Investigating Resistance to *Candida albicans* Infection through Genetic Mutations of *Caenorhabditis elegans*

A Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of the Degree of Bachelor of Science

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Abstract

Candidiasis is a serious disease caused by *Candida albicans* that affects over four billion people a year. Of those infected, 32,000 of them develop systemic infections in the US with a 30%-60% fatality rate. This type of infection is especially prevalent in immune-compromised individuals such as HIV-infected, diabetes and cancer patients. In this study, we mutagenized *Caenorhabditis elegans* and exposed them to *C. albicans* in order to isolate mutants resistant to the yeast infection. After survival tests of mutant *C. elegans* on *C. albicans*-containing media, three putative resistant strains were isolated for future analysis. Further characterization of these mutants may identify genes required for sensitivity to *C. albicans* infections and be useful in the study and prevention of Candidiasis.

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1. Introduction

Candidiasis is a yeast infection cause by *Candida albicans.* It is a serious disease that affects over four billion people a year worldwide.[1] In the United States, 32,000 individuals are infected with the systemic infection of *C. albicans* each year.[2] Most Candida infections occur on epithelial surfaces such as in the mouth, nail, vaginal, and skin regions. These surface infections are easily treatable using topical or oral anti-fungal agents.[1] In some cases, the Candida infection passes into the blood stream, becomes systemic and infects many vital organs. This systemic infection is commonly seen in patients that have compromised immune systems such as those with HIV, diabetes, cancer, or those who are recovering from an organ transplant or other major surgeries. Systemic Candidiasis is fatal with a 30%-60% mortality rate.[3]

Over the past decade the seriousness of Candida infections has increased, causing a necessity for a suitable treatment to battle these infections. Little is known about the effects that Candidiasis has on the body. It has been the interest of many different research organizations to create a model of what occurs in the infected body. By using the organism *Caenorhabditis elegans* as a model host, information about this disease can be discovered.

Our research sought to induce a mutation in *C. elegans,* a model organism, that might cause a resistance to infection by *C. albicans*. If resistant mutants are successfully isolated, the mutation can be identified to determine the mutated gene that is responsible for causing this resistance. The identified gene can then be compared to homologous genes in humans to help create a better understanding of the Candidiasis infection. By investigating the genes that could be responsible for resistance to Candida infections, an effective treatment for the systemic infections of Candidiasis could be created to help lower the mortality rate.

2. Background

2.1 *Candida albicans* Background and Infections

Candida albicans is the most common human fungal pathogen and is naturally found in healthy human digestive and reproductive organs. It can exist in two forms – as either small circular cocci yeast cells or branch-like hyphae cells.

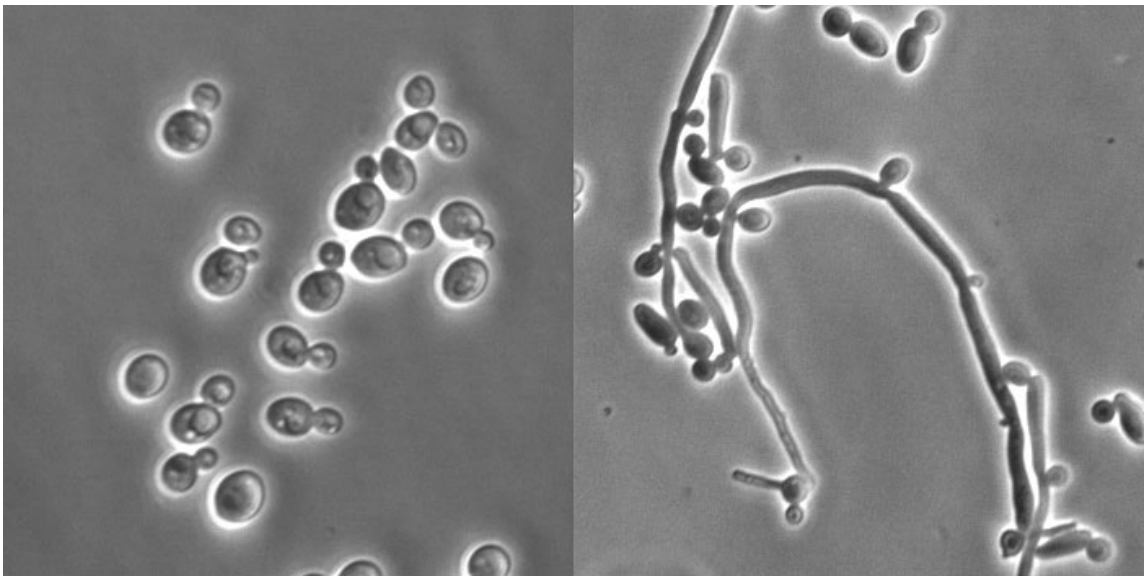

Figure 1: Two Forms of the *Candida albicans* Yeast[4]

The small cocci yeast cells, shown in the left panel of Figure 1, are often seen on the mucosal membranes in the body while the branch-like hyphae, shown in the right panel of Figure 1, are commonly seen in liquid cultures *in vitro*. Both shapes help *C. albicans* establish bloodstream infections. The small cocci yeast cells are ideal for passing through the epithelial membrane to reach the blood stream. Once in the blood stream, the yeast begins to form hyphae, which disrupts surrounding tissues, thus causing the harmful effects on many organs that are seen in systemic Candidiasis.[5]

