation in either 2 ml or 10 ml SP liquid media. The frequency of sporulated zygotes was found to be greater after incubation in 10 ml SP liquid media, contrary to the report by Dujon (personal communication). It was therefore concluded that the lack of sporulation was due neither to the experimental techniques employed, nor to the presence of a mit^- mutation within one parental strain. The problem appeared to result from some property of the op_1mit^- mutants or the grande tester strain, D6.

In a final effort to isolate $\propto OP_1mit^-$ meiotic segregants, D603-3B, D603-3B/8 and D603-3B/9 were mated to two different rho^+ tester strains (D41 and IL8-4D); zygotes were being produced by the method of Kotylak and Slonimski (1976). After incubation in 2 ml SP liquid media, D41 was found to increase the efficiency of sporulation of zygotes derived from D603-3B but had no detectable effect on zygotes derived from the two op_1mit^- mutants. IL8-4D further increased the efficiency of sporulation of zygotes obtained from D603-3B. A slight increase was also recorded with zygotes from D603-3B/9, although no noticeable difference was observed with zygotes from D603-3B/8. This problem of poor sporulation was never resolved.

Sporulated suspensions of cells from the four crosses:

D41 x D603-3B/8 and D603-3B/9 and IL8-4D x D603-3B/8 and D603-3B/9 were treated with snail enzyme and finally spread on complex glucose media as described in section 3:2. Resultant colonies were then mated to D603-3B, D6/ETH1 and D22/ETH1 and the diploid progeny scored for respiratory competence on N3 solid media. Those colonies that exhibited growth patterns characteristic of $\propto OP_1 mit^-$ and $\propto OP_1 rho^-$ strains (see Table 3:2) were retained. Since no suitable $aOP_1 rho^-$ strain, possessing the allele(s) defective in D603-3B/8 and D603-3B/9, was available, $\propto OP_1 mit^-$ and $\propto OP_1 rho^-$ mutants could not immediately be distinguished. In order to discriminate between these two cell types, the retained colonies were crossed to several $aop_1 mit^-$ mutants and the resultant diploids analysed for respiratory competence. The rationale being that $\propto OP_1 mit^-$ segregants would restore the rho^+ state when crossed to any $aop_1 mit^-$ strain except that possessing the same mit^- mutation. $\propto OP_1 rho^-$ mutants, however, would only be capable of restoring the rho^+ state if they retained the mit^+ allele that was defective in the $aop_1 mit^-$ tester. And, since spontaneous rho^- strains frequently possess large deletions of mtDNA, it was thought unlikely that $\propto OP_1 rho^-$ mutants would produce respiratory competent diploids when crossed to all $aop_1 mit^-$ testers.

A total of approximately 1300 colonies, from the four matings, were screened. From this, 29 were classified as $\propto OP_1 mit^-$ or $\propto OP_1 rho^-$. All of these proved to be $\propto OP_1 rho^-$, as judged by their inability to produce respiratory competent diploids when crossed to more than one $aop_1 mit^-$ tester strain. Since it was not possible to isolate $\propto OP_1 mit^-$ meiotic segregants of either D603-3B/8 or D603-3B/9, the question of allelism remains unanswered.

Two plausible explanations for the absence of $\propto OP_1 mit^-$ spores may be put forward. Firstly, there exists the possibility that all the observed tetrads originated from rho^+ diploids; one of the products of vegetative multiplication of the zygotes. If this were indeed the situation, then only rho^+ and spontaneously arising rho^- haploids would be detected among the meiotic progeny. This explanation implies that zygotes, containing both mit^- and rho^+ mitochondrial genomes, are incapable of sporulation. Although such zygotes have been induced to sporulate by several groups of workers (Tzagoloff et al., 1975c; Kotylak and Slonimski, 1976; R. J. Schweyen, personal communication), the postulated absence of this function, in the zygotes studied here, may be due to properties inherent in the parental strains. The second possibility is that the mit^- mutations present in D603-3B/8 and D603-3B/9 are very unstable, resulting in the production of a high proportion of rho^- cells. This instability would not be a major problem in haploid cells also possessing the nuclear op_1 mutation. However, when mated to a rho^+ strain and subsequently sporulated, the absence of the op_1 mutation in half of the meiotic progeny would permit the propagation of the rho^- state. It is therefore conceivable that $OP_1 mit^-$ cells, formed at meiosis, might rapidly produce $OP_1 rho^-$ progeny. The effect of this would be to continually reduce the frequency of $OP_1 mit^-$ cells within the population and therefore reduce the likelihood of their detection.

3:4 Summary

1.) No clear mitotic segregation of the mit^- mutations,

carried by D603-3B/8 and D603-3B/9, was observed upon crossing to two rho^+ tester strains. However, the ability to isolate mit^- segregants among the diploid progeny confirmed that such a segregation had occurred. The cytoplasmic nature of these lesions is therefore further established.

2.) No $\propto OP_1 mit^-$ meiotic segregants were detected after sporulation of zygotes produced by crossing D603-3B/8 and D603-3B/9 to two rho^+ tester strains. Thus tests, to confirm or refute the allelic nature of the two mit^- mutations, could not be attempted.

CHAPTER FOUR MAPPING OF THE MIT$^-$ MUTATIONS POSSESSED BY D603-3B/8 AND D603-3B/9.

4:1 Introduction

Since D603-3B/8 and D603-3B/9 were found to be capable of restoring the rho^{-+} state in crosses to several mit$^-$ tester strains, but not when mated to a putative rho^o tester, the mutations possessed by these strains were assumed to be mitochondrial. In the preceding chapter, the non-Mendelian character of these lesions was confirmed by the demonstration of a mitotic segregation of mit$^+$ and mit$^-$ alleles. However, attempts to prove or disprove the allelic nature of these two mutations were unsuccessful.

These mutants were considered worthy of further study since it seemed plausible that these strains carried lesions in regions of mtDNA controlling the biosynthesis of the OS-ATPase complex. It was therefore decided to map the mutations of D603-3B/8 and D603-3B/9 to other known mit$^-$ loci and subsequently to appropriate mitochondrial antibiotic resistance (ant^r) loci. This would permit the location of these lesions on a circular map of mtDNA. In addition, differences in the recombination frequencies of the two mutants with other well characterized strains might be reasonably argued to reflect the non-allelic nature of the mutations. Alternatively, similar frequencies would indicate a very close relationship and possible allelism.

4:2 Materials and Methods

a) Materials

Strains: The genotypes and origins of strains used in this section is presented in Table 4:1.

Chemicals: Oligomycin (a mixture of approximately 15% oligomycin A and 85% oligomycin B), erythromycin and L-arginine (free base) were obtained from Sigma London Chemical Company Ltd., Poole, England.

Media: NO, N3 and WO are as described in section 2:2. YPD, WO (10%) and SP are as described in section 3:2. Other media employed in this chapter:

Difco N3: 1% (w/v) Difco yeast extract
1% (w/v) Difco bacto-peptone
3% (w/v) glycerol
0.05 ml Na^+K^+ phosphate buffer, pH 6.25.

N30: As for Difco N3 media but also containing 3 μg/ml oligomycin.

N5E: As for Difco N3 media but also containing 5 mg/ml erythromycin.

WO plus Arginine: As for WO media but also containing 0.002% (w/v) L-arginine (free base).

Difco N3, N30 and N5E were solidified by the addition of 2.3% (w/v) Difco bacto-agar prior to autoclaving. WO plus arginine was solidified by the addition of 2.3% (w/v) Oxoid agar No. 3.

Table 4:1. List of Strains Employed in Chapter Four.

Name	Genotype: Nuclear	Genotype: Mitochondrial	Origin
D603-3B	a, ade 2, his 2, pet 9 (op_1)	ω^+, rho^+	Dr. J. Haslam
D6	α, arg, met	ω^+, rho^+	Dr. D. Wilkie
•D6/ETH1	α, arg, met	rho^o	This Thesis
D22/A15	a, ade 2	ω^+, rho^+ oli^r2-144	Dr. P. R. Avner (Avner and Griffiths, 1973a, b)
DL218-1C	α, arg	ω^+, rho^+ oli^r2-144	This Thesis
DL219-1A	α, arg	ω^+, rho^+	This Thesis
KL60-2C	α, aux	ω^+, rho^+ cap^r-321, ery^r-221, par^r1-454	Prof. P.P. Slonimski (Wolf et al., 1973)
+M5-16/2	α, ade 1	ω^+, mit^- (oxi 3)	Dr. G. Carignani (Tzagoloff et al., 1975c; Slonimski and Tzagoloff, 1976)
M17-231	α, met	ω^+, mit^- (cob 1)	Dr. A. Tzagoloff (Tzagoloff et al., 1976b)
M28-81	α, met	ω^+, mit^- (pho 1)	Dr. A. Tzagoloff (Foury and Tzagoloff, 1976b)

• The mitochondrial genotype of D6/ETH1 is putatively designated rho^o. The method of petite induction and a determination of the suppressivity of this strain is presented in the Appendix.

+ M5-16/2 is a spontaneous adenine auxotroph of M5-16.

b) General Methods:

These are described in section 2:2.

c) Special Methods:

Preparation of drug media: Oligomycin was added, from a 1 mg/ml stock solution prepared in methanol, after autoclaving and cooling Difco N3 media to 45 to 50°C.

