CHARACTERIZATION OF NEW MUTANTS IN THE 16S RIBOSOMAL SUBUNIT REGION OF THE MITOCHONDRIAL GENOME OF SACCHAROMYCES CEREVISIAE.

by

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This paper was prepared
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To:

My mother and father who trust and support me in all I do. Thanks.

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ABSTRACT

In *Saccharomyces cerevisiae*, the ribosomal region of the mitochondrial DNA codes for a 16S rRNA and a 21S rRNA, the rRNA components of the small and large subunits of the mitochondrial ribosomes. However, all mitochondrial ribosomal proteins, with one exception are encoded by nuclear DNA. Hence, the assembly of mitochondrial ribosomes raises interesting questions regarding the interactions between the nuclear and mitochondrial gene products. Paromomycin resistance in *S. cerevisiae* may result from mutations at the *par 1* locus. In this study, paromomycin resistant (P^R) mutants were generated using manganese mutagenesis and the mitochondrial mutants were tested for allelism. Three additional new P^R strains were constructed from the manganese mutants. Interestingly, 58% of our mutants were nuclear suggesting paromomycin resistance can be influenced by nuclear mutations in presumed mitochondrial ribosomal protein genes. Allelism tests of the mitochondrial mutants indicate some of our mutants are non-allelic. In this study, there was a lack of strong P^R mutants in contrast to those usually generated in the 21S rRNA. This suggests that the fidelity of the rRNA gene may not be as important for antibiotic binding in the 16S rRNA in comparison to the 21S rRNA. This hypothesis is supported by studies in the homologous *E.coli* gene where disruptions in the secondary structure of the 16S rRNA have been shown to affect paromomycin binding.

The characterization of new paromomycin resistant mutations in the 16S rRNA gene will help in studies of new mutations affecting the binding of antibiotics to the mitochondrial ribosome. Further genetic studies in conjunction with new biochemical methods for characterizing the rRNA molecule, will help to expand our current knowledge on the relationship between gene sequence, organelle function, protein synthesis and antibiotic binding.

INRODUCTION

S*accharomyces cerevisiae,* commonly known as baker's yeast, is a model eukaryote for genetic analysis as it is a simple unicellular organism that can be easily manipulated and controlled under laboratory conditions. Some of the characteristics that make *S. cerevisiae* an ideal organism for biochemical studies are rapid growth, dispersed cells, non-pathogenicity, ease of mutant isolation and a well-defined genetic system with numerous viable molecular markers (Sherman, 1998). *S. cerevisiae* has both a stable haploid and diploid state. Therefore, mutations can be isolated and expressed in haploid strains, and complementation tests can be carried out in the diploid strain. *S. cerevisiae* is also a facultative anaerobe as it can respire via aerobic respiration using a non-fermentable carbon source such as glycerol or by fermentation using a fermentable carbon source such as glucose. When grown on a fermentable carbon source such as glucose, *S. cerevisiae* cells use the fermentation pathway exclusively, and under these conditions, protein synthesis is inhibited in the mitochondria at the transcriptional level. This ability is not possible in most eukaryotes. Therefore, *S. cerevisiae* is ideal for studies of mitochondrial genetics and function because mutations affecting mitochondrial function are not lethal to the cells, so that strains with or without functional mitochondria can be maintained and studied in the laboratory.

Yeast Life Cycle

S. cerevisiae can reproduce both sexually and asexually and yeast strains are stable in both the haploid and diploid states. Sexual reproduction of two haploids to form a diploid cell can be easily carried out. Haploids exist as either a or α mating types and two different mating types must be brought together in order for sexual reproduction to occur. The mating haploid cells fuse to form diploid zygotes which subsequently grow by asexual reproduction. The diploid cells can be induced to undergo meiosis and sporulation under conditions of nitrogen starvation producing haploid spores from the diploid cells. During sporulation, the diploid cells undergo meiosis yielding four haploid daughter cells encapsulated as spores within a sac-like structure called an ascus. The a and α mating types are under the control of a pair of *MATa/MAT*α heterozygous alleles, therefore each ascus contains two *MATa* and two *MAT*α haploid daughter cells. The ascus walls can be ruptured with appropriate treatment releasing the spores which can then be separated and grown as separate a or α haploid colonies. Figure 1 illustrates the complete yeast life cycle.

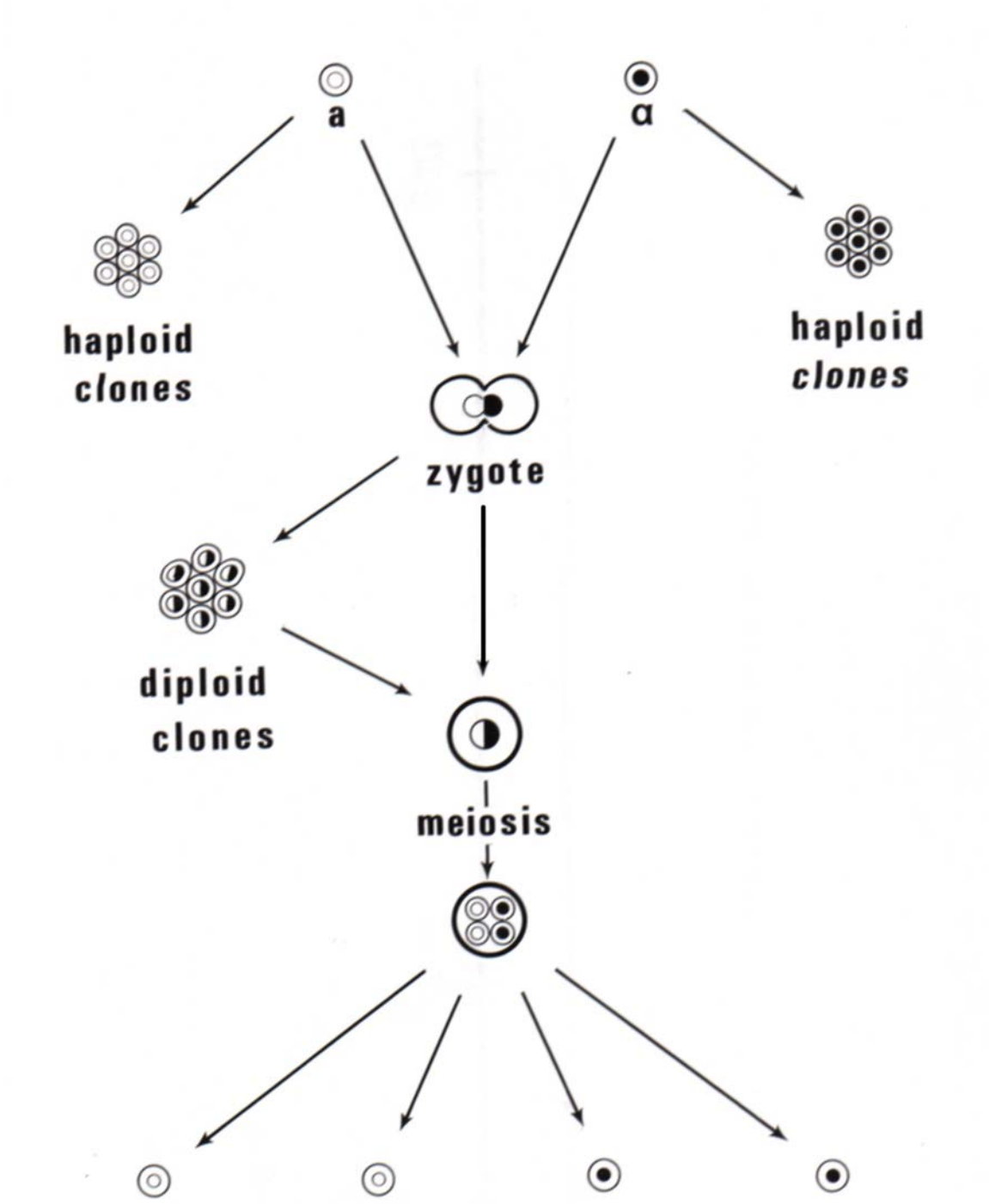


Figure 1: The Life Cycle of *Saccharomyces cerevisiae* (Knight, 1977)

Mitochondria

Mitochondria are complex organelles with a double membrane containing their own genome and protein synthesis machinery. Mitochondria arise only by growth and division of pre-existing mitochondria, and the mitochondrial genome is separate from the nuclear genome at all stages of the life cycle. The assembly of the mitochondria involves complex interactions between the mitochondrial and nuclear genetic systems. The mitochondrial genome encodes for a small number of proteins that are translated on mitochondrial ribosomes. Most of these proteins are hydrophobic components of the respiratory chain localized in the inner mitochondrial membrane. The nuclear-encoded protein components are translated on cytoplasmic ribosomes and then imported into mitochondria.

The principal function of the mitochondria is to produce energy for cellular processes in the form of ATP through aerobic respiration. This is done via a variety of complex pathways involving the citric acid cycle, the electron transport chain and oxidative phosphorylation. Mitochondria are also involved in the biosynthesis of cellular metabolites including some amino acids and lipids (Attardi and Schatz, 1988). In a set of reactions, pyruvic acid produces hydrogen atoms which are transported via coenzymes to the cristae where they donate electrons to the electron transport chain. The electron transport chain passes the electrons through a series of electron carriers. The final electron acceptor is oxygen and as the electrons flow through carriers down

the chain, protons are pumped into the intermembrane space of the mitochondria. The protons are then pumped back to the matrix via ATPase which is embedded in the inner membrane, in a process that is accompanied by the formation of one ATP molecule. The complex enzyme systems involved with the electron transport chain and oxidative phosphorylation are composed of polypeptide subunits, some of which are dually encoded by the nuclear genome and others by mitochondrial DNA.

Nuclear vs. Mitochondrial Inheritance

Mitochondrial inheritance does not obey the classical rules of Mendelian genetics. Therefore it is possible to distinguish a nuclear mutation from a mitochondrial one by observing the patterns of inheritance in progeny. Since nuclear genes are inherited in a classical Mendelian fashion, crossing a strain with a nuclear dominant allele to one with a recessive allele results in heterozygous progeny displaying the dominant phenotype.

