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ISOLATION AND CHARACTERIZATION OF CHLORAMPHENICOL RESISTANT MITOCHONDRIAL MUTANTS IN *SACCHAROMYCES CEREVISIAE.*

by

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

To my parents, Dr. Aziz Abdullah and Lubna Aziz. Without your unconditional love and support, none of this would ever have been possible.

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ABSTRACT

Chloramphenicol is a broad spectrum antibiotic, cheaply and easily available. It works by inhibiting protein synthesis in prokaryotes such as *E. coli*. Its main mode of action is via its binding to the large ribosomal subunit of the bacterial ribosome and inhibiting peptidyl transferase activity, thereby terminating protein chain elongation (Moazed and Noller, 1987).

Saccharomyces cerevisiae, commonly known as baker's yeast, is a model eukaryotic organism. It can be easily grown in the laboratory and is ideal for testing resistance against microbial drugs. This is because the structure of the mitochondrial genome is similar to that of prokaryotic microbes and thus allows for successful comparison. The mitochondrial genome of *S. cerevisiae* is a molecule of approximately 75 kb and codes for the two rRNA components of the mitochondrial ribosomes. Similar to *E. coli, S. cerevisiae* when exposed to chloramphenicol shows inhibited mitochondrial protein synthesis (Clark-Walker, 1967). Resistance to chloramphenicol can arise in yeast via point mutations in the mitochondrial genome. These mutations affect the structure of the large ribosomal subunit in such a way that the antibiotic can no longer bind to it and thus inhibit protein synthesis. A known site of resistance to chloramphenicol in that region of the mitochondrial genome is called *cap1*.

In this study spontaneous mutants of *S. cerevisiae* that showed resistance to chloramphenicol were generated from multiple strains. These mutants were then tested for their recombination frequencies with one another and with known *cap1* testers. Allelic and non-allelic mutants were determined, and some of those were selected for mitochondrial DNA isolation and sequencing. The purpose of this study was to not only confirm the published sequence of the *cap1* mutation but also to characterize and sequence new mutants that display additional sites of mutation. It is hoped that with a greater understanding of how chloramphenicol binds to the mitochondrial ribosome in yeast, new strategies for the development of synthetic antibiotics can be developed.

INTRODUCTION

Overview

Saccharomyces cerevisiae, commonly known as baker's yeast, is a model eukaryotic organism. Already well known because of its role in alcohol production in the making of wine and beer, this unicellular organism has been used extensively in molecular and cellular studies after being introduced as an experimental organism by H. Roman in the mid-1930s (Feldmann, 2010).

S. cerevisiae is a model system because it is a simple eukaryotic organism that has little genetic complexity. It can be readily grown in the laboratory on controlled media and its environment can be easily manipulated. Moreover, *S. cerevisiae* grows rapidly and its cells can be easily dispersed. *S. cerevisiae* can also be easily replica plated and mutant isolation can be carried out with relative ease. It was also the first eukaryote whose genome was completely sequenced, and this sequence is easily available today. This combined with its highly versatile DNA transformation system makes *S. cerevisiae* invaluable in its status as a model organism. Lastly *S. cerevisiae* is non-pathogenic and large quantities of it are cheaply and readily available (Sherman, 2002). This allows *S. cerevisiae* to be easily utilized in biological experiments.

Life Cycle of *Saccharomyces cerevisiae*

S. cerevisiae can exist in both haploid and diploid forms depending on the stage of its life cycle. These forms are referred to as the haplophase and the

diplophase, respectively. In heterothallic strains-where heterothallic indicates the formation of a diploid via two distinct haploid forms- two kinds of mating types are found. These are the a and α, and are the haploid forms of *S. cerevisiae* (Mortimer, 1981). The two mating types can only mate with the opposite mating type to form a diploid. The a and α cell types initiate mating by producing a peptide mating factor. In α cells this is called the α factor and is a 13 amino acid long peptide. In a cells, this factor is a 12 amino acid long peptide and is called the a factor. The mating factors inhibit growth of the cells, arresting them in G1 and thereby allowing mating to proceed. The α/a diploid does not produce either of these factors which prepare a cell for mating and thus does not mate. However when this diploid faces starvation in the form of a nutritional deficiency of nitrogen and carbon it will undergo sporulation and budding (Herskowitz, 1988).

Sporulation thus results in the production of four haploids, two a and two α, contained in the wall of the mother cell called the ascus. The segregation patterns of these haploids can then be analyzed (Mortimer, 1981). This kind of analysis is called tetrad analysis. However it is not a technique that will be utilized in this study. The spore capsule then bursts open releasing these haploids and the cycle can now repeat, as shown in Figure 1.

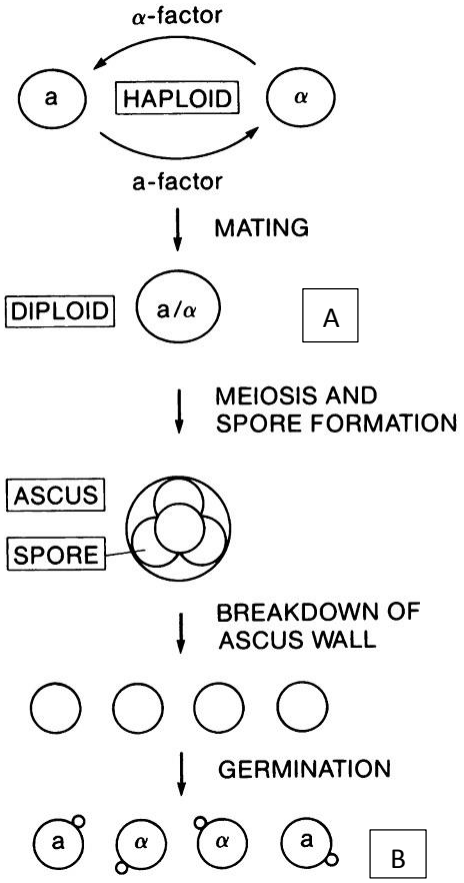

Figure 1: Life Cycle of *Saccharomyces cerevisiae* (Herskowitz, 1988)

S. cerevisiae has both a haploid stage and a diploid stage. It can transition between the two stages via mating and meiosis as shown. It can also maintain these states via mitosis, with the diploid state being maintained from stage A onwards and the haploid being maintained from stage B.

Additionally *S. cerevisiae* also has a homothallic life cycle. This basically means that a single haploid cell can give rise to diploid progeny. It is proposed that this happens when a single haploid cell of *S. cerevisiae* can reprogram its DNA in such a way that it now has the capability to produce both the a and α haploid mating type regardless of its own identity. As the cell continues to divide, its

colony then contains both mating types, i.e. α and a. These can then mate together to form the α/a diploid (Herskowitz, 1988).

Mitochondria of *Saccharomyces cerevisiae*

In this study, I will be concentrating on the mitochondrial mutants isolated from *S. cerevisiae*. The mitochondria of *S. cerevisiae* have four distinct parts: an outer membrane, an inner membrane, an intermembranal space and a compartment enclosed by the inner membrane called the matrix. The matrix is the site of the *S. cerevisiae* mitochondrial genome, where the it is packed with proteins and is attached to the inner membrane as nucleoids (Okamoto and Shaw, 2005). The mitochondrial genome is a molecule of approximately 75 kilo bases and is circular. It is also AT-rich with 82 percent of its genome being saturated by AT as compared to 60 percent of the chromosomal DNA (Fangman and Zakian, 1981).

The mitochondrion provides many functions for the yeast. It is not only a site of oxidative phosphorylation, through which it plays an essential role in respiration, but it also plays a role in apoptosis (Perocchi et al., 2006). All of these require essential proteins and enzymes. It is estimated that about 700-800 mitochondrial proteins are present in S. *Cerevisiae.* However only 8 of these proteins are encoded by the mitochondrial genome (Perocchi et al., 2006). The rest of these are encoded by the nuclear genome. The mitochondrial genome also specifies the RNA components of the mitochondrial ribosomes. The yeast mitochondrial

ribosomes, although comparable to the cytoplasmic ribosomes of the host cell in terms of their general features, do not share the same constituents. Moreover the *S. cerevisiae* mitochondrial ribosomes, just like the mitochondrial ribosomes of other animal and fungi, contain only two rRNA molecules. These are the 21S and the 15S ribosomal subunits (Graak and Wittmann-Liebold,1998).

The segment of the mitochondrial DNA in *S. cerevisiae* that encodes for the 21S subunit of the mitochondrial ribosomes is known as the R region. The gene encoding the 15S ribosomal subunit does not lie either within or near this region and the two genes are separated by about one-third of the genome (Knight, 1980). For the purposes of this study I will only concentrate on the R-region and thus only on the 21S subunit of the mitochondrial ribosome. The R- region is of particular importance in this study since it is the site for mutations that confer resistance to antibiotics such as chloramphenicol, erythromycin and spiramycin. The mutations that conferred resistance to chloramphenicol were all found to be in the gene specifying the 21S ribosomal unit and were at the loci *cap1, cap2, cap3* and *cap 4* (Knight, 1980). However, the sequence for only *cap1* is available and the rest still need to be characterized.

In addition to the ribosomal RNA genes, the mitochondrial DNA of yeast also specifies certain constituents of the cytochrome oxidase, coenzyme QH_2-cytochrome c reductase, ATPase and a full set of tRNAs. It also contains the *var1* gene, which codes for the var 1 protein that becomes a part of the small

ribosome subunit. These constituents are produced *in situ* inside the mitochondria and most are immediately incorporated in the electron transfer chain (Tzagoloff and Myers, 1986).