Erythromycin was added, in the solid form and dissolved, after autoclaving and cooling Difco N3 media as described above.

Determination of mit^- x mit^- recombination frequencies: Appropriate strains were inoculated, from agar slopes, into 10 ml YPD liquid media in boiling tubes. After approximately 40 hours incubation, without shaking, the cells were harvested and washed once in sterile distilled water. 5×10^6 cells of the two parental strains were then inoculated into 5 ml YPD liquid media in a preincubated 50 ml conical flask and shaken vigorously for 90 minutes. The cells were then pelleted in a conical centrifuge tube using a BTL bench centrifuge (swing-out rotor) at 2,500 r.p.m. for 5 minutes. The tube and contents were then incubated, without decanting the supernatant and without shaking, for 4 hours. After discarding the supernatant, the cells were washed once in 5 ml sterile distilled water. The cells were then resuspended in 0.5 ml distilled water and 0.01 ml inoculated into 5 ml WO (10%) liquid media in a boiling tube. After approximately 70 hours incubation, without agitation, the cells were collected, washed twice in sterile distilled water and plated on WO solid media to give approximately 100 to 200 cells per plate. After 3 days the resultant diploid colonies were velveteen replica plated onto NO and N3 solid media. The frequency of rho^+

recombinants was scored after a further 2 days.

Construction of DL218-1C and DL219-1A: D6 and D22/A15 were inoculated, from agar slopes, into 10 ml NO liquid media in boiling tubes and incubated overnight without shaking. A drop of each culture was then mixed on the surface of a WO plate as described by Coen and coworkers (Coen et al., 1970). After three days incubation, a loop of cells was inoculated into 10 ml SP liquid media in a 100 ml conical flask. Subsequent sporulation, tetrad digestion and plating of spores on NO solid media was essentially as described in section 3:2. After three days incubation colonies were picked off, patched out onto fresh NO solid media in gridded patterns and incubated for a further three days. The patches were then velveteen replica plated onto N3, N30, WO, WO plus arginine and lawns of D6 and D22 on NO solid media. Suitable α,arg,rho$^+$, oli^{r}2 and α,arg,rho$^+$,oli^{s}2 colonies were selected after a further three days. These were subcloned twice on N3 solid media and retested. Finally one α,arg,rho$^+$,oli^{r}2 colony (designated DL218-1C) and one α,arg,rho$^+$,oli^{s}2 colony (designated DL219-1A) were retained and stored on agar slopes.

Determination of ant^r x mit$^-$ recombination frequencies: Appropriate strains were inoculated, from agar slopes, into 10 ml NO liquid media in boiling tubes and grown overnight without shaking. The cells were then harvested, washed twice and resuspended in approximately 10 ml sterile distilled water. A drop of each of the two parental strains was then mixed on the surface of a WO plate. After three days incubation a loop of cells was resuspended in 10 ml sterile distilled water, diluted and plated

on WO solid media to give approximately 100 to 150 cells per plate. After a further three days the resultant diploid colonies were velveteen replica plated onto Difco N3 and N30 or N5E solid media. The numbers of rho^+ oli^S2 or rho^+ ery^S recombinants were then scored after 3 to 4 days and expressed as a percentage of the total number of rho^+ diploids.

Estimation of the reversion frequencies of op_1mit^- mutants to op_1rho^+: As described in section 2:2.

Sporulation of diploids, tetrad digestion and spore germination and the statistical treatment of results: As described in section 3:2.

Estimation of the reversion frequencies of OP_1mit^- mutants to OP_1rho^+: The same method was employed as that described for the estimation of the reversion frequencies of constructed mit^- diploids to rho^+, in section 3:2.

4:3 Results and Discussion

In an effort to determine the location of the mutations carried by D603-3B/8 and D603-3B/9 on a circular map of mtDNA, the frequencies of rho^+ recombinants, generated by crossing these op_1mit^- mutants to three OP_1mit^- strains, were examined. The results, obtained by crossing these mutants to M5-16/2, M17-231 and M28-81, are presented in Table 4:2. Since high frequencies of reversion of op_1mit^- to op_1rho^+ and/or OP_1mit^- to OP_1rho^+ might influence the frequency of rho^+ recombinants detected, the number of rho^+ cells present in each parental culture, prior to

Table 4:2 **The Frequencies of Rho$^+$ Recombinants Generated in Crosses of the Type: $op_1 mit^-$ x $OP_1 mit^-$.**

Cross	Number of rho$^+$ recombinants	Total number of diploids scored	% rho$^+$ recombinants
+ D603-3B/8 x M5-16/2	41	620	6.61 ± 2.00
• D603-3B/8 x M17-231	349	1063	32.83 ± 2.88
° D603-3B/8 x M28-81	38	1574	2.41 ± 0.77
+ D603-3B/9 x M5-16/2	41	625	6.56 ± 1.98
• D603-3B/9 x M17-231	189	745	25.37 ± 3.19
° D603-3B/9 x M28-81	20	1559	1.28 ± 0.57

The experiment was performed as described in section 4:2. The frequencies of rho$^+$ recombinants are expressed as a percentage of the total number of diploids scored ± 95% confidence limits, calculated as described in section 3:2. The reversion frequencies of the $op_1 mit^-$ (to $op_1 rho^+$) and $OP_1 mit^-$ (to $OP_1 rho^+$) parental strains were estimated in each experiment and are presented in Table 4:3.

+ Results obtained from a single experiment. • Results obtained from a combination of two individual experiments. ° Results obtained from a combination of three individual experiments. M5-16/2 possesses a lesion in oxi 3; M17-231 possesses a lesion in cob 1; M28-81 possesses a lesion in pho 1.

mating, was estimated in every experiment. These frequencies are displayed in Table 4:3. As previously discussed, accurate determination of the spontaneous reversion frequencies of D603-3B/8 and D603-3B/9 is hampered by the presence of the nuclear recessive op_1 mutation. Revertant cells may not be directly detected, despite the existance of the rho^+ state, since the op_1 lesion itself prevents growth on non-fermentable substrates. In order to estimate the numbers of rho^+ cells in an op_1mit^- population, a sample must be crossed to a putative rho^o tester strain and the resultant diploids analysed for respiratory competence. In such a test op_1mit^- cells would not give rise to respiratory competent diploids whereas op_1rho^+ cells would do so. This estimation is further confused by the appearance of spontaneous revertants arising within respiratory incompetent replicas. In situations where growth on non-fermentable media cannot be clearly attributed to either the presence of an op_1rho^+ revertant replica or to a cluster of revertant clones within a respiratory incompetent replica, the former is assumed.

A much more accurate estimation of the reversion frequencies of OP_1mit^- strains may be obtained by plating large numbers (usually 0.5 to 1.0 x 10^8) of cells directly onto non-fermentable media and scoring for respiratory competent clones after a suitable period of incubation. However, even in such instances problems may be encountered. For example, in contrast to other OP_1mit^- strains tested, M17-231 gave rise to a completely heterogeneous sized population of revertants. Only those that were clearly discernable from the background lawn of tiny colonies were scored as revertants. Large colonies are believed to result from the vegetative multiplication, on N1 or N3 solid media, of

Table 4:3 Frequencies of Reversion of $op_1 mit^-$ and $OP_1 mit^-$ (to $op_1 rho^+$ and $OP_1 rho^+$ respectively) Parental Strains Employed in Mit^- x Mit^- Crosses.

Strain	Experiment	Frequency of reversion (per cell)
D603-3B/8	A	less than 1.4×10^{-3}
	B	5.7×10^{-4}
	C	less than 3.0×10^{-4}
D603-3B/9	A	less than 9.1×10^{-4}
	B	3.1×10^{-4}
	C	5.7×10^{-4}
M5-16/2	C	less than 3.9×10^{-9}
M17-231	A	1.5×10^{-6}
	B	1.5×10^{-6}
M28-81	A	6.5×10^{-8}
	B	1.5×10^{-8}
	C	2.1×10^{-8}

Reversion frequencies of $op_1 mit^-$ and $OP_1 mit^-$ strains (to $op_1 rho^+$ and $OP_1 rho^+$ respectively) were estimated, on the same samples employed in mit^- x mit^- recombination experiments, as described in section 4:2. The frequencies presented for each experiment were determined on samples of between 1000 and 3500 cells in the case of D603-3B/8 and D603-3B/9 and on 1.0 to 2.5 x 10^8 cells in the case of M5-16/2, M17-231 and M28-81.

The letters A, B and C refer to three separate experiments.

$OP_1 rho^+$ revertant cells present in the parental culture prior to plating. The background lawn of tiny colonies is thought to be due to reversion of $OP_1 mit^-$ cells after plating on non-fermentable media. Trace amounts of fermentable substrates, within N1 and N3 solid media, are not considered responsible for the observed background of small colonies since this phenomenon was not observed with other $OP_1 mit^-$ strains. In addition, the availability of low levels of fermentable substrates would be expected to result in slight but confluent growth, not individual colonies. The possibility that the mitochondrial mutation present in M17-231 is 'leaky' may also be ruled out, since this too would be predicted to result in confluent growth.