Mitochondrial alleles continuously segregate during the mitotic cell divisions following zygote formation and this phenomenon is known as vegetative segregation. When two haploids, each with different mitochondrial DNA mate, their mitochondria fuse to allow recombination between the parental mitochondrial DNAs (Callen, 1974). The diploids are then described as heteroplasmic with two different types of mitochondrial DNA. Asexual reproduction by budding then occurs and within 20-25 generations, the

progeny will become homoplasmic, containing only one type of the parental mitochondrial DNA (Birky, 1978). Sporulation of a diploid clone thus results in segregation of the mitochondrial alleles in a 4:0 or 0:4 ratio among the haploid progeny. Therefore, all *S. cerevisiae* cells are homoplasmic with respect to their mitochondrial DNA as the heteroplasmic colonies create homoplasmic cells owing to vegetative segregation. One hypothesis suggested for the phenomenon of vegetative segregation is that the first bud off the zygote occurs at one end of the cell before the completion of cytoplasmic mixing which causes the diploid zygote to be uniformly heteroplasmic. Such a clone would already be mostly pure for one mitochondrial type and subsequent budding would quickly result in a homoplasmic cell (Strausberg and Perlman, 1978).

Yeast Mitochondrial Mutants:

Mit⁻ mutants

Mit⁻ mutant are respiratory incompetent mutants that arise due to the loss of function of one or more of the major mitochondrial enzyme complexes. They result from point mutations in the mitochondrial structural genes resulting in non-functional products of cytochrome oxidase, coenzyme QHz-cytochrome c reductase and oligomycin sensitive ATPase (Tzagoloff et. al., 1976, Borst and Grivell, 1978). *Mit ⁻* mutants have functional mitochondrial protein synthesis although they are respiratory deficient.

Syn- mutants

Syn- mutants are conditional mutants that arise from small or point mutation in the syn genes (Tzagoloff et al., 1976). The *syn* genes code for the small and large RNA subunits of mitochondrial ribosomes and transfer RNAs (Reijnders et. al, 1972, Casey et. al, 1974). Since these genes are necessary for functional mitochondrial protein synthesis machinery, *syn*- mutants are deficient for mitochondrial protein synthesis.

Petite mutants

Petite mutants are respiratory deficient mutants resulting from large deletions in the mitochondrial DNA. Rho minus (rho-) *petites* usually have more than fifty percent of the genome deleted and rho zero (rho^0) *petites* completely lack detectable mitochondrial DNA. Yeast rho^0 strains are viable on fermentable carbon sources since the mitochondrial DNA is not required for important cellular functions other than respiration. *Petite* mutants are useful in physical mapping of regions of the mitochondrial genome via deletion mapping. In this process, a collection of stable *petite* mutants are generated from the wildtype which consists of various retained or deleted combinations of the gene segment to be mapped. The mtDNA of each *petite* is then analyzed to determine the size and fraction of the rho^+ mtDNA retained. The approximate physical location of the mtDNA segments left in is then determined by measuring the overlap between different *petites* and by

comparison of the loci that are retained or lost from the *petites* (Nagley et. al, 1976). A locus retained in common between two or more *petites* is taken to lie within the retained mtDNA segment in question while a locus that is not present in a *petite* is taken to map outside of the entire genome segment retained by the *petite*.

Yeast Mitochondria

The mitochondrial genome of *Saccharomyces cerevisiae* is a circular DNA molecule 75 to 80kb in size with a molecular weight of about 5.0 $x10^7$ daltons (Fox et al., 1991). Mitochondria arise only by growth and division of preexisting mitochondria, and the mitochondrial genome is separate from the nuclear genome at all stages of the life cycle. It encodes eight proteins, a set of tRNAs, an RNA component of an RNase P-like processing enzyme involved in tRNA processing and a 16S rRNA and a 21S rRNA, the rRNA components of the small and large subunits of the mitochondrial ribosomes. Seven of the proteins coded by the mtDNA are subunits of respiratory chain enzyme complexes: cytochrome c oxidase subunits I, II and III, apo-cytochrome *b*, and subunits 6, 8 and 9 of the F_0 component of the mitochondrial ATPase. However, mitochondria are now completely genetically dependent on nuclear genome expression for maintenance of their function, as most mitochondrial proteins are encoded by nuclear DNA. The only other major protein coded by the mtDNA is VAR1, a component of the mitochondrial ribosomal small unit

essential for protein synthesis (Trepstra et al., 1979; Costanzo and Fox, 1991). Mitochondrial encoded proteins represent only about 5% of the total proteins found in mitochondria. The remaining components of the respiratory chain enzyme complexes as well as all other mitochondrial ribosomal proteins are the products of nuclear genes that are synthesized in the cytoplasm and transported to the mitochondria (Strausberg et al., 1978). This peculiar situation where a hundred or so nuclear genes are required to allow the expression of seven mitochondrial proteins suggests that nuclear and mitochondrial gene expression must be coordinated at some level to achieve normal balance of mitochondrial proteins (Costanzo and Fox, 1991).

Ribosomes

The ribosome is the site of protein synthesis in the cell. In eukaryotes, there are two kinds of ribosomes; the free ribosomes and the membrane bound ribosomes. The membrane bound ribosomes synthesize products for destinations outside the cytosol, and are attached to the endoplasmic reticulum to produce polypeptide chains that protrude towards the lumen. The free ribosomes include the cytoplasmic and the mitochondrial ribosomes. The cytoplasmic ribosomes synthesize protein for use in the cytoplasm and the mitochondrial ones synthesize proteins destined for the inner mitochondrial membrane (those involved in the electron transport chain and oxidative

phosphorylation). The mitochondrial ribosomes are found within the mitochondria and are quite different from other ribosomes.

Ribosomes consist of two subunits that work together as one unit to translate mRNA into a polypeptide chain during protein synthesis. Each subunit consists of one or two rRNAs and several smaller protein molecules.

Ribosomal subunits of prokaryotes and eukaryotes are similar. The size of ribosome particles is measured in Sveberg units (S), which is a measure of the sedimentation rate of a particle in a centrifuge. Prokaryotes have 70S ribosomes consisting of a small 30S subunit and a large 50S subunit while eukaryotes have 80S ribosomes consisting of a 40S small subunit and a 60S large subunit. The ribosomes found in both chloroplasts and mitochondria of eukaryotes more closely resemble that of prokaryotes with a sedimentation size of 70S, which is cited as evidence supporting the endosymbiotic theory of evolution. The difference between the prokaryotic and eukaryotic ribosomes is very important, as it is exploited by pharmaceuticals in creating drugs that target the prokaryotic 70S ribosome, but do not affect the eukaryotic 80S ribosome and therefore can target bacteria without affecting the mammalian host cells.

Mitochondrial Ribosomes

Mitochondrial ribosomes differ from cytoplasmic ribosomes in their nucleotide composition, the electrophoretic mobility of some of their

respective ribosomal proteins and their denaturation profiles (Morimoto and Halvorson, 1971). The similarities between the mitochondrial and prokaryotic ribosome can be seen in comparisons between the bacterium *Escherichia coli* and *S. cerevisiae.* In *E. coli*, the large subunit has a size of 50S and contains 5S and 23 S rRNA while the small subunit has an overall size of 30S and contains one 16S rRNA. In *S. cerevisiae*, the large mitochondrial ribosomal subunit has an overall size of 54S and contains one 21S rRNA while the small subunit contains the 16S rRNA and has an overall size of 38S. Mitochondrial ribosomes also share similar antibiotic sensitivity with prokaryotes. For example, cycloheximide is an antibiotic that has no effect on the function of the mitochondrial ribosome but, inhibits protein synthesis on the eukaryotic cytoplasmic ribosome. Similarly, protein synthesis in mitochondrial ribosomes as well as prokaryotic ribosomes is inhibited by the same drugs such as chloramphenicol, erythromycin, paromomycin and tetracycline.

Mitochondrial ribosomes are associated with the inner mitochondrial membrane. This is thought to be due to the hydrophobic nature of the membrane proteins that form part of the electron transport chain and protein synthesis elsewhere would require a lot of energy to transport the proteins across the aqueous mitochondrial matrix.

Var 1

The *var 1* gene encodes the only mitochondrial ribosomal protein known to be encoded by mitochondrial DNA. (Butow, et al., 1985). VAR 1 is a hydrophilic, basic protein which is unlike other mitochondrial proteins that are highly hydrophobic. Studies reveal that the VAR 1 polypeptide is first assembled into a small RNAse - sensitive particle which is a precursor in the formation of the 38S small ribosomal subunit. Trepstra et al. (1979), found VAR 1 to be present in roughly equal amounts as other proteins of the 38S subunit, and they suggest it has a role as an internal ribosomal protein, since it was difficult to remove by a high saline based wash. Erythromycin inhibition of protein synthesis also revealed that the small ribosomal subunit could not be assembled without the presence of the VAR 1 polypeptide. The production of VAR 1 raises interesting questions concerning its function and continued production as the only mitochondrial encoded ribosomal protein.

Antibiotic Resistance and the Ribosomal Region

Mitochondria are sensitive to antibiotics acting as inhibitors of mitochondrial biogenesis. These fall into two categories: those that prevent the cells undergoing aerobic respiration by interfering with the mitochondrial gene products that are part of the enzyme complexes of the electron transport chain and oxidative phosphorylation, and those that interfere with mitochondrial protein synthesis. Oligomycin affects the function of the ATP

synthetase of the electron transport chain while chloramphenicol, erythromycin and paromomycin interfere with protein synthesis. These last three antibiotics, all prevent protein synthesis by interacting with the rRNA regions of the mitochondrial DNA.

In an antibiotic sensitive cell, the antibiotic is able to bind to the surface of the mitochondrial ribosome and interfere with protein synthesis. A mutation in the mitochondrial rRNA could change the surface of the ribosome such that antibiotic binding is impossible (Spithill et al., 1978). Mutations resulting in resistance to drugs interfering with protein synthesis have been shown to be cytoplasmically inherited. The first antibiotics to be studied were chloramphenicol and erythromycin, which were shown to bind to bacterial ribosomes, inhibiting protein synthesis. In yeast, chloramphenicol and erythromycin were found to bind to the mitochondrial ribosome inhibiting mitochondrial protein synthesis without affecting the cytoplasmic ribosomes.

