ISOLATION AND CHARACTERIZATION OF NOVEL OLIGOMYCIN-RESISTANT *SACCHAROMYCES CEREVISIAE* MUTANTS OF THE MITOCHONDRIALLY ENCODED F_O ATPASE SUBUNITS

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This paper was prepared
under the direction of
Professor Jeffrey Knight
for eight credits.

To:

Valerie Anne Muchmore
6/11/1986 - 4/15/05
In loving memory of your smile and friendship

-and-

Lynn L'Archeveque, Class of 1997
The best science role model a sister could ask for

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ABSTRACT

The mitochondria of *Saccharomyces cerevisiae* are semi-autonomous organelles that rely on nuclear genes to function. Although most subunits of yeast ATPase are nuclear-encoded, the membrane-bound F_O portion is comprised of three mitochondrially-encoded subunits. F_O ATPase confers sensitivity to the antibiotic oligomycin, whereby mitochondrial metabolism is disrupted and ATP synthesis ceases.[1] There are four oligomycin resistance loci located on the mitochondrial DNA, namely *oli1*, *oli2*, *oli3*, and *oli4*. However, there is currently no fine structure genetic map describing these genes. Three hundred and eighty-eight oligomycin-resistant mutants were generated following manganese mutagenesis. Following preliminary testing, mutants were crossed to determine allelism. Random diploid analysis was used to score diploid progeny as either oligomycin-sensitive or oligomycin-resistant, and recombination frequencies were calculated. A fine structure genetic map of the *oli1-oli3* region of the mtDNA was generated, and mutants mapping outside this region were identified. Since mitochondria play an essential role in normal cellular growth and respiration, this study and others will help elucidate the resistance genes and the mechanisms of antibiotic binding and organelle function.

[1] Avner, P.R., Coen, D., Dujon, B., and P.P.Slonimski. 1973. Mitochondrial genetics. IV. Allelism and mapping studies of oligomycin resistant mutants in *S. cerevisiae*. *Molec. Gen. Genet,* 125**:** 9-52.

INTRODUCTION

Mitochondria are semi-autonomous organelles that retain some independence but rely on the nucleus and nuclear genes for normal function. As the cellular powerhouses in eukaryotic cells, mitochondria serve as the location of oxidative phosphorylation and Adenosine 5´-Triphosphate (ATP) synthesis. Over time, mitochondria have become almost entirely dependent on nuclear DNA for the synthesis of mitochondrial proteins, although they maintain their own mtDNA which codes for several genes involved in cellular processes (Graack and Wittmann-Liebold, 1998).

Since mitochondria are essential to normal cellular growth and respiration, it is of interest to understand the underlying genetic inheritance and expression of mitochondrial DNA. Significantly, mitochondrial inheritance is different from the better understood mechanisms of nuclear gene transmission. Mitochondrial DNA is a relatively simple system that can be used to effectively study cellular processes of replication, recombination, and mutation. Many mutations in the mitochondrial genome lead to various types of antibiotic resistance; the characterization of these mutations leads to further understanding of antibiotic binding and organelle function.

Model Organism

Study of the common baker's yeast, *Saccharomyces cerevisiae*, has led to a greater understanding of mitochondrial genetics, as it is a model eukaryote for study. It was the first eukaryotic organism to have its nuclear and mitochondrial genomes completely sequenced. *S. cerevisiae* is easy to maintain and manipulate in the laboratory, since it has rapid growth cycles, dispersed cells, is non-pathogenic, and has the ability to reproduce sexually and asexually. *S. cerevisiae* has a well-defined genetic system with many molecular markers, and mutants can easily be isolated and studied (Sherman, 1998). As a facultative anaerobe, *S. cerevisiae* can respire via aerobic respiration using a non-fermentable carbon source such as glycerol, or perform fermentation using a fermentable carbon source like glucose. Using the fermentation pathway exclusively, protein synthesis is inhibited in the mitochondria at the transcriptional level. Notably, mutations affecting mitochondrial function are not lethal in a facultative anaerobe, as strains with or without functional mitochondria can be maintained in the laboratory. Mitochondrial mutants lacking the ability to grow on glycerol can also be isolated to study mechanisms in mitochondrial gene transmission. The phenotype of the mitochondrial mutant can be determined by growing the mutant on a non-fermentable carbon source, forcing it to use aerobic respiration. In sum, *S. cerevisiae* is ideal for studying mitochondrial transmission genetics and antibiotic resistance.

Yeast Life Cycle

S. cerevisiae is both homothallic and heterothallic, and is easily maintained in both haploid and diploid states. Haploid cells exist in one of two cellular mating types *a* and α, which are controlled by alternative alleles at the mating type locus found on chromosome III as part of the nuclear DNA. Cells with the MAT*a* allele exhibit the *a* phenotype; likewise those with the MATα allele exhibit the α phenotype. It is possible for strains to switch mating types through genetic rearrangement using the cassette transposition mechanism (Herskowitz, 1988). Strains maintained in laboratory settings, however, generally maintain the same mating types over repeated generations, allowing for reliable use in matings.

Haploids may reproduce asexually by mitosis and budding and may also reproduce sexually. In order for the production of diploid progeny to occur, cells with opposite mating types must be mated together. The haploid cells fuse, thus creating a zygote with the MAT*a*/MATα genotype. Under normal conditions, cells will be maintained in the diploid state through repeated mitoses. Diploid cells growing under optimal conditions can complete a cell cycle in about 100 minutes (Hartwell, 1974). It is possible to induce formation of haploid cells, as diploid cells may undergo meiosis and sporulate during nitrogen starvation or when using acetate as a food source. The result is four haploid ascospores, two with each mating type. Figure 1 depicts the complete life cycle of *S. cerevisiae*.

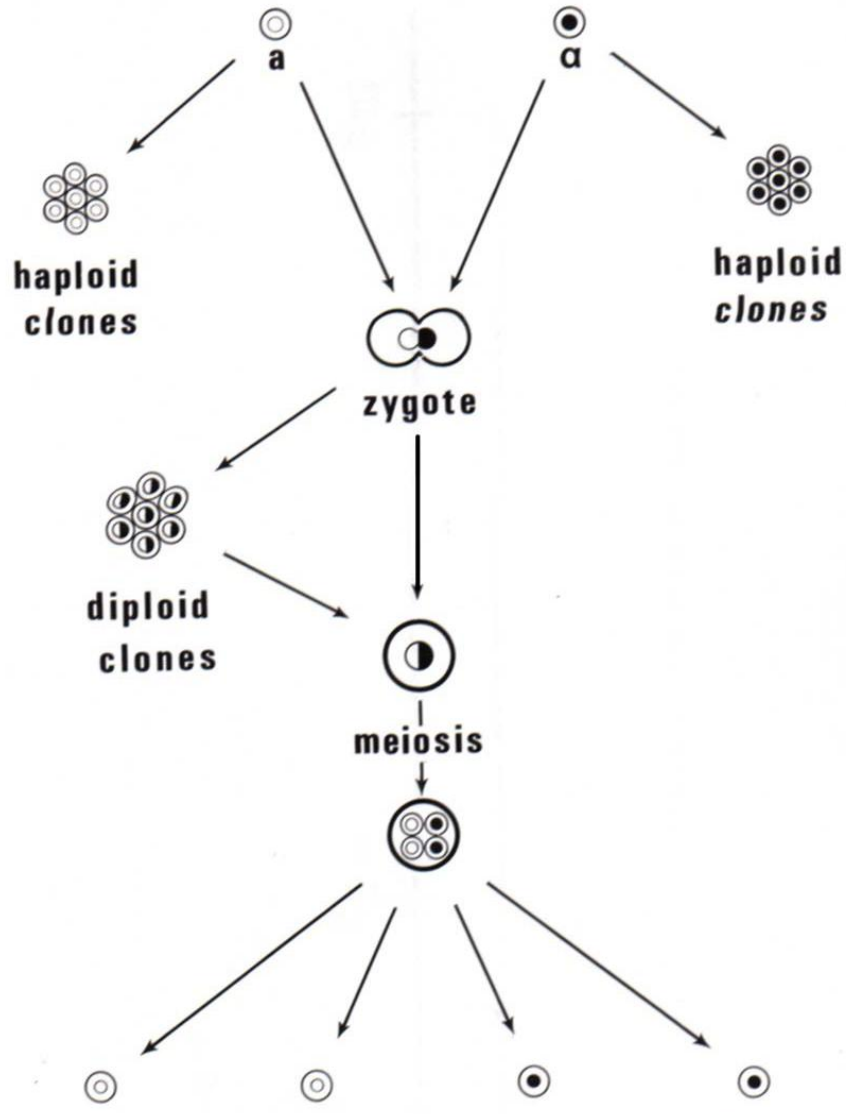

Figure 1: The life cycle of *Saccharomyces cerevisiae* (Knight, 1977).

Mitochondria

Mitochondria are membrane-bound organelles found in most eukaryotic organisms. Since they share features with prokaryotes, like ribosomes, DNA, and the ability to self replicate by growth and division of preexisting mitochondria, it is hypothesized that mitochondria arose evolutionarily as the result of an endosymbiotic relationship (Margulis and Dolan, 2002). Mitochondria retain their own genome, known as mitochondrial DNA (mtDNA), which remains separate from the nuclear genome over the complete life cycle. Since mitochondria retain their own protein synthesis machinery, the few proteins that mtDNA encodes are translated on mitochondrial ribosomes. The number of mitochondria within a cell varies with cell type and function, but haploid yeast cells generally contain approximately 100 mitochondrial genomes distributed in 5-40 mitochondria throughout the cell. In *S. cerevisiae*, mitochondria form a branched tubular network below the cell cortex, maintained by active actin-dependent transport in addition to membrane fusion and fission events (Dimmer et al., 2002).

Mitochondria possess a unique structure characterized by two membranes that separate the organelle into four distinct compartments (McBride et al., 2006). The outer mitochondrial membrane possesses a protein: phospholipid ratio of 1:1, similar to the eukaryote plasma membrane. It contains porins to facilitate passage of molecules under 5000 Daltons, as well as active transport mechanisms, such as the translocase of the outer membrane complexes (TOM), which transport larger molecules across the membrane. The inner mitochondrial membrane has a

protein: phospholipid ratio of 3:1, and does not contain porins. Therefore nearly all molecules wishing to transverse the inner membrane must utilize the translocase of the inner membrane complexes (TIM). The inner membrane is extensively folded into cristae, which increase the surface area of the membrane and serve as the location for the electron transport chain and ATPase. The intermembrane space, located between the two membranes, is chemically similar to the cytosol, owing to the permeable nature of the outer membrane. However, the protein composition differs significantly, as proteins require a certain amino acid sequence to cross the membrane. The matrix, enclosed by the inner membrane, contains about two thirds of the total protein content of the mitochondrion. Here is a highly concentrated mixture of enzymes, ribosomes, tRNA, and multiple copies of the mitochondrial genome. The division of mitochondria into compartments allows for a wide array of functionality. Figure 2 illustrates the nature of these compartments.

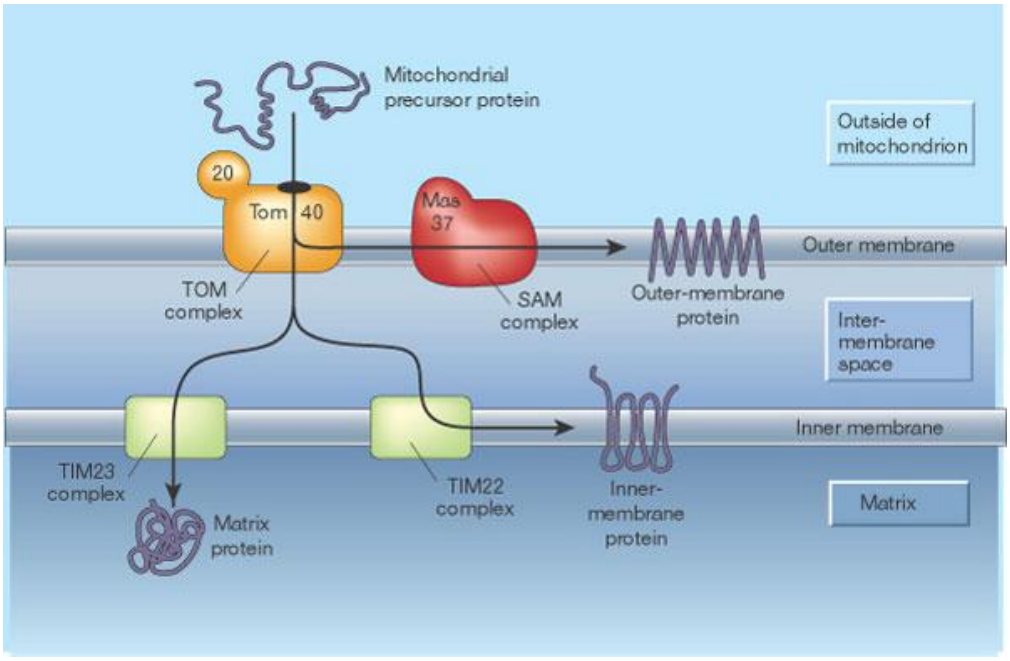

Figure 2. Inner and outer mitochondrial membranes depicting the location of the TIM and TOM complexes involved with protein transport across the mitochondrial membrane. The intermembrane space and matrix are also shown (Katsuyoshi, 2003).

Mitochondria enclose several metabolic functions, including reactions of the citric acid cycle, iron/sulfur cluster assembly (e.g. ferredoxins), and the biosynthesis of cellular metabolites, including some amino acids and lipids (Bian and Cowan, 1999; Dimmer et al., 2002). However, producing the majority of ATP for the cell remains the most notable role of mitochondria. During the anaerobic process of glycolysis, one molecule of glucose is converted into two molecules of pyruvic acid. Pyruvate is transported into the matrix and decarboxylated to form acetyl-CoA, which is used in the citric acid, or Krebs cycle. Oxidative phosphorylation follows, with the electron transport chain and synthesis of ATP.

In the electron transport chain, there are four complexes (NADH coenxyme Q reductase, succinate dehydrogenase, cytochrome bc_1 reductase, and cytochrome c oxidase) where the transfer of electrons is coupled to proton transfer across the inner membrane into the intermembrane space (Figure 3). Molecules produced in the Krebs cycle, like the coenzyme NADH and succinate, are oxidized which releases energy along the chain. The final electron acceptor is oxygen, which is reduced to water as a byproduct. The fifth and final complex, ATPase, uses the proton-motive force of usually three to four protons to generate one molecule of ATP from ADP and inorganic phosphate, P_i. This occurs as protons are pumped back into the matrix through the protein complex. These various complexes involved in oxidative phosphorylation are composed of polypeptide subunits, mostly encoded by nuclear genes. Notably, however,

cytochrome c oxidase, cytochrome bc_1 reductase, and F_1-F_O ATPase have polypeptide subunits encoded by both nuclear and mitochondrial DNA.

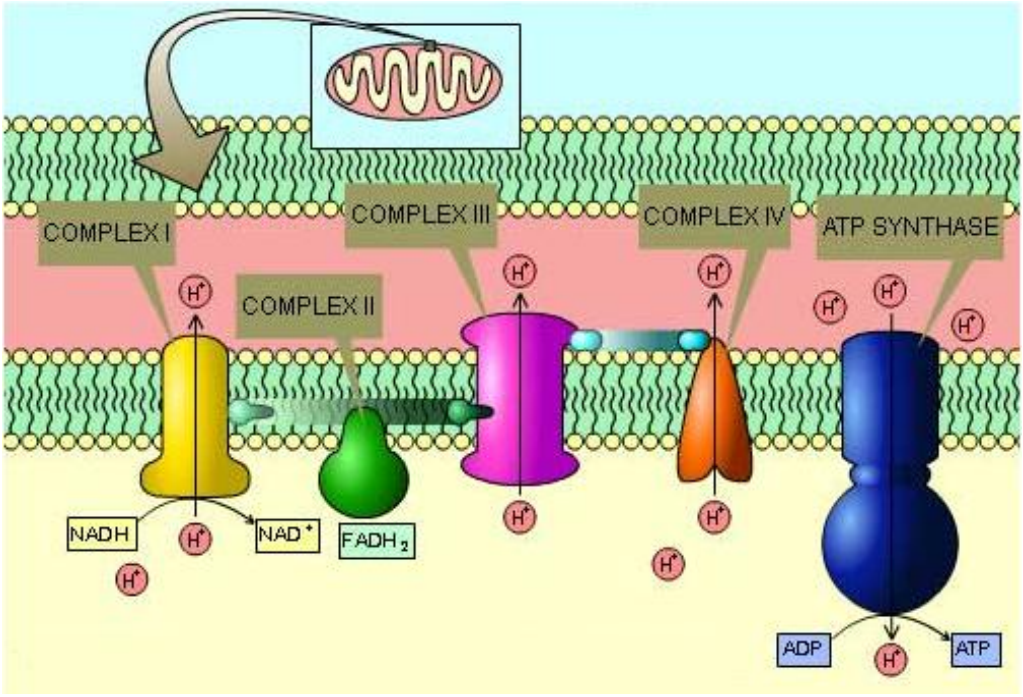

Figure 3. The protein complexes involved in the electron transport chain and oxidative phosphorylation, shown embedded in the inner mitochondrial membrane. Abbreviations for complexes are as follows: Complex I: NADH coenxyme Q reductase; Complex II: succinate dehydrogenase; Complex III: cytochrome bc_1 reductase; and Complex IV: cytochrome c oxidase (*Adapted from* HOPES, 2008).