The R Region and the Omega Intron of the Mitochondrial Genome

The sequence that codes for the mature rRNA of the large subunit is 3273 base pairs long. This sequence is found within the R-region and, similar to the rest of the mitochondrial genome, is AT-rich with its cytosine-guanine content being only 20.5 percent (Sor and Fukuhara, 1983).

The 21S rRNA gene is important to this study since it is a region that often has mutations that result in resistance to antibiotics, especially erythromycin, spiramycin and chloramphenicol. Three main loci have been isolated that undergo point mutations to provide resistance to these antibiotics. These are called *RIB1*, *RIBII* and *RIBIII* or otherwise commonly known as *cap1*, *spi1* and *ery1*, respectively (Netter et al., 1974).

Moreover there are three different forms of 21S sequence that are widely known today. These are Omega plus (ω+), Omega negative (ω-) and Omega neutral (ω_n). Omega (from now on referred to as ω) is an intron inserted into the middle of the 21S gene. It is an 1143 base pair long intron inserted after the 2689^{th} base pair of the 21S gene (Sor and Fukuhara, 1983). This intron is not transcribed during the transcription of the 21S gene thereby accounting for the shorter length of the code for the large subunit as compared to the

complete code of the gene. However there is also a 66 base pair mini-insert located 156 base pairs upstream to the ω intron. This sequence is transcribed into the mature RNA without excision (Dujon, 1980). Since the nature of this mini insert is palindromic it is hypothesized that it might be a mitochondrial transposable element. The ω intron encodes for a 235 amino acid polypeptide and encodes for a protein that exhibits double strand endonuclease activity that targets the ω- site in ω- strains (Colleaux et al., 1986).

The ω intron and the mini-insert are only present in strains of *Saccharomyces cerevisiae* that are ω+, strains that are ω- and ω_n do not have this intervening sequence or mini insert. Instead of the mini insert they have two additional adenine nucleotides between the 2534^{th} and 2535^{th} base pairs (Sor and Fukuhara, 1983). ω_n strains are different than both of these since they are a result of spontaneous mutation in ω- strains. They occur due to a base substitution very close to the point of insertion of the ω intron in ω+ alleles (Dujon, 1980). The difference between these three alleles is important because of a particular phenomenon called the polarity of recombination that occurs at the 21S rRNA gene.

In ω+ x ω+ or ω- x ω- crosses, also known as homopolar crosses, no polarity of recombination occurs. Reciprocal recombinants of the markers found in the region occur at expected frequencies. This is extremely important for determining the genetic map of the genome and the position of these markers. This procedure has been especially used to determine the distance between

the antibiotic resistance loci of erythromycin and chloramphenicol found in the region (Netter et al., 1974).

However, in ω+ x ω- crosses, also known as heteropolar crosses, polarity of recombination occurs. The two reciprocal recombinant types occur with extremely unequal frequencies with one of the recombinant types magnified disproportionately and the other repressed. Moreover the majority of the ω- alleles are converted to ω+. It is proposed that this happens because a heteroduplex forms between the ω+ and ω- strands. This results in the ω+ endonuclease excising the ω- strand at the ω insertion site, resulting in a gap directly opposite to the ω intron. Synthesis of the ω intron then follows filling in the gap and thus converting the ω- strand to a ω+, as shown in Figure 2 (Dujon, 1981).

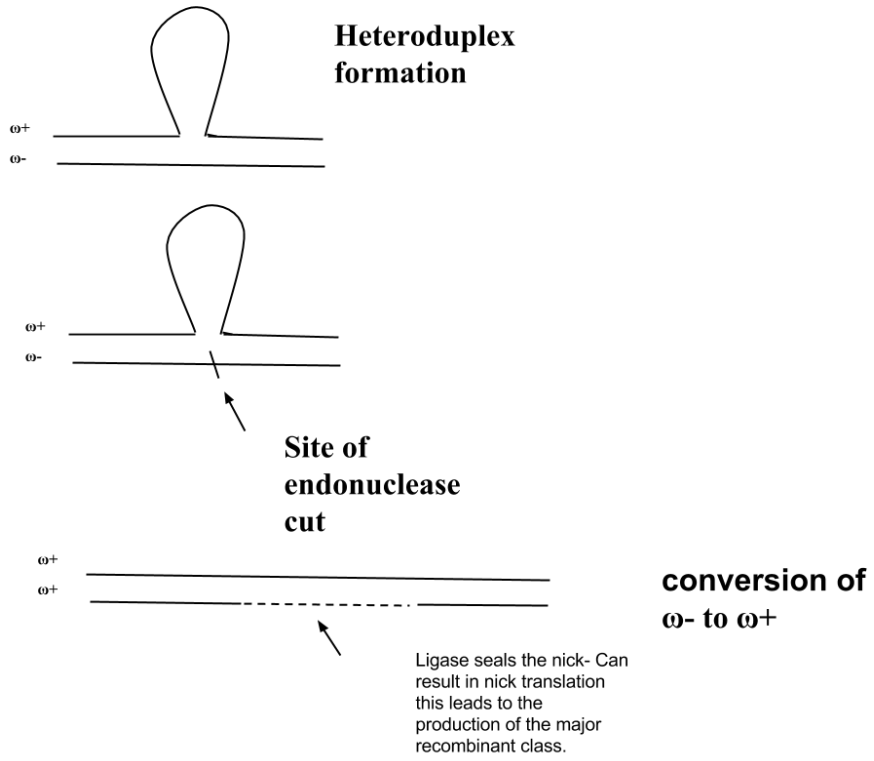

Figure 2: The formation of a heteroduplex during a heteropolar cross leading to the conversion of the ω- genotype to ω+.

A genetic map of the three *RIB* loci with respect to ω has been determined. All three of these loci are found near the ω region with *RIBI* being the nearest and *RIBIII* being the farthest, this is shown in Figure 3 (Netter et al., 1973).

RIBIII—ω—RIBI—RIBII

Figure 3: Positions of the three RIB loci with respect to omega.

The RIB loci are the site of antibiotic resistance mutations with RIBI containing mutations for chloramphenicol, RIBII for spiramycin and RIBIII for erythromycin.

Because of the proximity of these three loci and the number of antibiotic resistant markers found in these regions, polarity of recombination is an important consideration. It should be noted that polarity of recombination decreases as the distance from the ω intron increases, so the *RIBI* locus experiences the most effect in its markers and *RIBIII* the least (Netter et al., 1973). However the effect on all three is still very significant with frequencies between the markers present in *RIBI* and *RIBIII* respectively being highly affected between ω+ and ω- crosses (Dujon, 1980).

Chloramphenicol and *cap1*

Chloramphenicol is a broad spectrum antibiotic specifically known for inhibiting the synthesis of bacterial protein while not affecting other metabolic processes (Jardetzky, 1963). In yeast it inhibits amino acid incorporation by its mitochondrial ribosomes while leaving the cytoplasmic ribosomes unaffected. It was observed that *S. cerevisiae* grown on fermentable glucose in the presence of

chloramphenicol no longer forms cytochrome aa_3 and forms only trace amounts of cytochrome b (Huang et al., 1966).

Moreover, chloramphenicol affects *S. cerevisiae* in a way that makes the affected cells comparable to the respiratory deficient mutant yeast. A respiratory deficient *S. cerevisiae* is characterized by a lack of insoluble mitochondrial enzymes and cytochromes a, a_3 and b. Therefore it stands to reason that chloramphenicol reversibly inhibits translation of specific genes for mitochondrial proteins at the mitochondrial ribosome level (Clark-Walker, 1967).

Studies done on *E.coli* show that the drug inhibits protein synthesis by binding to the peptidyl transferase center of the large ribosome subunit. Through this binding, chloramphenicol then interferes with amino acid bond formation and terminates protein chain elongation (Moazed and Noller, 1987). It is this effect of chloramphenicol on the mitochondrial ribosome that is of particular interest to this study.

Chloramphenicol resistant mutations prevent the antibiotic from binding with the mitochondrial ribosome and thus interfering with mitochondrial protein synthesis. However, it is important to note that resistance to chloramphenicol does not only occur via mutations in the *S. cerevisiae* mitochondrial genome but can also occur via nuclear genome mutations. There are two kinds of such nuclear mutants. First are the permeability mutants that block the passage of the drug by either making the cell membrane or the mitochondrial membrane

impermeable. Second are those that have mutations in the genes that encode ribosomal proteins (Waxman et al., 1979). Moreover nuclear mutations can also act as suppressors of the mitochondrial mutations that confer resistance to chloramphenicol (Knight et al., 1984).

However, in this study I am only going to examine mitochondrial mutants, and all of these mutations occur in the 21S rRNA gene. A known mitochondrial mutation conferring resistance to chloramphenicol is a point mutation called *cap1*. This is also known as *RIBI* and results due to a A/T to C/G transversion at the 3884th base pair of the 21S rRNA gene (Sor and Fukuhara, 1983). This *cap1* point mutation has been also referred to as the C^{R}_{321} mutation and for the purposes of this study I will consider it as my main known mutation.