The ribosomal or 'R' region of the mtDNA was first characterized by Netter et al. in 1974. They identified genes that code for the large ribosomal RNA (21S gene) and the small ribosomal RNA (16S gene) which are separated by a distance of approximately 30 kilobases. Several antibiotic resistance loci have been mapped to the R region. See Figure 2 for a physical map of the mtDNA of *S. cerevisiae.*

The 21S rRNA gene of the R region has particularly been extensively studied. The loci conferring antibiotic resistance to chloramphenicol and

erythromycin have been mapped to this gene. Mutations that map to this region and confer antibiotic resistance presumably alter the RNA transcript in such a manner that modifies the resulting three dimensional structure of the ribosome so that the antibiotic can no longer properly bind to the ribosomal – antibiotic binding site (Grivell et al., 1973). Mutations conferring resistance to chloramphenicol and erythromycin map to the *cap1* and *ery1* loci in this region 95% of the time. The mutations conferring antibiotic resistance can occur at multiple sites on the gene which are separable by recombination. Other mutable sites mapped within the 21S gene include *cap2*, *cap3* and *cap4* (Knight, 1980) and *ery2* (Knight et. al, 1982).

Mutations mapping to the *spi1* locus can confer resistance to both spiramycin and erythromycin and different mutations that map to the *ery1* site may either erythromycin or chloramphenicol resistance. Figure 3 depicts the fine structure map of the 21S gene.

Antibiotic resistant phenotypes can also result from mutations in nuclear genes. According to Waxman et al., (1979), nuclear antibiotic resistance mutations can be grouped into two: permeability mutants in which the mitochondrial membrane is altered to partially or completely block the route of the drug and mutations in genes specifying mitochondrial proteins. Permeability mutations usually confer a low level of drug resistance and usually result in cross resistance to other drugs (Rank et al., 1975). It is also possible for nuclear mutations to suppress the mitochondrially determined

antibiotic resistant phenotype due to changes in a ribosomal protein. Three such genes: *mcr1, mcr2* and *mcr3* (modified chloramphenicol resistance) were identified by Waxman et al., (1979). These nuclear mutants were able to suppress expression of the antibiotic resistance phenotype conferred by mitochondrial mutations.

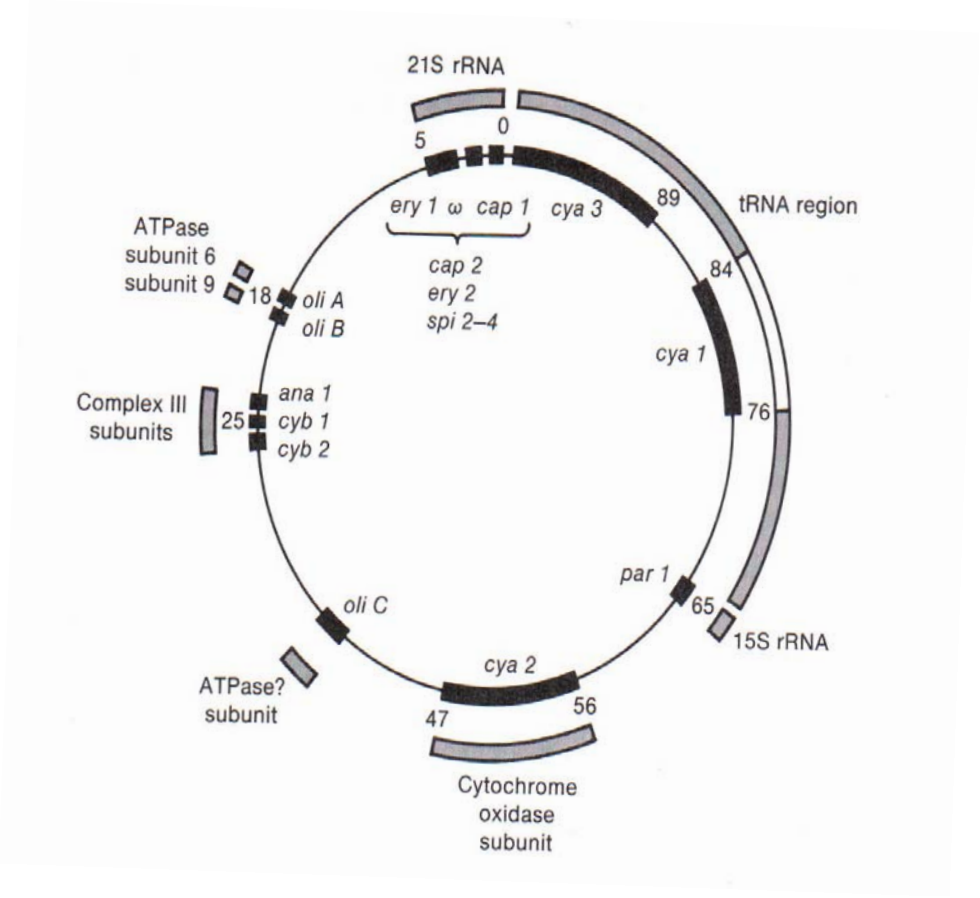


Figure 2: Genetic map of the mitochondrial genome of *Saccharomyces cerevisiae* showing mutant loci (black) and gene functions (grey). Mutants are labeled inside the circle (Adapted from Linnane, A., and Nagley, P., 1978).

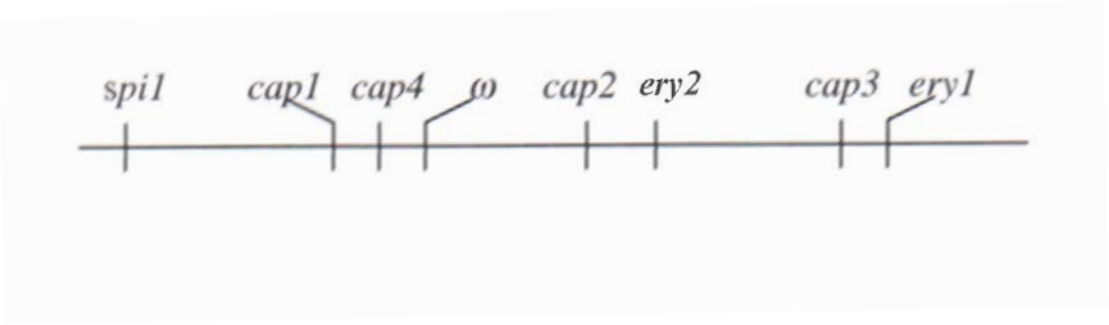


Figure 3: Fine structure map of 21S rRNA gene showing position of different mutable sites conferring antibiotic resistance. *Cap1, cap2, cap3* and *cap4* confer resistance to chloramphenicol with resistance mapping to *cap1* 95% of the time. *Ery1* and *ery2* confer erythromycin resistance and spiramycin resistance has been mapped to *spi1* (Knight, 1980).

The 16S rRNA gene

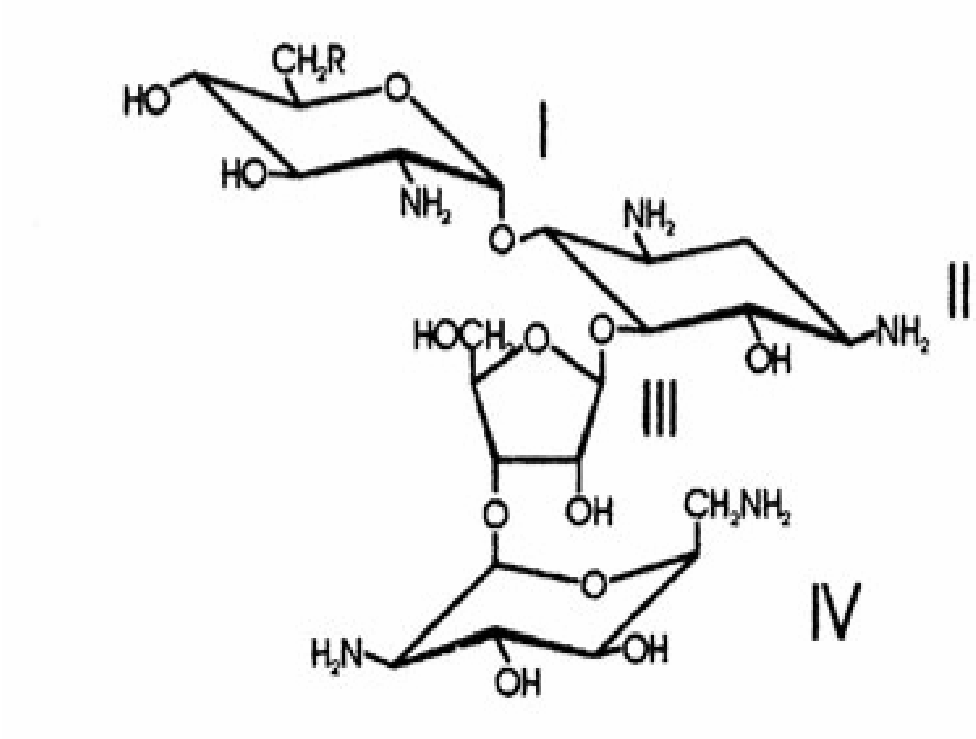
In the yeast system, Wolf et al. (1973) reported a paromomycin resistant mutant whose mutation mapped to the 16S rRNA gene, and comparative sequencing of the paromomycin resistant and sensitive strains of *S. cerevisiae* indicate that resistance to paromomycin arises from a point mutation in the 16S rRNA coding sequence described as *par1* (Li et al., 1982). This strongly suggests that the *par* locus is a true genetic marker of the 16S rRNA gene in *S. cerevisiae.*

Paromomycin

Paromomycin is a fermentation product of *Streptomyces rimosus* that belongs to the aminoglycoside group of antibiotics that also includes streptomycin, neomycin, kanamycin and gentamycin. Figure 4 shows the chemical structure of paromomycin. The antibacterial action of paromomycin relies on the binding of paromomycin to subunits of ribosomes. Aminoglycosides bind to RNA, their positive charge being attracted to the negatively charged RNA backbone (Schroeder et al., 2000). Aminoglycosides bind to the 30S subunit causing misreading of the genetic code and thereby inhibiting translation (Cundliffe, 1981). Aminoglycosides are excellent tools for analysis of RNA function as well as for therapeutic studies targeting the RNA. In prokaryotes they inhibit several catalytic RNAs such as RNase P,

self-splicing introns and small ribozymes *in vitro* and interfere with HIV replication by disrupting RNA-protein contacts. The mechanism of aminoglycoside action is not yet well understood, as their target site is usually rRNA. The complexity of the ribosome has made high resolution structure determination challenging (Schroeder et al., 2000).

Studies of antibiotic resistance in *E. coli* may contribute some insight into the occurrence of antibiotic resistance in *S. cerevisiae*. In *E. coli*, paromomycin binds specifically to the RNA oligonuceotide at the 30S A site, and studies using heteronuclear NMR found paromomycin to directly bind to the A-site of the 16S r RNA, resulting in a conformational change of the structure of the region (Fourmy et al., 1996). See Figure 5 for NMR structure of RNA binding site complexes with paromomycin in *E. coli.*



R= OH

Figure 4: Chemical structure of paromomycin (Schroeder et al., 2000).