Nuclear Versus Mitochondrial Inheritance

Mitochondrial inheritance is unique from nuclear inheritance because it is non-Mendelian in nature. In crosses between a strain with a nuclear dominant allele and a strain with a recessive allele, progeny displaying only the dominant phenotype will result, indicative of a nuclear mutation. If a cross results in a mixture of two phenotypes, the mutation is mitochondrial in nature. For example, matings between a naturally sensitive strain and an antibiotic resistant strain with a mitochondrial mutation are expected to result in a mixture of resistant and sensitive colony morphologies. Therefore, crosses can be performed to determine mode of inheritance for antibiotic resistance phenotypes.

Vegetative Segregation

Nuclear genes have been known to segregate, or separate the alleles from a heterozygous cell into daughter cells, only during meiotic divisions (Birky et al., 1978). Mitochondrial genes, however, regularly segregate during mitosis. This results in homoplasmic cells, or those with identical mitochondrial DNA molecules, from the progeny of a heteroplasmic cell. Following zygote formation in yeast, where the new heteroplasmic cell has mtDNA from both the α and a parents, the mtDNA molecules are able to mix and recombine through vegetative segregation. This phenomenon occurs continually following zygote formation in yeast, and pure cell lines are rapidly created in 20-25 generations (Dujon et al.,

1974). Consequently, both haploid and diploid cells can have homoplasmic mitochondrial DNA.

There are several theories that attempt to explain vegetative segregation. A popular explanation considers vegetative segregation the result of random mtDNA distribution between the parent and bud during cell division. A heteroplasmic cell can produce one or more homoplasmic cells if the segregation of organelles is random. Here, each bud obtains a few mtDNA molecules from the parent cell, which then undergo multiple rounds of replication to reach the approximate total of 100 mtDNA molecules. This process causes some genotypes to be lost through chance selection due to a small sample size of mitochondria. In further support of this theory, Strausberg et al. (1978) suggest that the first bud from the zygote may occur at the end of the cell before cytoplasmic mixing is complete. Without first becoming entirely heteroplasmic, the zygote would produce a bud that would be nearly pure for one mitochondrial type. After subsequent budding, a homoplasmic cell would soon result. It has also been observed that mitochondria from one parent tend to remain at one end of the fused zygote, while the mitochondria from the other parent remain at the opposite end following diploid formation (Callen, 1974). As a result, segregation could occur rapidly if buds are formed at the ends of the zygote as opposed to the site of fusion.

Recombination: Phage Analogy Model

Recombination of mitochondrial genes was first reported by Thomas and Wilkie (1968). Although used to effectively map the *Escherichia coli* genome, use of recombination has some limitations in the *S. cerevisiae* mitochondrial genetic system. The transmission and recombination observed in mitochondrial genes is different from genes located on nuclear chromosomes. In most nuclear systems, the maximum detectable recombination frequency between chromosomes is 50% (Birky et al., 1978). In yeast mitochondrial genes, however, the theoretical maximum recombination frequency is reduced to 20-25% for unlinked markers. This limit was originally suggested by Avner et al. (1973) and Wolf et al. (1973) and is continuously observed in laboratory studies of mitochondrial genetics. The Phage Analogy model first proposed by Dujon et al. (1974) addresses the reduction in recombination frequency with the following hypothesis: Within the zygote is an assemblage of mtDNA molecules equally contributed from each haploid parent. In these panmictic mating conditions, there will be several rounds of random homologous and heterologous pairings and recombination. Recombination that may result from homologous pairings will be undetected; therefore the maximum rate of recombination is reduced from 50% to the theoretical upper limit of 25%.

Genetic Mapping

Recombination frequencies are the standard genetic means by which a mutation is mapped. First used to map nuclear genes, this method has also been used for mtDNA. As a measurement of the frequency of recombination between two loci, recombination frequencies are especially useful when a strain with a known locus is crossed to a mutant of unknown locus. In this study, recombination frequencies are used to determine if mutations conferring resistance to an antibiotic occur at the same or different loci on the mtDNA. It is important to recall that the theoretical threshold recombination frequency of twenty-five percent indicates two unlinked loci. Since loci are technically linked, as they are on the same mtDNA molecule, this threshold can be problematic. Therefore, we say that two loci separable by twenty-five percent recombination are genetically unlinked by this method of analysis, as we cannot detect accurate linkage. If no recombinants in a population of 1000 or more random diploid progeny are observed, the mutant is said to be allelic to the marker. This technique is useful for markers separated by no more than 6000bp; for anything greater, other mapping techniques must be employed (Dujon et al., 1974).

Yeast Mitochondrial Genome

The mitochondrial genome remains distinct from the nuclear genome at all stages of the organism's life cycle (Birky et al., 1978). However, nearly all

proteins associated with yeast mtDNA are the product of nuclear genes, demanding a situation where many nuclear genes are required for the expression of seven mitochondrial genes (Constanzo and Fox, 1991). Therefore, mitochondrial and nuclear gene expression must be coordinated in some way to balance the subunit production. The mtDNA genome is a circular DNA molecule characterized by its relatively small size, which is useful for mapping genetic markers. The genome is 75 to 80kb and has a molecular weight of about $5.0x10^7$ Daltons (Borst and Grivell, 1978; Constanzo and Fox, 1991). For comparison, the human mitochondrial genome is about 17kb in size (Anderson et al., 1981). In most organisms, mitochondrial DNA lacks introns, but they are observed in yeast mtDNA. About 82% of base pairing is A/T, with approximately 200 C/G base pairs scattered across the genome (Wenzlau and Perlman, 1990). With regard to the genetic code and translation of mitochondrial mRNAs, the yeast and human systems differ from each other, and also from codon assignments found in the universal genetic code (Bonitz et al., 1980). In yeast, the usual UGA stop codon codes for tryptophan, AUA specifies methionine rather than isoleucine, and CUA codes for threonine instead of leucine.

Mitochondrial DNA codes for five percent of the proteins found in yeast mitochondria; the rest are encoded by nuclear genes, synthesized in the cytoplasm, and imported into mitochondria (Borst and Grivell, 1978). The mtDNA genes encode seven subunits of three major protein complexes of the electron transport chain required for oxidative phosphorylation. These genes code

for the cytochrome c oxidase subunits I, II, and III (*cox1*, *cox 2*, *cox 3*), apo-cytochrome *b* of the cytochrome bc_1 reductase complex (*cytb*), and subunits 6, 8, and 9 of the F_O ATPase (*atp6, atp8, atp9*) (Foury et al., 1998). The mtDNA also has genes coding for (large) 21S and (small) 16S rRNA subunits, a full set of 24 tRNAs, and the 9S RNA component of an RNase P-like enzyme, which is used in tRNA processing (Constanzo and Fox, 1991). Yeast mtDNA also encodes the ribosomal protein VAR1, which is a component of the small ribosomal subunit (16S) that is essential for mitochondrial protein synthesis. Finally, in strains with introns, there are several low abundance proteins encoded by the open reading frames of the genes coding cytochrome oxidase subunit I, cytochrome b, and the 21S rRNA. The roles of these proteins range from intron splicing to the transposition of the introns that encode them. Figure 4 depicts a map of the yeast mtDNA and its corresponding functional proteins.

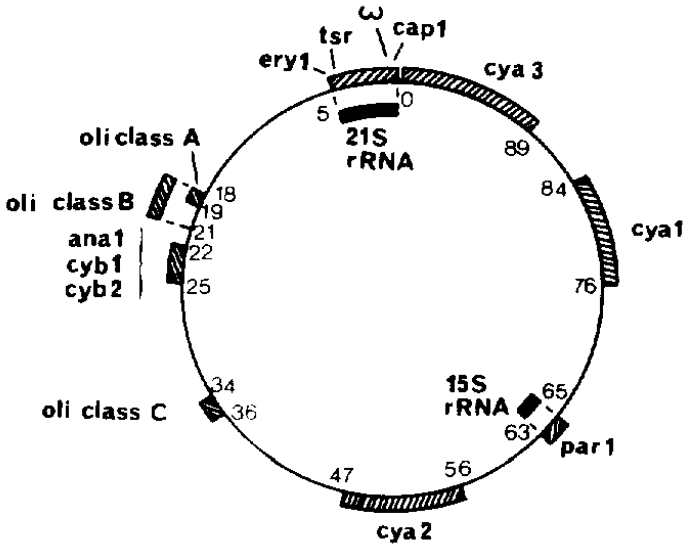

Figure 4a. *S. cerevisiae* physical mtDNA map showing genes and their respective map positions (Linnane and Nagley, 1978).

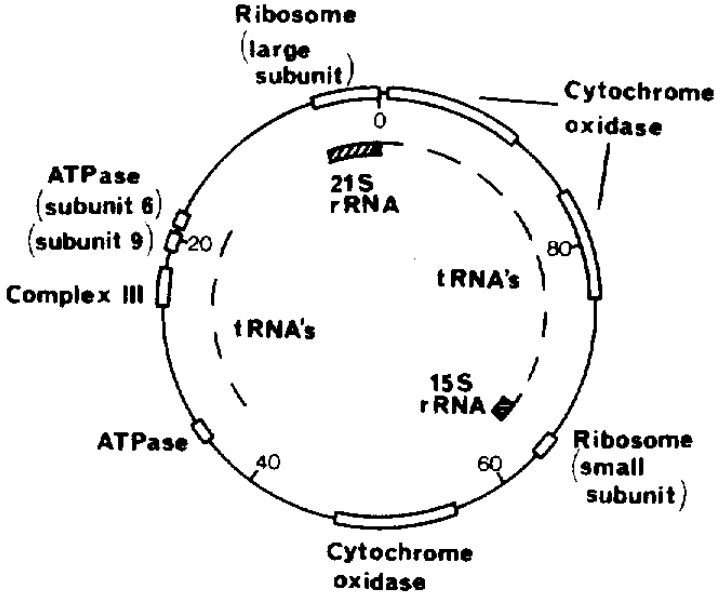

Figure 4b. Map of the functions encoded in the mtDNA based on the genetic loci and the proteins they encode (Linnane and Nagley, 1978).

ATP synthase

ATP synthases are a class of enzymes that catalyze the synthesis of ATP from ADP and P_i using the proton electrochemical gradient in aerobic respiration, and can also hydrolyze ATP (Giraud and Velours, 1994). There are several different types, which vary in function, structure, and the ions they transport. The F-ATPase complexes are important for ATP synthesis in most living organisms (Arnold et al., 1998). Found in inner mitochondrial membranes, chloroplast thylakoid membranes, and bacterial plasma membranes, these transmembrane F-ATPases are the primary producers of ATP for the organism.

F_1-F_O ATPases consist of two distinct domains, each with a separate function and location. (Arnold et al., 1998; Giraud and Velours, 1994). Owing to its hydrophobic nature, the F_O portion is embedded in the membrane and is involved in proton translocation. Here, protons flow from the intermembrane space into F_O, which generates energy in the central stalk from F_O rotation. The F_1 component, which is hydrophilic and extrinsically bound to F_O, is involved in ATP synthesis and hydrolysis. It is attached to the matrix side and uses the energy generated by the rotation of F_O to form ATP. Finally, a stator arm serves to connect the two domains (Tzagaloff et al., 2004). While the two domains are functionally coupled for normal ATP synthesis, F_1 can retain its hydrolyzing abilities if uncoupled. Figure 5 highlights a schematic representation of the F_1-F_O ATPase of *S. cerevisiae*.

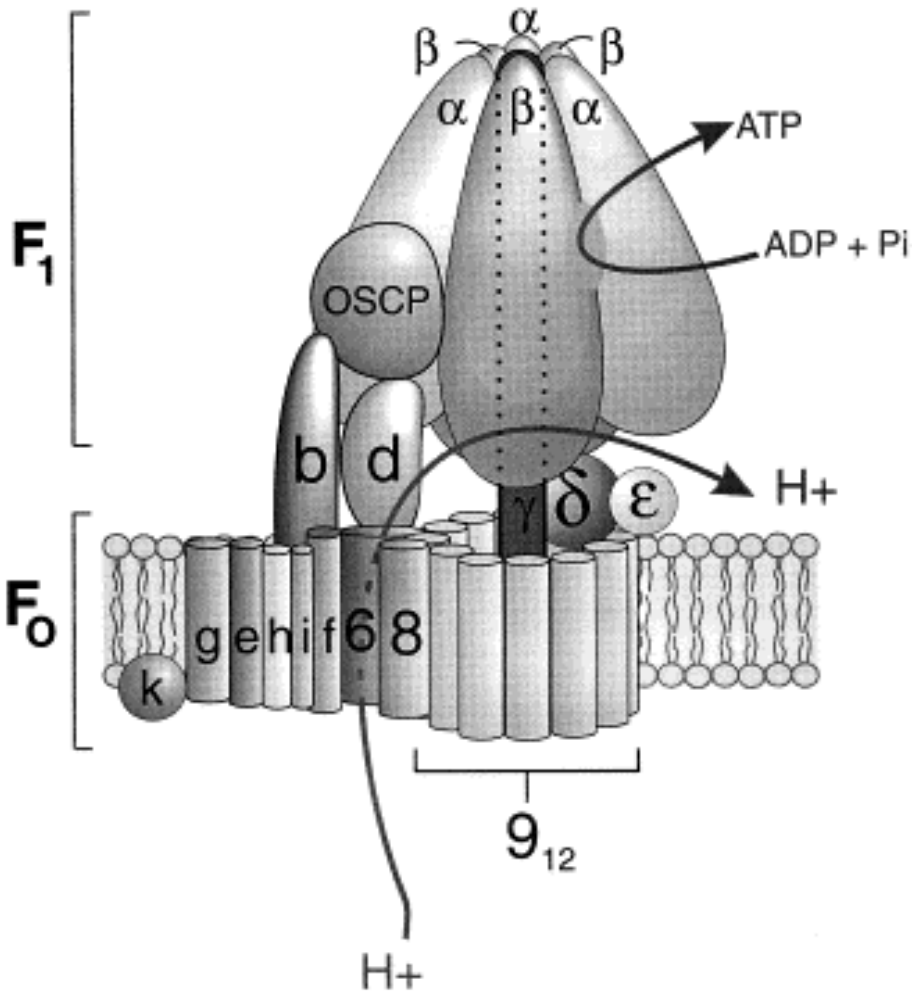

Figure 5. Schematic diagram of *S. cerevisiae* F_1-F_O ATPase embedded in the inner mitochondrial membrane. The central stalk and stator arm connecting F_1 with F_0 are depicted; the central stalk is comprised of subunits γ, δ and ε, while the stator arm (shown at left) is proposed to comprise OSCP together with subunit b and d. The F_O portion is shown with twelve subunits 9 comprising a ring, and subunits 6 and 8, involved in oligomycin binding, are also shown. Protons are depicted as passing through the inner mitochondrial membrane to reach the F_1 sector. ATP synthesis is indicated as occurring at the interface of the α and β subunits. (Devenish et al., 2000).

Yeast ATPase is complex and is comprised of numerous subunits distributed between the two domains. The F_O ATPase of *S. cerevisiae* contains eight subunits (Tzagoloff et al., 2004). An oligomer of the subunit Atp9p forms a rotating ring-like structure within the membrane bilayer, that when combined with Atp6p is able to drive proton transfer across the membrane. There are six other subunits, including Atp8p, Atp4p, the oligomycin sensitivity-conferring protein (OSCP) and its subunits d, f, and h, that form the stator arm connecting the two domains. F_1 is a hexamer formed by three α and three β subunits. The γ, δ, and ε subunits of F_1 form a central stalk and are responsible for linking the F_1 portion to the Atp9p ring. Finally, there are seven other proteins involved in the regulation, or oligomerization, of the ATPase complex.

Current research demonstrates that three of the subunits from the F_O portion, namely Atp6p, Atp8p, and Atp9p, or 6, 8, and 9 respectively, are mitochondrially-encoded and responsible for conferring oligomycin resistance (Arnold et al., 1998; Tzagoloff et al., 2004). Other F_O subunits and all F_1 subunits are encoded by the nucleus and synthesized in the cytosol. While the biogenesis of ATPase is not entirely understood, it has been demonstrated that the F_1 portion is assembled independently of F_O and attaches to the Atp9p ring, which is embedded in the membrane following its synthesis (Figure 6). Next, Atp8p, Atp4p, and others form the stator arm and attach. Final modification by Atp6p yields a functional ATPase complex, and it has been suggested that the late addition of Atp6p prevents an immature flux of protons across the membrane.

Arnold et al. (1998) demonstrated that F_1-F_O ATPase exists as a dimer to increase stability (Figure 7). In this way they propose that the F_1-F_O ATPase domains form into supracomplexes in order to enhance membrane stability and transduce energy in the most optimal fashion.

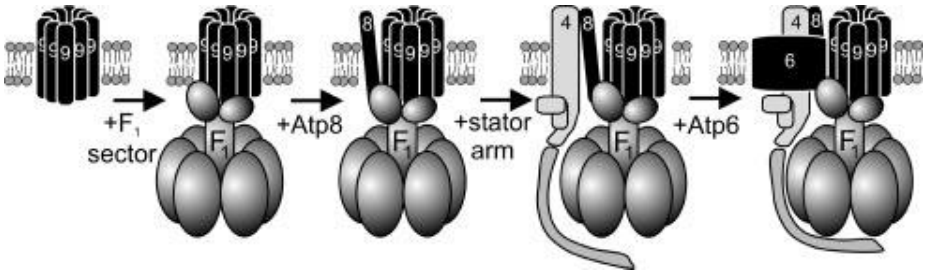

Figure 6. Diagram showing the assembly of F_1-F_O ATPase in *S. cerevisiae*. Mitchondrially-encoded subunits are shown in black, while nuclear-encoded subunits are in gray (Tzagoloff et al., 2004).