It was earlier thought that there were two main mutations in the RIBI region of the mitochondrial genome. These were referred to as C^{R}_{321} and C^{R}_{323}. Similar to C^{R}_{321}, C^{R}_{323} was also a point mutation but resulted via a G/C to A/T transition and was found to be 56 base pairs from the C^{R}_{321} mutation when ω_n and ω- strains were compared. This mutation was isolated in a ω_n strain but was thought to also exist at the *RIBI* locus (Dujon, 1980). However when the 21S region of the mitochondrial genome was sequenced it was seen that the mutation C^{R}_{323} was actually observed at the last few base pairs before the insertion of the ω intron, with the ω intron accounting for all bases between 2689-3831 on the gene and the mutation occurring at the 2685th base (Sor and Fukuhara, 1983). This realization, in lieu of the fact that the mutation

responsible for converting ω- strain to ω_n also occurs within a few base pairs before the insertion of the ω intron, calls into question whether this C^R_{323} mutation might just occur in ω_n strains. The two mutations as well as their proximity to the ω intron are shown in Figure 4.

Figure 4: Partial sequence of the mutated *21S rRNA* gene mutated at *cap1* (Sor and Fukuhara, 1983).

This shows the positions of the C^{R}_{323} and the C^{R}_{321} mutations marked with arrows at positions 2685 and 3884 respectively. The sequences pertaining to the omega intron are in lower case. Numbers on the right of the sequence state the base pairs in that particular line.

It is for that reason that in this study I will only consider C^{R}_{321} mutation as our *cap1*. The *cap1* mutation can occur regardless of the ω allele and has been recognized in many strains that can then serve as my *cap1* testers.

For the purposes of this study I will only be concentrating on the mitochondrial mutants. Therefore it is essential to distinguish between nuclear and mitochondrial mutants. This is easily achieved because of the differences between the modes of nuclear and mitochondrial inheritance.

Mitochondrial Versus Nuclear Inheritance

Nuclear traits are passed on through generations in what is known as Mendelian inheritance. In *Saccharomyces cerevisiae* this can be observed during sporulation when mating types are segregated. However the same cannot be said about the inheritance of the mitochondrial characteristics. In 1949, it was shown that some respiratory deficient mutants do not follow Mendelian rules of segregation but instead showed a mode of inheritance comparable to that of cytoplasmic particles. After further research this mode of inheritance was further understood and the mechanism was deemed as vegetative segregation (Dujon, 1981).

To understand vegetative segregation, it is first important to understand the concept of homoplasmic and heteroplasmic cells. An average rapidly growing yeast cell contains up to ten mitochondria, while a stationary phase cell can contain up to 50. A single mitochondrion can in turn contain up to 150 DNA molecules (Stevens, 1981). A yeast cell is called homoplasmic when all

mitochondrial DNA copies present in the cell are genetically identical. Heteroplasmic cells on the other hand do not have this feature and can have different mitochondrial DNA within the same cell. When a homoplasmic cell divides it gives rise to all homoplasmic cells; however when a heteroplasmic cell divides it gives rise to cells that compose of different mitochondrial genotypes. It is during the latter that we observe vegetative segregation.

Experiments have shown that cells produced by a heteroplasmic cell can either be heteroplasmic or homoplasmic (Dujon, 1981). However the cell undergoes segregation in a manner that ensures that the frequency of getting a heteroplasmic cell is very low and most if not all cells end up as homoplasmic. When a zygote is formed via the mating of two distinct homoplasmic cells, the resulting zygote itself is heteroplasmic. It contains both of the parental mitochondrial types and allows recombination to occur between the two genotypes and forms recombinants. However this is often accompanied by a rapid segregation of DNA molecules. Few mitochondria are transferred to the new buds which then further undergo fission to produce more mitochondria in the new bud. This continues for every new generation produced and after the production of 20-25 generations all cells become virtually homoplasmic (Dujon, 1981). However it should be noted that two cells found within the same generation need not have the same mitochondrial genome, yet every mitochondrial DNA molecule would be the same within each cell. This is illustrated in Figure 5 below.

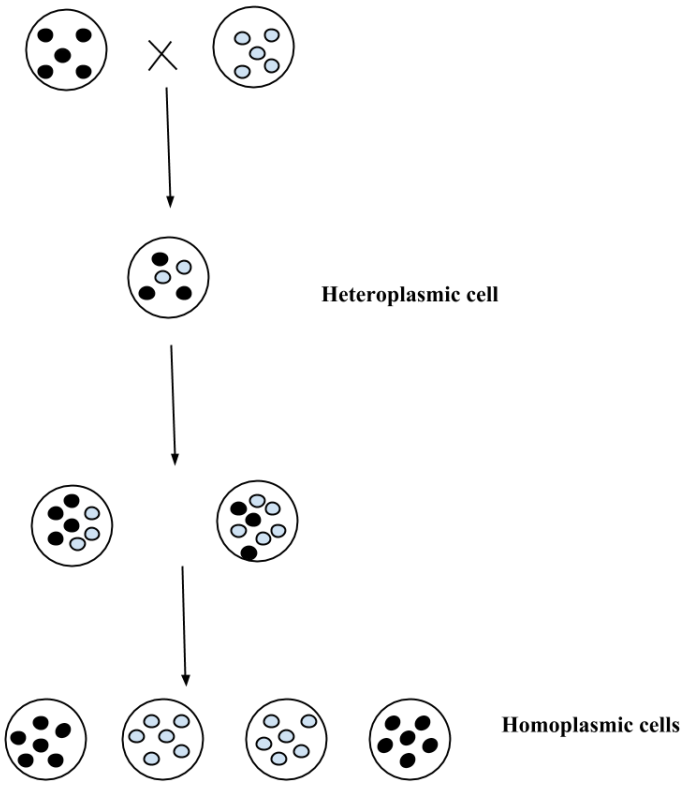

Figure 5: Vegetative Segregation

This Figure shows how a heteroplasmic cell with two different mitochondrial markers segregates over multiple generations to produce homoplasmic cells containing distinct markers from each other.

These mechanisms of recombination and vegetative segregation are very useful in scientific research. By allowing two mutants to mate and undergo recombination and vegetative segregation and then using cross streaking to separate distinct colonies, recombination frequencies between two different mutations can be calculated. This is one of the main methods that I have employed in my study.

My Study

In this study auxotrophic strains of *S. cerevisiae* were used, i.e. strains which can produce all amino acids and other growth factors except one or two. This allowed me to manipulate the media on which these strains grow in such a way that only the desired strains were allowed to grow. Also the progeny of two complementary auxotrophic strains are able to grow on minimal media while the parents would not. Four chloramphenicol sensitive haploid strains with different auxotrophic requirements (two with the *a* mating type and two with the α mating type) were chosen as parent strains from which to select spontaneous chloramphenicol resistant mutants. Mutants were tested for mitochondrial versus nuclear inheritance via random diploid analysis. The mitochondrial mutants were isolated and strategic matings were set up to determine recombination frequencies. My hope was to discover some mutants that were non-allelic to each other. These mutants then underwent a genetic analysis where their mitochondrial DNA was extracted and sequenced. The hope was to compare these sequences with the already published *cap1* sequence. It was the purpose of this study to not only reconfirm the sequence of *cap1* but also to find other point mutations in the R region of the mitochondrial genome of *S. cerevisiae*.

It should be noted that it is relatively easy to prepare *S. cerevisiae* in a way so that they use their mitochondria or not. If *S. cerevisiae* is grown on a fermentable carbon source then the organism would only use the fermentation pathway thereby not using its mitochondria. Alternatively, if it is grown on a non-

fermentable source like glycerol we can ensure that *S. cerevisiae* only uses its mitochondria, thereby allowing us to manipulate its respiratory environment (Sherman, 2002). Therefore, for the isolation of mitochondrial mutants YG plates (plates with the non-fermentable glycerol as a carbon source) and YG+C (plates with glycerol and chloramphenicol) would be used instead of the regular YD (plates with fermentable glucose). This is a relatively simple manipulation and once again emphasizes why *S. cerevisiae* is a model organism.

This study does not only provide evidence for new sites of mutation in yeast but also attempts to characterize them. It is hoped that with future research more can be known about the mutants studied.

MATERIALS AND METHODS

Media

Five main kinds of media were used. These were YD, YG, YG+C, MD and MD+AA. Each of these have unique properties that make them essential to this study.

YD

This provides the growing *Saccharomyces cerevisiae* with a fermentable carbon source in the form of glucose. It allows all viable strains to grow regardless of their mitochondrial mutations or nutrient deficiencies. YD media was made using the following constituents:

10 g/L yeast extract

20g/L bactopeptone

20g/L glucose

20/L agar

YG

This media provides only a non-fermentable carbon source to the growing *Saccharomyces cerevisiae* in the form of glycerol. It is useful for distinguishing respiratory competence/incompetence since the *S. cerevisiae* are forced to respire aerobically and use their mitochondria. *Petite* mutants cannot grow on this media. YG media was made using the following constituents:

10 g/L yeast extract

20 g/L bactopeptone

32 mL/L glycerol

20 g/L agar

YG+C

YG+C is the exact same media as the YG with the addition of one additional component, chloramphenicol. This antibiotic (0.5g/500 ml dissolved in methanol) was added to the YG media just prior to pouring the plate. This media selects against *S. cerevisiae* that are sensitive to the antibiotic. The only strains of *S. cerevisiae* that can grow on this media are those that have resistance to the antibiotic and are able to respire aerobically.

MD

This media is also known as minimal media. It contains only the most basic nutrients that the *S .cerevisiae* need to grow and survive and do not produce themselves while glucose serves as the carbon source. Only prototrophic yeast strains can grow on this media. This is very helpful in distinguishing diploids formed between complementary haploid auxotrophs and is used widely to determine whether mating has occurred. MD was made using the following constituents as:

10 mL/L buffer

10 mL/L salts

1 mL/L vitamins

1 mL/L calcium

0.1 mL/L trace minerals

Stock solutions of these were prepared as described by Wickerham (1946) as modified by Birky et al., (1975)

MD+AA

This media is a variation of the minimal media and can be altered according to the strains being used. The AA stands for amino acid and is used to confirm the auxotrophic requirements of the strains being used. The amino acid (0.1g) is dissolved in 10 ml of sterilized water and 200µL of this supplement is spread on a dry MD plate to create this media.