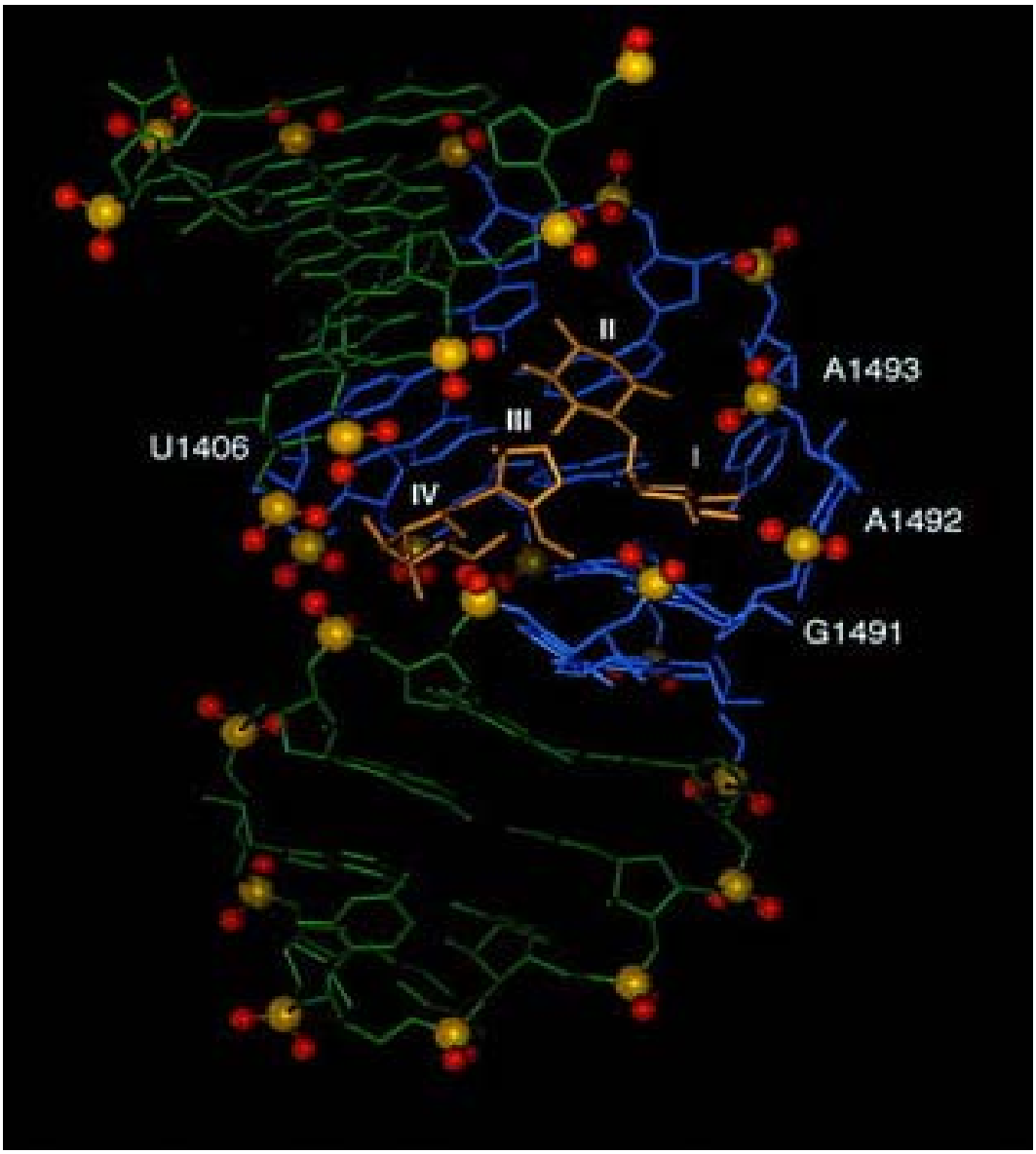


Figure 5: Single representative NMR structure of the decoding site of the 16S rRNA from *E.coli*. The A-site RNA complexed with paromomycin (Fourmy et al., 1996, 1998b)

Mutageneses

Mitochondrial mutations can be generated through a variety of techniques. The most commonly used method of generating antibiotic resistance mutants is manganese mutagenesis (Putrament et al., 1973; Knight, 1980). DNA polymerases require a divalent cation activity for proper function and Mg^{2+} is considered the physiological activator of the enzyme. The enzyme has a highly specific base-pairing mechanism accompanied by a 3'to5' proofreading exonuclease function. Studies in E.coli by El-Dairy et. al (1984), show that substitution of Mg^{2+} with Mn^{2+} causes an increased frequency of error by increasing incorrect base-pair pairing during DNA replication and also decreasing the 3'to5' proofreading exonuclease function. Manganese mutagenesis is specifically favorable for mitochondrial mutants, as Mn^{2+} primarily interacts with mtDNA polymerase (Putrament et al., 1973), making it specific for induction of mitochondrial mutants.

Ethidium bromide mutagenesis can also be used to generate mitochondrial mutants. Ethidium bromide is used to generate rho^- mutants lacking functional mitochondrial DNA. Ethidium Bromide intercalates into the mtDNA causing fragmentation via removal of free nucleotides (Dujon, 1981).

Another mutagen is ethyl methane sulfanate (EMS), which is used to generate nuclear mutants without affecting the mitochondrial genome.

Mutations in yeast are also commonly generated spontaneously. Spontaneous mutations result from endogenous DNA lesions and have a strong selection bias in the site they affect and the type of mutations they cause. Their site specificity provides some insight into the areas of the DNA sequence that are most prone to mutagenesis (Maki, 2002). Studying spontaneous mutations that inhibit antibiotic binding on the nuclear ribosome can provide some insight into the areas that are most important for antibiotic targeting and function.

Genetic Mapping

Recombination frequencies are used to map the distance between a new mutant and a previously known locus on a gene. Two mutations are considered allelic when no recombination is observed.

A useful alternative approach to genetic mapping of the drug resistance loci in yeast has been the analysis of the frequencies of retention or deletion of markers in *petite* mutants derived from the wildtype strain (Nagley et.al, 1976). *Petite*s can also be used in conjunction with restriction–enzyme analysis to for genetic mapping. The mtDNA is cut into fragments of various sizes which are then hybridized to cRNA. For example, if a *petite* with chloramphenicol resistant marker hybridizes with fragment A from a restriction enzyme digest and fragment B from another digest, the locus

conferring chloramphenicol resistance is located within the region of overlap of the two fragments.

The Phage Analogy Model

The maximum interchromosomal recombination frequency that can be observed is 50%. However, the maximum observable recombination frequency that can occur between mitochondrial alleles is only 20-25%. Dujon et al., (1974) proposed a model to explain this phenomenon, based on an analogy observed in the phage infected cell. The phage analogy model hypothesizes that within the zygote is a random pool of mitochondrial DNA contributed in roughly equal parts by both parents. In such a random mating pool of genetic information, some of the recombinations taking place will be homologous in nature while others will be heterologous. Homologous recombinant pairings would not be detectable thereby reducing the theoretical maximum recombination frequency of 50% by half making the observed limit of recombination frequency 25%.

Project Objectives

Investigations of new mutants affecting the binding sites of antibiotics such as chloramphenicol, erythromycin and paromomycin to the mitochondrial ribosome provide an insight to nucleo-mitochondrial interactions and will help to expand our current knowledge on the relationship

between mitochondrial gene sequence, organelle function, and antibiotic action on protein synthesis.

The objective of this study is first to attempt to generate new mutants that arise from the ribosomal region of the mitochondrial DNA of *S. cerevisiae* conferring resistance to the antibiotic paromomycin and, to characterize their allelic relationship with respect to the *par 1* locus described in the literature.

The second objective of this study is to generate a putative fine structure genetic map of the 16S gene similar to that derived for the 21S gene reviewed via recombination frequency analysis.

MATERIALS AND METHODS

Yeast Strains

A complete list of all the yeast strains used in this study along with their nuclear and mitochondrial markers can be found in Table 1. All strains were grown at 30^0C.

Media

The compositions of the media used in this study to grow yeast are listed below:

YD media: This is a complete medium containing glucose as the fermentable carbon source. It was used in maintenance of all the strains.

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

YG media: This is a complete medium containing glycerol as the non-fermentable carbon source that must be metabolized by the mitochondria. This medium was used to determine the respiratory capability of the strains.

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

YG + C media: This is the YG media supplemented with the antibiotic chloramphenicol. This is done after autoclaving the YG media so as not to inactivate the drug. This medium is used in identifying chloramphenicol resistant and sensitive strains. Chloramphenicol is added to the medium in the amount of 1.5g in 5ml of methanol to 0.5 liters of YG media just before pouring plates.

YG + E media: This is the YG media supplemented with the antibiotic erythromycin. This is done after autoclaving the YG media so as not to inactivate the drug. This medium was used in selecting erythromycin resistant and sensitive strains. The antibiotic is added to the medium in the amount of 0.5g of erythromycin in 5ml of methanol to 0.5 liters of YG media just before pouring plates.

YG + O media: This is the YG media supplemented with the antibiotic oligomycin. This is done after autoclaving the YG media so as not to inactivate the drug. This medium used in identification of oligomycin resistant and sensitive strains. The antibiotic is added to two liters of YG in the amount of 3mg of oligomycin in 8ml of methanol.

YG + P media: This is the YG media supplemented with the antibiotic paromomycin. This is done after autoclaving the YG media so as not

to inactivate the drug. This medium is used in selecting paromomycin resistant and sensitive strains. Three different concentrations of the antibiotic were dissolved in water and added to YG media just before pouring plates.

- 0.5g paromomycin in 5ml water added to 0.5 liters of YG
- 0.75g paromomycin in 5ml water added to 0.5 liters of YG
- 1.0g paromomycin in 5ml water added to 0.5 liters of YG

MD: This is a minimal medium prepared as described by Wickerham (1946). This medium contains glucose as a fermentable carbon source and is used for matings and random diploid analysis.

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

MD + amino acid: This is the MD plate supplemented with the appropriate amino acid to determine the auxotrophic requirement of the haploid strains. One-twentieth of a gram of amino acid was placed in 5ml of sterile water and 200 ug of the mixture was spread on each MD plate and allowed to dry.