The observed reversion frequency of the mit^- mutation present in M5-16/2 is in good agreement with the reported value of less than 10^{-8} per cell (Tzagoloff *et al.*, 1975c). Reversion frequencies of M17-231 and M28-81 have not been published. Also in agreement with published observations (Foury and Tzagoloff, 1976b) is the finding that M28-81 is capable of growth, albeit less efficiently than rho^+ strains, on non-fermentable media.

Reversion frequencies, of the order set out in Table 4:3, were considered too low to interfere with the estimation of the numbers of rho^+ recombinants arising from $op_1 mit^- \times OP_1 mit^-$ crosses.

The results presented in Table 4:2, involving recombination in a non-polar region of the mitochondrial genome, clearly show a tight linkage of the mutations carried by D603-3B/8 and D603-3B/9 to that carried by M28-81. In addition, the mitochondrial mutations present in these two $op_1 mit^-$ strains appear loosely linked to the lesion carried by M5-16/2. Since the

percentages of rho^+ recombinants were found to be manifestly greater in crosses of D603-3B/8 and D603-3B/9 to M5-16/2 and M17-231 than in crosses to M28-81, the results may be interpreted as follows. The mitochondrial mutations induced in the two op_1mit^- mutants are considered to lie between the oxi 3 and cob 1 regions of the genome, close to and possibly within the pho 1 segment as depicted in Figure 4:1. From the results presented in Table 4:2 it is not possible to categorically state whether or not the mutations, carried by the two op_1mit^- mutants, are allelic with the mit^- mutation carried by M28-81. Whilst the detection of a low frequency of rho^+ recombinants in crosses of D603-3B/8 and D603-3B/9 with M28-81 might indicate a lack of allelism, it is conceivable that the observed rho^+ diploids arose by reversion of the mit^- determinants to mit^+. However, this is considered unlikely since, as shown in Table 4:3, the mitochondrial mutations present in these strains do not appear to have high frequencies of reversion. In conclusion the author feels that the mutations carried by D603-3B/8 and D603-3B/9 are distinct from that carried by M28-81. However, the possibility that the lesions, possessed by these op_1mit^- mutants, are located at other loci within the pho 1 region cannot be eliminated. It is also plausible that these mutations represent new loci in an, as yet, uncharacterized region of the mitochondrial genome, close to but distinct from the pho 1 region.

A comparison between the numbers of rho^+ recombinants observed in crosses of M5-16/2, M17-231 and M28-81 with D603-3B/8 and D603-3B/9 is also considered necessary. Essentially identical values were discovered in crosses of the two op_1mit^- mutants

Figure 4:1. Schematic Representation of the Location of the Mit$^-$ Mutations Possessed by D603-3B/8 and D603-3B/9 as Deduced by Mit$^-$ x Mit$^-$ Recombination Data.

The hatched areas represent the mit$^-$ regions designated: oxi 3, pho 1, cob 1, cob 2 and pho 2. The antibiotic resistance loci oli^{r}1 and oli^{r}2 are shown to occupy positions within the pho 2 and pho 1 segments respectively. Although evidence has been presented that the oli^{r}1 and pho 2 mutations lie within the same gene (Coruzzi *et al.*, 1978), this association has not been proven for the oli^{r}2 and pho 1 mutations. However, a close linkage of these latter two determinants has been established (Foury and Tzagoloff, 1976b; Somlo *et al.*, 1977).

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to M5-16/2. With M28-81, the frequencies of rho^+ recombinants did not differ significantly (i.e. the $\pm$ 95% confidence limits overlap). A difference was observed however with crosses involving M17-231; D603-3B/8 producing a greater frequency of rho^+ recombinants than D603-3B/9. Since the frequencies obtained by crossing the two $op_1 mit^-$ mutants to either M5-16/2 or M28-81 were similar, it is not clear why they differed when M17-231 was the tester strain. It should be pointed out that if the observed differences with M17-231 are genuine, then these results provide circumstantial evidence that the mit^- mutations carried by D603-3B/8 and D603-3B/9 are dissimilar. Identical, allelic mutations in strains possessing the same nuclear genotype would be expected to behave similarly.

Since the mutations in the two $op_1 mit^-$ strains had been located close to, and possibly within, the pho 1 region, the relationship of these lesions to two mitochondrial drug resistance loci was investigated. These studies would further establish the location of the mutations on a map of mtDNA. The $oli^r 2$ and ery^r drug resistance loci were chosen for this series of experiments; the former because of the close linkage of this marker to the pho 1 region (Foury and Tzagoloff, 1976b; Somlo et al., 1977) and the latter as an unlinked mitochondrial marker. The frequencies of oligomycin-sensitive and erythromycin-sensitive rho^+ diploids, expressed as a percentage of the rho^+ population, obtained by crossing D603-3B/8 and D603-3B/9 to DL218-1C and KL60-2C are presented in Tables 4:4 and 4:5 respectively.

As may be observed in Table 4:4, a low frequency of oligomycin-sensitive rho^+ recombinants was found when either $op_1 mit^-$ mutant was crossed to DL218-1C, a strain carrying the $oli^r 2$

Table 4:4 Frequencies of $oli^r2\ rho^+$ and $oli^s2\ rho^+$ Colonies Issued from Crosses of the Type: $op_1\ oli^s2\ mit^-$ x $OP_1 oli^r2\ rho^+$.

Cross	Number of $oli^r2\ rho^+$ colonies	Number of $oli^s2\ rho^+$ colonies	Number of rho^+ colonies scored	% $oli^s2\ rho^+$ recombinants
$^+$D603-3B ($oli^s2\ rho^+$) x DL218-1C ($oli^r2\ rho^+$)	369	256	625	40.96 ± 3.93.
$^\bullet$D603-3B/8 ($oli^s2\ mit^-$) x DL218-1C ($oli^r2\ rho^+$)	813	9	822	1.11 ± 0.73
$^\bullet$D603-3B/8 ($oli^s2\ mit^-$) x DL219-1A ($oli^s2\ rho^+$)	0	578	578	100.00
^{o}D603-3B/9 ($oli^s2\ mit^-$) x DL218-1C ($oli^r2\ rho^+$)	819	11	830	1.33 ± 0.80
^{o}D603-3B/9 ($oli^s2\ mit^-$) x DL219-1A ($oli^s2\ rho^+$)	0	1438	1438	100.00

The experiment was performed as described in section 4:2. The frequencies of $oli^s2\ rho^+$ recombinants are expressed as a percentage of the total number of rho^+ diploids scored ± 95% confidence limits, calculated as described in section 3:2. + Results obtained from a single experiment. • Results obtained from a combination of two individual experiments. o Results obtained from a combination of three individual experiments.

Table 4:5. Frequencies of ery^r rho^+ and ery^s rho^+ Colonies Issued from Crosses of the Type: op_1 ery^s mit^- x OP_1 ery^r rho^+.

Cross	Number of ery^r rho^+ colonies	Number of ery^s rho^+ colonies	Number of rho^+ colonies scored	% ery^s rho^+ recombinants
+D603-3B (ery^s rho^+) x KL60-2C (ery^r rho^+)	17	355	372	95.43 ± 2.17
•D603-3B/8 (ery^s mit^-) x KL60-2C (ery^r rho^+)	73	64	137	46.72 ± 8.53
°D603-3B/9 (ery^s mit^-) x KL60-2C (ery^r rho^+)	286	99	385	25.71 ± 4.45

The experiment was performed as described in section 4:2. The frequencies of ery^s rho^+ recombinants are expressed as a percentage of the total number of rho^+ diploids scored ± 95% confidence limits, calculated as described in section 3:2. + Results obtained from a single experiment.
• Results obtained from a combination of two individual experiments. ° Results obtained from a combination of three individual experiments.

drug resistance marker. These values of 1.11 ± 0.73% and 1.33 ± 0.80%, for D603-3B/8 and D603-3B/9 respectively, are clearly not significantly different. In addition, these values are very similar to those obtained by other workers, for crosses between pho 1 mutants and strains carrying the oli^{r}2 drug resistance locus (Foury and Tzagoloff, 1976b; Somlo et al., 1977), by essentially similar methods. Thus it seems likely that the mit$^-$ mutations present in the two op$_1$mit$^-$ strains lie in the pho 1 region of the mitochondrial genome.

Also presented in Table 4:4 are the results of crossing D603-3B/8 and D603-3B/9 to DL219-1A, a strain carrying a sensitive allele at the oli 2 locus. As might be predicted, no oligomycin-resistant rho$^+$ diploids were ever detected. Evidence of the non-Mendelian character of the oli^{r}2 drug resistance mutation is also presented in this Table. A clear mitotic segregation (oli^{s}2 : oli^{r}2, 40.96 : 59.04) of the oligomycin resistance determinant was demonstrated upon crossing D603-3B with DL218-1C.

The observation that, in crosses of D603-3B/8 and D603-3B/9 with DL218-1C and DL219-1A, slower growing respiratory competent diploids were occasionally detected, is also thought worthy of comment. The majority of these were found to be oligomycin-sensitive, although oligomycin-resistant diploids have also been observed. Such diploids were not detected among the progeny of the control cross (i.e. D603-3B x DL218-1C). These slower growing diploids were not thought to be mit$^-$ since mit$^-$ diploids, constructed by crossing D603-3B/8 or D603-3B/9 to D6, fail to exhibit growth on non-fermentable media. The slow growing colonies were believed to be either oli^{s}2 rho$^+$ or oli^{r}2 rho$^+$, and were scored as such. Recombination, between oli^{s}2 mit$^-$

and oli^{r}2 rho$^+$ or oli^{s}2 rho$^+$ genomes, resulting in slightly imperfect rho$^+$ copies of mtDNA is considered a possible explanation for these observations. The detection of slower growing diploids, in crosses of mit$^-$ mutants with rho$^+$ strains, has not previously been reported.