Isolation of Healthy Strains and Confirmation of Auxotrophic Requirements

Haploid strains were selected based on their availability in the laboratory. All strains used were auxotrophic in nature which means that they did not have the ability to produce one or more amino acids needed for growth and thus could only grow in the presence of those nutrients. Strains were plated on fresh YD and allowed to grow overnight in the incubator set at 30℃. Six *a* and 6 α strains were chosen for their vigorous growth and then a 15 µL drop of each strain was dropped on an MD and an MD+AA plate to test their stated auxotrophic requirements. All of the 12 strains did not grow on MD but grew on plates

supplemented with their required amino acid. These strains were then chosen for further study. Table I indicates these strains and their properties.

Table I: Strains selected for Study

This depicts all the healthy strains isolated and selected for further research.

Strain Name	Mating Type	Auxotrophic Requirements
CCD6-1	a	met-
CCD6-2	a	arg-
HA2	a	ade-
D22-5	a	ade-
55R2	a	ura-
CD655-3	a	arg- ura-
DPI-1B	α	his- trp-
CD655-10	α	arg- ura-
CD655-8	α	met-
ID4-1	α	ade- trp-
IL126	α	his-
D6-5	α	met-

Testing for Healthy Maters

The 12 strains listed above were tested for their mating efficiencies. All possible a x α crosses with complementary auxotrophic requirements were set up on MD plates. These were done by first making a cloudy suspension of each strain in 5mL of sterilized water and then by putting two 15 µL drops of each strain on the plate. One drop of each strain was put on the same spot as the previous strain while the other spot was put in an isolated area. This was to ensure that the strains were still auxotrophic and that all growth on the plate was only due to diploids. This procedure is demonstrated in Figure 6 below. The plates were then placed in the incubator at 30℃ for three days and then observed.

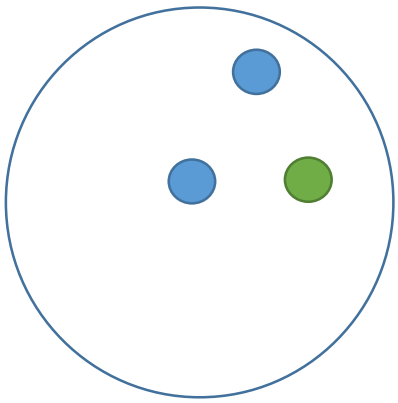

Figure 6: A typical mating plate.

This shows a typical mating plate. The blue spots indicate revertant control spots of an a and α strain. The green spot indicates both drops of a and α superimposed on each other. A successful mating will show a lot of growth on the green spot after three days with none at the blue spots.

Drop tests for Sensitivity to Chloramphenicol

In order to select strains to screen for spontaneous mutations, strains sensitive to chloramphenicol were needed. Drop tests were performed on all strains to ensure sensitivity. These drop tests were conducted by putting 15 µL drops of each strain, prepared in 5 mL of sterilized water, on two plates in the same order (YG and YG+C). These plates were kept in the incubator for three days and the levels of growth were assessed. All strains grew on YG, however those that were sensitive to chloramphenicol did not grow on YG+C.

Plating for Spontaneous Mutagenesis

Strains that then demonstrated both high mating efficiencies and sensitivity to chloramphenicol were chosen to be plated for spontaneous mutations. Both of the *a* strains 55R2 and D22-5 as well as the two α strains DPI-IB and D6-5 were chosen. CD655-8 was kept as a reserve in case the other two α strains did not show any spontaneous mutations; otherwise, two α and two a strains were enough for the purposes of this study.

To plate for spontaneous mutagenesis, a very cloudy suspension of each strain was made in 5 ml of sterilized water. A100 µL drop of the strain was then dropped on a YG+C plate and spread using a sterilized glass spreader. Five plates for each strain were prepared in this manner and placed in the 30℃ incubator for two weeks. At the end of this time mutant colonies were observed.

Streaking for Colony Isolation

This technique was used to purify strains and their mutants. The mutants obtained to purify from spontaneous mutagenesis were purified in order to ensure that all cells from the mutants were exactly the same.

To streak for colony isolation, the colony in question was picked from the plates by using a sterilized loop and then suspensions were made in 5 mL of sterilized water. A loop full of each suspension was streaked on a YD plate as to approximate serial dilution, as shown in Figure 7. The plates were kept in the incubator for two days and a healthy colony was selected from each plate and re-plated on a fresh YD plate.

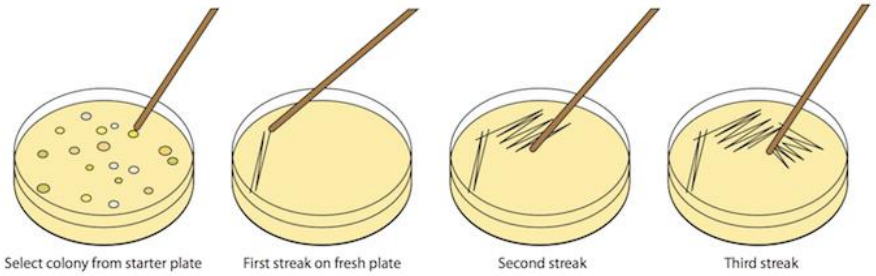

Figure 7: Streaking for colony isolation
(http://www.shmoop.com/prokaryotes/postulate-petri-dish.html)

Determining Mitochondrial Versus Nuclear Inheritance

The mode of inheritance of the mutation in each mutant strain was determined by setting up crosses between the mutants and the sensitive parental type, as shown in Table II, followed by random diploid analysis.

Table II: Matings for determining inheritance patterns.

This shows the crosses set up between the mutants and the sensitive parental strains. The C superscript shows the sensitivity or resistance of each strain while the amino acid or nucleic acid annotation following the strain depicts the auxotrophic requirement.

Cross	Mutant	Wild Type
a	α D6-5 (#1,#2,#3) C^Rmet	a 55R2 C^S ura
b	a 55R2 (#1,#2,#3,#4,#5,#6) C^Rura	α D6-5 C^S met
c	α DPI-IB (#1,#2,#3,#4,#5) C^Rade2	a 55R2 C^S ura

Random Diploid Analysis

Random diploid analysis is one of the major tools in this study. It does not only help determine whether mutants are mitochondrial or nuclear in nature but also serves as the main tool for determining recombination frequencies. There are two main techniques involved in random diploid analysis:

1) Cross Streaking

This technique allows for individual colonies of the diploids to be separated via serial dilution. Each resulting diploid patch from the chosen matings is

first mixed on the plate and then used to create a very light, perceptibly cloudy suspension. A light loop full of the suspension is streaked on an MD plate in horizontal lines until it is even. Then the plate is rotated 90 degrees and the process repeated. This is done to create 5 similar plates for each diploid. These plates are then left in the incubator to grow for three days. Numerous colonies can be observed on each plate and replica plating can be carried out for each cross streaked plate.

2) Replica Plating.

This technique allows the determination of sensitive versus resistant colonies resulting from a particular mating. It is done by creating "replicas" of the cross streaked plates on two different plates, YG and YG+C. These replicas are made by using sterilized velvets which go over a replica plater. The cross streaked plate is first pressed lightly on this velvet allowing the colonies to touch the velvet. A fresh YG plate is then pressed on the same velvet allowing the impression of the colonies to map out on the plate. This is then repeated for the YG+C plate. These plates are placed in the incubator at 30℃ for three days to allow the colonies to grow.

Determination of Recombination Frequencies

Once a set of mitochondrial mutants was established, crosses between the mutants are set up to determine whether the mutations are allelic or non-allelic to each other. All possible matings are set up and random diploid analysis is carried out

by cross streaking and replica plating. Each mating is cross streaked on about 20 plates and then each cross streak is replica plated. Every colony on the replica plate is then characterized as either sensitive or resistant by comparing its growth on the YG plate with its counterpart on the YG+C plate. The number of observed sensitive colonies then allow for the calculation of the recombination frequencies.

Additionally, two strains with known *cap1* mutations were further selected to perform recombination tests. These were the IL126 and the 5-1/6 strains and had ω genotypes that allowed for homopolar crosses with their respective mutants.

Calculation of Recombination Frequency

Recombination frequencies were calculated using the formula below:

$$\text{Recombination Frequency} = \left(\frac{\#\ of\ C^S\ colonies\ x\ 2}{\#T.C}\right) x\ 100$$

Where C^S stands for chloramphenicol sensitive colonies and T.C stands for total colonies.

Isolation of Mitochondria and DNA extraction

In an overnight culture of 40 mL of liquid YD, each yeast strain was grown via shaking. The contents of these flasks were centrifuged at 1500 x g at 4℃ for ten minutes to harvest the cells. The pellets obtained were washed with 50 mL of ice cold water by vortexing and then re-centrifuged to form pellets. They were washed with 25 mL ice-cold 1.2M sorbitol and centrifuged once again to reform the pellets. The pellets were suspended in 30 mL CL Buffer (0.5M sorbitol,

10mM EDTA, 50mM TRIS. Cl pH 7.4, 2% (v/v) 2-mercaptoethanol) with 0.25 mg/mL zymolyase 20T (ICN Biomedicals, USA). These were incubated at 37℃ for 1 hour to allow for successful lysis. Tubes were taken out and vortexed. Cell debris was removed through two rounds of centrifugation at 1500 x g at 4℃ and at 2100 x g at 4℃ respectively for ten minutes each. The supernatant was transferred to a fresh tube and the pellet was resuspended in 20 mL of CL Buffer again for 30 minutes to increase the yield. Centrifugation for ten minutes at 2100 x g at 4℃ then followed and the supernatant obtained was added to the previously stored supernatant. The mitochondria were then collected by a last round of centrifugation for 15 minutes at 16000 x g at 4℃. These were then washed three times with 4 mL of ice-cold MW Buffer (0.5 M sorbitol, 1 mM EDTA, 50 mM TRIS.Cl, pH 7.4).