YD + EB: This is the YD plate supplemented with ethidium bromide. 5mg/L of ethidium bromide is added to 0.1M sodium phosphate and added to YD just before pouring. Plates are stored in the dark.

Pre-sporulation media: This is a complete medium rich in glucose as a fermentable carbon. It is used to prepare diploids for sporulation.

8g/L yeast extract
3g/L bactopeptone
100g/L glucose
20g/L agar

Sporulation media: This is a complete medium containing sodium acetate and a small amount of glucose as a fermentable carbon source. It is used to induce sporulation in diploids.

10g/L sodium acetate
1g/L yeast extract
0.5g/L glucose
20g/L agar

Drop Testing

Drop tests were performed to check the auxotrophic requirements and antibiotic sensitivities of each strain used in the study. A heavy suspension of the haploid to be tested was prepared in 5ml of sterile water. A single drop of the suspension was then placed on MD plates, supplemented MD, and drug plates. After 48 hours, the colonies were scored as either antibiotic resistant or sensitive, and the auxotrophic requirements were noted.

Mating procedure

Using a sterile toothpick, parent haploids were picked and patched overnight on YD plates in preparation for mating. A heavy suspension of each parent haploid was made in 5ml water. A single drop of the suspension of each of the a and α haploid parent was placed together on an MD plate. As a control for prototrophic revertants, a single drop of each haploid parent was also placed on a separate location on the MD plate. The plates were then incubated at 30^{o}C for 48-72 hours and observed for growth of diploid progeny.

Sporulation

Sub-cloned diploid strains to be sporulated were grown overnight on YD media. Some of the cells were then transferred to pre-sporulation media for 48 hours and after that time, were transferred to sporulation media for 48-72 hours. After this, the efficiency of the sporulation was determined by looking for the presence of the asci under the microscope. After asci were identified, a heavy suspension of the sporulated cells was prepared in 5ml sterile water. 0.125ml of glusulase was added to the suspension and the mixture was incubated at 30^{0}C for one hour. The mixture was centrifuged at 8,000rpm for 10 minutes and the resulting pellet washed twice with 5ml of sterile water before finally being re-suspended in 5ml of sterile water. The suspension was sonicated at a microtip limit of 4 watts for 1 minute to release

the haploid spores. The suspension was diluted 2 fold and cross-streaked on several YD masters using a sterile loop. The plates were then incubated at 30^0C for a minimum of 72 hours after which presumptive haploid colonies were identified by morphology.

Haploid Characterization

The characterization of sporulated haploids involves various tests to determine the auxotrophic requirements of the strains, their mating types and confirmation of their mitochondrial genotype.

The auxotrophic requirements of the presumptive haploids are determined by patching them out onto a YD master plate, growing them overnight, and then replica-plating on to an MD plate(control) as well as onto MD plates supplemented with all possible combinations of growth factors required by the auxotrophic parents.

The mating types were determined by replica-plating the haploids onto mating lawns with tester strains of both the a and α mating types. The mating lawns were made by spreading a heavy suspension of the a and α tester strains on MD plates. The mating type could then be deduced by observing which matings occurred as that indicated the haploid in question was of the opposite mating type.

The mitochondrial antibiotic resistance genotype was determined by replica plating YD masters to YG, YG+C, YG+E, YG+ O and YG+P plates or, drop testing the haploids onto the mentioned drug plates.

All plates were incubated for 72 hours at 30^0C and scored for auxotrophic requirement, mating type and mitochondrial antibiotic resistance phenotype.

Ethidium Bromide Mutagenesis

Several strains were grown overnight on YD plates and light suspensions were prepared in 10mL sterile water. The cells were then cross-streaked on YG + EB plates and incubated in the dark for three days to allow for generation of rho^0 mutants.

To verify deletion of all mitochondrial DNA in rho^0 strains constructed, the rho^0 haploids are crossed to complementary tester strains. Such a cross is expected to yield progeny expressing the mitochondrial phenotype of the tester parent only, since the rho^0 strains lack mitochondrial DNA. Therefore, the mitochondrial genotype of progeny can be deduced as all respiratory competent progeny will contain the mtDNA of the other parent.

Manganese Mutagenesis

The strains described in Table 1 were patched overnight on YD media. The cells were then re-suspended in 50mL liquid YD and diluted to an optical

density (O.D.) of 0.1. The cells were allowed to grow at 30°C on a shaker for 4-5 hours to an O.D. of about 0.4 at which point they are in log phase. 1ml of 1M $MnCl_2$ and 0.5ml of 0.1M $MgCl_2$ were added to the cells. The magnesium is added with the manganese mutagen to decrease the amount of rho$^-$ *petites* and to overcome the growth inhibition of the manganese (Putrament, et al., 1973). After shaking for 5 more hours at 30°C, the cells were centrifuged. The pellet for each strain was washed twice with 20ml 0.1M $MgCl_2$ and re-suspended in 20ml sterile water. 100ul of the suspension was plated onto each of several YG + P plates using a spreader. The plates were incubated for 7-10 days after which paromomycin resistant mutants were picked and sub-cloned onto YD media. A single colony from each sub-clone was then picked and isolated for further analysis.

Random diploid analysis

A sample of the growing diploid cells was taken using an inoculating loop and suspended in 10ml water to create a "just perceptibly cloudy" suspension of cells. A loopful of the diploid suspension was then cross streaked across the entire surface of a MD plate to ensure complete and even distribution of cells across the plate. Each plate was expected to give rise to approximately 200 diploid colonies after 48-72 hours of incubation at 30°C. Therefore, for each diploid, a total of six MD plates were cross streaked to

give a predicted total of about 1200 diploid clones. At least 1000 colonies are needed to provide reliable data in recombination analysis and allelism tests.

The colonies on the master MD plates were imprinted onto a piece of sterile velvet and then replica-plated to plates of YG (control) and YG + appropriate drug in order to determine the antibiotic phenotype of the colonies. After 2-3 days, each colony is scored as antibiotic resistant or sensitive (Figure 6).

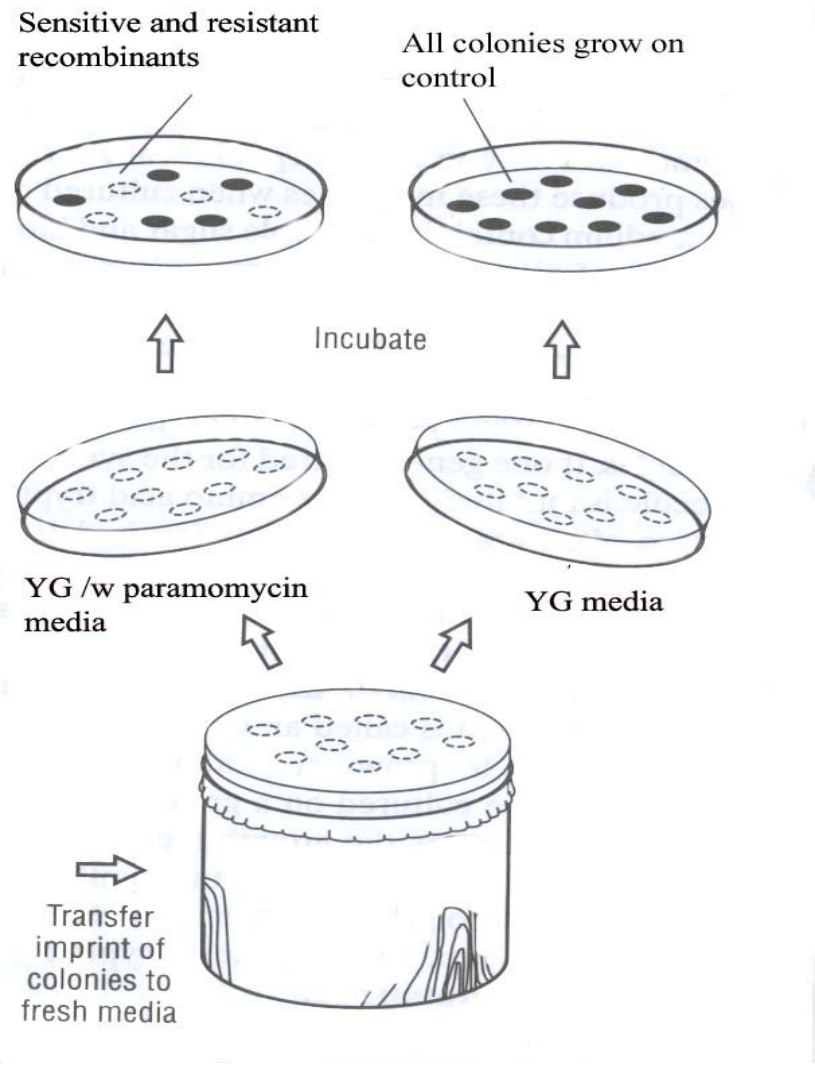


Figure 6: Random Diploid Analysis

RESULTS

Manganese Mutagenesis

Manganese mutagenesis was performed on the following strains after verifying they were paromomycin sensitive: 5-1/6, CCD6-1, D6-5, HAT, HB2, HBT, IL126-1B and MtS-14 (Table 1). After ten days, 24 P^R colonies each from the HAT, HB2 and IL126-1B strains were isolated and patched onto YD and sub-cloned for further studies. Fifteen colonies were also isolated from each of the following strains: 5-1/6, CCD6-1, D6-5, HBT, and MtS-14. The isolated colonies were sub-cloned onto YD media for further studies.

From the sub-clones, four HAT mutants were further characterized as twenty of the HAT P^R mutants were prototrophic revertants or did not mate well in subsequent crosses. None of the HB2 P^R mutants were characterized as all but one were prototrophic revertants. Twenty-two of the IL126-1B P^R mutants were further characterized. Tests for respiratory sufficiency in HBT P^R mutants did not yield distinctive P^R colonies so these were discontinued from further analysis. Four of the fifteen CCD6-1 P^R mutants were prototrophic revertants, and seven of the MtS-14 P^R mutants did not yield distinctive P^R colonies post sub-cloning.

In total, 147 mutants were isolated from mutagenesis and 77 of these were further characterized (Table 2).