Table 4:5 presents the results obtained by crossing D603-3B, D603-3B/8 and D603-3B/9 to KL60-2C. The first point to note is the segregation of the ery^r drug resistant determinant (ery^s : ery^r, 95.43 : 4.57) in the cross with D603-3B. When this research was initiated the mitochondrial genotype of D603-3B, with respect to omega, was unknown. In a recent experiment (W.E. Lancashire, personal communication) no polarity of recombination was detected when D603-3B was crossed to IL8-4D, an ω^+, rho$^+$, cap^r-321, ery^r-514 strain (Avner *et al.*, 1973). D603-3B is therefore assumed to carry the ω^+ allele. Since KL60-2C also possesses the ω^+ determinant (Wolf *et al.*, 1973), the observed asymmetric proportions of ery^r and ery^s progeny cannot be due to polarity of recombination. It seems likely therefore that the results are due to differences in the transmission of the two alleles.

High and differing frequencies of ery^{s}rho$^+$ recombinants were detected when D603-3B/8 and D603-3B/9 were crossed to KL60-2C. Again these results are believed to be due to differences in the transmission of the various alleles. Of the four possible genotypes resulting from these crosses (i.e. ery^r rho$^+$, ery^s rho$^+$, ery^r mit$^-$ and ery^s mit$^-$), only two (ery^r rho$^+$ and ery^s rho$^+$) were scored. The reason for this being the inability to distinguish ery^r mit$^-$ from ery^s mit$^-$ diploids. Since the percentages of all four mitochondrial genotypes are unknown, it

is not possible to determine the transmission of the various alleles. However, once again the author feels that these differences, in the frequencies of recombinants, reflect a possible difference between the mit^- mutations carried by D603-3B/8 and D603-3B/9.

As with crosses of the two $op_1 mit^-$ mutants to DL218-1C and DL219-1A, slower growing respiratory competent diploids were occasionally detected in crosses with KL60-2C. The majority of these were erythromycin-sensitive, although erythromycin-resistant ones were also observed. As previously, no slow growing diploids were detected in the control cross (i.e. D603-3B x KL60-2C). These colonies were scored as either ery^s rho^+ or ery^r rho^+ and are thought to arise by a similar mechanism to that proposed for $oli^r 2$ rho^+ and $oli^s 2$ rho^+ slow growing diploids.

4:4 Summary

1) The mit^- mutations carried by D603-3B/8 and D603-3B/9 have been mapped, with respect to the mit^- lesions possessed by M5-16/2, M17-231 and M28-81, by recombination frequency analysis. These are found to be located between the cob 1 and oxi 3 regions, close to and possibly allelic with the pho 1 segment of the mitochondrial genome.

2) D603-3B/8 and D603-3B/9 have also been crossed to an $oli^r 2$ strain. $oli^s 2$ rho^+ recombinants were detected at a frequency of 1.0 to 1.5% in such crosses, indicating a close linkage of these two mit^- mutations to the $oli^r 2$ locus.

3) Different frequencies of rho^+ and ery^s rho^+ recombinants were observed when these two $op_1 mit^-$ mutants were crossed to

M17-231 and KL60-2C respectively suggesting that the two mit^- lesions may be non-allelic.

4) The occasional detection of slower growing respiratory competent diploids in crosses of D603-3B/8 and D603-3B/9 to DL218-1C (oli^r2 rho^+), DL219-1A (oli^s2 rho^+) and KL60-2C (ery^r rho^+) is reported and discussed.

CHAPTER FIVE. PRELIMINARY STUDIES ON THE CONSTRUCTED MIT$^-$ DIPLOIDS DL223 AND DL224.

5:1 Introduction

In the previous chapter the location of the mit$^-$ mutations possessed by D603-3B/8 and D603-3B/9 has been discussed. These lesions have been positioned, by mit$^-$ x mit$^-$ recombination analysis, close to and possibly within the pho 1 region of the mitochondrial genome. In addition, a tight linkage to the oli^{r}2 antibiotic resistance determinant has been established.

It has been reported (Foury and Tzagoloff, 1976b) that certain pho 1 mutants (i.e. M28-81 and M28-82) are capable of slow growth in glycerol liquid media. Since D603-3B/8 and D603-3B/9 probably contain lesions in the pho 1 region, the effect of these mitochondrial mutations on aerobic growth in liquid media was examined. Due to the presence of the nuclear recessive op$_1$ mutation, which results in the inability to utilize non-fermentable substrates for growth, the op$_1$mit$^-$ mutants could not be employed in these studies. Diploids, constructed as previously described, were therefore used. The cytochrome absorption spectra of these strains are also presented.

5:2 Materials and Methods

a) Materials:

Strains The genotypes and origins of strains used in this section is presented in Table 5:1.

Chemicals 2,3,5-triphenyl tetrazolium chloride (TTC) was obtained from BDH Chemicals Ltd., Poole, England.

Table 5:1. List of Strains Employed in Chapter Five.

Name	Genotype		Origin
	Nuclear	Mitochondrial	
DL220		rho^+	This Thesis
(A diploid segregant obtained by crossing D603-3B to D6)			
DL221		mit^- (oxi 3)	This Thesis
(A diploid segregant obtained by crossing D603-3B/2 to D6)			
DL222		rho^+	This Thesis
(A diploid segregant obtained by crossing D603-3B/8 to D6)			
DL223		mit^- (unknown)	This Thesis
(A diploid segregant obtained by crossing D603-3B/8 to D6)			
DL224		mit^- (unknown)	This Thesis
(A diploid segregant obtained by crossing D603-3B/9 to D6)			

Media NO and N3 are as described in section 2:2. Difco N3 is as defined in section 4:2. N2 media is essentially as described by Lancashire (1974). For convenience, the recipe for this is presented below:

N2: 1% (w/v) Difco yeast extract
1% (w/v) Oxoid bacteriological peptone
3% (w/v) glycerol
0.1% (w/v) glucose
0.05 M Na^+ K^+ phosphate buffer, pH 6.25.

N2 media was solidified by the addition of 2.3% (w/v) Oxoid agar No. 3, prior to autoclaving. Other media:

Difco NO: 1% (w/v) Difco yeast extract
1% (w/v) Difco bacto-peptone
2% (w/v) glucose
0.05 M Na^+ K^+ phosphate buffer, pH 6.25.

b) General Methods

These are as described in section 2:2.

c) Special Methods

Growth curves Appropriate strains were inoculated, from agar slopes, into 50 ml NO liquid media in 250 ml conical flasks and incubated on a rotary shaker. After approximately 48 hours, the cells were harvested, washed twice and resuspended in sterile distilled water. 5×10^7 cells were then inoculated into either 50 ml Difco NO liquid media or 50 ml Difco N3 liquid media in 250 ml baffled conical flasks, with side arms. The cultures were then agitated on a rotary shaker and the growth followed turbidometrically using an EEL colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) and filter number 607 (maximum

transmission peak at 600 nm). Absorbances were measured against either Difco NO or Difco N3 liquid media.

Samples of each culture were withdrawn both prior to inoculation of the cells into either Difco NO or Difco N3 liquid media and after the final turbidometric reading had been recorded. From these the cells were harvested, washed twice and finally resuspended in sterile distilled water. For rho^+ strains the suspensions were serially diluted and plated on N2 solid media to give approximately 150 to 200 cells per plate. After 4 to 5 days incubation the plates were overlayed with 0.1% TTC, as described below, in order to determine the rho^- frequencies. For mit^- strains the suspensions were diluted appropriately and plated to give approximately 10^8 cells per plate on N3 solid media and 100 to 150 cells per plate on NO solid media. The results were recorded after 3 to 5 days incubation.

Determination of rho^- frequencies by the 2,3,5-triphenyl tetrazolium chloride overlay technique (Ogur et al., 1957)

Media containing 0.1% (w/v) glucose and 1.5% (w/v) Difco bacto-agar was autoclaved and cooled to approximately 45°C. A solution of 2,3,5-triphenyl tetrazolium chloride (TTC), in a small volume of distilled water, was then added to give a final concentration of 0.1% (w/v). This media was then carefully poured over the surface of an N2 plate containing 100 to 200 colonies. After the overlay had solidified, the plates were incubated for 2 to 3 hours and the results scored. Grande colonies stained red whilst rho^- petites remained white.

Cytochrome absorption spectra of constructed rho^+ and mit^- diploids

Rho^+ and mit^- diploid cells were grown and the cytochrome

absorption spectra of these recorded as described for $op_1 mit^-$ strains in section 2:2.

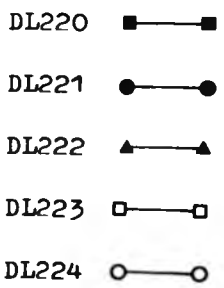
Prior to recording the spectra samples of each mit^- cell suspension were withdrawn. These were diluted appropriately and plated to give approximately 10^8 cells per plate on N3 solid media and 100 cells per plate on NO solid media. The results were scored after 3 to 4 days incubation.