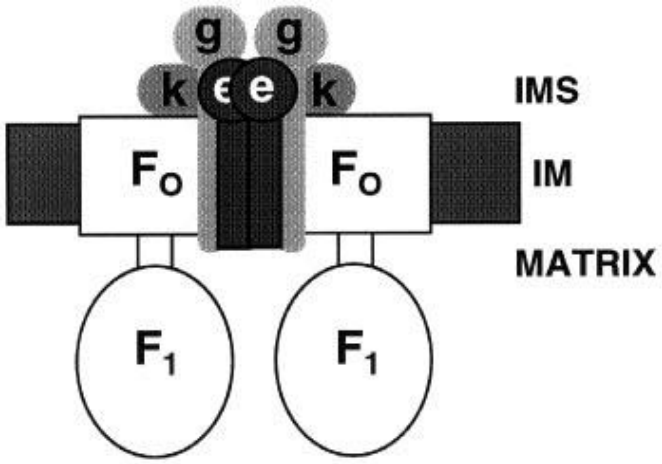

Figure 7. Schematic diagram of F_1-F_O ATPase dimerization occurring between the two F_O domains involving three subunits. Abbreviations: e, Su e/Tim11; g, Su g; k, Su k; IM, inner membrane; IMS, intermembrane space (Arnold et al., 1998).

Yeast Mitochondrial Mutations

Petite Mutants

Petite mutants, designated ρ^-, are those missing a large part of the mtDNA genome, ranging from 20% to 99.9%. Mutants that entirely lack mtDNA are designated ρ^0. These *petite* mutants have normal mitochondrial membranes but abnormal cristae, resulting in an overall smaller size of the mitochondrion. However, ρ^- mutants maintain the same amount of mtDNA as the wild type strains, as they are able to amplify the remaining segments in tandem repeats. *Petites* can mate with wild type strains, allowing for the transfer of their remaining mtDNA markers to the resulting diploids (Borst and Grivell, 1978). As a result of the genome deletions, *petites* are unable to carry out mitochondrial protein synthesis, and they are therefore respiratory deficient and unable to grow on non-fermentable carbon sources. On glucose, however, the strains may be maintained by using the fermentative pathway exclusively. *Petite* colonies are characterized by small, white colonies in comparison to wild type strain morphologies.

Mit$^-$ and Syn$^-$ Mutants

Mit$^-$ mutations affect synthesis of major mitochondrial membrane complex components, but mitochondrial protein synthesis is unaffected (Borst and Grivell, 1978). Syn$^-$ mutants are those affecting mitochondrial protein synthesis by targeting the synthesis machinery. These mutations, generally point mutations or

deletions, can be induced by exposure to Mn^{2+}. Both types of mutants are respiratory deficient, but mit^- mutants are distinguishable from *petites* because mit^- mutants retain active mitochondrial protein synthesis. When crossed, mit^- and syn^- mutants are able to either complement each other or undergo recombination to produce the wild type phenotype.

Antibiotic Resistance Mutants

Most wild type *S. cerevisiae* strains are unable to grow on a non-fermentable carbon source in the presence of antibiotics, although some display a natural resistance to treatment with an antibiotic. Antibiotics affect mitochondria in two main ways: namely by inhibiting protein synthesis or by disrupting oxidative phosphorylation. In a wild type, or antibiotic sensitive yeast, antibiotic binding on the surface of the mitochondrial ribosome can interfere with protein synthesis. However, if the antibiotic resistance marker is mutated, antibiotic interaction at the binding site may be impossible, thereby conferring resistance (Spithill et al., 1978). These mutations have been shown to be cytoplasmically inherited. Antibiotics such as erythromycin, chloramphenicol, spiramycin and paromomycin interfere with protein synthesis in this way. Analyses of *S. cerevisiae* strains with markers on the ribosomal region have resulted in the identification of multiple genetic markers on the 21 S ribosomal RNA subunits. With these data, a genomic map has been constructed of the mitochondrial DNA, and a fine structure genetic map exists for the 21 S rRNA gene (Netter et al.,

1974; Knight, 1980). The antibiotics that cause disruption to the electron transport chain and ATP production, like oligomycin, have been less extensively studied. There is currently no fine structure genetic map of the mutable sites conferring oligomycin resistance in *S. cerevisiae*.

Oligomycin

Oligomycin, a macrolide antibiotic, is an inhibitor of the ATPase complex associated with the inner mitochondrial membrane. It is isolated from *Streptomyces diastatochromogenes* and is found in three isomers, namely A, B, and C (Figure 8). Oligomycin is highly specific for the disruption of mitochondrial metabolism, observed both *in vitro* and *in vivo* (Avner et al., 1973). It is believed that oligomycin binds to the oligomycin sensitivity-conferring protein (OSCP) at F_O subunits 6 and 9, found in the stalk (Kim and Berdanier, 1999). Oligomycin blocks the synthesis of ATP as coupled to the electron transport chain in oxidative phosphorylation, and inhibits the energy linked reversal reactions in the electron transport chain when ATP is the energy source (Avner et al., 1973). Resistance to oligomycin can arise in both a nuclear and cytoplasmic fashion, each with variations in their cross resistance to mitochondrial inhibitors and uncouplers. Class I mutants arise from the mutation of a nuclear gene, thereby resulting in varying degrees of cross resistance to other antibiotics including oligomycin. This is due to changes in general membrane permeability, which prevent many antibiotics from crossing membranes and

interacting with their respective binding sites. There are also mutations in nuclear genes that can affect whether antibiotics bind to their targets. Therefore, Class I mutants are resistant to several mitochondrial uncouplers and inhibitors of mitochondrial protein synthesis (Griffiths and Houghton, 1974). Class II, or cytoplasmic mutants, however, are resistant specifically to oligomycin and related mitochondrial inhibitors like rutamycin and ossamycin. In contrast to Class I, these mutants are the result of cytoplasmic inheritance, and the resistance allele is located on the mitochondrial DNA.

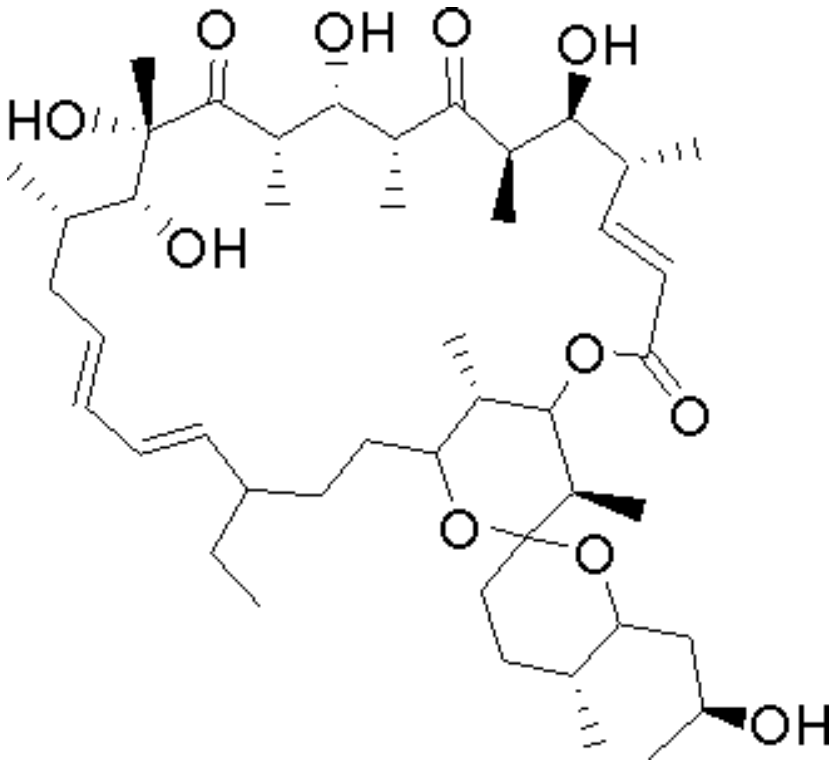

Figure 8. Chemical structure of oligomycin from *Streptomyces diastatochromogenes*, shown as a mixture of the A, B, and C isomers (Sigma-Aldrich, 2008).

In 1968, Parker et al. found that oligomycin resistance (O^R) in *S. cerevisiae* was controlled by at least two genes, whereby a single gene is responsible for resistance in any one mutant. Later in a study of rutamycin resistant *S. cerevisiae* mutants, Tzagoloff and Meagher (1971) describe the oligomycin/rutamycin sensitive ATPase, or F_O complex, as having nine distinct subunits. Five of these polypeptide subunits, namely 1, 2, 3, 4, and 8, are shown to also be a part of the F_1 portion. Subunits 5, 6, 7, and 9, however, are unique to the rutamycin-sensitive complex and are responsible for sensitivity to the antibiotic.

Avner et al. (1973) demonstrated that all oligomycin resistant mutants analyzed could be sorted into two allelism groups, representing two loci, O_I and O_{II} . These loci were shown to be either unlinked or very weakly linked, with a recombination frequency around 10 percent[1], with the majority of the mutations mapping to the O_I locus. The O_I group was characterized by a maximum recombination frequency of 0.02 percent, indicating that mutants with this mutation exhibit recombination at very low frequencies. Avner et al. concluded that the O_I and O_{II} loci represented two distinct genes that control oligomycin sensitivity exclusively.

Lancashire and Griffiths (1975) presented evidence for a third mitochondrial locus, named OLI III, which was found to be nonallelic with both

[1] Recombination frequencies in Avner et al. (1973) were calculated as follows: [(#O^S/ #O^R) x 100]. This is in contrast to the frequencies calculated in this study, where the frequencies of sensitive recombinants were then multiplied by 2 to account for undetectable resistant recombinants, following the formula: [((#O^S/ #O^R) x2) 100].

OL I and OL II. Furthermore, they demonstrated that OL I and OL III are closely linked. Clavilier (1976) isolated the *oli4* locus, which was the first mitochondrial mutant of the locus O^R_9 to not confer resistance at the ATPase level. Therefore, O^R_9 mutants can have a sensitive ATPase, which is seemingly because the mutation does not modify a peptide involved in an oligomycin-binding peptide. The locus *oli4* is found in wild type strains and is speculated to be different from *oli1* and *oli3*, since the latter are linked. Clavilier found that *oli4* mutants would probably not be found in a normal selection for O^R mutants, since they are less resistant and grow in the presence of reduced levels of oligomycin. Clavilier then proposed that each of the four *oli* loci may code for four of the ATPase subunits. However, the study did not conclusively demonstrate that each locus represents a discrete gene, whereby each of the theoretical mitochondrial genes would code for a discrete protein. Clavilier suggests that it may be proved that each locus represents one of the ATPase structural genes by confirming that each allele in each gene gives an independent modification of the oligomycin-sensitive ATPase complex. Tzagoloff et al. (1975) demonstrated that oligomycin sensitivity is controlled by four polypeptide subunits that are synthesized on mitochondrial ribosomes and are assumed products of the mtDNA. However, Griffiths and Houghton (1974) presented evidence that oligomycin sensitivity is due to two mitochondrially synthesized ATPase subunits and indicated that further studies must be performed to understand the nature of all four mitochondrially synthesized subunits.

In 1979 Somlo and Krupa described the ATPase loci as being confined to two unlinked regions, the first containing the closely linked OLI 1, OLI 3, and PHO 2 loci, and the second containing the OLI 2, OLI 4, and PHO 1 loci. The *oli2* and *oli4* genes are located only 200 base pairs apart on the genome and recombine with a frequency of 4.2%, while *oli1* and *oli2* are separated by 14 kilo bases (Novitski et al., 1984). John et al. (1986) describe *oli1* as coding for subunit 9 of ATPase and *oli2* coding for subunit 6. The genes coding for the remaining mitochondrially-encoded subunit 8 is also resolved. Subunit 8 is coded for by the *aap1* gene (ATPase associated protein) as part of the mtDNA (Macreadie et al., 1983). Due to the subunits they encode, the *oli1*, *oli2*, and *aap1* genes are also referred to as *atp9*, *atp6*, and *atp8*, representing subunits 9, 6, and 8 respectively (Foury et al., 1998). Current literature agrees that the three polypeptide subunits of ATPase encoded by mitochondrial DNA are subunits 6, 8, and 9. However, there are discrepancies in terminology of these loci which will be addressed later.

Manganese Mutagenesis

Mitochondrial mutations conferring antibiotic resistance may be induced by a variety of techniques. A frequently used method, and the one employed in this study, is manganese mutagenesis (Putrament et al., 1973; Knight, 1980). Manganese mutagenesis is specific for mitochondrial mutations as Mn^{2+} interacts primarily with mtDNA polymerase. DNA polymerases of all types utilize a divalent cation, namely Mg^{2+}, for their function (El-Deiry et al., 1984). Others

like Mn^{2+}, Co^{2+}, Be^{2+}, and Ni^{2+} can act in place of Mg^{2+} as physiological activators. When the carcinogen and mutagen Mn^{2+} is introduced, an increased frequency of *in vitro* incorporation is observed. This disrupts DNA polymerase's highly accurate base pairing mechanism, specifically affecting the base selection specificity by the polymerase and functionality of the 3´, 5´ proofreading exonuclease. This effect has been observed in *Escherichia coli* by El-Deiry et al. (1984), where Mn^{2+} altered the specificity of the 3´, 5´ proofreading exonuclease activity for a mismatched primer terminus. Their study suggests that Mn^{2+}'s mutagenicity is the result of interaction with the enzyme-template complex, with possible alterations at the polymerase and endonuclease active sites. If magnesium is also used in the mutagenesis procedure, the growth of respiratory sufficient cells is increased, thereby decreasing the appearance of *petites* (Putrament et al., 1973). Putrament et al. (1975) demonstrated that manganese specifically induces mitochondrial mutations in *S. cerevisiae*, as most mutations are extranuclear.

Project Objectives

There are currently four oligomycin resistance loci that have been mapped on the mitochondrial DNA: *oli1*, *oli2*, *oli3*, and *oli4*. Subunit 9 is encoded by *oli1* (*oli3* or *atp9*), subunit 6 is encoded by *atp6* (*oli2*, *oli4* or *pho1*), and subunit 8 is encoded by *atp8*, or *aap1*. The regulatory regions and binding sites have been defined: *oli1* at 46,723 to 46,953 base pairs; *apt6* at 28,487 to 29,266 base pairs;

and *atp8* at 27,666 to 27,812 base pairs (Teixeira et al., 2006). Although the positions have been verified, there are no fine structure genetic maps of these functional genes.

The goal of this study is to isolate new oligomycin resistant mutants using manganese mutagenesis, to affect these mitochondrial genes that confer oligomycin resistance. Following rudimentary analyses, these mutants will be characterized in respect to their allelic relationships with each other. It is hoped that mutants will be generated from mutations in each of these genes, and that tester strains may be identified.

The second goal of the project is to generate a fine structure genetic map of the genes conferring oligomycin resistance. Using the calculated recombination frequencies from the crosses, we will be able to place the mutants into complementation groups for these genes, and map the locations of the mutations along the genes. A fine structure map of the oligomycin resistance genes will be useful in understanding the loci and their functions. Studies to date have shown that fairly simple techniques of manganese mutagenesis and classical transmission genetics are useful for identifying mutable sites within the 21S and 16S rRNA genes that confer mitochondrial antibiotic resistance in *S. cerevisiae* (Netter et al., 1974; Knight, 1980). This project looks to use the same techniques to develop a fine structure map that highlights the structural genes of the ATPase subunits and those that confer oligomycin resistance.

MATERIALS AND METHODS

Yeast Strains

Table 1 lists the *Saccharomyces cerevisiae* strains used in this study. Strains are listed as haploids with their nuclear genotypes indicated, including mating type and auxotrophic requirement. Mitochondrial genotypes for oligomycin resistance and sensitivity are also noted. All strains were incubated at 30ºC unless otherwise noted.

Table 1: *Saccharomyces cerevisiae* strains used in this study, with their mating types, nuclear genotypes, and mitochondrial markers indicated.

Strain	Nuclear Genotype	Mitochondrial O^R/O^S Marker
D6-5	α arg	O^S
HB2	α ade	O^S
CCD6-1	α arg	O^S
CCD6-7	α arg met	O^R
IL126-1B	α his	O^S
CS*L	α ade his	O^S
HAT	*a* trp	O^S
55R2	*a* ura	O^S
MtS-14	*a* arg	O^S
5DSSCO	*a* ura	O^S

Media

Several types of media were used in experimental procedures to selectively or non-selectively grow and maintain the yeast strains. Their compositions are as follows:

YD Media: is a complete medium containing glucose, a fermentable carbon source, used for the maintenance of all strains.

10g/L yeast extract
20g/L bactopeptone
20g/L glucose

YG Media: is a complete medium that contains the non-fermentable carbon source glycerol, which must be metabolized by the mitochondria. Growth on this medium confirms respiratory competency.

10g/L yeast extract
20g/L bactopeptone
32ml/L glycerol

YG+O Media: is YG media supplemented with 1.5mg of the antibiotic oligomycin per liter YG media. Oligomycin was obtained from Sigma-Aldrich in a mixture of A, B, and C isomers. Oligomycin is dissolved in 95% ethanol and added to the media after autoclaving to ensure integrity of the antibiotic. It is used for selecting and distinguishing oligomycin resistant and sensitive strains.

MD Media: is a minimal medium that contains a fermentable carbon source, glucose, to induce yeast to use the fermentative pathway exclusively. It is used for matings, random diploid analysis, and the determination of auxotrophic requirements when supplemented with growth factors, such as amino acids. It is prepared using Wickerham's minimal salts and Wickerham's minimal praline medium with low sulfate as described in Wickerham (1946) and as modified by Birky (1975) to a pH of 6.8 and lower calcium concentration.