The mitochondrial DNA extraction was performed by using the DNeasy Blood and Tissue Kit from Qiagen©. The mitochondrial pellet was first dissolved in 200 µL of PBS and 20 µL of Proteinase K was added. 200µL of buffer AL was added and thoroughly mixed via vortexing. 200 µL of 95 percent ethanol followed and the mixture was again vortexed. This was transferred to a DNeasy mini spin column placed in a 2mL collection tube. The collection tubes were centrifuged at 6000 x g for 1 minute at room temperature. The flow through was discarded and a fresh collection tube was placed beneath the spin columns. 500 µL of AW1 Buffer was added and the tubes were re-centrifuged for the same time and speed. The collection tubes were once again discarded and replaced with new ones. 500

µL of AW2 buffer served as the second wash and the tubes were centrifuged at 2000 x g for 3 minutes at room temperature allowing for the DNeasy membrane to dry. The spin columns were now placed in new 1.5 mL tubes and 200 µL of AE Buffer was added at the center of the membrane to elute the DNA. The tubes were left to stand for one minute for efficient elution and the tubes were centrifuged at 6000 x g for 1 minute. This process was repeated with 200 µL of additional AE Buffer to increase the DNA yield. The final concentrations of the mitochondrial DNA obtained were then determined using a nanodrop spectrophotometer.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Once the DNA was obtained, the region of the 21S rRNA gene believed to carry chloramphenicol resistance mutations was amplified using the polymerase chain reaction. One forward primer, and two reverse primers were selected that spanned the *cap1* mutation as well as the surrounding nucleotides. The selected primers are shown in Figure 8. These primers were chosen for their high GC content and are about 20 base pairs long.

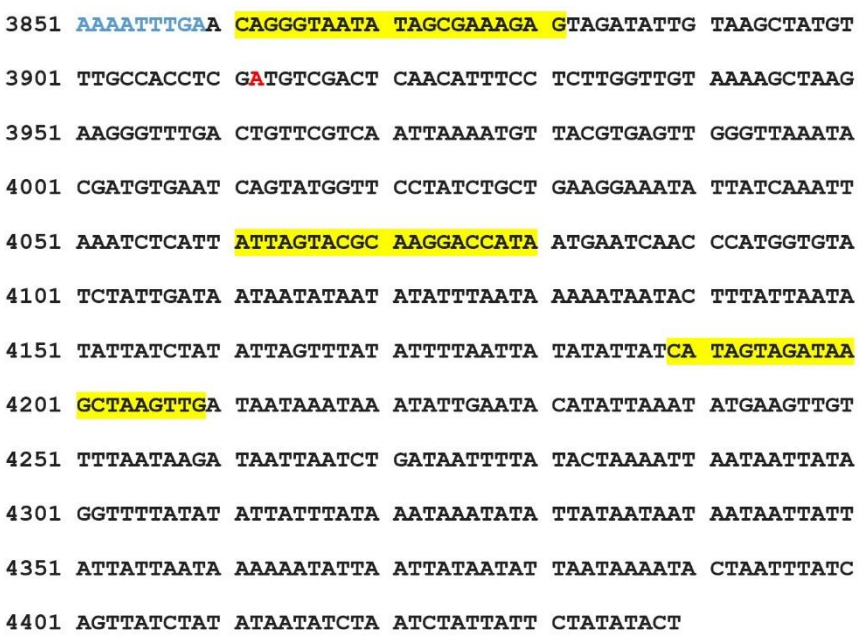

Figure 8: Selected primers for PCR.

This Figure shows a part of the 21S gene. The highlighted yellow sequences are the primers with the first being the forward primer, second being the reverse primer number 1 and the third being reverse primer number 2. The sequence in blue are the last few bases of the ω intron and the base pair in red is the *cap1* mutation.

Forward Primer: 5'->3'= **CAGGG TAATA TAGCG AAGAG**

Reverse Primer 1: 3'->5'= **TATGG TCCTT GCGTA CTAAT**

Reverse Primer 2: 3'->5'= **CAACT TAGCT TATCT ACTATG**

The 50µL PCR master mix was prepared for each sample by adding 10 µL of Taq 5X Master mix (New England BioLabs), 10 µL of template DNA, 1 µL of forward primer, 1 µL of reverse primer and 28 µL of deionized water. The PCR tubes were placed in a thermo cycler and the PCR cycle was set as shown in Table III.

Table III: PCR Cycle

Step	Temperature (°C)	Duration
Initial denaturation	95	5 min
35 cycles: denaturation annealing extension	95 49 68	30s 30s 30s
Final extension	68	5 min
Hold	4	-

After the PCR, a 5 % agarose gel was prepared by dissolving 0.5 g of agarose in 50 mL of 1X TAE Buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA) and 5 µL of Ethidium Bromide was added to allow for successful staining of the DNA. The gel was allowed to set in the electrophoresis chamber and once set was submerged in more TAE Buffer. 16 µL of the PCR product was mixed with 4 µL of gel loading dye and was loaded into the gel. A 100 base pair ladder was added as a reference and the gel was run at 100 Volts for 45 minutes. The gel was then viewed under ultra violet light, imaged and the PCR bands were cut from the gel by using a sharp clean blade.

DNA Purification

DNA purification from the gel was carried out by using the MinElute Gel Extraction Kit from Qiagen©. Cut gel fragments were transferred to an empty 1.5 mL tube and then each gel fragment was weighed by subtracting the weight on an empty 1.5 mL tube from the weight of the gel + tube. 3 volumes of buffer QG were added for 1 volume of the gel (100mg = 100µL). The tubes were incubated at 50℃ for ten minutes and were vortexed every 2-3 minutes during incubation to

completely dissolve the gel. 1 gel volume of isopropanol was added to the tube and the tubes were mixed by inverting. The sample was transferred to a MinElute column placed in a 2 mL collection tube and centrifuged for 1 minute at 13000 rpm at room temperature. The flow through was discarded and the same collection tube was placed beneath the MinElute column once again. This was repeated until all of the sample had passed through. 500 µL of Buffer QG were added and the sample was centrifuged again for 1min at 13000 rpm at room temperature. The flow through was once again discarded and the collection tube placed back again. 750 µL Buffer PE were added to the MinElute column and the column was left to stand for 5 minutes. The sample was centrifuged for 1 minute again at 13000 rpm at room temperature and then the flow through was discarded and centrifugation was repeated for 1 minute to completely remove the ethanol from the sample. Each MinElute column was placed in a fresh labeled 1.5 mL collection tube and 10 µL of Buffer EB was added to the center of the column for elution of the DNA. The samples were left to stand for 1 minute and were then centrifuged for 1 minute at 13000 rpm at room temperature. Buffer EB was added again and the steps above were repeated to increase the yield of the DNA. The concentration of the DNA was determined using a nanodrop spectrophotometer.

Sequencing

The samples were diluted with Buffer EB to 1 ng/µL for sequencing. 10 µL of each diluted sample was sealed in small PCR tubes. The forward primer was diluted to 5µM and 5µL of the primer for each sample were put in a separate

small PCR tube and sealed. The pre-defined sequencing option was selected at GENEWIZ, Inc. and the samples were shipped to the company.

RESULTS

Testing for mating efficiencies

Strains that showed successful mating are shown in Table IV.

Table IV: Efficient Maters:

The highlighted strains are those that showed high mating efficiencies. These strains were then used for further research.

Strain Name	Mating Type	Auxotrophic Requirements
CCD6-1	a	met-
CCD6-2	a	arg-
HA2	a	ade-
D22-5	a	ade-
55R2	a	ura-
CD655-3	a	arg- ura-
DPI-1B	α	his- trp-
CD655-10	α	arg- ura-
CD655-8	α	met-
ID4-1	α	ade- trp-
IL126	α	his-
D6-5	α	met-

Conducting Drop Tests on the Strains

The results of the drop tests conducted on the strains are shown in Table V.

Table V: Sensitive Strains:

Results of the drop tests done to determine sensitivity to chloramphenicol.

Strain Name	Mating Type	Sensitivity to Chloramphenicol
CCD6-1	a	Resistant
CCD6-2	a	Reverted to its prototrophic type
HA2	a	Resistant
D22-5	a	Sensitive
55R2	a	Sensitive
CD655-3	a	Resistant
DPI-1B	α	Sensitive
CD655-10	α	Resistant
CD655-8	α	Sensitive
ID4-1	α	Sensitive
IL126	α	Resistant
D6-5	α	Sensitive

Plating for Spontaneous Mutations and Determining Inheritance Pattern

Strains 55R2, D22-5, DPI-IB AND D6-5 were selected for spontaneous mutagenesis and were plated according to the methods described above. After two weeks of incubation some colonies appeared for each strain except D22-5. These colonies were picked and purified via colony isolation and then purified colonies were patched onto YD. Each colony was numbered and the number of colonies obtained for each strain are indicated in Table VI. D22-5 was then re-plated for spontaneous mutagenesis; however, when still no mutants were observed after two weeks the strain was discarded from the study.