5:3 Results and Discussion

The growth curves of five constructed rho^+ and mit^- diploids, in glucose liquid media, are presented in Figure 5:1. The biphasic growth patterns of DL220 and DL222, both rho^+ strains, may be clearly observed. Initially glucose is fermented; the synthesis of several respiratory components being repressed. After approximately twelve hours, when most of the glucose has been metabolised to ethanol, the second phase of growth ensues. The inflection in the growth curve represents the period of respiratory adaptation. This repression, by glucose, of respiratory activity has been extensively studied (Jayaraman *et al.*, 1966). The slightly slower growth rate of DL222, as compared to DL220, may be attributed to the difference in the spontaneous rho^- frequencies of these two strains. Rho^- cells were distinguished from rho^+ ones by plating on N2 solid media (petite determination media) and confirmed by the tetrazolium overlay technique (Ogur *et al.*, 1957). The spontaneous rho^- frequencies of DL220 and DL222, determined on a sample taken at the end of the growth curve, are presented in Table 5:2. This difference in growth rates manifests itself more clearly during the second, oxidative phase of growth. DL221, DL223 and DL224 exhibited very similar growth patterns.

Figure 5:1. Growth Curves of DL220, DL221, DL222, DL223 and DL224 in Glucose Liquid Media.

The experiment was performed as described in section 5:2.



EEL Absorbance

. DL223 and

section 5:2.

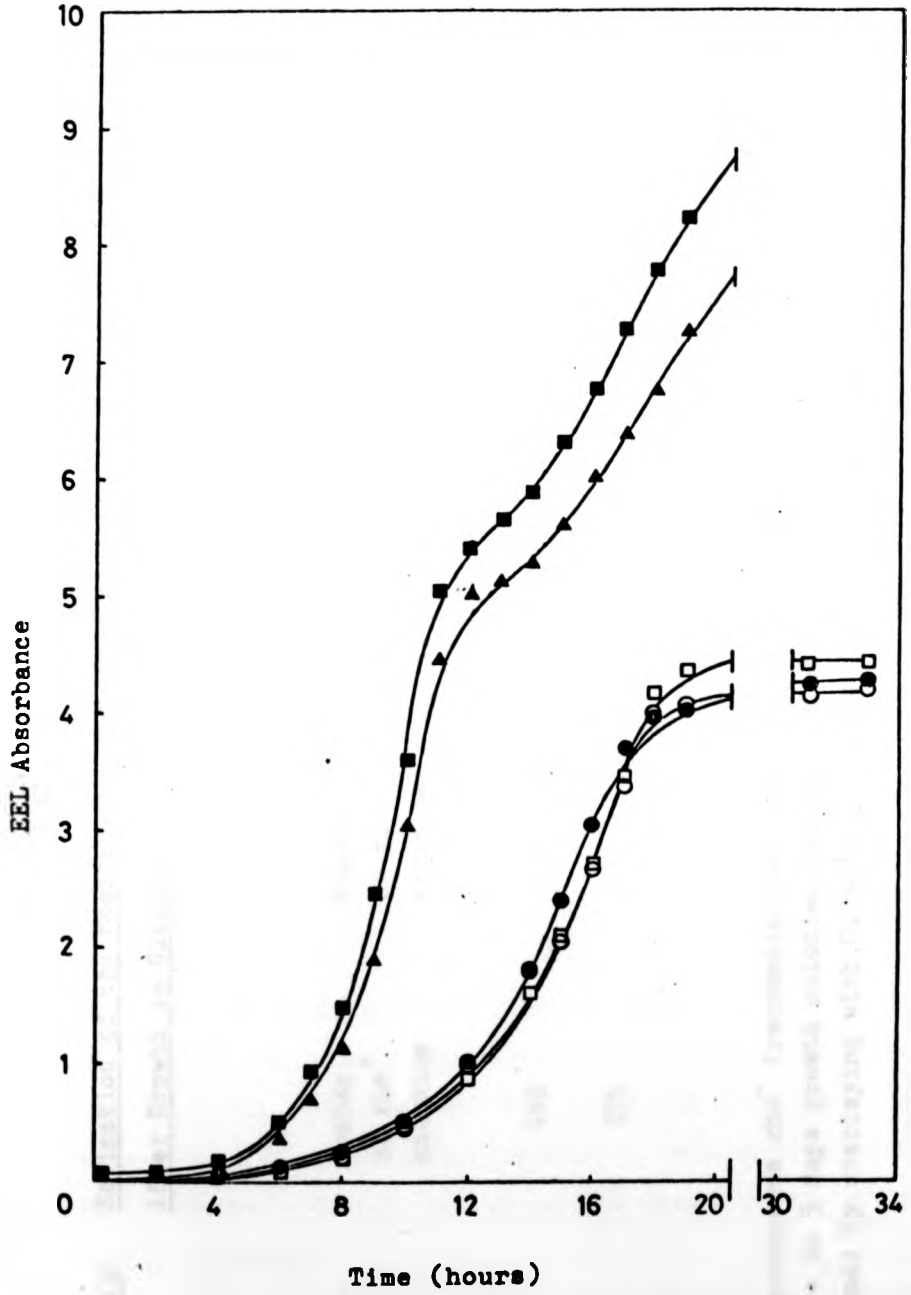


Table 5:2. **Estimation of the Spontaneous Rho$^-$ Frequencies of Two Rho$^+$ Diploids, DL220 and DL222, After Growth in Glucose.**

Strain	Number of rho$^+$ colonies	Number of rho$^-$ colonies	Number of colonies scored	% rho$^-$ colonies
DL220	418	12	430	2.79
DL222	315	74	389	19.02

Spontaneous rho$^-$ frequencies were determined by plating a sample of cells onto N2 solid media. After 4 to 5 days growth colonies were scored as either grande or petite. Petite colonies were confirmed by overlaying with 0.1% (w/v) TTC as described in section 5:2.

These mit$^-$ diploid segregants are only found capable of fermentative growth, they do not display the second, aerobic phase. Growth stops when all the glucose in the media is depleted.

A further point that requires comment is the difference in the initial rates of growth between the rho$^+$ and mit$^-$ strains, in glucose. It is clear that mit$^-$ diploid segregants grow more slowly. This may result from the imperfect nature of the repression by glucose, permitting growth of the rho$^+$ strains on both glucose and ethanol. In contrast, mit$^-$ cells would only be capable of utilizing glucose. An alternative explanation would be that the growth rate of mit$^-$ cells, and presumably also rho$^-$ cells, is inherently slower than that of rho$^+$ cells. This reduced growth rate might be due, either directly or indirectly, to the presence of incomplete mitochondria.

The spontaneous rho$^-$ frequencies of DL221, DL223 and DL224 obviously cannot be determined by the standard methods, since even mit$^-$ cells are incapable of utilizing the glycerol in N2 solid media. Therefore, in an attempt to estimate the rho$^-$ frequencies of these three mit$^-$ diploids, cells were plated to give single colonies on NO solid media. It was anticipated that colonies might then be classified as either mit$^-$ or rho$^-$ according to their colour. Mit$^-$ colonies might be expected to resemble rho$^+$ colonies in colour, since the mutations present in such strains are known to result in only minor mitochondrial modifications. Induction of the rho$^-$ mutation leads to pleiotropic changes and, in particular, a drastic alteration of the cytochrome contents. This results in the characteristic white colour of rho$^-$ petites.

It was however, found impossible to estimate the spontan-

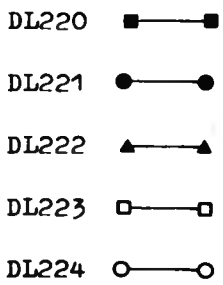
eous rho$^-$ frequencies of mit$^-$ diploids by this method. It seems probable that this was due to the instability of the mit$^-$ mutations, resulting in the production of large numbers of rho$^-$ cells. Mit$^-$ colonies would therefore contain a high proportion of rho$^-$ cells and thus the distinction between mit$^-$ and rho$^-$ colonies would be clouded. After plating of either DL221, DL223 or DL224 on NO solid media and incubation until individual colonies could be clearly detected, a population heterogeneous both with respect to size and colour resulted. Although a few colonies could be unequivocally classified as either mit$^-$ or rho$^-$, the majority could not be similarly grouped.

As previously discussed in chapter three, DL221, DL223 and DL224 could not have been rho$^-$ diploids since revertants, albeit at a very low frequency, were detected. The frequencies of spontaneous rho$^+$ revertants estimated for DL221, DL223 and DL224 prior to inoculation into Difco NO liquid media were as follows: 2.8×10^{-8}, 2.9×10^{-8} and less than 0.4×10^{-8} per cell. At the end of the growth curve the reversion frequency of DL224 was still found to be less than 0.4×10^{-8}, but the values for DL221 and DL223 were 2.9×10^{-5} and 1.5×10^{-5} per cell. The increased frequency of rho$^+$ revertants, observed with DL221 and DL223, presumably resulted from the multiplication of those present in the inoculum. It appears that no revertants were present in the DL224 inoculum and that no significant number arose during the time course of the growth curve.