10ml/L MD buffer
10ml/L MD salts
1ml/L MD vitamins
1ml/L MD calcium
0.1ml/L MD trace minerals
20g/L glucose

Pre-Sporulation Media: A complete medium rich in fermentable carbon, used to prepare diploids for sporulation.

8g/L yeast extract
3g/L bactopeptone
100g/L glucose

Sporulation Media: A complete medium containing sodium acetate and a small amount of fermentable carbon, used to induce the sporulation of diploids.

10g/L sodium acetate
1g/L yeast extract
0.5g/L glucose

In order to prepare plates, 20g of agar is added to each liter of liquid media before autoclaving.

Sporulation

To generate haploid colonies from diploid strains, the diploid strains are grown overnight on YD media, then transferred to glucose-rich pre-sporulation plates for 48 hours. Strains are then grown on sporulation media containing sodium acetate and few nutrients, which induce the cells to form asci. After 48-72 hours incubation at 30 ºC, a cell suspension is made and examined under the microscope at 100x to check for the presence of asci in tetrads. If asci are identified, a heavy suspension of the sporulated cells is prepared in 5ml of distilled water. Glusulase, a mix of snail digestive enzymes, is added to the suspension at a concentration of 0.025ml per ml of distilled water to release the spores from the asci walls. The suspension is vortexed and placed in the incubator/shaker for 1 hour at 30ºC. The suspension is centrifuged at 8,000 rpm for 10 minutes and the resulting pellet is rinsed twice with distilled water and

resuspended in 5ml of distilled water. The suspension is sonicated at a micro tip limit of 4 watts for 1 minute to complete the release of the haploid ascospores. Then a 1:10 suspension (about 10^6 per ml concentration) is made and cross streaked on several YD master plates. Plates are incubated at 30ºC for 72 hours, at which point necessary tests for nuclear auxotrophic markers, mating type, and mitochondrial genotype can be performed.

Haploid Identification

Following sporulation, small, non-white colonies are picked and patched onto YD media. After 24 hours they are replica-plated onto MD and YG plates. Colonies that fail to grow on MD, but show confluent growth on YG are identified as auxotrophic respiratory sufficient haploids. To characterize sporulated haploid strains, the auxotrophic requirement and mating type must be determined.

To establish auxotrophic requirements, cells are patched onto a YD master plate and then replica plated onto an MD plate (the control), as well as MD plates supplemented with all combinations of amino acid growth factors, purine, and pyrimidine potentially required by the auxotroph. The supplemented plates contain MD media with the addition of 0.1ml of a 1% amino acid or nucleic acid solution. It is expected that no strains will grow on the MD plates alone, while growth should be apparent on the plates supplemented with the strain's auxotrophic requirement.

Mating types are determined by crossing the ambiguous haploid with tester strains with known *a* or α mating types. Positive mating results indicate that the haploid in question is of the opposite mating type. For example, if the mating of the haploid with an *a* strain is successful, we can deduce that the new haploid is of the α mating type.

Drop Testing

To determine a strain's natural sensitivity or resistance to oligomycin, and to confirm the resistance of isolated haploid mutants, drop testing is performed. Heavy suspensions of each strain are prepared in 5ml of water, and single drops are placed on MD, YG and YG + O plates. This allows for comparison of growth for equal concentrations of cells on each plate. After 48 hours, the strains are scored as sensitive or resistant to oligomycin. It is expected that resistant haploid strains will grow well on YG and YG + O plates, with no growth on the MD plates.

Manganese Mutagenesis

Selected strains are grown overnight in 50mL liquid YD and diluted to an optical density (O.D.) between 0.05 and 0.15 (an optical density of 0.1 is equivalent to 2×10^7 haploid cells per ml). The cells are allowed to grow at 30ºC for 4-5 hours while shaking until an O.D. of 0.40 is obtained, indicative of log phase growth. One ml of 1M manganese chloride solution and 0.5ml 0.1M

magnesium chloride solution are added to the flask to yield a final concentration of 20mM manganese and 1.0mM magnesium. Addition of magnesium serves to decrease the number of ρ^- *petites* and overcome the growth inhibition caused by manganese (Putrament et al. 1973). After being returned to the incubator/shaker for 5 hours, the cells are centrifuged for 10 minutes at 8,000rpm. The pellets are washed twice with 20ml 10mM magnesium chloride solution and resuspended a third time in 20ml sterile water. Two hundred micro liters are spread on each of several YG+O plates, which are and incubated for 7-10 days, after which oligomycin resistant colonies are picked and subcloned onto YD media.

Subcloning

Subcloning is used to purify new mutant strains and to purify those strains with evidence of revertants or contamination. A full loop of heavy suspension is streaked across a YD plate at 90º turns to streak for isolation. Plates are incubated for 48 hours or until confluent growth is apparent. The healthy colonies that are free of revertants are picked and patched onto YD plates. They can then be used for further analysis.

Plate Matings

Oligomycin resistant haploid mutants are picked and patched overnight on YD plates so they are in log phase growth in preparation for mating. The haploid parent strains are suspended in 5ml water and a single drop (about 7.5 micro

liters) of the suspension of each *a* and α haploid parent are placed together on an MD plate. As a control for phototrophic revertants, single drops of each haploid parent are also placed on separate locations on the MD plate. The plates are then incubated for 48-72 hours and observed for growth of diploid progeny. Strains showing revertants are subcloned and the mating procedure repeated. Successful matings are further analyzed using random diploid analysis.

Random Diploid Analysis

Random diploid analysis is used to distinguish mitochondrial inheritance from nuclear inheritance and score O^R and O^S colonies for allelism testing. Following a successful mating, the following steps are used to analyze the progeny (Figure 9):

1. Cross Streaking: a sample of the diploid progeny is collected with an inoculating loop and suspended in 10mL of sterile water to yield a "just perceptibly cloudy" suspension (cell concentration approximately 10^6 cells per ml). Not quite a full loop of the suspension is cross-streaked across the entire surface of an MD plate. Each plate is expected to yield 100-300 diploid colonies, and therefore 5-6 MD plates are cross-streaked to yield a total of about 1000 independent colonies. Each colony is pure for a mitochondrial type.
2. Replica-Plating: after 3 days of incubation, sterile velvet is used to make an imprint of the cross-streaked MD master plates, which is then used to

transfer the yeast to both YG and YG+O plates. The YG+O plate distinguishes resistance or sensitivity to oligomycin, while the YG plate acts as a control for *petite* progeny.

3. Counting and Scoring: after 2-3 days of incubation, the colonies are counted and scored as either oligomycin sensitive or resistant. This produces the average genetic result of hundreds of matings. A minimum of 1000 colonies is needed to provide reliable allelism data.

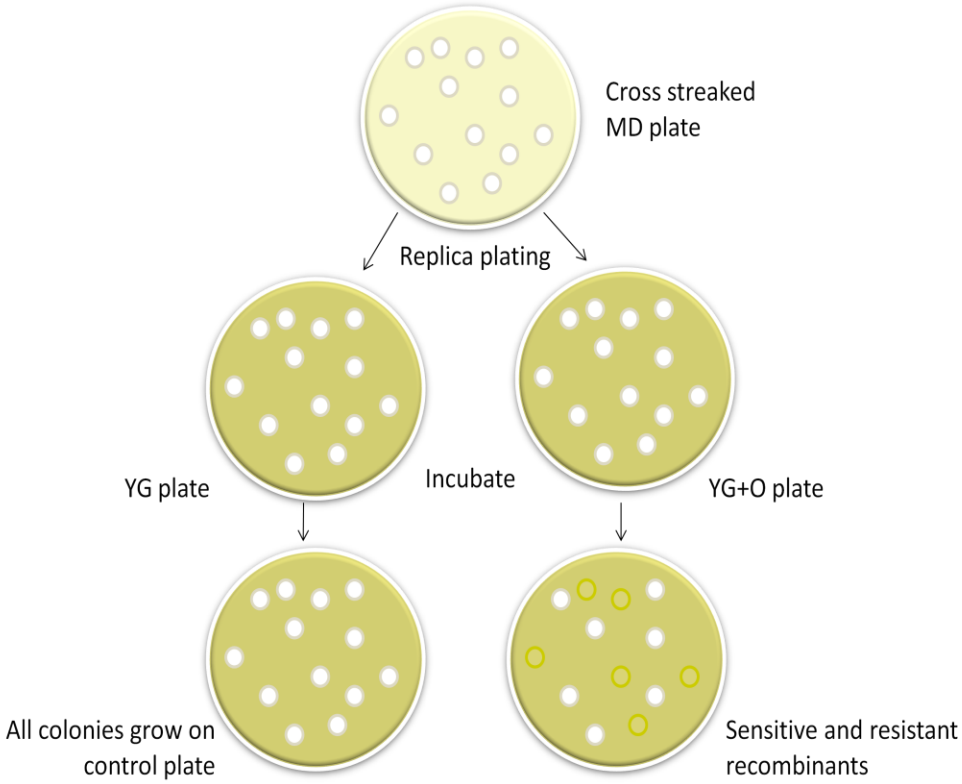

Figure 9. Diagram of random diploid analysis following mating and cross streaking procedures. At least 1000 colonies are then scored as sensitive or resistant using the YG+O plate.

Determination of Mitochondrial Inheritance

After mutagenesis, the antibiotic resistant haploid strains are characterized for whether the resistance is conveyed via mitochondrial or nuclear inheritance. The haploids are mated with a strain of known antibiotic sensitivity, and the vegetative diploid progeny are analyzed. After random diploid analysis, the presence of both resistant and sensitive colonies on the drug plates signifies mitotic segregation of alleles conferring resistance, and therefore indicates mitochondrial inheritance. If the drug plate yields all sensitive or all resistant colonies for a particular cross, we can consider the mutation to be the result of nuclear genes, and the strain is eliminated from further testing.

Allelism Testing

Once O^R mutants are isolated and determined acceptable for study, they are crossed with other O^R mutants of opposite mating types and complimentary auxotrophic requirements. Random diploid analysis is used to generate at least 1000 colonies that are scored as either oligomycin resistant or sensitive. By calculating recombination frequencies for each cross, allelism is determined for the mutations. Recombination frequencies are calculated with the following formula: [(# O^S/ #O^R) x100] x2. The lack of O^S recombinants shows that the mutations are allelic at that locus (Figure 10). Allelism is defined as a mutation in the same base pair or within three to four surrounding bases.

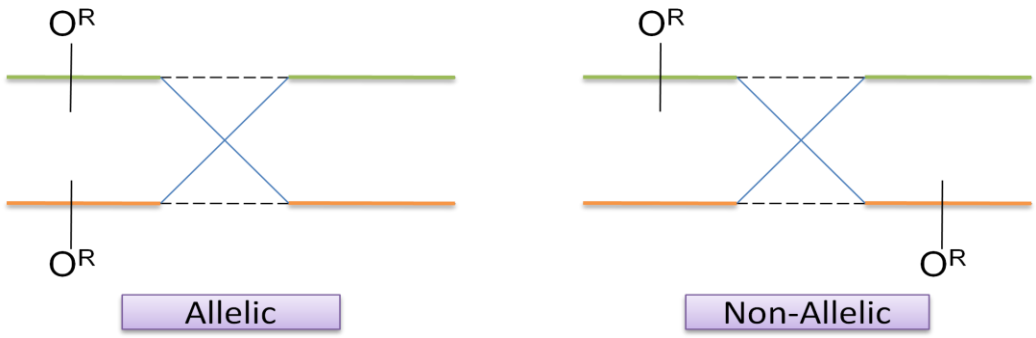

Figure 10. Diagram showing allelic (oligomycin resistant) and non-allelic (oligomycin sensitive) mtDNA molecules following recombination.

RESULTS

Manganese Mutagenesis and Mutant Characterization

Three rounds of manganese mutagenesis were performed on the master strains 55R2, HB2, D6-5, CCD6-1, Mts-14, and HAT, after they were confirmed to be sensitive to oligomycin via drop testing (Table 1). Master strains were selected for their ability to mate well with other strains, their retention of auxotrophic nature, and an equal distribution of auxotrophic requirements and mating types. In the first mutagenesis procedure, no HAT O^R mutants grew following the plating and ten day incubation. In the second, two mutants HAT O^R 1 and O^R 2 were isolated following the mutagenesis. Strain CCD6-1 O^R also showed relatively low growth, with only sixteen colonies isolated. All other strains showed the expected sensitive background growth with numerous resistant colonies following the ten day incubation period, with 44-154 mutants isolated for each strain. Three hundred and eighty-eight total mutants were isolated from the three procedures, and these were further tested to determine their properties (Table 2).

Mutants were first subcloned to confirm their purity, and then mutants were drop tested onto MD, YG, and YG+O plates to ensure that strains were haploid, auxotrophic, respiratory sufficient, and resistant to oligomycin. One hundred and sixty-nine mutants were then identified as being viable O^R mutants (Table 2). These strains were crossed with known O^S tester strains to determine

whether the O^R mutants' resistance mutation was nuclear or mitochondrial in nature.

Both HAT O^R strains showed growth in matings when crossed with CCD6-1 and IL126-1B strains; however, MD plates showed no growth when the diploid colonies were cross streaked. Therefore, these matings were inconclusive and HAT was not used in allelism studies.

Twenty-nine 55R2 O^R mutants were eliminated due to prototrophic revertants, failure to mate, or loss of oligomycin resistance. The fifteen remaining 55R2 O^R mutants were crossed with CCD6-1, and eleven of these were shown to have mitochondrial mutations (Appendix; Table 8). In several instances, the growth of O^R colonies on plates containing oligomycin was not sufficiently robust to be considered true resistance. The decision was made to only proceed with mutants demonstrating strong resistance to oligomycin.

Thirteen CCD6-1 O^R mutants were crossed with 5DSSCO, and one mutant was of mitochondrial nature (Appendix; Table 9). While two other crosses showed 3-4 possible sensitive recombinants out of a total 80-82 colonies, the ratio was considered to be sufficiently biased so as to cause doubt about the mitochondrial location of the mutations, and the strains were not used further.

Seventy-six HB2 O^R mutants were isolated but only nineteen were deemed viable for study. Of these nineteen crossed with the tester Mts-14, fifteen were determined to carry mitochondrial mutations and were further characterized

(Appendix; Table 10). The mutant HB2 O^R 11 did not show the strong colony growth needed to demonstrate true resistance and was not studied further.

Similarly, of the one hundred and fifty-four D6-5 O^R mutants isolated, only thirty-six were suitable for use in determining mode of inheritance. These thirty-six D6-5 O^R mutants were crossed with 5DSSCO and twenty-five were shown to be mitochondrial (Appendix; Table 11). In one instance, growth of the O^R colonies was not sufficient enough to be considered genuine resistance, and the mutant D6-5 O^R 46 was not used in future characterizations.

Eighty-six Mts-14 O^R mutants were shown to be acceptable for study (Appendix; Table 12), and were crossed with IL126-1B and CS*L testers. None of the matings were successful, and no other strains were identified as complementary maters for Mts-14, due to revertants and limitations in variable auxotrophic requirements. As a result, it is currently unknown how many of the total eighty-six Mts-14 O^R mutants are carrying a mitochondrial mutation for oligomycin resistance.

In total, 62.7 percent of mutants tested for nuclear versus mitochondrial inheritance were, indeed mitochondrial. Additionally, 30.1 percent were determined to be of nuclear inheritance, and 7.2 percent were inconclusive due to a weak demonstration of strong antibiotic resistance.

Allelism Testing

Following these initial characterizations, fifty-one O^R mitochondrial mutants were identified for use in allelism studies (Table 2), and Table 3 lists all theoretical combinations of matings. While every precaution was made to ensure viability of strains before use in allelism testing, there continued to be revertants and several strains failed to mate over the duration of study. Due to these issues, allelism data were not obtained for all fifty-one O^R mitochondrial mutants.

Mutants 55R2 O^R and HB2 O^R were crossed in combination. However, none of the matings showed any diploid growth, and no allelsim data were obtained for any of the HB2 O^R mutants.

Mutant CCD6-1 O^R 10 was crossed with the eleven viable 55R2 O^R mutants (Appendix; Table 13). Recombination frequencies ranged from 0 to 86.9 percent. CCD6-1 OR 10 was shown to be allelic with 55R2 O^R 7, 55R2 O^R 15, 55R2 O^R 19, and 55R2 O^R 20.

All viable D6-5 O^R mutants were crossed with viable 55R2 O^R mutants in a total of 193 matings (Appendix; Tables 14- 22). Recombination frequencies ranged from 0 to 135.0 percent. From these crosses, fifteen mutants were found to be allelic with each other (Table 4). Mutants D6-5 O^R 17, D6-5 O^R 57, and 55R2 O^R 12 also demonstrated allelism, although 55R2 O^R 12 was not allelic with any other D6-5 O^R or CCD6-1 O^R mutants. While most crosses were successful, some strains were eliminated at various points to prototrophic revertants. Data for crosses between several 55R2 O^R mutants (55R2 O^R 7, O^R 11, O^R 15, O^R 40, and

O^R 41) and D6-5 O^R mutants are missing in some crosses for this reason. Following subcloning of these strains, all but 55R2 O^R 7 and 55R2 O^R 41 were purified and are currently available for use.

Several mutants listed in Table 5 demonstrated no allelism with any strains following allelism crosses. Many of these crosses resulted in recombination frequencies much higher that the theoretical threshold of twenty-five percent.

In most crosses, a minimum of 1000 colonies were scored following the initial mating. If less than 1000 were obtained, the matings were repeated and the new data were combined with the initial crosses. Some matings, however, resulted in less than the desired number and were unable to be repeated due to time restraints. Although recombination frequencies were still calculated, it must be noted that these data are not significant, and further analyses must be performed. Similarly, in some cases where the recombination frequencies were higher than the theoretical threshold of twenty-five percent, crosses were repeated, and the new data were added to existing data, allowing for the recalculation of frequencies.