Table VI: Mutants obtained from selected strains.
This shows the number of mutants isolated from each strain. Each mutant is referred from now on via its strain name and assigned number.

Strain	Mating Type	Omega	Mutants
D6-5	α	ω-	#1,#2,#3
DPI-IB	α	ω-	#1,#2,#3,#4,#5
55R2	a	ω+	#1,#2,#3,#4,#5,#6
D22-5	a	ω+	-

Each mutant was then tested for mitochondrial versus nuclear inheritance via random diploid analysis. Mutants were crossed with sensitive parental strains and then the frequency of sensitive diploids were noted. For mitochondrial

inheritance, due to the mechanism of vegetative segregation there should be a 1:1 ratio between sensitive and resistant progeny. In the case of nuclear inheritance, however, Mendelian patterns should be observed with all progeny being either phenotypically sensitive or resistant. Table VII below shows the results of random diploid analysis and the mitochondrial mutants obtained.

Table VII: Mitochondrial versus Nuclear Mutants
This depicts the mutants determined as nuclear or mitochondrial for each particular strain.

Strain	Mating Type	Mitochondrial Mutants	Nuclear Mutants
D6-5	α	#1,#2,#3	-
DPI-IB	α	#1,#2,#3,#5	#4
55R2	a	#1,#3,#5	#2,#4,#6

The mitochondrial mutants were then selected for allelism tests between each other and the known *cap1* mutants.

Allelism Tests between 55R2,DPI-IB and D6-5 mutants

Allelism tests were carried out between all of the mitochondrial mutants obtained given that they had opposite mating types. All three strains had complementary auxotrophic requirements to one another and therefore allowed for successful mating. The results of the allelism tests are indicated in Table VIII.

Table VIII: Allelism tests between mutants

This indicates sensitive recombinants observed in the crosses between mutants.

The asterisk (*) indicates inconclusive results.

Cross Number	Cross	# of Sensitive Colonies (C^S)	# of Resistant Colonies (C^R)	# of Total Colonies (T.C)
1	55R2(3) x D6-5(2)	61	832	893
2	55R2(3) x D6-5(1)	22	867	889
3	55R2(1) x D6-5(1)	85	762	847
4	55R2(1) x D6-5(2)	59	727	786*
5	55R2(1) x DPI-IB (1)	-	-	- *
6	55R2 (1) x DPI-IB (2)	6	535	541*
7	55R2 (1) x DPI-IB (3)	4	865	869
8	55R2 (1) x DPI-IB (5)	110	783	893
9	55R2(3) x DPI-IB (1)	15	868	883
10	55R2 (3) x DPI-IB (2)	357	620	977
11	55R2 (3) x DPI-IB (3)	24	805	829
12	55R2 (3) x DPI-IB (5)	36	855	891

At least 800 diploids were counted and characterized for each mating except for cross numbers 4, 5 and 6. This is because the mutant 55R2-1 reverted during the process of random diploid analysis and thus more diploids could not be produced and counted as there was no way left to distinguish between the haploid 55R2-1 and its diploids. According to these results, a significant number of sensitive recombinants was observed between the crosses, but due to the heteropolar nature of the crosses the recombination frequencies were not calculated. However, the high number of sensitive progeny observed for all of these crosses, especially for crosses 8 and 9, serve to indicate that these mutations are non-allelic to one another and thus good candidates for sequencing.

Allelism Tests between mutants and *cap1* testers IL-126 and 5-1/6

Allelism tests were also carried out between known *cap1* testers that would allow for homopolar crosses. These testers were IL126 (ω +) and 5-1/6 (ω -) r

Table IX: Allelism tests between *cap1* testers

Cross Number	Cross	# of Sensitive Colonies (C^S)	# of Resistant Colonies (C^R)	# of Total Colonies (T.C)
1	55R2 (1) x IL126	40	583	623
2	55R2 (3) x IL126	0	888	888
3	DPI-IB (2) x 5-1/6	18	840	858
4	DPI-IB (3) x 5-1/6	-	-	-
5	DPI-IB (1) X 5/1/6	-	-	-
6	DPI-IB(5) x 5-1/6	-	-	-
7	D6-5 (1) x 5-1/6	0	646	646
8	D6-5 (2) x 5-1/6	54	822	876

For these allelism tests I endeavored to score at least 800 diploids for each cross. However the reversion 55R2-1 to its prototrophic type as well as the continued contamination of the 5-1/6 strain made that goal difficult.

For crosses 4-6, the matings showed three phenotypes for the progeny, namely resistant, slightly resistant and sensitive. The unclear distinction between slightly resistant and sensitive led to a difficulty in characterizing any progeny correctly and thus showed no results. Similarly for cross 7, 5-1/6 continued to throw off

revertants at a huge frequency, necessitating multiple purifications. This resulted in a lower total diploid count than expected.

However, looking at the above results it can be deduced that 55R2 (3) and D6-5 (1) are allelic at *cap1* yet in the case of D6-5 (1) this needs to be further confirmed by a characterization of some more diploids. Nonetheless, this makes both of these mutants' good candidates for sequencing for the reconfirmation of the *cap1* sequence.

Crosses 1, 3 and 8 on the other hand indicate that mutants 55R2 (1), DPI-IB (2) and D6-5 (2) are non-allelic at *cap1* due to the number of sensitive progeny observed. Recombination frequencies were calculated for the above crosses and the results are shown in Table X.

Table X: Recombination Frequencies

Cross Number	Cross	# of Total Colonies	Recombination Frequencies
1	55R2 (1) x IL126 C	623	13.7
2	55R2 (3) x IL126 C	888	0
3	DPI-IB (2) x 5-1/6 C	858	4.3
4	DPI-IB (3) x 5-1/6 C	-	
5	DPI-IB (1) X 5/1/6 C	-	
6	DPI-IB(5) x 5-1/6 C	-	
7	D6-5 (1) x 5-1/6 C	646	0
8	D6-5 (2) x 5-1/6 C	876	13.1

The recombination frequencies observed indicate once again that 55R2 (3) and D6-5 (1) are allelic at *cap1*. Mutant DPI-IB is non-allelic at *cap1*, with the two mutational sites being separated by about 4 map units. However, for mutants 55R2 (1) and D6-5 (2) the recombination frequency is above ten percent. This suggests that D6-5 (2) might be a nuclear mutant. However I cannot conclude the same for 55R2 (1), since the total diploid colonies scored was significantly below the 800 mark. Once more colonies are counted this frequency could change significantly.

Since only one recombination frequency within ten percent was observed, a structural genetic map would serve to be of limited use and was thus not constructed.

Mitochondrial DNA isolation of 55R2(1), 55R2 (3) and IL-126

Mutants 55R2 (1), 55R2 (3) and the *cap1* tester IL-126 were selected for sequencing, and mitochondrial DNA isolation was carried out using the techniques proposed previously. Once the DNA was isolated it was quantified using a nanodrop spectrophotometer and the concentrations as well as their 260/280 values were noted as indicators of purity. These are shown in Table XI.

Table XI: Concentrations of isolated mitochondrial DNA

Strain	Concentration ng/µl	260/280 (Purity Value)
55R2-3	19.6	2.28
55R2-1	58.5	2.16
IL126	58.5	2.21

The significantly high concentrations as well as the purities values (260/280 value) close to 2 show that DNA isolation was successful. This isolated DNA was then amplified using PCR.

PCR, Gel Electrophoresis and DNA purification

A polymerase chain reaction was set up for each template DNA twice for two sets of primers. The PCR products were then subject to gel electrophoresis in order to determine the success of the PCR. The gel image is shown in Figure 9.

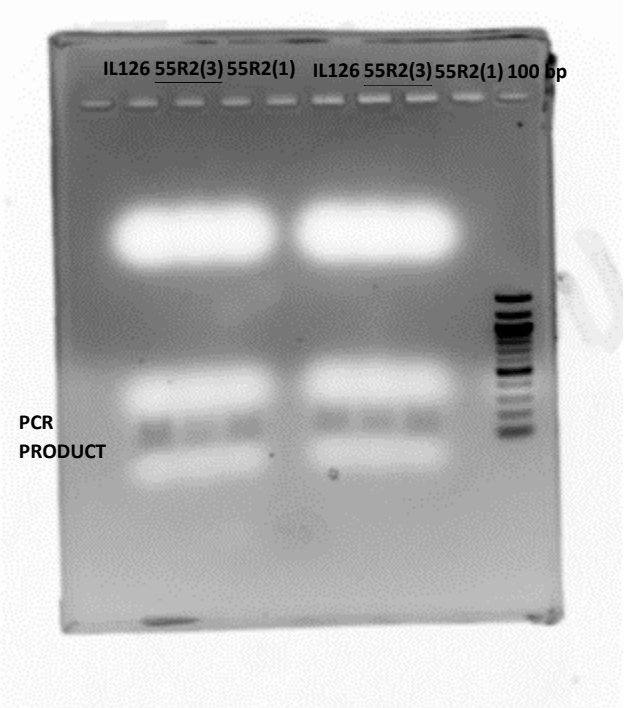

Figure 9: Electrophoresis Gel Image

This shows the image of the gel. Thick bands can be observed near the 300 base pair point of the base pair ladder for all 6 samples. The three lanes closest to the ladder are products with reverse primer 1 while the next three are those with reverse primer 2.

According to the gel the PCR was successful. The bands were cut from the gel and purified. The concentrations of the DNA were once again determined using a nanodrop spectrophotometer and concentration of the PCR products is shown in Table XII.