Nuclear vs. Mitochondrial Inheritance

Initial crosses between complementary P^R mutants appeared to indicate that all the mutants were non-allelic. Also, many of the matings showed over 25% recombination. This suggested nuclear inheritance of some or all of the P^R mutants, as 25% is the highest percentage of recombination possible within the yeast mitochondrial genome. Therefore, the P^R mutants were tested for mode of inheritance. Fifteen 5-1/6 P^R mutants were tested by crossing them to a HBT tester for determination of the mode of inheritance of the P^R marker. Thirteen of the fifteen mutants were found to arise from mitochondrial mutations (Table 3). However, the 5-1/6 strain did not give clear paromomycin resistance results therefore not used for further analysis.

Eleven CCD6-1P^R mutants were tested for mode of inheritance by crossing with a 55R2 tester. Six of the eleven CCD6-1 P^R mutants being characterized were found to arise from mitochondrial mutations (Table 4). The fifteen isolated D6-5 P^R mutants were crossed with tester HBT and fourteen were found to result from nuclear mutations, and only one from a mitochondrial mutation (Table 5). The four HAT P^R mutants isolated were also crossed to the HBT strain and all four were found to be mitochondrial in nature (Table 6). Twenty-two IL126-1B P^R mutants were crossed with tester 55R2 to determine mode of inheritance. Twenty of these were found to arise from nuclear mutations and two from mitochondrial mutations (Table 7). Ten

of the P^R mutants obtained from the MtS-14 strain were tested for mode of inheritance by crossing to a HBT tester strain. Six of these were found to be mitochondrial in nature, three nuclear recessive and one was found to be nuclear dominant in nature (Table 8). The mitochondrial mutants arising from the MtS-14 strain did not exhibit strong resistance to paromomycin. They therefore were not further analyzed.

A total of thirteen mitochondrial P^R mutants were identified from the 77 P^R mutants isolated and characterized from manganese mutagenesis (Table 9). Six of these were selected for further study.

Allelism Tests of P^R mutants

In the absence of a *par1* tester in the lab, crosses were performed between the four P^R HAT strains and the two P^R IL126-1B strains that were verified to be mitochondrial in nature. In the first round of testing, the P^R mutants were found to be non-allelic. Crosses between P^R HAT mutants and the P^R 5 IL126-1B mutant resulted in recombination frequencies of about 2% suggesting the HAT P^R 1, HAT P^R 3 and HAT P^R 5 mutants are allelic (Table 10).

Crosses between P^R HAT mutants and the P^R 6 IL126-1B mutant resulted in recombination frequencies of between 0.6%-14.5%. Due to the lack of additional P^R mutants of complementary mating types it was not possible to further extensively test the observed recombination results.

The crosses between the isolated and characterized P^R HAT mutants and P^R 6 IL126-1B mutants were repeated at a later time. At this time, the P^R HAT mutant # 4 had reverted to its prototrophic form. In this second round of allelism tests, crosses between P^R HAT mutants and the P^R 5 IL126-1B mutant, mainly resulted in recombination frequencies of about 0% (Table 11). Crosses between the P^R HAT mutants and the P^R 6 IL126-1B mutant resulted in recombination frequencies between 0- 2 %.

Construction of New Strains

In an attempt to enlarge the number of strains available for the allelism tests, strains of complementary mating types to the viable P^R HAT and P^R IL126-1B mutants were created using rho^0 strains created via ethidium bromide mutagenesis. The respiratory deficient strain 55R2ρ^0 was crossed with the P^R 5 IL126-1B mutant to generate a P^R mutants that would be complementary to the α P^R 5 IL126-1B mutants. The D6-5 ρ^0 strain was crossed with the P^R HAT mutants to generate α mutants that would be complementary to the a P^R HAT mutants previously generated via manganese mutagenesis. The new strains were characterized and different a strains with were isolated and used for further analysis. The constructed strains were named TOY55-1, TOY55-2 and TOY55-3 respectively (Table 12).

The P^R constructs TOY55-1, TOY55-2 were crossed with α P^R IL126-1B mutants (TOY55-3 was sterile). The cross of TOY55-1 to IL126-1B P^R5

and P^R 6 gave recombination frequencies of 1.62% and 0% respectively while the cross of TOY55-2 to IL126-1B P^R5 and P^R 6 gave recombination frequencies of 0% and 18.59% respectively indicating that TOY55-1 and IL126-1B P^R5 are non-allelic, while TOY55-2 and IL126-1B P^R6 are also non-allelic.

Table 1: Strains of *Saccharomyces cerevisiae* used in this study, including their mating types, auxotrophic requirements (nuclear markers) and, paromomycin resistance phenotype (mitochondrial marker).

Strain	Nuclear markers	Mitochondrial Markers
5 - 1/6	α ade	P^S
55R2	a ura	P^S
55R2 ρ^0	a ura	----- ----
CCD6-1	α arg	P^S
D6-5	α arg	P^S
D6-5ρ^0	α arg	---- ----
HAT	a trp	P^S
HB2	α ade	P^S
HBT	α trp	P^S
IL-126-1B	α his	P^S
MtS-14	a arg	P^S

Table 2: Summary of total number of P^R mutants isolated from manganese mutagenesis and number selected for further studies after preliminary observations.

Strain	Total number of P^R mutants isolated	Total number of P^R mutants further studied
5 - 1/6	15	15
CCD6-1	15	11
D6-5	15	15
HAT	24	4
HB2	24	0
HBT	15	0
IL-126-1B	24	22
MtS-14	15	10
Total	147	77

Table 3: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant 5-1/6 with tester HBT

Mutant	P^R	P^S	Total Colonies
1	31	439	470
2	0	370	370
3	11	221	232
4	27	402	429
5	10	320	330
6	12	102	114
7	7	117	124
8	27	299	326
9	7	117	124
10	10	193	203
11	1	114	115
12	0	133	133
13	87	34	121
14	3	166	169
15	0	83	83

Table 4: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant CCD6-1 with tester 55R2

Mutant	P^R	P^S	Total Colonies
1	0	113	113
2	0	167	167
3	0	328	328
4	0	335	335
5	7	408	415
6	0	155	155
7	0	154	154
8	4	245	249
9	4	129	133
10	5	234	239
11	6	301	307

Table 5: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant D6-5 with tester HBT

Mutant	P^R	P^S	Total Colonies
1	0	178	178
2	0	57	57
3	0	137	137
4	0	98	98
5	0	114	114
6	0	154	154
7	0	105	105
8	0	30	30
9	0	89	89
10	0	237	237
11	0	142	142
12	37	129	166
13	0	48	48
14	0	113	113
15	5	154	159

Table 6: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant HAT with tester HBT

Mutant	P^R	P^S	Total Colonies
1	51	174	225
2	145	180	325
3	61	131	192
4	87	154	241

Table 7: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant IL126-1B with tester 5522

Mutant	P^R	P^S	Total Colonies
1	0	139	139
2	1	88	89
3	0	217	217
4	0	194	194
5	16	213	229
6	128	21	149
7	0	45	45
8	0	101	101
9	0	387	387
10	0	110	110
11	0	65	65
12	0	40	40
13	0	136	136
14	0	176	176
15	0	243	243
16	0	303	303
17	0	54	54
18	0	76	76
19	0	111	111
20	0	150	150
21	0	139	139
22	1	88	89

Table 8: Result of test to determine mitochondrial vs. nuclear inheritance of paromomycin resistant mutant MtS-14 with tester HBT

Mutant	P^R	P^S	Total Colonies
1	94	14	108
2	0	131	131
3	6	52	58
4	30	56	86
5	40	36	76
6	0	26	26
7	0	94	94
8	8	34	42
9	36	0	36
10	9	47	56

Table 9: Results of test to determine mitochondrial vs. nuclear inheritance of selected P^R mutants. Mutants of 5-1/6, CCD6-1 and MtS-14 were not used as they did not give clear results

Strain	Total number of P^R mutants tested	Total number of mitochondrial P^R mutants
5 - 1/6	15	13
CCD6-1	11	6
D6-5	15	1
HAT	4	4
IL-126-1B	22	2
MtS-14	10	6
Total	77	32

Table 10: Result of allelism test from the mating of paromomycin resistance mutants HAT P^R and IL126-1BP^R, showing calculated recombination frequencies.

HAT	IL126-1B	P^S	Total Colonies	Frequency	Percent Recombination
1	5	11	1069	1.03×10^{-2}	2.06
3	5	12	1120	1.07×10^{-2}	2.14
5	5	8	732	1.09×10^{-2}	2.18
3	6	5	1199	4.17×10^{-2}	0.83
4	6	83	1229	6.75×10^{-2}	13.50
5	6	3	930	3.23×10^{-2}	0.65

Percent recombination is calculated by multiplying the frequency of sensitive mutants by two to account for the undetectable resistant recombinants, then by 100% to convert to percentage.

Table 11: Result of second set of allelism test from the mating of paromomycin resistance mutants HAT P^R and IL126-1BP^R showing calculated recombination frequencies.

HAT	IL126-1B	P^S	Total Colonies	Frequency	Percent Recombination
1	5	2	704	2.80×10^{-3}	0.56
3	5	0	1035	0	0
5	5	0	811	0	0
1	6	0	421	0	0
3	6	0	709	0	0
5	6	9	886	0.10	2.00

Percent recombination is calculated by multiplying the frequency of sensitive mutants by two to account for the undetectable resistant recombinants, then by 100% to convert to percentage.

Table 12: Constructed strains of *Saccharomyces cerevisiae* used in this study, including parental strains, mating types, auxotrophic requirements (nuclear markers) and, paromomycin resistance phenotype (mitochondrial marker).

Strain	Parent strains	Nucelar Markers	Mitochondrial Markers
TOY55-1	55R2ρ^0 x IL126-1BP^R5	a ura	P^R
TOY55-2	55R2ρ^0 x IL126 -1B P^R6	a ura	P^R
TOY55-3	D6-5ρ^0 x HAT P^R6	a arg	P^R

Table 13: Result of allelism test from the mating of constructed paromomycin resistance mutants TOY55 P^R to IL126-1BP^R mutants showing calculated recombination frequencies.

TOY55	IL126-1B	P^S	Total Colonies	Frequency	Percent Recombination
1	5	8	977	0.0081	1.62
2	5	0	716	0	0
1	6	0	610	0	0
2	6	79	850	0.093	18.59

Percent recombination is calculated by multiplying the frequency of sensitive mutants by two to account for the undetectable resistant recombinants, then by 100% to convert to percentage.