Oli1 Test Crosses

Following drop tests, CCD6-7 was selected as a naturally O^R master strain with an *oli1* mutation (Appendix; Table 23). It was crossed with 55R2 O^R 12, 55R2 O^R 19, 55R2 O^R 20, and 55R2 O^R 43. The 55R2 strains were selected for

the following reasons: two strains that were consistently allelic with each other (55R2 O^R 19, 55R2 O^R 20) and two strains that were consistently non-allelic with each other (55R2 O^R 12, 55R2 O^R 43). CCD6-7 was found to be allelic with both 55R2 O^R 19 and 55R2 O^R 20 at *oli1* (Appendix; Table 23). CCD6-7 was not allelic with 55R2 O^R 12 or 55R2 O^R 43, showing 8.01 and 51.7 percent recombination respectively.

Average recombination frequencies were calculated (Table 6) and sixteen mutants were determined to be allelic with *oli1* following these calculations (Table 7). In addition to these, eight other mutants were determined to be closely linked to the *oli1* locus, indicating the location of twenty-four mutants in the *oli1-oli3* region. Eleven mutants demonstrated recombination frequencies over the theoretical twenty-five percent. These mutants were assigned to the *oli2-oli4* region, as they presumably map outside of the *oli1-oli3* region (Figure 11). As there are no *oli2* or *oli4* tester strains in the laboratory, this study was unable to definitively confirm this assignment.

Stock Strain Data

Following initial drop testing to determine sensitivity or resistance to oligomycin, four strains showed natural resistance: 5-1/6, CCD6-2, CCD6-3, and CCD6-4. At this time, HBT and HART also showed very slight oligomycin resistance. When this test was repeated two years later, some changes were observed. Strains 5DSSCO, D22-5, and CCD6-7 were shown to be resistant to

oligomycin in addition to those previously noted. In order to clarify these results, it will be necessary to subclone the strains and re-test to ensure the purity of the resistance markers.

Fine Structure Genetic Map

A fine structure genetic map was obtained of the *oli1-oli3* region using recombination data (Figure 12). The *oli1* locus was used as the basis for determining the other loci. Average recombination frequencies were used to construct the final map (Table 6). All values were used in calculating the averages granted they were under the theoretical twenty-five percent threshold of recombination. In the absence of tester strains and data from additional mating combinations, it was difficult to resolve the exact positions of the loci; however, this fine structure genetic map represents a potential distribution of map positions for the *oli1-oli3* region.

Table 2: Summary of the total number of O^R mutants isolated from manganese mutagenesis and the number of mutants selected for further characterization after preliminary testing.

Strain	Total number O^R mutants isolated	Total number O^R mutants further studied	Total number O^R mutants used in allelism testing
55R2	44	15	11
Mts-14	96	86	0
CCD6-1	16	13	1
HB2	76	19	15
D6-5	154	36	25
HAT	2	0	0
Total	**388**	**169**	**51**

Table 3. List of all potential mating combinations from the mutants generated by manganese mutagenesis.

a **Strain**	***α*** **Strain**	**Successfully Performed**
55R2 O^R	HB2 O^R	
	D6-5 O^R	X
	CCD6-1 O^R	X
HAT O^R	HB2 O^R	
	D6-5 O^R	
	CCD6-1 O^R	
Mts-14 O^R	HB2 O^R	

Table 4. List of allelic mutants determined from crosses between D6-5 O^R and 55R2 O^R mutants.

Allelic Mutants	
D6-5 O^R 2	D6-5 O^R 91
D6-5 O^R 17	D6-5 O^R 99
D6-5 O^R 39	D6-5 O^R 140
D6-5 O^R 55	55R2 O^R 15
D6-5 O^R 57	55R2 O^R 19
D6-5 O^R 58	55R2 O^R 20
D6-5 O^R 84	55R2 O^R 42
D6-5 O^R 87	

Table 5. List of mutants that demonstrated no allelism in any crosses performed.

Non-Allelic Mutants	
D6-5 O^R 5	D6-5 O^R 137
D6-5 O^R 6	D6-5 O^R 138
D6-5 O^R 68	D6-5 O^R 141
D6-5 O^R 89	D6-5 O^R 145
D6-5 O^R 104	D6-5 O^R 149
D6-5 O^R 113	55R2 O^R 6
D6-5 O^R 129	55R2 O^R 40
D6-5 O^R 132	55R2 O^R 41
D6-5 O^R 133	55R2 O^R 43

Table 6. Average recombination frequencies between *oli1* and non-allelic mutants.

Strain	Number of values used in calculation	Average Recombination Frequency
55R2 O^R 6	7	10.3
55R2 O^R 12	10	5.52
55R2 O^R 40	8	4.99
55R2 O^R 41	8	8.65
55R2 O^R 43	12	6.74
D6-5 O^R 5	4	9.38
D6-5 O^R 6	4	1.41
D6-5 O^R 68	4	62.3
D6-5 O^R 89	2	16.2
D6-5 O^R 104	4	84.3
D6-5 O^R 113	4	58.5
D6-5 O^R 129	3	68.1
D6-5 O^R 132	4	73.6
D6-5 O^R 133	3	67.6
D6-5 O^R 137	4	64.4
D6-5 O^R 138	4	76.5
D6-5 O^R 141	4	60.9
D6-5 O^R 145	4	79.8
D6-5 O^R 149	4	58.5

Table 7. Mutants mapping to the *oli1-oli3* and *oli2-oli4* regions.

***oli1-oli3* Region**	***oli2-oli4* Region**
CCD6-1 O^R 10	D6-5 O^R 68
55R2 O^R 6	D6-5 O^R 104
55R2 O^R 7	D6-5 O^R 113
55R2 O^R 12	D6-5 O^R 129
55R2 O^R 15	D6-5 O^R 132
55R2 O^R 19	D6-5 O^R 133
55R2 O^R 20	D6-5 O^R 137
55R2 O^R 40	D6-5 O^R 138
55R2 O^R 41	D6-5 O^R 141
55R2 O^R 42	D6-5 O^R 145
55R2 O^R 43	D6-5 O^R 149
D6-5 O^R 2	
D6-5 O^R 5	
D6-5 O^R 6	
D6-5 O^R 17	
D6-5 O^R 39	
D6-5 O^R 55	
D6-5 O^R 57	
D6-5 O^R 58	
D6-5 O^R 84	
D6-5 O^R 87	
D6-5 O^R 89	
D6-5 O^R 91	
D6-5 O^R 99	
D6-5 O^R 140	

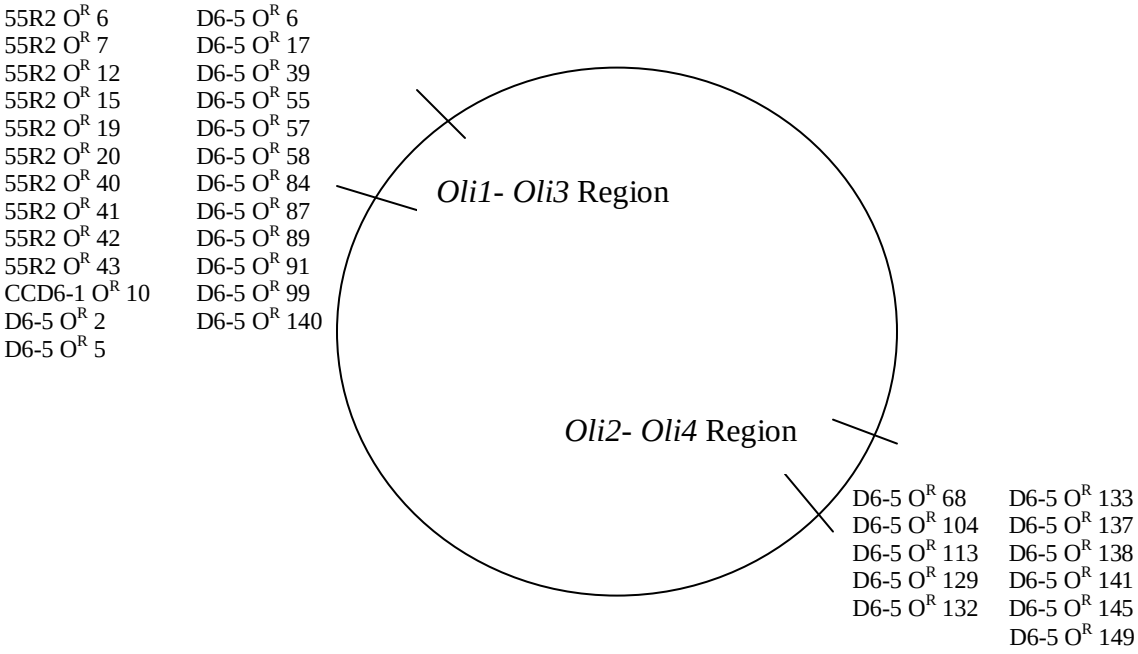

Figure 11. Schematic diagram of S. cerevisiae mtDNA showing the *oli1-oli3* and *oli2-oli4* regions and the mutants from this study that mapped to the regions respectively.

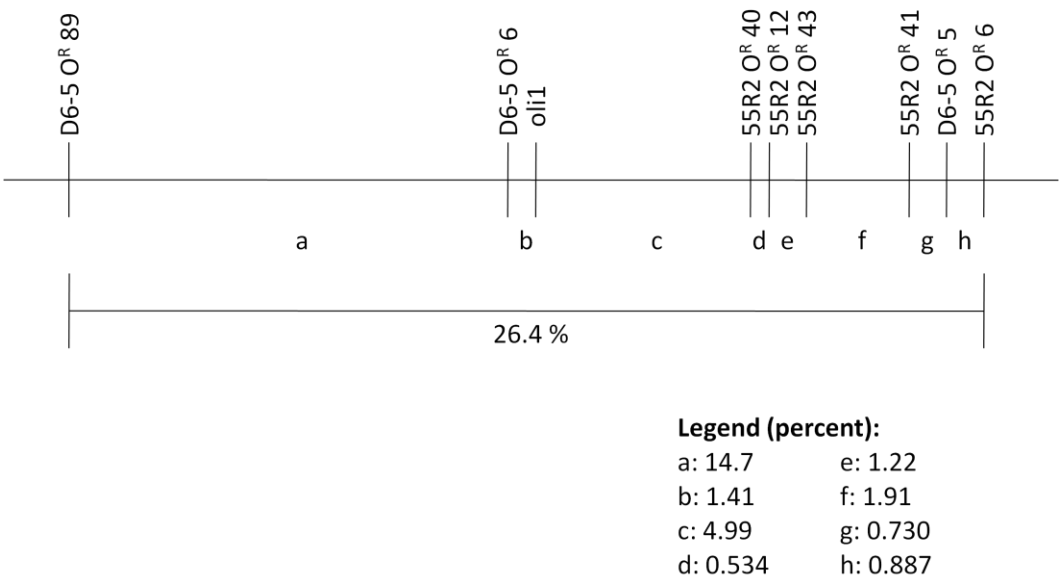

Figure 12. Fine structure genetic map of the *oli1-oli3* region, assembled using average recombination frequencies. Map distances between markers are indicated by the legend across the total 26.4 percent, or map units. Marker *oli1* is representative of the 16 mutants determined to be allelic at *oli1*.

DISCUSSION

Overview

The mtDNA of *S. cerevisiae* is unique in several ways, and discerning its properties and function are important to understand the overall role of mitochondria. While the products of mitochondrial protein synthesis represent only about five percent of total cellular protein synthesis, these proteins are essential for several functions, including the assembly of functional inner mitochondrial membrane complexes. Encoding three subunits of cytochrome c oxidase and one subunit of cytochrome bc_1 reductase, mtDNA is essential for a functional electron transport chain. The dually encoded F_1-F_O ATPase complex is especially crucial for the production of ATP via oxidative phosphorylation, and it is of interest to understand its functionality.

Oligomycin binds to the F_O portion of the F_1-F_O ATPase which prevents cells from performing successful oxidative phosphorylation and interferes with ATP synthesis. Mutations in any of the *oli* loci, however, confer resistance to the antibiotic, and allow normal function to progress. While we now know which genes code for which three mitochondrially-encoded subunits of the F_O portion, the nature of mutations conferring resistance are roughly understood. Further characterization of these regions is necessary to clarify the structure/function relationships between these genes and their polypeptide products.

While there are several methods that may be used to elucidate the location of genetic markers, fine structure genetic mapping is a popular genetic tool for analyzing different mutable sites within a single functional gene. As shown by the studies on 21S rRNA gene, this technique can yield important data for understanding the location of various markers along a gene. The fine structure genetic map that exists for the 21S rRNA gene is the model on which this study has been predicated (Figure 13). First characterized by Netter et al., (1974), the 21S rRNA functional gene encodes the large ribosomal subunit of mitochondrial ribosomes in *S. cerevisiae*. This gene includes several loci for antibiotic resistance to chloramphenicol, erythromycin, and spiramycin that are separable by recombination, as well as the ω marker. These antibiotics all interfere with mitochondrial protein synthesis. Grivell et al. (1973) proposed that mutations in these sites alter the RNA transcript so that the three dimensional structure of the ribosome is altered. This renders the organism resistant to an antibiotic, as the drug can no longer bind to the ribosomal binding site specific for that antibiotic. Knight (1980) resolved additional markers within this gene that led to further understanding about the function of the gene. The resolution of this map has allowed for the insight into the interaction of several antibiotics with the large ribosomal subunit.

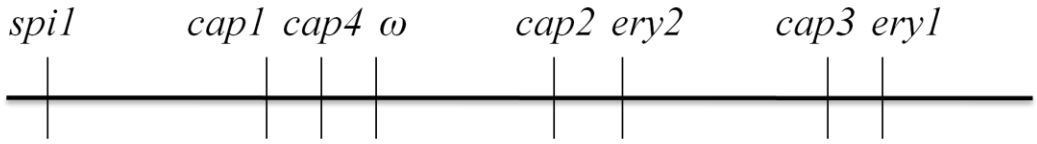

Figure 13. Fine structure genetic map of the 21S rRNA gene showing the positions of various mutable sites that confer antibiotic resistance, as well as the ω marker. Markers *cap1, cap2, cap3* and *cap4* confer resistance to chloramphenicol, *ery1* and *ery2* confer erythromycin resistance and *spi1* confers spiramycin resistance (Knight, 1980).

Although the specificities of the antibiotics that map to the 21S rRNA region are different from oligomycin, the antibiotics erythromycin, spiramycin, and oligomycin are all macrolides whose mechanisms are only beginning to be elucidated (Gaynor and Mankin, 2005). Some macrolides like erythromycin are extensively used in clinical practice, although over 50 years of research has provided limited information as to the exact mechanisms for the inhibition of ribosomal activity. The common use of some macrolide antibiotics has led to antibiotic resistant bacterial strains, which necessitates the need for an understanding of the structure-function relationships between the 21S ribosomal subunit and various antibiotics. Even less is known about oligomycin's interaction with F_1-F_O ATPase in *S. cerevisiae*, but some researchers have worked out various aspects of the protein-drug interaction. A study performed on beef heart mitochondria by Glaser et al. (1972) indicated that oligomycin binds to the F_O portion of the F_1-F_O ATPase in a 1:1 ratio, whereby one mole of oligomycin is needed to inhibit one mole of F_O in the absence of the F_1 portion. They further suggest that the oligomycin binding site is present as a monomer and describe that binding is influenced by the functional state of the ATPase. It is important to perform genetic studies of the regions conferring resistance to the antibiotic so that we may have a basic understanding of the structural genes on which to base future studies of protein function and antibiotic binding.

Project Analysis

Since fine structure genetic maps have been constructed using antibiotic resistance mutations, the goal of this research was to establish a fine structure genetic map for one or more of the *oli* loci of the mtDNA, with the intent of beginning to elucidate the structure-function relationship of oligomycin binding to the F_O ATPase. As observed by Knight (1980), manganese mutagenesis was specific for mitochondrial mutations in seventy-one percent of chloramphenicol resistant mutants and ninety percent of erythromycin and spiramycin resistant mutants. This study saw slightly less specificity, with about sixty-three percent exhibiting mitochondrial oligomycin resistance mutations. However, this was strain dependent and mutagenesis of some strains produced more mitochondrial mutants than others. The thirty percent represented by nuclear oligomycin resistance mutations are likely due to two types of mutations. These may be located in a nuclear structural gene or in nuclear genes that affect membrane permeability and confer cross resistance to multiple antibiotics (Avner et al., 1973). These figures confirm that it is necessary to test for nuclear versus mitochondrial inheritance prior to use in allelism testing.

Growth on oligomycin-supplemented YG plates is generally characterized by strong resistant and sensitive colony morphologies following random diploid analysis. Surprisingly in some instances, observed both in testing for mode of inheritance and allelism crosses, colony morphologies were ambiguous as to the nature of their resistance. Specifically, colonies that would be considered resistant

did not display strong growth, while colonies usually considered sensitive showed more growth than usual; these ambiguous colonies fell somewhere between the two colony morphologies. This is likely due to uneven replica plating, where more of the MD master colony is transferred to the YG+O plate. Hence the observed "growth" is likely residual from the MD colonies, and does not represent oligomycin resistance. A photograph of such colony morphologies can be seen in Figure 14, which illustrates the varying growth following random diploid analysis. In this study, the colonies were consistently scored as sensitive if they exhibited significantly less growth than the growth on the control plates. In the case of Figure 14, all highlighted colonies were scored as sensitive. While this sliding scale may have slightly affected the allelism data, the values can be considered reliable because the scoring procedure remained constant. In the future, it will be necessary to define these different morphologies prior to analysis, and to ensure that scoring is kept consistent. Likewise, it may be useful to experiment with a slightly higher concentration of oligomycin to determine if this helps characterize colonies more definitively.