Table XII: DNA concentration of PCR Products

The PCR products using reverse primer 1 are indicated by (1/1) and the products with reverse primer 2 are indicated by (1/2).

DNA	**Concentration (ng/ul)**	**260/280**
55R2-1 (1/1)	10.7	1.99
55R2-3 (1/1)	13.9	2.48
IL126 (1/1)	4.3	1.63
55R2-1 (1/2)	3.6	1.68
55R2-3 (1/2)	19.98	1.55
IL126 (1/2)	7.3	1.75

Sequencing

Sequencing did not divulge conclusive results due to a failure in the ability of the primer to anneal to the sequence. The first four samples listed in Table XII, provided no sequence. The last two samples namely 55R2-3 (1/2) and IL126 (1/2) showed a partial sequence which is shown in Figure 10. This sequence proved to

be of limited use due to a large portion of nucleotides not being sequenced, thus making it incomparable to the mitochondrial sequence of interest.

55R2-3 (1/2)

NNNNNNNNNNNNNNNNCCGANGAANCCTCTTANTNCGACNGNNAAAATGCCNCTTGANN
NTCAAAACTGTCACCTAAATNNNNGNNNNNNAAGNGNTCAAACTGTNTCCTGTGTGAGT
TTGTTATCCTCTCGANNTTCCANCGACGTANNATGAGGANNAANNACGTGTAAANCCNG
NGGTGCCCANAGANTGGGCTN

IL126 (1/2)

NNNNNNNNNNNNNNNNNNNANAANCNCTAGANTCNCCTGNTANTCCCCNCNTGTGTNC
CNAAANTGNCCCTAAATNANNANAANNNGNNGGNNNAAGNNATATCAGGNNNNTNTAGT
NANNNGNNNCGGATTCCNCNCTNCTTACGAGCCGGAGATNAAGGTGTANGTCCTGNGGT
GCCTAATGGGTNGGCTNN

Figure 10: Partial Sequences obtained for Mutants.

Partial sequences obtained for 55R2-3 (1/2) and IL126 (1/2). The sequences are dominated by undetermined base pairs shown by N.

DISCUSSION

Spontaneous Mutagenesis

When strains 55R2, IL126, D22-5 and D6-5, were plated on YG+C plates, growth of mutant colonies was observed for all strains except D22-5. This was not surprising since it has been previously observed that there is substantial variation between strains regarding their susceptibility to mutations in the mitochondrial genome. It has been seen that D22-5 carries certain nuclear genes that can suppress resistance mutations in certain parts of the mitochondrial genome (Waxman et al., 1979). Although this observation implies that it was in fact the exposure to the antibiotic that resulted in the induction of these mutants, all the antibiotic in fact does is allow for intracellular selection for the resistant mitochondria. In 1943, using the fluctuation test, it was shown that in *E. coli* resistance to virus did not develop upon exposure but rather was present before the infection (Luria and Delbrück, 1943). In 1973 Birky allowed the application of this to yeast when he used the Newcombe method to demonstrate this in *S. cerevisiae.* He observed that replating some colonies of *S. cerevisiae* on a fresh plate and then exposing both the original and the fresh plate to antibiotic resulted in more colonies on the freshly plated plate (Birky, 1973).

Resistant mitochondria that arise via spontaneous mutations do not become apparent in yeast growing on media without antibiotic. This is because the resistant mitochondria are actually at a selective disadvantage since they perform

normal mitochondrial functions marginally less efficiently than the wild type. However, when that yeast is grown on media supplemented with antibiotic, in this case chloramphenicol, sensitive mitochondria do not function at all, and the mitochondrial DNA is not replicated. This then results in the intracellular selection for the resistant mitochondria. As the yeast colonies continue to grow, we can then observe resistant colonies which are homogeneous for the resistant mitochondria (Birky, 1973). Strains 55R2, IL126 and D6-5 all underwent this intracellular selection and thus resulted in mitochondrial mutants.

For strain D22-5 this could be one of the reasons as to why no resistant colonies were observed. As the antibiotic does not induce mutagenesis, no resistant colonies might be observed if the population of D22-5 had no mutant mitochondrial DNA molecules to begin with. This combined with the fact that the strain might be not be as susceptible to mutations as other strains being used could have led to the observed result.

Allelism Tests between Mutants- Heteropolar Crosses

Heterpolar crosses display a distinctive polarity of recombination. Instead of producing equal frequencies of the parental types and the recombinant types, heteropolar crosses produce these in markedly unequal frequencies (Perlman and Birky, 1974). During this process, one of the two recombinant types is magnified while the other is repressed. When a heteroduplex is formed and the endonuclease cuts the ω- strand, polarized gene conversion can happen as a result of gap filling

and nick translation of the bases found on the ω- strand using the ω+ as a template (Wolf et al., 1974). This polarized gene conversion can either occur for the entire ω- strand or the ligase can seal the nick midway allowing for only a partial sequence to be converted as shown in Figure 11.

The knowledge of this phenomenon combined with the results of the allelism tests bring to light important conclusions regarding the mutations. Firstly, the presence of any sensitive progeny indicates the sensitive phenotype was in fact the major recombinant type. Since there are sensitive progeny present the mutations must be non-allelic to one another with the mutation on the ω+ mutant being farther away from the ω intron as compared to the mutation found on the ω- strain. Consider cross number 10, shown in Table VIII, where 55R2 (3) is the ω+ mutant and DPI-IB (2) is the ω- mutant. The cross resulted in 357 sensitive progeny, showing that the major recombinant type was in fact the sensitive phenotype. Allowing for selective gene conversion (polarized gene conversion) this cross then shows that not only are the two mutants non-allelic to one another but the mutation for DPI-IB (2) is closer to the ω intron than that for 55R2 (3). This is shown in Figure 11. It should also be noted that the parental type cannot be distinguished in this cross because its resulting phenotype as well as the genotype would be similar to the ω+ parental strain.

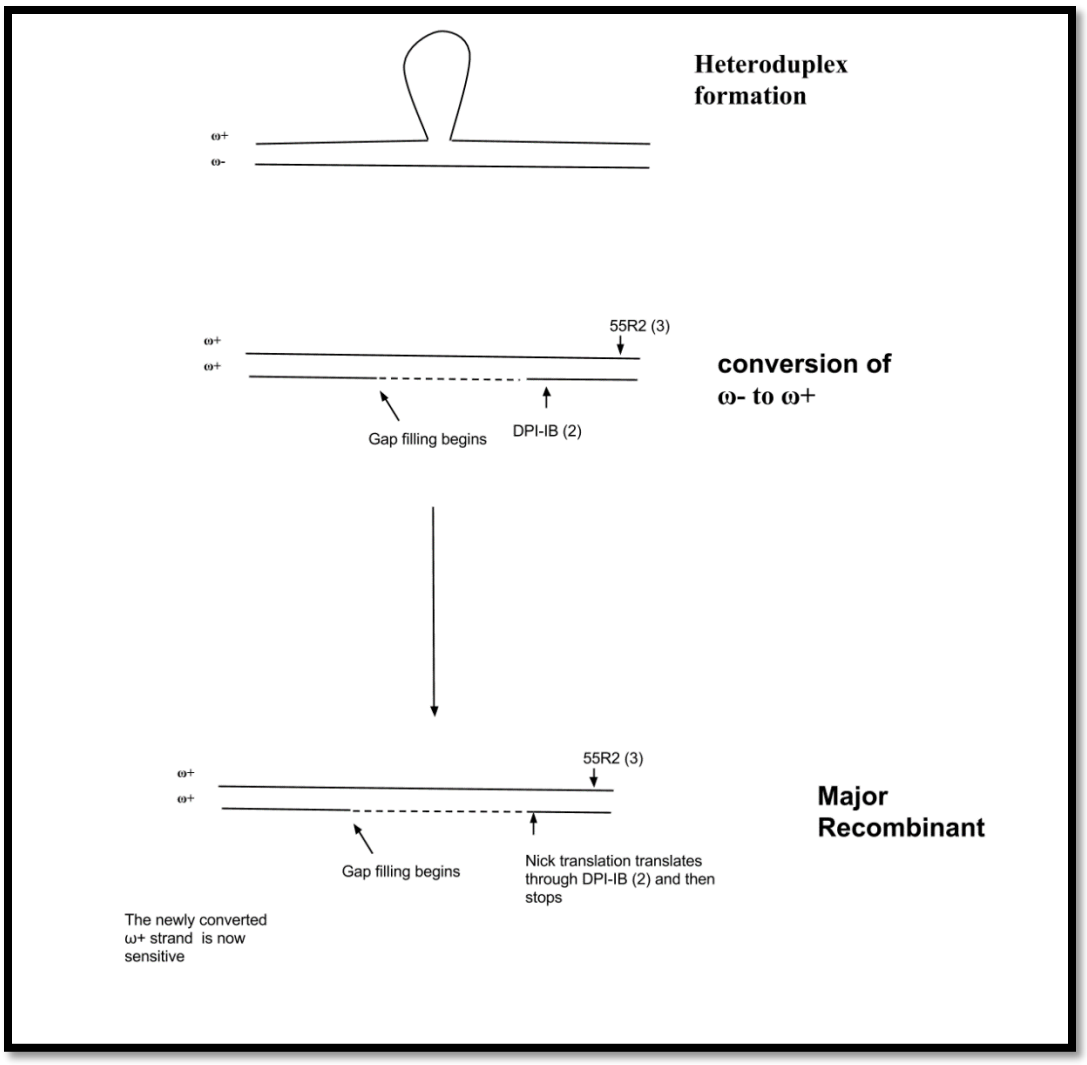

Figure 11: Proposed mechanism of recombination

Proposed mechanism of recombination for the cross between 55R2 (3) and DPI-IB (2). The major recombinant type is the sensitive phenotype which was observed and counted.