DISCUSSION

Based on the specificity of manganese mutagenesis in inducing mitochondrial mutants and previous studies by Knight (1980) in which 71% of manganese induced chloramphenicol resistant mutants and 90% of erythromycin and spiramycin resistant mutants were mitochondrial in origin, we assumed that mutants generated in this study would be primarily mitochondrial. This assumption proved to be incorrect, since most of the paromomycin mutants isolated following manganese mutagenesis in this study proved to be nuclear in nature. The test for mitochondrial vs. nuclear inheritance of paromomycin resistance involves crossing the new P^R mutants to P^S testers. If the P^R mutation is nuclear, the progeny would all display the P^R phenotype if the mutation resulted from a nuclear dominant allele. If the P^R allele is recessive, the progeny would all display the P^S phenotype. Mitochondrial inheritance of the P^R determinant would result in approximately equal numbers of P^S and P^R progeny. Most of our test crosses resulted in all P^S progeny, indicating that the mutations are nuclear recessive in nature. Some results showed a very small ratio of P^R progeny compared to P^S progeny, and since we would expect roughly equal numbers of P^S and P^R mutants in the case of mitochondrial inheritance, these mutations were also likely to be the result of nuclear inheritance. The high percentage of nuclear mutants

generated in this study was unexpected and further study to determine elucidate this observation is necessary.

A number of the P^R mutants generated were prototrophic revertants and therefore could not be used for further studies. For example, of the 24 HAT P^R mutants, 20 were revertants and 23 of the 24 HB2 P^R mutants were also prototrophic revertants. It is important to note that the *ade* requiring mutants displayed a high number of prototrophic mutants. Studies of regulatory mutants in the *his1* locus of yeast strains have indicated that prototrophic mutants arise from second-site alterations that cause recovery of original auxotrophic parental alleles (Lax et. al, 1979). It is possible that the prototrophic revertants observed in this study are generated from similar second-site mutations in the gene specifying the auxotrophic requirement, leading to the restored function of the affected gene.

Allelism tests of P^R mutants

Allelism can be determined by counting at least 1000 diploid progeny from a random diploid analysis. In a cross between two P^R mutants, if no P^S colonies are observed in the diploid progeny, the mutations are said to be allelic. By definition, allelic mutations can not be separated by recombination. However, if the mutations are non–allelic and recombination occurs, some recombinant progeny will posses mutations in the *par1* locus while others will

result from crossing over of sections of parental DNA that do not contain the *par1* locus and these recombinants will be paromomycin sensitive (Figure 7).

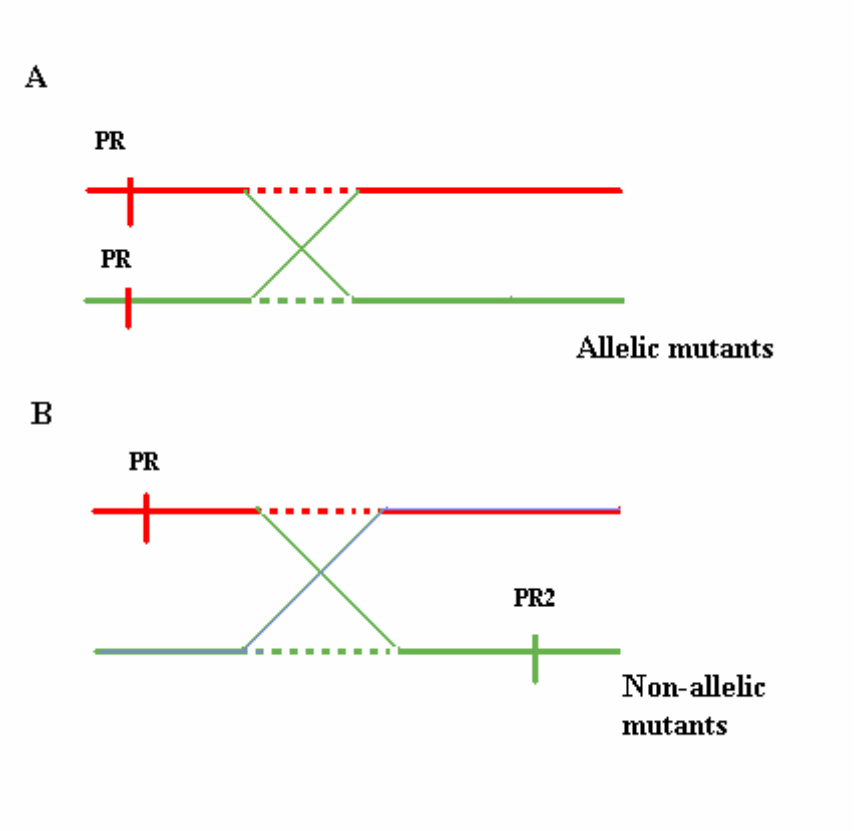


Figure 7: Schematic representation of cross-over during recombination and allelism. In 6A, crossing over cannot occur between the P^R loci because they are allelic. In 6B, the P^R loci are non-allelic and recombination can take place between them, resulting in P^S progeny without either P^R locus.

In the absence of a *par1* tester in the lab, crosses were performed between the four P^R HAT strains and the two P^R IL126-1B strains. From the first set of tests, all isolated HAT P^R mutants and IL126-1B P^R mutants appeared to be non-allelic with recombination frequencies of about 2.0% in crosses of HAT P^R mutants to the IL126-1B P^R 5 mutant, and frequencies of 0.65%-13.50% in crosses of HAT P^R mutants to IL126-1B P^R 6mutants. This suggests that at least one of our P^R mutants is non-allelic at *par 1.* The similar recombination frequencies observed in crosses between all HAT P^R mutants to IL126-1B P^R mutants suggest that the HAT P^R mutants (mutants 1, 3 and 5) may result from changes at the same locus and are allelic. The recombination frequencies of 0.83% and 0.65 % observed in crosses of the HAT P^R mutants 3 and 4 to the IL126-1B P^R 6 mutant support the suggestion that the HAT P^R mutants 3 and 4 are allelic. The HAT P^R mutant 4 could be non-allelic to other HAT P^R mutants. However, this could not be studied due to prototrophic reversion of the mutant before it could be mated to the IL126-1B P^R5 mutant.

To determine if the results observed were truly due to non-allelic mutations in the mitochondrial DNA, random diploid analyses of the paromomycin resistant HAT and IL126-1B mutants were repeated. The second set of results did not correlate with the previous findings that suggest non-allelism of the new mutants at the putative *par 1* (Table 11). The matings between the HAT P^R mutants 1, 3 and 5 showed a percent recombination of 0.56%, 0% and 0% respectively when crossed with the IL126-1B P^R5 mutant

suggesting that the mutants are allelic to the IL126-1B P^R mutant. The results still suggest that the HAT P^R mutants could be allelic to each other, as they display similar recombination frequencies when crossed to the same P^R mutant.

The matings of the HAT P^R mutants 1, 3 and 5 to the IL126-1B P^R6 mutant showed percent recombinations of 0%, 0% and 2.0% respectively, indicating that the HAT P^R mutants 1 and 3 are likely allelic to the putative *par1* locus.

The differences observed between the first and second set of allelism tests could be due to the slightly different definitions applied in counting the diploids. In the first test, colonies showing moderate sensitivity as well as those showing very high sensitivity to paromomycin were scored as P^S whereby only those showing very high sensitivity to the drug were scored as P^S in the second set of tests. Hence, the results from the second set of allelism test are probably a truer reflection of the recombination frequencies between the HAT and IL126-1B mutants.

Some of the data may be inaccurate as the number of diploid progeny counted was not always up to the minimum 1000 required for allelism tests to be considered significant. A larger size of allelism tests of P^R mutants should be conducted to further elucidate allelism at the *par 1* locus as there is not information to determine whether observed mutations are truly non-allelic or

not. However, the small number of mitochondrially inherited paromomycin resistant mutants generated made this difficult.

Allelism can also be studied by crossing new P^R mutants to testers with known antibiotic resistance loci. The use of such tester strains greatly enhances recombination analysis by confirming or disproving that new antibiotic resistant mutants have mutations that are allelic to the antibiotic resistance mutation present in the tester strain. However, because allelism at *par1* has not been studied, there was a lack of *par 1* tester strains available to perform such allelism tests in this study.

Allelism Tests of Constructed Strains

All constructed strains were of the a mating type and therefore were crossed to the two α IL126-1B P^R mutants generated via manganese mutagenesis. The mutant TOY55-1, showed 1.62% recombination when crossed to IL126-1B P^R 5 and 0% when crossed to IL126-1B P^R6. The mutant TOY55-2, showed 0% and 18.59% recombination when crossed to IL126-1B P^R 5 and P^R6 mutants respectively. The frequencies observed are interesting as they do not support expectations. As constructs of the IL126-1B P^R5 and P^R6 mutants, the TOY55-1 and TOY55-2 mutants would be expected to be allelic to the IL126-1B P^R5 and IL126-1B P^R6 mutants respectively. The frequencies observed may be a result of scoring less than 1000 progeny in all cases. The

TOY55-3 construct was sterile and therefore unsuitable for further genetic analysis.

Characterization of P^R Mutants

Characterization of progeny following random diploid analysis was challenging for P^R mutants. Unlike the mutants showing resistance to the more extensively studied antibiotics chloramphenicol, erythromycin and spiramycin, paromomycin resistant mutants rarely displayed uniformly strong drug resistance. Rather, characterized diploids displayed several levels of drug resistance. Some were highly resistant to paromomycin, some displayed partial resistance, and others were completely sensitive (See Figure 8). In this study, only diploids displaying at least 75% sensitivity to the drug as estimated by control colony size were considered drug sensitive in the allelism tests.

Paromomycin Resistance and the 16S rRNA Gene

The Ribosomal RNA Mutation Databases provide lists of mutated positions in 16S and 16S-like ribosomal RNA and their identities, listed according to the *E .coli* numbering system. According to this list, various base pair alteration positions can result in different levels of paromomycin binding to the 16S ribosomal subunit. A C1407G/G1494C alteration results in 'severely impaired paromomycin binding', a C1409G/G1491C alteration

results in ‘slightly reduced interaction with paromomycin’ and a 1491 G->U alteration results in a ‘weakened interaction with paromomycin’ (Triman et al., 1998). This suggests that different levels of paromomycin resistance could be observed based on the position of the alteration in the 16S rRNA gene.