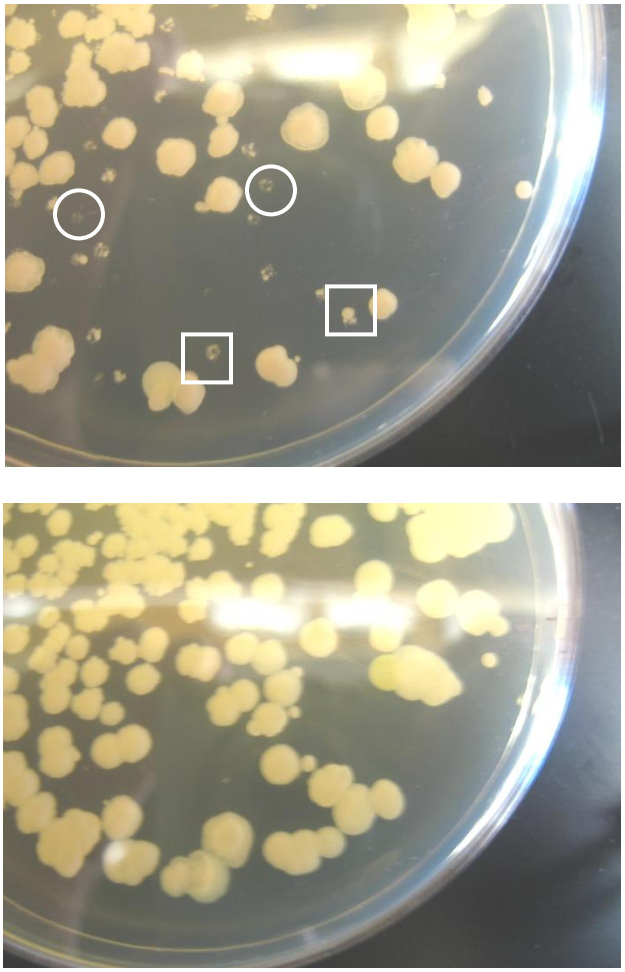

Figure 14. YG+O plate (top) and YG control plate (bottom) showing various colony morphologies following allelism testing. Sensitive and resistant colonies are shown, with circles highlighting two sensitive colonies and square representing two colonies with ambiguous resistance.

The reason for consistently unsuccessful matings between some strains is unknown. Mts-14 O^R mutants were unable to successfully mate when crossed with O^S strains of opposite mating type and complementary auxotrophic requirements. These strains had demonstrated the ability to mate previous to selection for mutation, yet after the mutagenesis procedure, the strains failed to do so. Interestingly, HAT O^R mutants showed diploid growth following matings, but no colonies were apparent on cross streaked MD plates. Furthermore, HB2 O^R mutants failed to produce diploid growth when mated with 55R2 O^R mutants, but were successful when mating with other O^R strains. It is possible that the mutagenesis procedure caused a mutation to genes necessary for mating, but it is odd that all aforementioned mutants would consistently demonstrate this mutation. Since it was unclear if these strains demonstrated nuclear or mitochondrial inheritance, they were eliminated from allelism testing, which significantly limited the number of crosses able to be performed. Attempting to control for this phenomenon in the future, perhaps by performing more extensive preliminary testing on additional strains, would ensure that a larger number of crosses would be able to be scored.

Despite issues with some strains, a large number of crosses were performed that allowed for an analysis of the amount of recombination between oligomycin resistance markers in numerous strains (Appendix; Tables 13-22). Sixteen resistant mutants consistently demonstrated allelism with each other when crossed, which we predicted to carry mutations at the *oli1* locus since most

mutations map to this marker (Avner et al., 1973). This hypothesis was later confirmed when mutants were crossed with a known *oli1* tester (Appendix; Table 23). In addition, many crosses yielded recombination frequencies below twenty percent, with many averaging at or below ten percent. These values were indicative of linked markers, or at the very least, markers located in the same *oli* region. When individual data are considered (Appendix; Tables 13 and 14), and not the averages using in the mapping analysis (Table 6), multiple recombination frequencies were at or below one percent, which indicates strong associations between markers. These data were highly significant in constructing a fine structure genetic map of the region defined as *oli1*-*oli3*.

In this study, a considerable number of crosses resulted in recombination frequencies that exceeded the theoretical threshold of twenty-five percent, thus indicating that the mutational loci were genetically unlinked by this assay. Several explanations may account for these results. First, crosses between unlinked nuclear mutations may result in recombination frequencies exceeding the values as we obtained. This explanation likely accounts for the calculated frequencies over one hundred percent (Appendix; Tables 18 and 19), whereby a nuclear mutant was mistaken for a mitochondrial one. To control for this in the future, it may be necessary to score a larger number of plates following mode of inheritance testing. Similarly, crosses between an O^R and O^S strain could yield frequencies of this range. Previous to use in allelism testing, however, all strains

were determined to feature mitochondrial oligomycin resistance mutations and to clearly demonstrate O^R in drop testing.

It is likely, therefore, that these high recombination frequencies are the result of another occurrence. Since mapping via recombination frequencies is useful only for mutations separated by no more than 6000 base pairs (Dujon et al., 1974), it is probable that crosses resulting in high frequencies are the result of strains carrying mutations separated by this distance or greater, presumably in the *oli2-oli4* region which is separated from *oli1* by 14 kilobases (Novitski et al., 1984). The mtDNA molecule is highly recombinogenic, which supports the observation that some crosses display frequent recombination and generate high frequencies. In the absence of *oli2* and *oli4* tester strains in the laboratory, it is currently unknown if the mutant strains definitively exhibit these mutations. However, future studies should include these test crosses to determine if, indeed, these mutations map to unlinked regions. While these high recombination frequencies are ineffective for fine structure genetic mapping within our defined *oli1-oli3* region, they provide information as to the location of other O^R loci. Significantly, many of the recombination frequencies were well within the theoretical threshold value of twenty-five percent, which were used to construct the fine structure map of the *oli1-oli3* region (Figure 12). Therefore, all data were essential for understanding the nature of these novel oligomycin resistant mutants.

In several instances, shown in Tables 21 and 22 (Appendix), 1000 colonies were unable to be scored due to limited growth and time restrictions for

repeating the crosses. While the frequencies calculated may not be an accurate portrayal of the amount of recombination, we can assume that the percentages would remain relatively stable if repeated. Therefore, these crosses were used to determine if the mutations mapped to the *oli1-oli3* or *oli2-oli4* regions. In all instances, values indicated that mutations mapped to the latter. This supports the observation that the *oli1-oli3* and *oli2-oli4* regions are genetically unlinked (Clavilier, 1976).

It is also interesting to note that in two instances when allelism crosses were repeated, different recombination frequencies were obtained. As shown in Tables 14 and 15 (Appendix), there were two occasions where repeated crosses between mutants resulted in differing values. Two crosses between D6-5 O^R 5 and 55R2 O^R6 produced frequencies of 59.0 and 36.5 percent. Similarly, crosses between D6-5 O^R 39 and 55R2 O^R 6 yielded frequencies of 25.9 and 41.2 percent. While these values differ, they do not point to inconsistencies with the procedure or necessarily indicate unviable strains; instead, values are indicative of unlinked mutations. Any recombination frequencies over twenty-five percent essentially represent the same data; namely that loci are unlinked by this analysis. Inconsistencies in recombination frequencies from cross to cross have also been observed by others (Avner et al., 1973; Netter et al., 1974), which suggests that a similar occurrence might be detected if more crosses were repeated. This observation is significant when considering the application of these data. Since recombination occurs frequently and there is the possibility for recombination

frequencies to be inconsistent, large recombination frequencies should be used primarily to define complementation groups and provide a rudimentary basis for fine structure genetic mapping. Values within the theoretical maximum of twenty-five percent are typically consistent and meaningful. Recombination frequencies under twenty percent, however, are the most reliable and can be confidently used to construct fine structure genetic maps of defined functional genes.

The CCD6-7 O^R tester strain, resistant via a mutation at *oli1*, provided a method of determining which, if any, mutants isolated in this study were allelic at the *oli1* locus. Although all mutants were not crossed to the tester strain due to time constraints, a representative sample of four mutants was selected and crossed to the tester strain (Appendix; Table 23). As expected, the two allelic mutants (55R2 O^R 19, 55R2 O^R 20) were allelic with *oli1*, which characterized the large group of sixteen allelic mutants as having the *oli1* mutation. Similarly, mutants that were consistently non-allelic did not map to the *oli1* locus; 55R2 O^R 12 and 55R2 O^R 43 displayed recombination frequencies of 8.01 and 51.7 percent recombination respectively. The distance between *oli1* and 55R2 O^R 12 was also relatively supported by the other allelism crosses (Table 6), where recombination frequencies between markers averaged at 5.52 percent. The recombination frequency of 51.7 percent between CCD6-7 and 55R2 O^R 43 would indicate that the mutation of the latter is likely in the *oli2-oli4* region, but this was not supported by other crosses; the mutation was found to be located in the *oli1-oli3* region with an average recombination frequency of 6.74 percent (Table 6).

Perhaps repeating this cross, or crossing 55R2 O^R 43 to other *oli1* O^R strains could help clarify this difference.

The derivation of a fine structure genetic map of the *oli1-oli3* region based on allelism data is significant. No such map has been published, and this study represents an effective basis for future analyses to resolve these map positions definitively. Following numerous crosses between 55R2 O^R, CCD6-1 O^R, and D6-5 O^R mutants, a fine structure genetic map was obtained for eight 55R2 O^R and D6-5 O^R mutants (Figure 12). Furthermore, allelism with *oli1* was confirmed for sixteen additional mutants. This position served as the basis from which recombination frequencies were used to place the additional eight mutants along the region. A total recombination frequency of 26.4 percent was obtained for the *oli1-oli3* region.

The map (Figure 12) shows the majority of markers mapping to the right of the *oli1* locus, with one marker, D6-5 O^R 89 mapping 16.1 map units to the left of *oli1*. Based on the map, it appears that most of the mutations to the right of the *oli1* locus are most likely situated within the *oli1* region. Mutant D6-5 O^R 89 may be representative of the *oli3* end of the *oli1-oli3* region, since it shows a higher recombination frequency and is separated from the other mutants by a considerable distance. This would also be supported by Avner et al. (1973) who observe that most mutations map to *oli1*. Using the limited combinations of crosses, I was unable to definitively place markers to the right or left of *oli1*, so it

must be remembered that these positions represent a preliminary analysis of the data.

Since the frequencies used to compile the map are based on averages, the total recombination of 26.4 percent along the map may fluctuate in future studies, but it provides an estimate of the amount of recombination. Given that the map defines a region containing two loci, the observed recombination may be slightly higher than the theoretical threshold of twenty-five percent. Therefore, it may be subjective to consider these values as absolute, since values over twenty-five percent represent unlinkage in this classification. When the two mapped mutants separated by the average 26.4 percent (D6-5 O^R 89 and 55R2 O^R 6) were crossed, the observed frequency was 38.8 percent which confirms this speculation. The 21S rRNA gene exhibits approximately ten percent recombination across the entire functional gene, which is a smaller segment of mtDNA than the more than 38,000 base pairs representing the oli1-oli3 region (Teixeira et al., 2006). When mutants can be mapped specifically to either the *oli1* or *oli3* regions, it will be possible to calculate the amount of recombination within *oli1* and *oli3* respectively.

While this study has proved useful in describing a fine structure genetic map based on several crosses, the data do not allow for a comprehensive analysis of the mutations. It will be necessary to perform reciprocal crosses between mutants mapping to the same complementation groups in order to resolve their map positions. For example, two *oli1* 55R2 strains must be crossed to determine if

they are allelic, if one represents a mutation at another location on the gene, or potentially an *oli3* mutation. However, this is unfeasible in our current laboratory stock, since all mutants generated from the same strain carry the same auxotrophic requirements and mating types, and therefore will not successfully mate. In order to perform these crosses, stock construction must be performed to expand the number of viable strains for use in allelism testing. Stock construction involves using ethidium bromide mutagenesis to select for ρ^0 mutations in strains of opposite mating types and auxotrophic requirements that can be crossed to current O^R strains. We can then mate our original strain, 55R2 O^R (*a*, ura) with a complementary ρ^0 strain (for example, *α*, arg). The mating types and auxotrophic requirements in these diploids will independently segregate, but the O^R marker from the 55R2 O^R strain will remain, since the ρ^0 strain contains no mtDNA. Following sporulation, we can select for haploids that are characterized by oligomycin resistance, the *α* mating type, and arg auxotrophic requirement. These new haploids can then be crossed against the 55R2 O^R strains. This will allow for a larger number of crosses to be performed and for data to be collected and used to compile more accurate maps.

Several limitations have been highlighted in this study, which indicate the need for future investigations to truly elucidate the nature of the generated mutants. In order to further resolve the location of mutations in the mtDNA, deletion mapping can be employed. The technique of deletion mapping, or the use of *petites* with known deletions in crosses, allows for the determination of the

precise location of mutations. Deustch et al. (1974) demonstrated that the ρ^+ factor represents the entire mtDNA molecule, in which antibiotic resistance markers reside. When strains are mutated and ρ^- *petites* are derived, the known deletions and remaining mtDNA may be used to study the relation between antibiotic resistance markers. The technique involves crossing mutants to characterized ρ^- strains to determine if the antibiotic resistance mutation is located within the *petite* deletion. Molloy et al. (1975) confirmed that crosses involving these ρ^- strains were useful in ordering genetic markers that were difficult to place using other means.

Since the order of the *oli* loci have been resolved, the focus of future studies should be primarily on the nature of particular mutations within these genes. Specifically, it will be useful to identify the specific base pairs these mutations are located in to obtain a picture of the various mutations conferring oligomycin resistance. DNA sequencing would provide a direct and relatively simple way to understand the location and nature of mutations observed in this study. Since the wild type mtDNA has been fully sequenced and published, a comparison of mutant strains to this model would allow for a discernment of type and location of O^R mutations.

Nomenclature

It is necessary to address the inconsistencies in the literature regarding the terminology used to describe the *oli* loci, as they present confusion when

considering the loci in an applied context. Avner et al. (1973) described two oligomycin allelism groups, loci named O_I and O_{II}, which they portray as two separate functional genes separable by high recombination. These same loci were mentioned by Lancashire and Griffiths (1975), but they called them OL I and OL II, and they identified a third locus, OL III. Their study describes OL I and OL III as being linked and nonallelic. Linnane et al. (1976) depict a mitochondrial DNA map showing three oligomycin resistance loci: O_I, O_{II}, and O_{III}. Here, the loci O_I and O_{III} are shown with corresponding markers of oli17 and oli1 respectively. These are shown to be in close proximity on the map, while locus O_{II} is separated by considerable distance. When Cavilier publishes in 1976, she cites Avner et al. (1973) and Lancashire and Griffiths (1975), but calls the three loci *oli1*, *oli2*, and *oli3*. She then identifies an *oli4* locus as well, which she considers linked to *oli2*.

By 1979, Somlo and Krupa designate loci by the uppercase designation. Namely, they characterize the oligomycin loci by two unlinked regions, the first containing the closely linked OLI 1, OLI 3, and PHO 2 loci, and the second containing the OLI 2, OLI 4, and PHO 1 loci. While this nomenclature is relatively traceable throughout the literature, it has created problems when establishing the functional genes that encode F_O ATPase subunits. In the late 1970's and 1980's, *oli1* and *oli2* were identified as structural genes that encoded subunit 9 and subunit 6 of the F_O ATPase respectively (Novitski et al., 1984; John et al., 1986). However, maps show genes for subunits 6 and 9 as being closely located, in the positions previously occupied by *oli1* and *oli3* (Figure 4b).

Similarly, the region for subunit 8, defined as ATPase in Figure 4, is located where the *oli2* locus was previously. Clearly, there is a discrepancy in the nomenclature that has confused the functional genes and their gene products. While the subunit 9 is consistently defined by the *oli1* locus, it is unclear whether subunit 6 is encoded by *oli3*, as pictured, or by *oli2*, as described.

Research teams from two major laboratories conducted most of the early genetic studies in *S. cerevisiae*. P. Slonimski, of the Centre de Génétique Moléculaire du Centre National de la Recherché Scientifique in France, and A. W. Linnane from the Monash laboratory in Australia are well-known pioneers in the field, but they did not get along. Lack of communication and establishment of individual nomenclature systems may have fueled these differences, which have translated into confusing and somewhat arbitrary names for these loci. Recent advances in sequencing the mtDNA and characterizing these loci have been helpful, but they have failed to address these nomenclature inconsistencies.

The YEASTRACT database, which has catalogued the genes of the mtDNA, lumps the following loci together: Subunit 9 is encoded by *oli1* (*oli3* or *atp9*), subunit 6 is encoded by *atp6* (*oli2*, *oli4* or *pho1*), and subunit 8 is encoded by *atp8*, or *aap1* (Teixeira et al., 2006). While four oligomycin resistance loci have been mapped, recent studies indicate that *oli1* and *oli3* represent the same structural gene, as do *oli2* and *oli4*. Again, this would place *oli1-oli3* and *oli2-oli4* in close proximity, with the *atp8* gene located in the "ATPase" locus (Figure 4b). These distinctions are inconsistent with the genetic data that place *oli1* and *oli2* at

considerable distance separable by recombination. Clearly there is a need for the correction of any misnomers and a unification of nomenclature for future research. In order to work within these terms, this study used *oli1*, *oli2*, *oli3*, and *oli4* to represent the four genetically characterized loci on the mtDNA. While subunits 6, 8, and 9 were discussed in the context of the most recent nomenclature, the conflict between these and the genetic data were recognized.