For all the other heteropolar crosses, the same mechanism can be used to account for the results. This brings forth the conclusion that all the 55R2 mutants are farther away from the ω intron than the mutants for the DPI-IB and the D6-5

strains. Furthermore all the DPI-IB mutants are non-allelic to the 55R2 mutants and all the D6-5 mutants are also non allelic to the 55R2 mutants. However since the major recombinant numbers are magnified due to the polarity of recombination it cannot be ascertained how far away these mutants are to one another, nor can it be determined whether different mutants from one strain are actually allelic or non-allelic.

Allelism Tests between Mutants – Homopolar Crosses

Mutants were also crossed with known homopolar *cap1* testers in order to calculate their recombination frequencies with respect to *cap1*. Unfortunately 55R2 (1) reverted to prototrophy, and as a result far fewer diploids could be scored in the random diploid analysis. However, since 55R2 (1) does show a significant number of sensitive progeny in this cross, it does appear to be non-allelic and was thus selected for sequencing. 55R2 (3) showed no sensitive progeny when crossed with the *cap1* tester, IL126. This indicated that the mutant was in fact allelic at *cap1*, and it was chosen for sequencing alongside the *cap1* tester to reconfirm the *cap1* sequence. Furthermore, the recombination frequency of 0 combined with the results of the allelism tests conducted between mutants allows for the conclusion that the mutations for the DPI-IB mutants and the D6-5 mutants are upstream from the *cap1* mutation.

According to studies done by Knight in 1980, three new loci of chloramphenicol resistance were discovered. These were *cap2, cap3* and *cap4* respectively.

Recombination tests between mutants at these loci and *cap1* mutants showed that the *cap4* locus was the only one closely linked to the *cap1* locus while the others were separated by the ω intron (Knight, 1980). However, after sequencing of the 21S gene it was discovered that the ω intron actually lies upstream of *cap1* as well. In light of this discovery and the genetic map constructed by Knight, as shown in Figure 12, it is expected that the mutants from DPI-IB and D6-5 might also be non-allelic at the loci *cap2*, *cap3* and *cap4*, since all of these new mutations lie upstream from 55R2 (3), which is allelic at *cap1*. However for the mutation 55R2 (1) it is possible that it might be allelic at any of these above mentioned loci as it is downstream to the mutations obtained from the other strains.

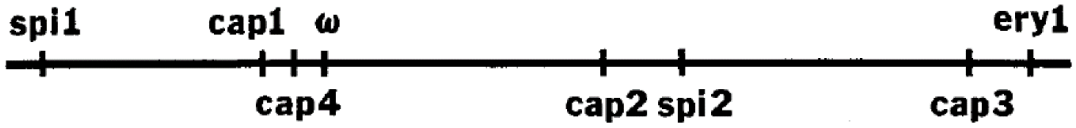

Figure 12: Fine-Structure genetic map of the chloramphenicol resistant loci. Fine-Structure genetic map of the chloramphenicol resistant loci where ω indicates the omega intron (Knight, 1980). The ω intron in actuality lies to the left of *cap1*.

Furthermore, according to the recombination frequency calculation for the cross between DPI-IB (2) and IL126 (*cap1* tester), the recombination frequency is

approximately 4.3%. This is very different from the recombination frequencies calculated for crosses between *cap1* and the discovered loci *cap2, cap3* and *cap4* as shown in Table XIII. This difference in recombination frequencies further indicates that the mutation DPI-IB (2) is non-allelic at *cap2, cap3* and *cap4.*

Table XIII: Recombination frequencies for crosses between cap1 and other loci. (Knight, 1980).

Recombination frequencies for all the crosses below are either significantly higher or lower than 4.3%, indicating the uniqueness of DPI-IB (2)

Cross	Recombination Frequency (%)
cap1 x cap2	13.2
cap1 x cap3	6.0
cap1 x cap4	0.38

Sequencing

The sequencing failed because of insufficient annealing of the primer to the sequence. The sequencing needs to be repeated again by using the reverse primers instead. It can be seen from the partial sequences in Figure 9 that the majority of the sequence is unknown. This has made it very hard to determine which part of the genome has been amplified. Repeating the sequencing with the respective reverse primers can help to determine that.

Importance of the Study

The mitochondrial genome of *S. cerevisiae* is comparable to genomes found in many other organisms, be they unicellular organisms like *E. coli* or multicellular organisms like mice. There is a high degree of homology between their genomes especially in the regions encoding for the large ribosomal RNA component of the ribosome (mitochondrial ribosomes in mammals).

When looking at the *E. coli* 23S gene (large ribosomal RNA gene) and comparing it to that of the 21S gene of *S. cerevisiae*, there are highly conserved sequences between the two genomes. Moreover, the two main chloramphenicol resistance mutational sites characterized in yeast, namely C^{R}_{321} and C^{R}_{323}, can be assigned to positions 2447 and 2503 respectively in *E. coli*, where homology allows for the two genes to be placed together from positions 2307-2621. Furthermore, these two sites in *E. coli* also correspond to the peptidyl transferase function of the gene further drawing parallels between the two organisms (Brosius et al., 1980). This then shows that any information regarding resistant mechanisms discovered in *S. cerevisiae* can be directly used to understand similar mechanisms in *E. coli.*

Additionally, similar homology can be observed between the 21S gene of *S. cerevisiae* and the 16S gene of mice. The 16S gene is the region encoding for the large mitochondrial ribosomal RNA in mice. When mitochondrial DNA from chloramphenicol resistant mouse cells was isolated and sequenced it was

discovered that this resistance was also a result of a single base pair change (an A-G transition) 243 nucleotides away from the 3' end of the coding region. This site of mutation lies in a 10 nucleotide region that is completely homologous to not only the *S. cerevisiae* genome but also the human genome. Additionally this mutation on the mouse genome is only one nucleotide away from the analogous mutation (C^{R}_{321}) on the *S. cerevisiae* genome (Blanc et al., 1981). This is not only suggestive of highly conserved genetic function but demonstrates how the finding of this study can directly impact how we understand antibiotic resistance in even mammalian organisms.

Possible Sources of Error

There are many possible sources of error in this study but most pertain to the counting of colonies during allelism tests. Most of the crosses with 5-1/6 led to the presence of three phenotypes, thereby making characterization difficult. It is possible that the colonies counted for other crosses might have yielded inaccurate results due to a partially resistant colony being counted as sensitive. There is a need to repeat these crosses and count a greater number of progeny in order to reconfirm these results. Secondly, the bands obtained after PCR were all seen near the 300 base pair ladder. Although these bands were thick and clearly visible the two sets of primers should have yielded bands of different lengths, with the mix containing reverse primer one yielding bands of approximately 220 base pairs and thus visible near the 200 base pair ladder while those containing reverse primer 2 resulting in bands near the 300 base pair ladder due to a sequence length of 349

base pairs. Lastly, candidates for sequencing were chosen after preliminary colony counting. This resulted in the selection of mutant 55R2 (1) due to a possibility of non allelism at *cap1*. However as the mutant later reverted to prototrophy, more colonies could not be counted yet DNA extraction had already been carried out. There is hope that DNA sequencing would reveal this mutant to be non-allelic, due to a high recombination frequency observed.

Conclusions

- The mutant 55R2-3 is allelic at *cap1* due to the lack of sensitive recombinants observed between the mutant and the *cap1* tester.
- The 55R2 mutants are farther away from the ω intron as compared to the mutants obtain from DPI-IB and D6-5. This can be seen via the sensitive recombinants observed due to it being the major recombinant type.
- Further evidence to support the conclusion is the lack of any observed recombination frequencies similar to those observed for the downstream *cap2, cap3* and *cap4* loci.
- The recombination frequency of DPI-IB (2) mutant with the *cap1* locus is 4.3 %. This indicates in light of the above conclusions that the mutation lies about 4.3 units upstream from the *cap1* locus.

Future directions

There are a variety of directions this research can go in the future. Firstly, there is a need to sequence the mutants obtained in this study using the reverse primer. It

has already been suggested by this study that mutants obtained from strains DPI-IB and D6-5 are non- allelic and lie closer to the ω intron than those obtained from the strain 55R2 and thus should be confirmed by sequencing. It is expected that the mutations from all these strains should lie relatively close to each other; therefore, another direction this study can take is the isolation of mutants in strains that are homopolar to the existing set of mutations. Calculation of recombination frequencies for such crosses would then allow for the construction of a fine-structure genetic map as well as serve to identify more mutants as good candidates for sequencing. Lastly, manganese mutagenesis can be used to induce mutations in various strains that would allow for resistance to chloramphenicol. Manganese induces these mutations in the mitochondrial genome of yeast by interacting with the mitochondrial DNA polymerase (Putrament et al., 1973). These mutants can then be characterized and sequenced and then compared with the sequence already available. It is possible that mutation clusters discovered via this method might be different from those that have been isolated via spontaneous mutagenesis. This might then explain why none of the mutants obtained in this study seem to be allelic at *cap2, cap3* and *cap4*, since those loci were discovered via manganese mutagenesis. In conclusion, this study has a lot of potential and would be valuable for the further understanding of how chloramphenicol binds to mitochondrial ribosomes. Any such insights might also aid our understanding of the binding of this drug to ribosomes in organisms like *E. coli*, mice and other mammals.

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