The growth curves of DL220, DL221, DL222, DL223 and DL224 in glycerol liquid media are presented in Figure 5:2. The results clearly show that DL220 and DL222 are capable of metabolising glycerol, whereas DL221, DL223 and DL224 are not. The

Figure 5:2. Growth Curves of DL220, DL221, DL222, DL223 and DL224 in Glycerol Liquid Media.

The experiment was performed as described in section 5:2.



2, DL223

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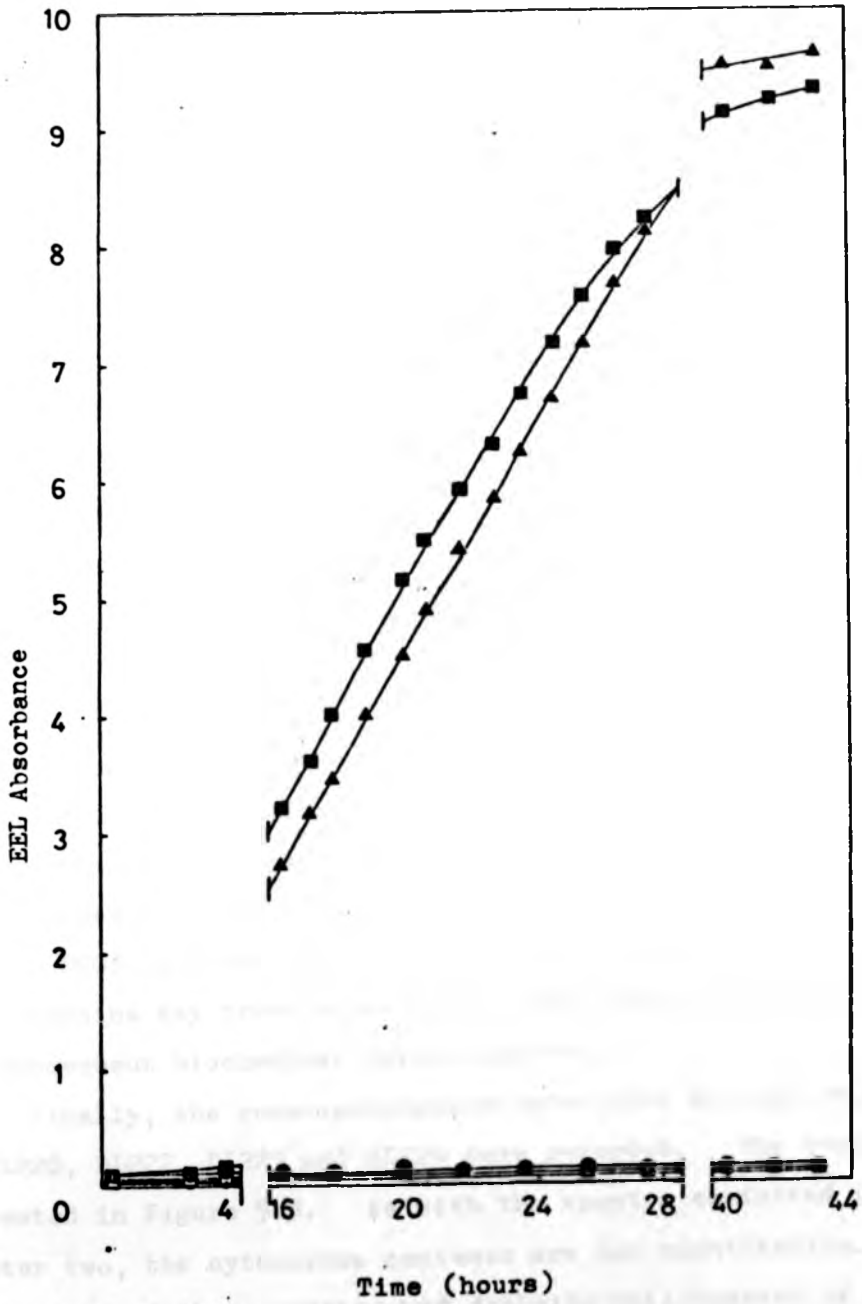


spontaneous rho$^-$ frequencies of the two rho$^+$ diploids, estimated after growth in glycerol liquid media, are shown in Table 5:3. These values appear lower than the corresponding ones obtained after growth in glucose liquid media (see Table 5:2). This is as one might anticipate. Spontaneous rho$^-$ petites produced in glucose liquid media would be capable of multiplication, whereas those arising in glycerol liquid media would not.

Foury and Tzagoloff (1976b) have reported that two pho 1 mutants (M28-81 and M28-82) exhibit slow residual growth in glycerol liquid media. These workers conclude that the mutants may possess the capacity for oxidative phosphorylation, albeit with a greatly reduced efficiency. It seems plausible that DL223 and DL224 also possess mutations in the pho 1 segment of the mitochondrial genome. The observation therefore that these mit$^-$ diploids fail to exhibit growth in glycerol liquid media, even after 43 hours, requires comment. This author believes that the slow growth of certain pho 1 strains is not a characteristic of all pho 1 mutants. It is thought that residual growth is a consequence of the mit$^-$ mutation being 'leaky'; 'leaky' oxi 3 mutants having been isolated and described in chapter two of this thesis. The mit$^-$ lesions possessed by DL223 and DL224 (and by D603-3B/8 and D603-3B/9) are not 'leaky'. As a result, these strains may prove more useful than those of Tzagoloff in any subsequent biochemical investigations.

Finally, the room-temperature cytochrome absorption spectra of DL220, DL222, DL223 and DL224 were recorded. The results are presented in Figure 5:3. As with the spectra exhibited in chapter two, the cytochrome contents are not quantitative. Suspensions of these constructed diploids will consist of more

Table 5:3. Estimation of the Spontaneous Rho$^-$ Frequencies of Two Rho$^+$ Diploids, DL220 and DL222, after Growth in Glycerol.

Strain	Number of rho$^+$ colonies	Number of rho$^-$ colonies	Number of colonies scored	% rho$^-$ colonies
DL220	438	2	440	0.45
DL222	321	5	326	1.53

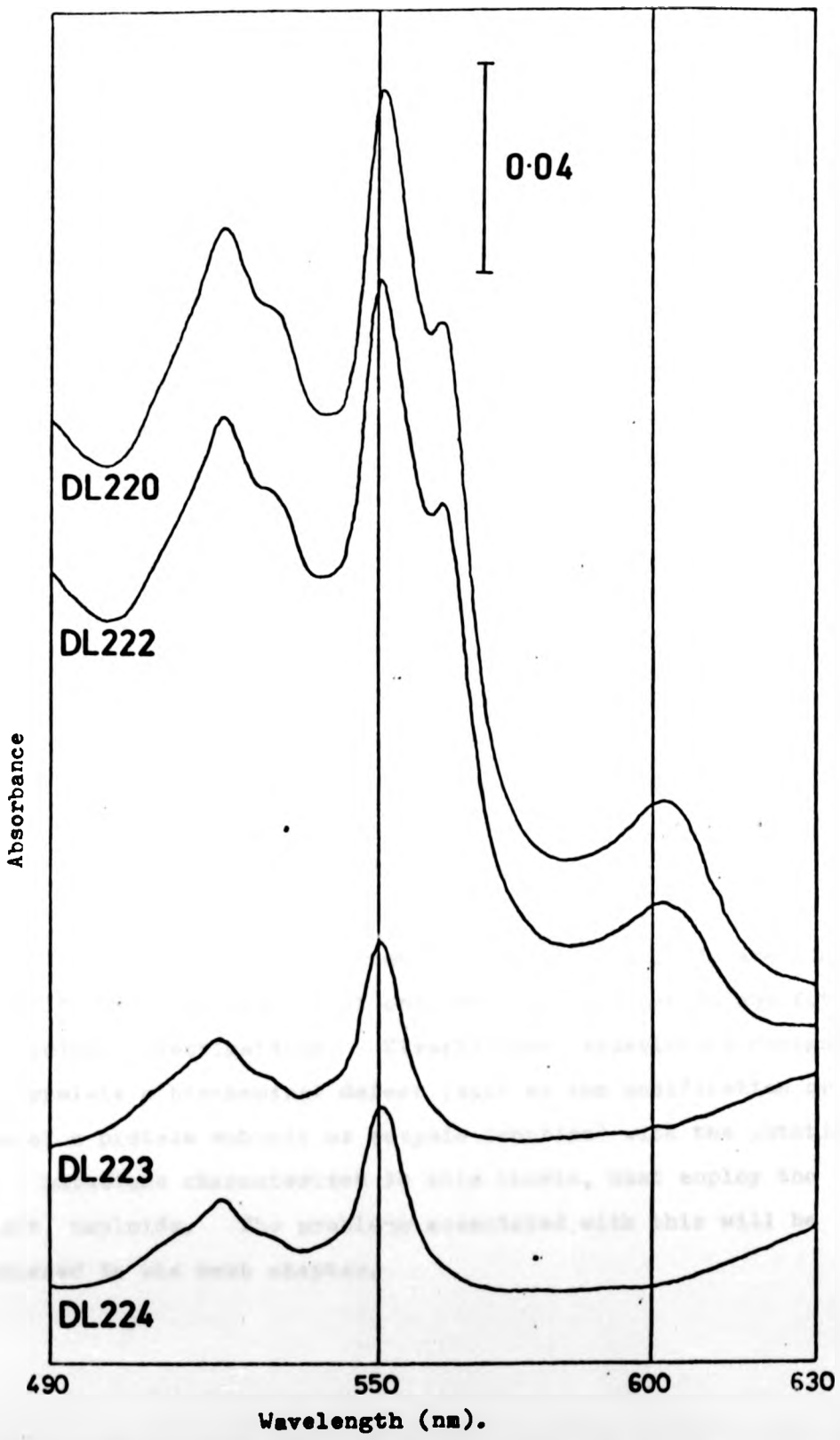
Spontaneous rho$^-$ frequencies were determined by plating a sample of cells onto N2 solid media. After 4 to 5 days growth colonies were scored as either grande or petite. Petite colonies were confirmed by overlaying with 0.1% (w/v) TTC as described in section 5:2.