There is also some discrepancy regarding the terminology of F_1-F_O ATPase. Studies on *S. cerevisiae* originally denoted the F_O subunit with the letter "O" to represent the effects of oligomycin on normal function (Cammack, 2004). However, some studies denote this same subunit with the number zero, as in F_1-F_0 ATPase. It appears that this is often due to preference, and may stem from the lack of typeset options in the past. This phenomenon is also seen in other enzyme nomenclature systems (Cammack, 2004). Acknowledging these differences, F_1-F_O ATPase was used to denote the complex in this study, as the vast majority of references cited use this terminology.

Medical Applications

It is well established that *S. cerevisiae* is an excellent single celled eukaryote through which to view more advanced eukaryotic systems. This is especially true in studies involving mitochondrial genetics, since *in vivo* manipulation of this system in a facultative anaerobe does not involve death to the organism. Yeast and humans share conserved mitochondrial features, including

DNA replication, recombination, and repair; RNA transcription and translation; intracellular tracking; general metabolism; and mitochondrial biogenesis (Barrientos, 2003). Since both systems have their genomes sequenced, the shift in research is one toward functional genomics in order to determine the relationship between the role and function of proteins. Since yeast lends itself well to studying mitochondrial genetics, and up to thirty percent of its genes are homologs with genes implicated in human disease, it is an excellent model organism for such studies.

Perhaps the largest body of research is found in using yeast to study mitochondrial biogenesis and human mitochondrial disorders resulting from disrupted mitochondrial metabolism. Mutations in the oxidative phosphorylation system are the most common causes of mitochondrial diseases, including Leigh syndrome and MELAS, a neurodegenerative disease (Barrientos, 2003). While most mutations currently known affect complexes III and IV of the electron transport chain, they are important to understanding the entire functional role of oxidative phosphorylation and its role in disease.

Yeast has also been used in pharmaceutical research for new anti-cancer drugs and anti-prion disease drugs (Mager and Winderickx, 2005). Advances in understanding cellular mechanisms of diseases like Huntington's, Alzheimer's, and Parkinson's disease have also been noted. In many cases, this system provides an arena for experimenting with pharmaceutical products to interfere or halt these degenerative cellular events. There is considerable work being performed in an

effort to understand the function of mitochondrial membranes in hope of generating more effective drug delivery (Murphy and Smith, 2000). It has been proposed that selective targeting of the mitochondria will be necessary for intervention in certain medical instances. Using targeted antioxidants to protect mitochondria from neurodegenerative diseases and ageing, as well as studies regulating apoptosis, show promise for the future.

Yeast, therefore, demonstrates a remarkable promise as a model organism for understanding the genetic and functional relationships of numerous human diseases. This study, looking at the function of an antibiotic lethal to humans, follows in this trend toward humanized research; it elucidates eukaryotic genetic function in a simple organism that can later be applied to human mitochondrial genetics. Since mitochondria are so essential to normal metabolic function in eukaryotes, understanding all aspects of their function will be necessary for confronting the human mitochondrial disorders and related diseases. However, the basic genetic and functional relationships must be established, which this study and others are developing.

Conclusions and Future Directions

This research has generated a fine structure genetic map of the *oli1-oli3* region of the *S. cerevisiae* mitochondrial DNA, which represents an important step in the elucidation of structure-function relationships of the *oli* functional genes, F_O ATPase, and oligomycin binding. A working stock of oligomycin

resistant mutants has been generated in the laboratory that will be useful in continuing this analysis. With the addition of *oli2*, *oli3*, and perhaps *oli4* testers in the laboratory, it will be possible to confirm the complementation groups to which mutants in this study have been assigned, and potentially map additional regions with greater accuracy. While perfection of the genetic map will require future analyses, obtaining both allelism and DNA sequencing data, this represents a useful background on which to predicate similar studies.

APPENDIX

Table 8: Results of crosses between oligomycin resistant 55R2 mutants and oligomycin sensitive strain CCD6-1 to determine mitochondrial versus nuclear inheritance.

55R2 O^R Mutant	Total O^R colonies	Total O^S colonies	Total colonies	Mitochondrial
6	65	72	137	X
7	54	53	107	X
11	71	38	109	X
12	82	37	119	X
15	51	25	76	X
19	70	63	133	X
20	87	75	162	X
37	50	33	83	*
38	54	60	114	*
39	48	21	69	*
40	40	46	86	X
41	48	33	81	X
42	80	41	121	X
43	56	46	102	X
44	0	123	123	

*O^R colonies did not exhibit strong, confluent growth. Therefore, they were not used for further testing even though the exhibited both resistant and sensitive recombinants.

Table 9: Results of crosses between oligomycin resistant CCD6-1 mutants and oligomycin sensitive strain 5DSSCO to determine mitochondrial versus nuclear inheritance.

CCD6-1 O^R Mutant	Total O^R colonies	Total O^S colonies	Total colonies	Mitochondrial
1	115	0	115	
2	120	0	120	
3	97	0	97	
4	76	4	80	
5	79	3	82	
6	89	0	89	
7	143	0	143	
9	70	0	70	
10	56	28	84	X
11	59	0	59	
12	104	0	104	
13	56	0	56	
15	94	0	94	

Table 10: Results of crosses between oligomycin resistant HB2 mutants and oligomycin sensitive tester Mts-14 to determine mitochondrial versus nuclear inheritance.

HB2 O^R Mutant	Total O^R colonies	Total O^S colonies	Total colonies	Mitochondrial
2	66	50	116	X
3	99	48	147	X
4	65	68	133	X
7	75	57	132	X
8	43	58	101	X
10	56	34	90	X
11	61	23	84	*
12	31	32	63	X
16	96	50	146	X
20	90	58	148	X
21	80	50	130	X
22	56	58	114	X
23	90	101	191	X
29	50	55	105	X
31	40	31	71	X
33	100	0	100	
36	39	18	57	X
40	70	0	70	
41	55	0	55	

Table 11: Results of crosses between oligomycin resistant D6-5 mutants and oligomycin sensitive tester 5DSSCO to determine mitochondrial versus nuclear inheritance.

Mutant	Total OR colonies	Total OS colonies	Total colonies	Mitochondrial
1	54	0	54	
2	100	20	120	X
4	60	2	62	*
5	52	18	70	X
6	38	11	49	X
10	67	0	67	
14	97	0	97	
17	94	16	110	X
34	80	0	80	
39	71	8	79	X
43	46	0	46	
46	115	9	124	*
49	0	90	90	
51	140	0	140	
53	76	0	76	
55	68	7	75	X

*O^R colonies did not exhibit strong, confluent growth. Therefore, they were not used for further testing even though the exhibited both resistant and sensitive recombinants.

Table 11 continued: Results of crosses between oligomycin resistant D6-5 mutants and oligomycin sensitive tester 5DSSCO to determine mitochondrial versus nuclear inheritance.

Mutant	Total OR colonies	Total OS colonies	Total colonies	Mitochondrial
56	46	0	46	
57	90	6	96	X
58	145	14	159	X
68	37	154	191	X
84	149	21	170	X
87	130	25	155	X
89	152	43	195	X
91	172	30	202	X
99	220	30	250	X
104	297	46	343	X
113	99	175	274	X
129	65	99	164	X
132	87	133	220	X
133	176	164	340	X
137	61	147	208	X
138	71	123	194	X
140	178	16	194	X
141	218	23	241	X
145	86	185	271	X
149	81	118	199	X

Table 12. List of Mts-14 O^R mutants where mutations are ambiguous as to their nuclear or mitochondrial inheritance.

Mts-14 O^R 3	**Mts-14 O^R 4**	**Mts-14 O^R 5**	**Mts-14 O^R 6**
Mts-14 O^R 7	Mts-14 O^R 8	Mts-14 O^R 9	Mts-14 O^R 10
Mts-14 O^R 11	Mts-14 O^R 12	Mts-14 O^R 13	Mts-14 O^R 14
Mts-14 O^R 15	Mts-14 O^R 16	Mts-14 O^R 17	Mts-14 O^R 18
Mts-14 O^R 20	Mts-14 O^R 21	Mts-14 O^R 22	Mts-14 O^R 23
Mts-14 O^R 25	Mts-14 O^R 26	Mts-14 O^R 27	Mts-14 O^R 28
Mts-14 O^R 29	Mts-14 O^R 30	Mts-14 O^R 31	Mts-14 O^R 32
Mts-14 O^R 33	Mts-14 O^R 34	Mts-14 O^R 35	Mts-14 O^R 36
Mts-14 O^R 37	Mts-14 O^R 38	Mts-14 O^R 39	Mts-14 O^R 40
Mts-14 O^R 41	Mts-14 O^R 42	Mts-14 O^R 43	Mts-14 O^R 44
Mts-14 O^R 45	Mts-14 O^R 46	Mts-14 O^R 47	Mts-14 O^R 48
Mts-14 O^R 49	Mts-14 O^R 50	Mts-14 O^R 51	Mts-14 O^R 52
Mts-14 O^R 53	Mts-14 O^R 54	Mts-14 O^R 55	Mts-14 O^R 56
Mts-14 O^R 57	Mts-14 O^R 58	Mts-14 O^R 59	Mts-14 O^R 60
Mts-14 O^R 61	Mts-14 O^R 62	Mts-14 O^R 63	Mts-14 O^R 65
Mts-14 O^R 67	Mts-14 O^R 68	Mts-14 O^R 69	Mts-14 O^R 70
Mts-14 O^R 71	Mts-14 O^R 72	Mts-14 O^R 73	Mts-14 O^R 74
Mts-14 O^R 75	Mts-14 O^R 78	Mts-14 O^R 79	Mts-14 O^R 81
Mts-14 O^R 82	Mts-14 O^R 83	Mts-14 O^R 84	Mts-14 O^R 85
Mts-14 O^R 86	Mts-14 O^R 87	Mts-14 O^R 88	Mts-14 O^R 89
Mts-14 O^R 91	Mts-14 O^R 92	Mts-14 O^R 93	Mts-14 O^R 95
Mts-14 O^R 96			

Table 13. Allelism data from matings between oligomycin resistant mutants CCD6-1 O^R and 55R2 O^R with calculated recombination frequencies.

CCD6-1 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
10	6	45	1043	4.30×10^{-2}	8.63
10	7	0	1083	0	0
10	12	5	1197	8.35×10^{-3}	0.835
10	15	0	1170	0	0
10	19	0	1184	0	0
10	20	0	1229	0	0
10	40	6	1060	5.66×10^{-3}	1.13
10	41	8	1272	6.29×10^{-3}	1.26
10	42	135	1067	1.27×10^{-1}	25.3
10	43	638	2039	3.13×10^{-1}	62.7

Table 14. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 2, 5, 6 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
2	6	71	1435	4.95×10^{-2}	9.90
2	12	9	1226	7.34×10^{-3}	1.46
2	15	0	1166	0	0
2	19	0	1417	0	0
2	20	0	1038	0	0
2	40	29	1179	2.46×10^{-2}	4.92
2	41	68	1271	5.35×10^{-2}	10.7
2	42	0	1087	0	0
2	43	47	1272	3.69×10^{-2}	7.39
5	6	343	1162	2.95×10^{-1}	59.0
		197	1079	1.83×10^{-1}	36.5
5	12	123	1233	9.98×10^{-2}	19.9
5	15	114	1060	1.08×10^{-1}	21.5
5	19	9	1214	7.41×10^{-3}	1.48
5	20	50	1265	3.95×10^{-2}	7.91
5	40	11	1099	1.00×10^{-2}	2.00
5	41	23	1122	2.05×10^{-2}	4.10
5	42	38	1146	3.31×10^{-2}	6.63
5	43	8	1159	6.90×10^{-3}	1.38
6	6	75	1489	5.04×10^{-2}	10.1
6	12	19	1613	1.18×10^{-2}	2.36
6	15	20	1487	1.34×10^{-2}	2.69
6	19	1	1069	9.35×10^{-4}	0.187
6	20	1	1286	7.78×10^{-4}	0.156
6	40	32	1213	2.64×10^{-2}	5.28
6	41	10	1093	9.14×10^{-3}	1.83
6	42	16	1229	1.30×10^{-2}	2.60
6	43	64	1239	5.17×10^{-2}	10.3

Table 15. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 17, 39, 55 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
17	6	12	1101	1.09×10^{-2}	2.18
17	12	0	1107	0	0
17	15	0	1131	0	0
17	19	0	1038	0	0
17	20	0	1118	0	0
17	40	13	1118	1.16×10^{-2}	2.33
17	41	18	1199	1.50×10^{-2}	3.00
17	42	0	1046	0	0
17	43	16	1144	1.40×10^{-2}	2.80
39	6	165	1273	1.30×10^{-1}	25.9
		281	1365	2.06×10^{-1}	41.2
39	12	59	1242	4.75×10^{-2}	9.50
39	15	0	1271	0	0
39	19	0	1096	0	0
39	20	0	1153	0	0
39	40	39	1083	3.60×10^{-2}	7.20
39	41	29	1132	2.56×10^{-2}	5.12
39	42	0	1214	0	0
39	43	24	1174	2.04×10^{-2}	4.09
55	6	141	1329	1.06×10^{-1}	21.2
55	12	89	1163	7.65×10^{-2}	15.3
55	15	0	1057	0	0
55	19	0	1118	0	0
55	20	0	1124	0	0
55	40	30	1136	2.64×10^{-2}	5.28
55	41	80	1154	6.93×10^{-2}	13.9
55	42	0	1029	0	0
55	43	19	1133	1.68×10^{-2}	3.35

Table 16. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 57, 58, 68 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
57	6	96	1174	8.18×10^{-2}	16.4
57	12	0	1092	0	0
57	15	0	1134	0	0
57	19	0	1155	0	0
57	20	0	1045	0	0
57	40	17	1029	1.65×10^{-2}	3.30
57	41	201	1358	1.51×10^{-1}	30.3
57	42	0	1159	0	0
57	43	67	1177	5.69×10^{-2}	11.4
58	6	192	1058	1.81×10^{-1}	36.3
58	12	27	1143	2.36×10^{-2}	4.72
58	15	0	1148	0	0
58	19	0	1048	0	0
58	20	0	1079	0	0
58	40	28	1030	2.72×10^{-2}	5.44
58	41	83	1160	7.16×10^{-2}	14.3
58	42	0	1165	0	0
58	43	31	1057	2.93×10^{-2}	5.87
68	6	416	1260	3.30×10^{-1}	66.0
68	12	393	1307	3.01×10^{-1}	60.1
68	15	597	1344	4.44×10^{-1}	88.8
68	19	289	1174	2.46×10^{-1}	49.2
68	20	409	1251	3.27×10^{-1}	65.4
68	40	462	1230	3.76×10^{-1}	75.1
68	41	546	1246	4.38×10^{-1}	87.6
68	42	304	1327	2.29×10^{-1}	45.8
68	43	324	1357	2.39×10^{-1}	47.8

Table 17. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 84, 87, 89 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
84	6	78	1062	7.34×10^{-2}	14.7
84	12	12	1140	1.05×10^{-2}	2.11
84	15	0	1230	0	0
84	19	0	1073	0	0
84	20	0	1276	0	0
84	40	39	1209	3.22×10^{-2}	6.45
84	41	25	1222	2.05×10^{-2}	4.09
84	42	0	1177	0	0
84	43	33	1234	2.67×10^{-2}	5.35
87	6	71	1226	5.79×10^{-2}	11.6
87	12	44	1192	3.69×10^{-2}	7.38
87	15				
87	19	0	1047	0	0
87	20	0	1246	0	0
87	40	23	1187	1.94×10^{-2}	3.88
87	41	117	1386	8.44×10^{-2}	16.9
87	42	0	1218	0	0
87	43	67	1301	5.15×10^{-2}	10.3
89	6	294	1517	1.94×10^{-1}	38.8
89	12	200	1393	1.44×10^{-1}	28.7
89	15				
89	19	80	1158	6.91×10^{-2}	13.8
89	20	118	1278	9.23×10^{-2}	18.5
89	40	247	1646	1.50×10^{-1}	30.0
89	41	173	1231	1.41×10^{-1}	28.1
89	42	204	1311	1.56×10^{-1}	31.1
89	43	204	1566	1.30×10^{-2}	26.1

Table 18. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 91, 99, 104 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
91	6	139	1266	1.10×10^{-1}	21.9
91	12	26	1214	2.14×10^{-2}	4.28
91	15				
91	19	0	1101	0	0
91	20	0	1061	0	0
91	40				
91	41				
91	42	0	1104	0	0
91	43	31	1098	2.82×10^{-2}	5.65
99	6	117	1119	1.05×10^{-1}	20.9
99	12	34	1117	3.04×10^{-2}	6.09
99	15				
99	19	0	1047	0	0
99	20	0	1104	0	0
99	40				
99	41				
99	42	0	1174	0	0
99	43	37	1109	3.34×10^{-2}	6.67
104	6	734	1087	6.75×10^{-1}	135.0
104	12	142	1155	1.23×10^{-1}	24.6
104	15	497	1650	3.01×10^{-1}	60.2
104	19	907	1506	6.02×10^{-1}	120.5
104	20	427	1550	2.75×10^{-1}	55.1
104	40				
104	41				
104	42	616	1215	5.07×10^{-1}	101.4
104	43	628	1047	6.00×10^{-1}	120.0