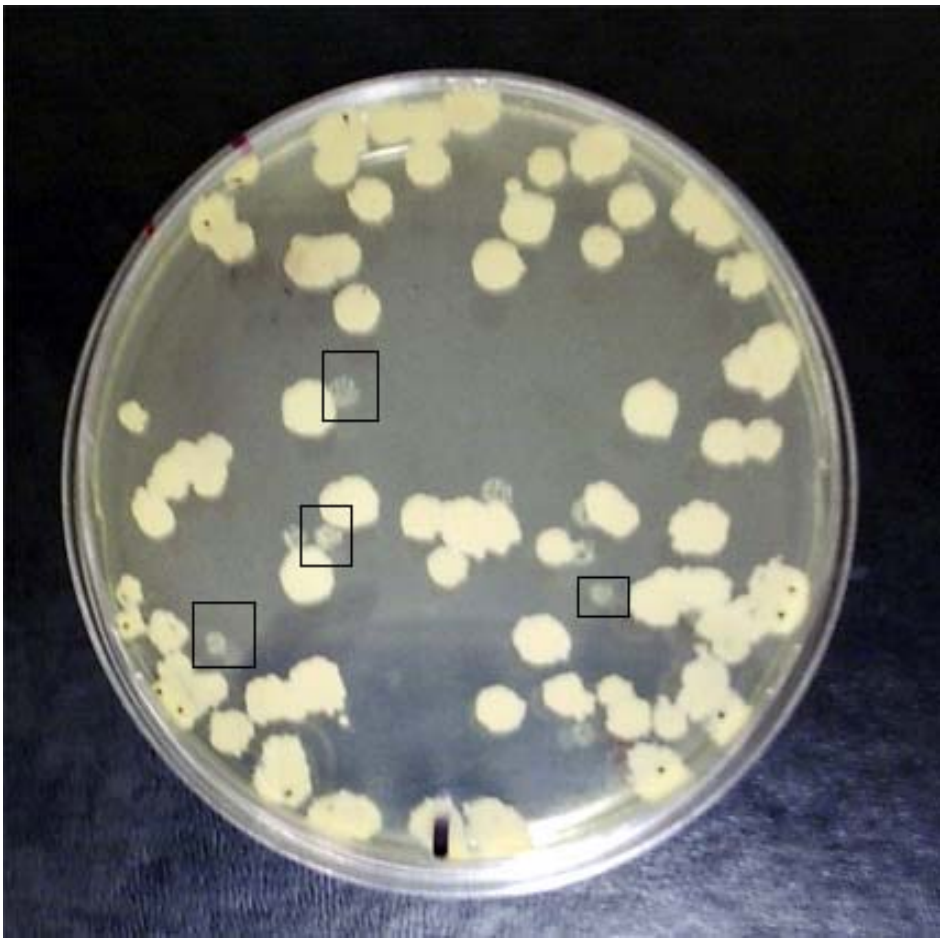


Figure 8: YG + P plate showing P^R and P^S colonies and differential expression of paromomycin resistance.

Comparisons with *E.coli*

Paromomycin resistance has been localized near the 3'end of the 16S rRNA gene in *E. coli,* a region that exhibits significant homology and has been highly conserved in prokaryotes and eukaryotes (Li et al., 1982; Edlind, 1989). Therefore studies of the 16S rRNA of other homologous organisms especially *E. coli* should elucidate the yeast 16S rRNA. Sequencing of a paromomycin resistant and sensitive yeast strain by Li et. al., (1982) revealed a single C-> G base pair substitution at position 1514 of the gene. Mutation and NMR structure studies have shown that paromomycin resistance results from disruption of the CG base pair at the 1409-1491 position in *E.coli (*Edlind, 1989; Recht et. al., 1998; Carter et. al, 2000). The 1514 position is 1409 in the *E. coli* numbering system, indicating the high homology between the regions conferring paromomycin resistance to both strains. See Figure 9 for secondary structures of *E.coli* and *S. cerevisiae.*

Studies in *E.coli* suggest that the paromomycin binding near the 3' terminus of the RNA is fairly accessible at the surface of the 30S ribosomal subunit, suggesting that the secondary or even tertiary structure of the gene is important to association with paromomycin (Chapman et. al, 1984; Li et al, 1982). The hypothesis that paromomycin resistance results from a change in RNA structure rather than sequence is supported by findings in the protozoan *Tetrahymena,* where a homologous G->A transition disrupts the first base pair

of a large hairpin loop located near the 3'end of the 17S rRNA (Spangler and Blackburn, 1985). These studies are relevant to this study, as they could explain the difficulty encountered in characterizing the *par 1* locus in the 16S rRNA gene and the reason why no other loci conferring resistance to the antibiotic have been found in the gene. This is in contrast to the 21 S rRNA gene where several antibiotic resistant loci conferring resistance to chloramphenicol, erythromycin and spiramycin have been characterized.

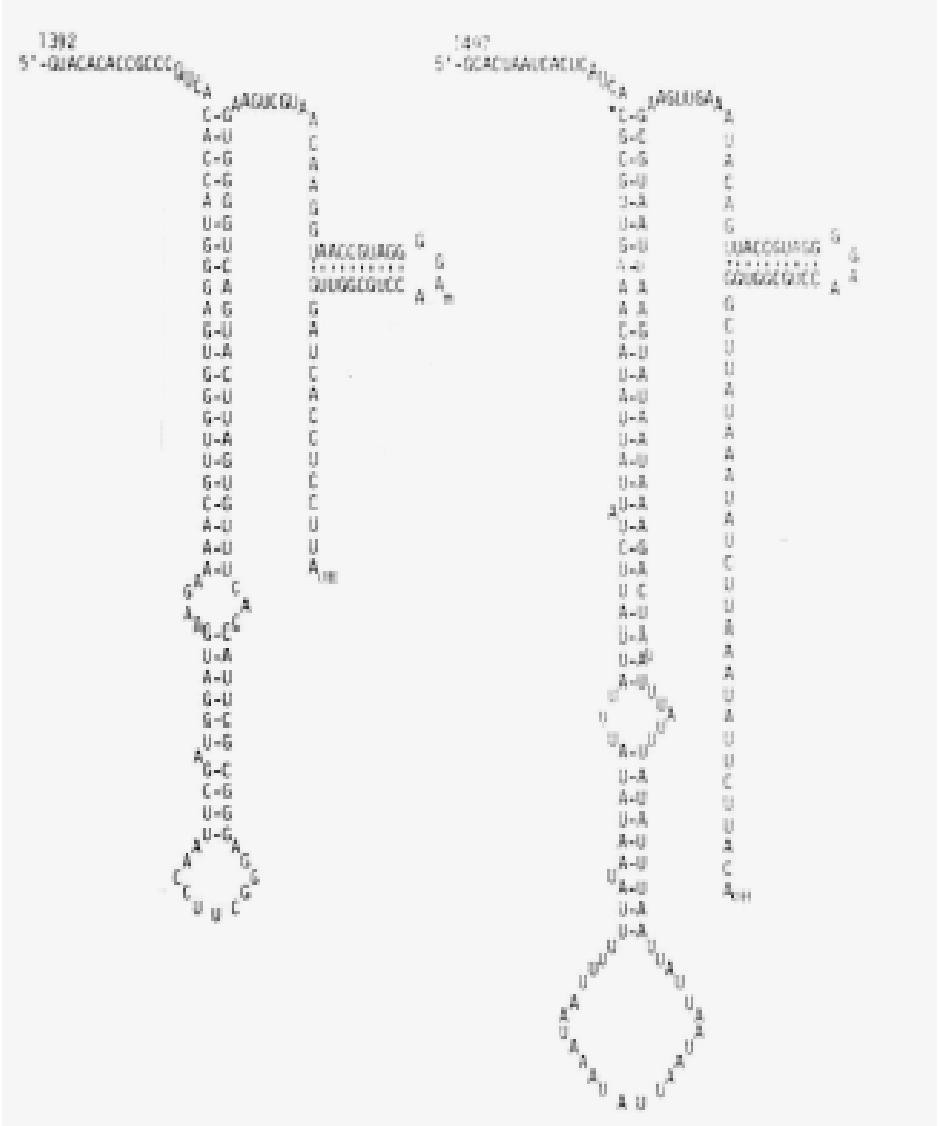


Figure 9: Secondary structure models of *E.coli* and *S. cerevisiae* mitochondrial rRNA showing gene homology. *E.coli* structure is on the left and *S. cerevisiae* structure is on the right (adapted from Li et al, 1982).

Nucleo-mitochondrial Interactions

Nuclear genes can also affect expression of antibiotic resistance. Wolf (1973) showed that nuclear genes can decrease resistance to paromomycin. Several studies have shown that nuclear-mitochondrial interactions can affect drug resistance in yeast. (Wolf, 1973; Cohen et al., 1979; Waxman et al., 1979). Such studies show that among strains that carry the mitochondrial P^S allele, differential phenotypic expression of the allele can be observed due to phenotypic interactions between the mitochondrial allele and the nuclear genes, which can influence phenotypic expression of drug resistance. Paromomycin phenotypic expression has been shown to be modified by a single dominant nuclear modifier MPR1 capable of suppressing mitochondrial paromomycin resistance (Waxman et al., 1979). These studies suggest that our P^R mutants might have been suppressed by nuclear modifiers and second-site mutations that masked expression of paromomycin resistance.

The generation of some non-allelic mutants provides some evidence that other mutable sites conferring resistance to paromomycin other than *par1* exist within the 16S rRNA gene. To further analyze this, one suggestion is to sequence the 16S rRNA gene of non-allelic mutants to see where the mutations fall and what type of disruptions are causing the paromomycin resistant phenotype. Another suggestion is to study the suppressors of mitochondrial paromomycin resistance, as the presence of such modifiers can lead to misinterpretation of data. Similar future studies should consider the use

of isogenic strains of yeast to eliminate differences in expression that could be strain specific.

Although this study has shown only limited success in generating a fine structure map of the 16S rRNA gene, it generates some interesting observations and questions concerning the nature of the 16S rRNA gene and its associations with protein synthesis and paromomycin binding. The most interesting hypothesis suggests that, unlike the more extensively studied antibiotics (chloramphenicol, erythromycin and spiramycin), paromomycin resistance in yeast is primary the result of disruptions in the secondary and tertiary structures of the 16S rRNA subunit. This hypothesis is supported by studies of the secondary and crystal structure of paromomycin binding in homologous small ribosomal subunits of *E. coli* and *T. thermophilus* as well as those showing that changes in the rRNA gene do not always affect paromomycin resistance. Continued studies of the fine structure map of the 16S rRNA gene in *E. coli* and *S. cerevisiae* in association with new biochemical characterization of the small rRNA should further elucidate the structure and function of the small ribosomal subunit in protein synthesis and antibiotic resistance.

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