Figure 5:3. Room-Temperature Cytochrome Absorption Spectra of DL220, DL222, DL223 and DL224.

The experiment was performed as described in section 5:2. The cell concentration of DL220 was approximately 0.85×10^9 cells per ml. The concentration of the other three samples was approximately 10^9 cells per ml.

Spectra

ection
tely
other



than one cell type. For DL220 and DL222, rho$^+$ and rho$^-$ cells will be present. For DL223 and DL224, mit$^-$, rho$^-$ and possibly a low frequency of rho$^+$ revertants will exist. Obviously, the frequencies of the different cell types will influence the resultant spectra. These results should therefore only be considered from a qualitative point of view.

Clearly and predictably DL220 and DL222, the two rho$^+$ diploid segregants, were found to possess cytochromes c, b and a + a_3, absorption maxima being observed at 551, 562 and 602 nm respectively. DL223 and DL224 were only found to exhibit an α peak at 550 to 551 nm, corresponding to cytochrome c. No shoulder at 562 nm and no peak at 602 nm, corresponding to cytochromes b and a + a_3 respectively, were detected. The spectra resemble that of a rho$^-$ petite. These traces suggest that the mit$^-$ mutations present in DL223 and DL224 are highly unstable, resulting in the production of large numbers of rho$^-$ cells. This explanation has not been experimentally established since, as mentioned earlier in this section, rho$^-$ colonies could not be clearly distinguished from mit$^-$colonies on NO solid media.

If, as believed, these constructed mit$^-$ diploids are highly unstable then they will be of only very limited use in any future biochemical investigations. Clearly then, experiments designed to correlate a biochemical defect (such as the modification or loss of a protein subunit or enzymic function) with the putative pho 1 mutations characterized in this thesis, must employ the op$_1$mit$^-$ haploids. The problems associated with this will be discussed in the next chapter.

5:4 Summary

1.) The growth of DL220, DL221, DL222, DL223 and DL224 in both glucose and glycerol liquid media has been studied. In glucose, DL220 and DL222 exhibit a typical biphasic pattern of growth. In contrast, DL221, DL223 and DL224 display only the first, fermentative phase. These mit^- diploids fail to exhibit any detectable growth in glycerol liquid media.

2.) The room-temperature cytochrome absorption spectra of DL220, DL222, DL223 and DL224 are also presented. DL220 and DL222 display absorption maxima corresponding to cytochromes *c*, *b* and *a* + a_3. DL223 and DL224 exhibit cytochrome absorption spectra typical of a rho^- petite. These latter results are interpreted in terms of the instability of the mit^- mutations, resulting in the production of large numbers of rho^- cells.

CHAPTER SIX GENERAL DISCUSSION

The importance of mutants in elucidating the individual steps constituting biochemical pathways and processes is widely realized. Initially bacteria, and Escherichia coli in particular, were employed in such investigations. More recently yeast, a relatively simple eukaryote, has been applied to biochemical-genetic studies of organelle function. This organism has many of the advantages of bacteria (rapid growth, amenability to genetic manipulation etcetera) and has become invaluable in investigations of mitochondrial biogenesis and the function of mtDNA.

This research was initiated in an effort to answer two important questions concerning the mitochondrial OS-ATPase complex. Firstly, work in this laboratory has, for several years, been directed towards a more complete understanding of this enzyme complex. In particular the mode of action of various antibiotics, the function(s) of the mitochondrially synthesized polypeptides and the role played by this multisub-unit enzyme in the intricate process of oxidative phosphorylation has been investigated. In this context the isolation, genetic characterization and subsequent biochemical analysis of cyto-plasmically-inherited yeast mutants resistant to inhibitors of oxidative phosphorylation has been fundamental. To date however, this approach has met with only limited success. Therefore, additional mutants possessing deletions of mtDNA within genes responsible for the biosynthesis of the OS-ATPase complex, possibly resulting in major modifications of the enzymic structure, were sought. Secondly, the isolation and

subsequent genetic analysis of a series of mitochondrial mutants might provide information as to the number of genes on mtDNA responsible for the biosynthesis of this enzyme complex.

In this thesis the isolation of cytoplasmic mutants of S. cerevisiae, defective in either respiration or oxidative phosphorylation, is presented. Two of these mutants, D603-3B/8 and D603-3B/9, have been extensively studied. The mitochondrial nature of the mutations, carried by these two strains, has been clearly established and their approximate location on a map of mtDNA determined. These mit^- lesions have been found to be closely linked to the oli^r2 drug resistance locus (Avner et al., 1973). In addition these mutations are found to be linked to and possibly allelic with the pho 1 segment of the mitochondrial genome (Foury and Tzagoloff, 1976b). The possibility that the mutants described here represent a new mit^- region, distinct from pho 1, cannot be excluded. This however is considered unlikely since the frequencies of rho^+oli^s2 recombinants, detected when D603-3B/8 and D603-3B/9 are crossed to a rho^+oli^r2 tester strain, are similar to those observed for established pho 1 mutants (Foury and Tzagoloff, 1976b; Somlo et al., 1977). Even if these mit^- mutations are localized within the pho 1 segment of the mitochondrial genome, they may still prove to be distinct from other lesions mapped in this region and therefore of considerable importance.

It is relevant to point out that the mit^- mutations, carried by D603-3B/8 and D603-3B/9, do not permit even residual growth on non-fermentable media when the op_1 nuclear determinant is rendered recessive. These results are in contrast with those obtained by Foury and Tzagoloff (1976b) for two pho 1 mutants,

M28-81 and M28-82. This author suggests that the ability of these strains, to grow slowly on glycerol, is a characteristic of the particular mutations carried by them and not of pho 1 mutants in general. M28-81 and M28-82 are therefore considered to be 'leaky' mit$^-$ mutants.

DL223 and DL224, two mit$^-$ diploids constructed by crossing D6 to D603-3B/8 and D603-3B/9 respectively, were found to be unstable. These strains will therefore be of only limited use in any future biochemical investigations. Thus, until the nuclear op$_1$ mutation can be eliminated, either by meiosis or the construction of stable diploids, consequences of the induced mitochondrial lesions will have to be studied in the original op$_1$mit$^-$ mutants. The utilization of such strains presents an additional problem. When comparing the properties of op$_1$mit$^-$ mutants with the op$_1$rho$^+$ parental, two factors must be taken into consideration. Firstly, any experimental differences may be directly attributable to the mit$^-$ mutations themselves. It is also conceivable that differences may arise indirectly, through an interaction of the mit$^-$ lesions with the nuclear op$_1$ determinant.

Clearly however, mutants of this type will be of great value in future biochemical studies of the yeast OS-ATPase complex. Preliminary investigations of pho 1 mutants have already been published (Tzagoloff *et al.*, 1975c; Foury and Tzagoloff, 1976b; Somlo *et al.*, 1977), although to date it has not been possible to identify a defective gene product.

The research presented in this thesis also attempted to answer the question: how many subunits of the OS-ATPase complex does mtDNA specify? Radioactive labelling experiments have

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The research presented in this thesis also attempted to answer the question: how many subunits of the OS-ATPase complex does mtDNA specify? Radioactive labelling experiments have

indicated that, in yeast, four polypeptides of this enzyme are synthesized on the mitoribosomes (Tzagoloff and Meagher, 1972) and are therefore, presumably mitochondrially coded. These components exhibit molecular weights of 29,000, 22,000, (Tzagoloff and Meagher, 1971), 12,000 (Tzagoloff et al., 1973) and 7,800 daltons (Tzagoloff and Akai, 1972). To date however, only two regions of mtDNA have been implicated in the biosynthesis of the OS-ATPase complex, these have been designated pho 1 (Foury and Tzagoloff, 1976b) and pho 2 (Tzagoloff et al., 1976c). Mit$^-$ mutants of both classes fail to exhibit a mitochondrial oligomycin-sensitive ATPase activity. Mutations in the pho 2 region have been correlated with a modification of subunit 9, the 7,800 dalton component of the OS-ATPase complex (Coruzzi et al., 1978; A. Tzagoloff, personal communication). As previously mentioned, the defective gene product in pho 1 mutants has not yet been identified.