Table 19. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 113, 129, 132 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
113	6	426	1577	2.70×10^{-1}	54.0
113	12	367	1248	2.94×10^{-1}	58.8
113	15	294	1114	2.64×10^{-1}	52.8
113	19	376	1456	2.58×10^{-1}	51.7
113	20	303	1145	2.65×10^{-1}	52.9
113	40				
113	41				
113	42	674	1758	3.83×10^{-1}	76.7
113	43	370	1152	3.21×10^{-1}	64.2
129	6	577	1252	4.61×10^{-1}	92.2
129	12	485	1398	3.47×10^{-1}	69.4
129	15				
129	19	566	1583	3.58×10^{-1}	71.5
129	20	427	1524	2.80×10^{-1}	56.0
129	40				
129	41				
129	42	530	1384	3.83×10^{-1}	76.6
129	43	959	1729	5.55×10^{-1}	111.0
132	6	507	1275	3.98×10^{-1}	79.5
132	12	248	1208	2.05×10^{-1}	41.1
132	15	424	1206	3.52×10^{-1}	70.3
132	19	411	1156	3.56×10^{-1}	71.1
132	20	373	1198	3.28×10^{-1}	65.6
132	40				
132	41				
132	42	503	1150	4.37×10^{-1}	87.5
132	43	395	1141	3.46×10^{-1}	69.2

Table 20. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 133, 137, 138 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
133	6	444	1186	3.74×10^{-1}	74.8
133	12	383	1103	3.47×10^{-1}	69.5
133	15				
133	19	415	1265	3.28×10^{-1}	65.6
133	20	346	1173	2.95×10^{-1}	58.9
133	40				
133	41				
133	42	530	1358	3.90×10^{-1}	78.1
133	43	509	1515	3.36×10^{-1}	67.2
137	6	463	1222	3.79×10^{-1}	75.8
137	12	277	1187	2.33×10^{-1}	46.7
137	15	357	1085	3.29×10^{-1}	65.8
137	19	326	1264	2.58×10^{-1}	51.6
137	20	413	1213	3.40×10^{-1}	68.1
137	40				
137	41				
137	42	404	1118	3.61×10^{-1}	72.3
137	43	522	1162	4.49×10^{-1}	89.9
138	6	597	1289	4.63×10^{-1}	92.6
138	12	393	1161	3.39×10^{-1}	67.7
138	15	448	1247	3.59×10^{-1}	71.9
138	19	504	1318	3.82×10^{-1}	76.5
138	20	489	1212	4.03×10^{-1}	80.7
138	40				
138	41				
138	42	468	1215	3.85×10^{-1}	77.0
138	43	384	1106	3.47×10^{-1}	69.4

Table 21. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 140, 141 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
140	6	45	1054	4.27×10^{-2}	8.54
140	12	18	1016	1.77×10^{-2}	3.54
140	15	0	1171	0	0
140	19	0	1005	0	0
140	20	0	1098	0	0
140	40				
140	41				
140	42	0	1113	0	0
140	43	45	1174	3.83×10^{-2}	7.67
141	6	260	1151	2.26×10^{-1}	45.2
141	12	221	648	3.41×10^{-1}	68.2
141	15	125	1040	1.20×10^{-1}	24.0
141	19	462	1155	4.0×10^{-1}	80.0
141	20	351	1180	2.97×10^{-1}	59.5
141	40				
141	41				
141	42	512	1279	4.00×10^{-1}	80.1
141	43	402	1264	3.18×10^{-1}	63.6

Table 22. Allelism data from matings between oligomycin resistant mutants D6-5 O^R 145, 149 and 55R2 O^R with calculated recombination frequencies.

D6-5 O^R	55R2 O^R	O^S colonies	Total colonies	Frequency	Percent Recombination
145	6	448	1331	3.37×10^{-1}	67.3
145	12	279	1099	2.54×10^{-1}	50.8
145	15	276	1253	2.20×10^{-1}	44.1
145	19	426	1099	3.88×10^{-1}	77.5
145	20	489	1000	4.89×10^{-1}	97.8
145	40				
145	41				
145	42	628	1258	4.99×10^{-1}	99.8
145	43	411	1089	3.77×10^{-1}	75.5
149	6	52	260	2.0×10^{-1}	40.0
149	12	26	406	6.40×10^{-2}	12.8
149	15	78	439	1.78×10^{-1}	35.5
149	19	228	645	3.53×10^{-1}	70.7
149	20	192	540	3.56×10^{-1}	71.1
149	40				
149	41				
149	42	108	382	2.83×10^{-1}	56.5
149	43	128	391	3.27×10^{-1}	65.5

Table 23. Allelsim data from crosses between CCD6-7 O^R and four selected 55R2 O^R mutants.

	55R2 O^R	O^S	Total Colonies	Frequency	Percent Recombination
CCD6-7	12	46	1148	4.01×10^{-2}	8.01
CCD6-7	19	0	1303	0	0
CCD6-7	20	0	1131	0	0
CCD6-7	43	380	1471	2.58×10^{-1}	51.7

REFERENCES

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and I. G. Young. 1981. Sequence and organization of the human mitochondrial genome. *Nature*, 290(5806): 457-465.

Arnold, I., K. Pfeiffer, W. Neupert, R.A. Stuart, and H. Schagger. 1998. Yeast mitochondrial F_1-F_O ATP synthase exists as a dimer: identification of three dimer-specific subunits. *The EMBO Journal*, 17(24): 7170-7178.

Avner, P.R., D. Coen, B. Dujon, and P.P.Slonimski, 1973. Mitochondrial genetics. IV. Allelism and mapping studies of oligomycin resistant mutants in *S. cerevisiae*. *Molec. Gen. Genet*, 125**:**9-52.

Barrientos, A. 2003. Yeast models of human mitochondrial diseases. *Life*, 55: 83-95.

Bian, S., and J. A. Cowan. 1999. Protein-bound iron-sulfur centers: form, function, and assembly. *Coordination Chemistry Reviews*, 190-192: 1049-1066.

Birky, C. W., Jr. 1975. Effects of glucose repression on the transmission and recombination of mitochondrial genes in yeast (*Saccharomyces cerevisiae*). *Genetics*, 80: 695-709.

Birky, C. W., Jr., Strausberg, R. L, Forster, J. L., and P.S. Perlman, 1978. Vegetative segregation of mitochondria in yeast: estimating parameters using a random model. 158: 251-261.

Bonitz, S. G., Berlani, R., Coruzzi, G., Li, M., Macinot, G., Nobrega, F. G., Nobrega, M. P., Thalenfield, B. E., and A. Tzagoloff. 1980. Codon recognition rules in yeast mitochondria. *Proc. Natl. Acad. Sci.*, 77(6): 3167-3170.

Borst, P., and L. A. Grivell. 1978. The mitochondrial genome of yeast. *Cell*, 15:705-723.

Callen, D. F. 1974. Segregation of mitochondrially inherited antibiotic resistance genes in zygote cell lineages. *Molec. gen. Genet.*, 134: 65-76.

Cammack, R. 2004. Systematic Names for Systems Biology. In: Hicks, M.G., and C. Kettner [eds], Experimental Standard Conditions of Enzyme Characterizations. Ruedesheim, Germany.

Clavilier, L. 1976. Mitochondrial genetics. XII. An oligomycin-resistant mutant localized at a new mitochondrial locus in *Saccharomyces cerevisiae*. *Genetics*, 83:227-243.

Constanzo, M. C., and T. D. Fox. 1991. Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. *Annu. Rev. Genet*., 24:91-113.

Deutsch, J., Dujon, B., Netter, P., Petrochilo, E., Slonimski, P.P., Bolotin-Fukuhara, M., and D. Coen. 1974. Mitochondrial Genetics VI. The *petite* mutation in *Saccharomyces cerevisiae*: Interrelations between the loss of the ρ^+ factor and the loss of the drug resistance mitochondrial genetic markers. *Genetics*, 76: 195-219.

Devenish, R. J., Prescot, M., Roucou, X., and P. Nagley. 2000. Insights into ATP synthase assembly and function through the molecular genetic manipulation of subunits of the yeast mitochondrial enzyme complex. *Biochimica et Biophysica Acta*, 1458: 428-442.

Dimmer, K.S., Fritz, S., Fuchs, F., Messershcmitt, M., Weinbach, N., Neupert, W., and B. Westermann. 2002. Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell*, 13: 847-853.

Dujon, B., Slonimski, P. P., and L. Weill. 1974. Mitochondrial Genetics IX: A Model for recombination and segregation of mitochondrial genomes in *Saccharomyces cerevisiae*. *Genetics*, 78: 415-437.

El-Deiry, W. S., Downey, K. M., and A. G. So. 1984. Molecular mechanisms of manganese mutagenesis. *Proc. Natl. Acad. Sci*., 81: 7378-7382.

Foury, F., Roganti, T., Lecrenier, N., and B. Purnelle. 1998. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Letters*, 440: 325-331.

Gaynor, M., and A.S. Mankin. 2005. Macrolide antibiotics: binding site, mechanism of action, resistance. *Frontiers in Medicinal Chemistry*, 2: 21-35.

Glaser, E., Norling, B., Kopeky, J., and L. Ernster. 1972. Comparison of the effects of oligomycin and dicyclohexylcarbodiimide on mitochondria1 ATPase and related reactions. *Eur. J. Biochem*., 121: 525-531.

Giraud, M-F., and J. Velours. 1994. ATP synthase of yeast mitochondria: Isolation of the F_1 δ subunit, sequence and disruption of the structural gene. *Eur. J. Biochem*., 222: 851-859.

Graak, H.R., and B. Wittmann-Liebold, 1998. Mitochondrial ribosomal proteins (MRPs) of yeast. *Biochem J,* 329: 433-448.

Griffiths, D.E., and R.L. Houghton. 1974. Studies on energy-linked reactions: modified mitochondrial ATPase of oligomycin-resistant mutants of *Saccharomyces cerevisiae*. *Eur. J. Biochem*, 46: 157-167.

Grivell, L.A., Netter,P., Borst, P., and P.P. Slonimski. 1973. Mitochondrial antibiotic resistance in yeast: ribosomal mutants resistant to chloramphenicol, erythromycin and spiramycin. *Biochim. Biophys. Acta.* 312: 358-167.

Hartwell, L.H. 1974. *Saccharomyces cerevisiae* cell cycle. *Bacterio.l Rev.*, 38(2): 164-198.

Herskowitz, I. 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiological Reviews,* 52(4): 536-553.

HOPES, Stanford University. 2008. Available: http://www.stanford.edu/group/hopes/treatmts/ebuffer/f_j13electtrans.jpg).

John, U. P., Willson, T. A., Linnane, A. W., and P. Nagley. 1986. Biogenesis of mitochondria: DNA sequence analysis of mit- mutations in the mitochondrial oli2 gene coding for mitochondrial ATPase subunit 6 in *Saccharomyces cerevisiae*. *Nucleic Acids Research,* 14(18): 7437-7451.

Katsuyoshi, M. 2003. Cell biology: moving inside membranes. *Nature*, 424: 505-506.

Kim, S-B., and C. D. Berdanier. 1999. Oligomycin sensitivity of mitochondrial F_1-F_O ATPase in diabetes-prone BHE/cdb rats. *Am. J. Physiol. Endocrinol. Metab*., 277: 702-707.

Knight, J.A. 1977. Isolation and characterization of new mutants in the ribosomal region of the mitochondrial genome of *Saccharomyces cerevisiae.* Ph.D. Thesis. The Ohio State University. Columbus, Ohio.

Knight, J.A., 1980. New antibiotic resistance loci in the ribosomal region of yeast mitochondrial DNA. *Genetics*, 94: 69-92.

Lancashire, W. E., and D. E. Griffiths. 1975. Studies on energy-linked reactions: genetic analysis of venturicidin-resistant mutants. *Eur. J. Biochem*., 51: 401-413.

Linnane, A.W., Lukins, H.B., Molloy, P. L., Nagely, P., Rytka, J., Sriprakash, S., and M.K. Trembath. 1976. Biogenesis of mitochondria: molecular mapping of the mitochondrial genome of yeast. *Proc. Natl. Acad. Sci. USA,* 73(6): 2082-2085.

Linnane, A.W., and Nagley, P. 1978. Mitochondrial genetics in perspective: the derivation of a genetic and physical map of the yeast mitochondrial genome. *Plasmid.* 1: 324-345.

Macreadie, I. G., Novitski, C. E., Maxwell, R. J., John, U., Ooi, B-G., McMullen, G. L., Lukins, H. B., Linnane, A. W., and P. Nagley. 1983. Biogenesis of mitochondria: the mitochondrial gene (*aapl*) coding for mitochondrial ATPase subunit8 in *Saccharomyces cerevisiae*. *Nucleic Acids Research*, 11(13): 4435-4451.

Mager, W.H., and J. Winderickx. 2005. Yeast as a model for medical and medicinal research. *TRENDS in Pharmacological Science*, 26(5): 265-273.

Margulis, L., and M. F. Dolan. 2002. Early life: evolution on the precambrian earth. 2nd ed. Sudbury, MA: Jones and Bartlett Publishers.

McBride, H.M., Neuspiel, M., and S. Wasiak. 2006. Mitochondria: more than just a powerhouse. *Current Biology*, 16: R551-R560.

Molloy, P.L., Linnane, A.W., and H.B. Lukins. 1975. Biogenesis of mitochondria: analysis of deletion of mitochondrial antibiotic resistance markers in petite mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, 122(1): 7-18.

Murphy, M.P., and R.A.J. Smith. 2000. Drug delivery to mitochondria: the key to mitochondrial medicine. *Advanced Drug Delivery Reviews*, 41: 235-250.

Netter, P., Petrochilo, E., Slonimski, P.P., Bolotin-Fukuhara ,M., Coen, D., Deutsch, J., and B. Dujon. 1974. Mitochondrial genetics. VII. allelism and mapping studies of ribosomal mutants resistant to chloramphenicol, erythromycin and spiramycin in *S. cerevisiae*. *Genetic,* 78: 1063-100.

Novitski, C. E., Macreadie, I. G., Maxwell, R. J., Lukins, H. B., Linnane, A. W., and P. Nagley. 1984. Biogenesis of mitochondria: genetic and molecular analysis of the *oli2* region of mitochondrial DNA in *Saccharomyces cerevisiae. Current Genetics*, 8: 135-146.

Parker, J. H., Trimble, I. R. Jr., and J. R. Mattoon. 1968. Oligomycin resistance in normal and mutant yeast. Biochemical and Biophysical Research Communications, 33(4): 590-595.

Putrament, A., Baranowska, H., and W. Prazmo. 1973. Induction by manganese of mitochondrial antibiotic resistance mutations in yeast. *Molec. Gen. Genet*. 126: 357-366.

Putrament, A, Baranowska, H., Ejchart, A., and W. Prazmo. 1975. Manganese mutagenesis in yeast V. A practical application of manganese for the induction of mitochondrial antibiotic resistance mutations. *J. Gen. Microbiol*. 62: 265-270.

Sherman, F., 1998. An introduction to the genetics and molecular biology of the yeast *Saccharomyces cerevisiae*. University of Rochester Medical School, Rochester, New York.

Sigma-Aldrich Co. 2008. Available http://sigmaaldrich.com.

Somlo, M., and M. Krupa. 1979. The mitochondrially encoded "Phantom ATPase" of *S. cerevisiae*. *Molec. gen. Genet*., 167: 329-335.

Spithill, T.W., M.K. Trembath, H.B. Lukins, and A.W. Linnane, 1978. Mutations of the mitochondrial DNA of *Saccharomyces cerevisiae* which affect the interaction between mitochondrial ribosomes and the inner mitochondrial membrane. *Molec. Gen. Genet.*, 164: 155-162.

Strausberg, R.L., R.D. Vincent, P.S. Perlman and R.A. Butow, 1978. Asymmetric gene conversion at inserted segments on yeast mitochondrial DNA. *Nature*, 276: 577-583.

Teixeira, M. C., Monteiro, P., Jain, P., Tenreiro, S., Fernandes, A.R., Mira, N.P., Alenquer, M., Freitas, A. T., Oliveira, A. L., and I. Sá-Correia. 2006. The YEASTRACT database: a tool for the analysis of transcription regulatory associations in *Saccharomyces cerevisiae*, *Nucl. Acids Res.*, 34: D446-D451.

Thomas, D.Y., and D. Wilkie. 1968. Recombination of mitochondrial drug-resistance factors in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communication*, 30(4): 368-372.

Tzagoloff, A., and P. Meagher. 1971. Assembly of the mitochondrial membrane system: V. properties of a dispersed preparation of the rutamycin-sensitive Adenosine Triphosphatase of yeast mitochondria. *J. Biol. Chem.*, 246(23): 7328-7336.

Tzagoloff, A., Akai, A., and R.B. Needleman. 1975. Assembly of the mitochondrial membrane system: isolation of nuclear and cytoplasmic mutants of *Saccharomyces cerevisiae* with specific defects it mitochondrial functions. *J. Bacteriol*., 122(3): 826-831.

Tzagoloff, A., Barrientos, A., Neupert, W., and J. M. Herrmann. 2004. Atp10p assists assembly of Atp6p into the F_O unit of the yeast mitochondrial ATPase. *J. Biol. Chem*., 279(19): 19775-19780.

Wenzlau, J. M, and P. S. Perlman. 1990. Mobility of two optional G+C rich clusters of the *var1* gene of yeast mitochondrial DNA. *Genetics*, 126: 53-62.

Wickerham, L. J. 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. *J. Bacteriol*., 52:293-301.

Wolf, K., Dujon, B., and P. Slonimski. 1973. Mitochondrial Genetics V: Multifactorial mitochondrial crosses involving a mutation conferring paromomycin-resistance in *Saccharomyces cerevisiae*. *Mol. Gen. Genet*, 125: 53-90.