If mutations in the pho 1 segment are found to result in the modification or deletion of a single subunit of the enzyme, then clearly the question arises: from where do the remaining two mitochondrially synthesized components originate? Four tentative solutions may be put forward.

i) The possibility exists that the pho 1 and/or pho 2 region specifies more than one polypeptide of the complex. If these segments of mtDNA represent single genes, then either post-transcriptional modification of messenger ribonucleic acid (mRNA) or post-translational cleavage of a precursor protein molecule must also be postulated. However, it is quite conceivable that one or both of these regions contain more than one gene. Although it has been demonstrated (Coruzzi et al., 1978)

that pho 2 mutations result in the modification of a single polypeptide, only two such mutants have been studied. Only the isolation of a large number of such mit$^-$ mutants, followed by an analysis of their mitochondrially synthesized products will confirm or refute this supposition.

ii) The conclusion that four components of the OS-ATPase complex were mitochondrially synthesized, and hence presumably coded by mtDNA, was based upon the observation that their production was blocked by chloramphenicol (CAP), a specific inhibitor of mitochondrial protein synthesis (Lamb et al., 1968). CAP might also be expected to prevent the translation of mRNA species, other than mitochondrial mRNA, given that the mitoribosomes and mitochondrial tRNAs are capable of recognising the foreign messenger. It is plausible therefore, that one or more of the subunits are specified by nuclear DNA. However, as far as the author is aware, evidence of nuclear mRNA import into the mitochondrion is non-existent.

iii) A third alternative may be proposed, in which loss or modification of certain mitochondrially specified components of the OS-ATPase complex results in lethality. It would thus prove impossible to isolate mit$^-$ mutants possessing such lesions.

iv) Finally, it is possible that the subunit analysis of the enzyme or the radioactive labelling data is artifactual. Such spurious results may arise, for example, as a consequence of enzyme purification.

In conclusion, it is firmly believed that this ambiguity will only be resolved through the use of mutants. Correlation of defective mitochondrial translation products with mutations that have been accurately mapped on mtDNA will categorically

establish the number and location of the genes responsible for the biosynthesis of the OS-ATPase complex. The mutants described in this thesis were isolated with this goal in mind.

APPENDIX. INDUCTION AND DETERMINATION OF THE SUPPRESSIVITY OF TWO RHO^{o} TESTERS

Introduction

Since the two rho^{o} tester strains, D6/ETH1 and D22/ETH1, have been used extensively throughout this thesis, it was felt that they merited a section to themselves. These strains have been designated as putative. A characteristic of true rho^{o} strains is the complete lack of mtDNA. This is usually experimentally established by extraction of DNA from a mitochondrial preparation and subsequent analysis in CsCl density gradients. This procedure was not applied to D6/ETH1 and D22/ETH1.

A further property of rho^{o} strains is the absence of suppressivity (for a discussion of suppressivity see section 1:6), such petites being referred to as 'zero suppressive'. The degree of suppressivity of the two putative rho^{o} testers was therefore determined. A high or low degree of suppressivity would indicate that D6/ETH1 and D22/ETH1 still retained mtDNA. If, however, these strains proved to be 'zero suppressive', then it would be reasonable to conclude that they lacked mtDNA.

This appendix therefore presents the method of induction of D6/ETH1 and D22/ETH1 and a determination of their degree of suppressivity.

Materials and Methods

a) Materials

Strains: D6 (α, arg, met, ω^{+}, rho^{+}) and D22 (a, ade2, ω^{+}, rho^{+})

were employed in these experiments. These strains were kindly donated by Dr. D. Wilkie.

Chemicals 2,7-diamino-10-ethyl-9-phenylphenanthridium bromide (Ethidium bromide) was obtained from Calbiochem Ltd., London, England.

Media NO, N3 and WO media are as described in section 2:2.

b) General Methods

These are as described in section 2:2.

c) Special Methods

Induction of D6/ETH1 and D22/ETH1 A loop of cells of either D6 or D22 was inoculated, from an agar slope, into 50 ml NO liquid media containing 15μg/ml ethidium bromide (EtBr) in a 250 ml conical flask. The flask was then covered with aluminium foil to omit light (Lancashire, 1974). After 24 hours growth on a rotary shaker, 0.1 ml of the cell suspension was inoculated into a further 50 ml NO liquid media containing 15μg/ml EtBr in a similar vessel. The cells were grown, as described above for a second period of 24 hours, and then harvested and washed twice in sterile distilled water. After appropriate dilution the cells were spread on NO solid media to give approximately 50 cells per plate and incubated for 3 days. A single colony was then picked, subcloned twice on NO solid media and finally stocked on an agar slope. These petites were designated D6/ETH1 and D22/ETH1 respectively.

Determination of the degree of suppressivity Appropriate strains were inoculated, from agar slopes, into 10 ml NO liquid media in boiling tubes and incubated, without shaking, for approximately

40 hours. After harvesting and washing once in sterile distilled water, 5×10^6 cells of two parental strains were inoculated into 5 ml NO liquid media in a preincubated 50 ml conical flask. The flask and contents were then vigorously shaken on a water bath. After 90 minutes the cells were pelleted in a conical centrifuge tube using a BTL bench centrifuge (swing-out rotor) at 2,500 r.p.m. for 5 minutes. The tube was then incubated, without decanting the supernatant, for thirty minutes. After discarding the supernatant the cells were resuspended in 5 ml NO liquid media. The suspension was transferred to a sterile preincubated 50 ml conical flask, which was vigorously shaken on a water bath for a further 90 minutes. The cells were then harvested, as previously described, and washed twice in sterile distilled water. Subsequently the cells were resuspended in 1 ml distilled water, serially diluted and each dilution plated out onto WO solid media. After 3 days incubation, zygotic clones were velveteen replica plated onto NO and N3 solid media. The results were scored after a further 2 days.

Note that plating of the cell suspension was always completed within 2½ to 3 hours of zygote formation.

The degree of suppressivity, defined as the probability that a zygote will produce an entirely petite clone, was calculated from the equation of Ephrussi and Grandchamp (1965) where:

$$S = \frac{X - Y}{100 - Y} \times 100$$

S is the degree of suppressivity (%), X is the percentage of entirely petite zygotic clones and Y is the spontaneous frequency of petites observed in the grande tester strain.

Determination of the spontaneous rho$^-$ frequencies of D6 and D22

This was performed by plating between 100 and 150 cells per plate, of a D6 or D22 culture prior to mating, on N2 solid media. After 5 to 6 days growth colonies were scored as either grande or petite. Petite colonies were confirmed by overlaying with 0.1% (w/v) TTC as described in section 5:2.

Results and Discussion

The results of the determination of the degree of suppressivity of D6/ETH1 and D22/ETH1 are presented in Tables A1 and A2 respectively. Both of these values were found to be negative, i.e. the spontaneous petite frequency of the grande tester strain was greater than the frequency of entirely petite zygotic clones. These negative results are believed to indicate that the two petites are indeed 'zero suppressive'. It is thought that the negative values result through not having scored a sufficiently large number of zygotic clones or from not having determined the spontaneous rho$^-$ frequencies on a sufficiently large rho$^+$ sample.

It is important to point out that even if D6/ETH1 and D22/ETH1 contained mtDNA, this would not invalidate the work presented in this thesis. If these two strains retained some informational sequences then only two aspects of this research would be affected. Firstly, it is conceivable that certain op_1mit$^-$ mutants, possessing lesions in regions that are present in D6/ETH1, would not be detected. Such mutants would be dissimilar to those reported in this thesis. Secondly, the presence of coding sequences in D22/ETH1 might block attempts to isolate an $\propto OP_1$mit$^-$ meiotic segregant after crossing D603-3B/8 or D603-3B/9 to a grande tester strain. If this were the case then this

Table A1. Determination of the Degree of Suppressivity of D6/ETH1

Cross	Number of grande zygotic clones	Number of petite zygotic clones	Number of zygotic clones scored	% petite zygotic clones
D6/ETH1 x D22	561	28	589	4.75

Spontaneous rho$^-$ frequency

Strain	Number of grande colonies	Number of petite colonies	Number of colonies scored	% petite colonies
D22	539	30	569	5.27

$$\% S = \frac{4.75 - 5.27}{100 - 5.27} \times 100.$$ Negative i.e. 'zero suppressive'

The experiment was performed as described in Materials and Methods.
The spontaneous rho$^-$ frequency of D22 was determined by plating on N2 solid media. After 5 to 6 days growth, petites were confirmed by overlaying with 0.1% (w/v) TTC as described in section 5:2.

Table A2. Determination of the Degree of Suppressivity of D22/ETH1.

Cross	Number of grande zygotic clones	Number of petite zygotic clones	Number of zygotic clones scored	% Petite zygotic clones
D22/ETH1 x D6	255	10	265	3.77

Spontaneous rho$^-$ frequency

Strain	Number of grande colonies	Number of petite colonies	Number of colonies scored	% petite colonies
D6	515	28	543	5.16

$$\% S = \frac{3.77 - 5.16}{100 - 5.16} \times 100.$$ Negative i.e. 'zero suppressive'.

The experiment was performed as described in Materials and Methods.
The spontaneous rho$^-$ frequency of D6 was determined by plating on N2 solid media. After 5 to 6 days growth, petites were confirmed by overlaying with 0.1% (w/v) TTC as described in section 5:2.

putative rho^{o} strain would have to possess the sequence defective in the op$_{1}$mit^{-} mutant. These two possibilities are not considered likely.

In conclusion the results indicate that D6/ETH1 and D22/ETH1 are 'zero suppressive'. This author therefore suggests that these petites lack mtDNA. As previously discussed, this may only be refuted or proven by biochemical analysis of the types of DNA species present in these strains.

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