2.1.1 Topical Infections of *Candida albicans*

C. albicans prefers the presence of moist areas of the body, which is why it is usually found in the genital and oral regions. Many other microorganisms grow in healthy mucus membranes in the body. However, when other organisms become depleted, the yeasts are able to out compete for the limited resources. When the yeast takes over in the mouth or throat, it is often called 'thrush' and causes a sore throat along with a white coating in the mouth, as seen in Figure 2 below.[6] When the infection is in the female genital regions, it is called vulvovaginitis and can cause extreme itching and burning sensations along with possible white discharge.[2]

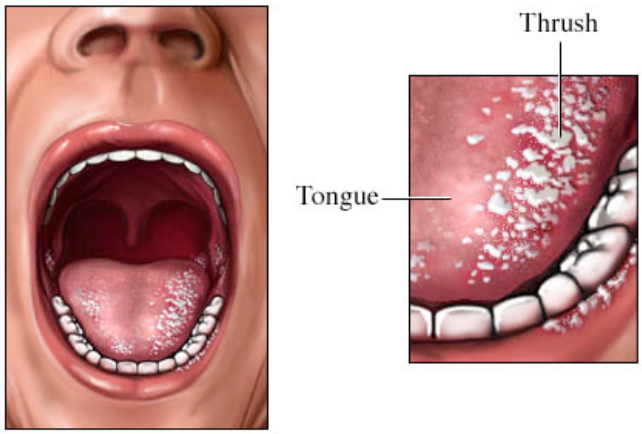

Figure 2: Oral "Thrush" Infection of *Candida albicans*[7]

In normal healthy individuals, these infections are quickly resolved with the use of topical and oral anti-fungal agents. However, these treatments are ineffective once the infection enters the blood stream as seen in systemic Candidiasis patients.

2.1.2 Systemic Candidiasis

Of all of the systemic Candidiasis infections that occur, 90% of them are caused by the *C. albicans* yeast, making it the fourth most common blood stream infection in hospitalized patients in the United States.[2,3] Thrush or vulvovaginitis infections rarely becomes a systemic Candidiasis infection unless the patient has the necessary risk factors that affect the body's ability to fight off infections. These include patients with compromised immune systems, physical trauma, poor nutrition, or previous treatment with steroids or antibiotics. Once the infection has reached the blood stream, diagnosis can be difficult due to a wide range of symptoms. The main symptoms are fever and chills, which are normally associated with a bacterial infection. This sometimes causes misdiagnosis and mistreatment. The rest of the symptoms vary depending on the organs that have become infected.[2] Candidiasis is normally diagnosed though blood cultures that look for the presence of yeast. The *C. albicans* that is normally seen in the blood culture is the hyphal form, which disrupts the surrounding tissues of vital organs.

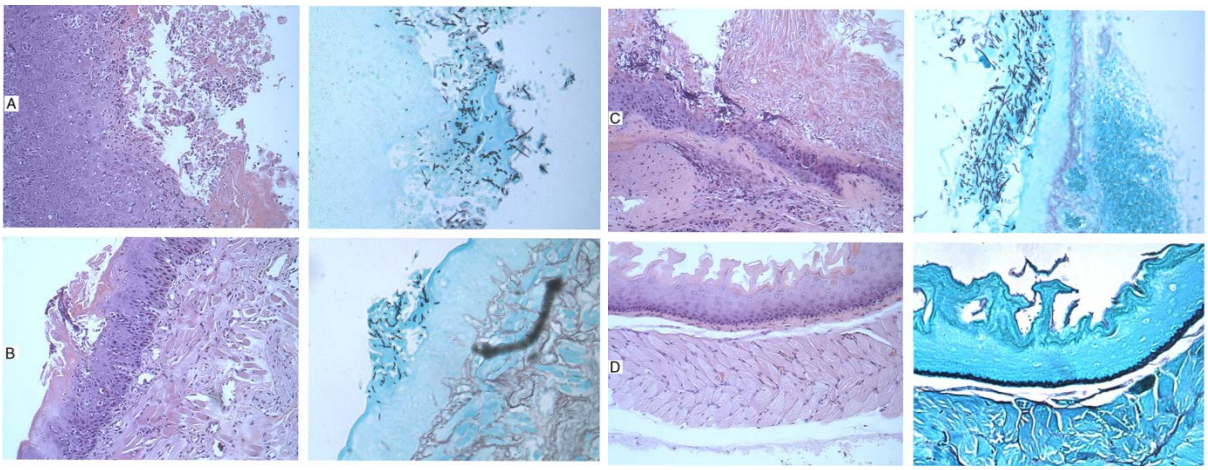

Figure 3: Histopathology of the Tongue and Esophagi of Mice infected with Active *C. albicans* and Null Heat Killed *C. albicans* Strains[8]

Figure 3 shows the tongue (A&B) and esophagi tissues (C&D) infected with active *C. albicans* (A&C) and a null heat killed *C. albicans* (B&D). The tissues that were infected with the active yeast showed a greater inflammatory reaction, seen by the increased amount of black spotting, demonstrating how organs react when infected with Candidiasis. Although panel B shows black spots, it is significantly less than panel A, illustrating the difference between active and heat killed *C. albicans*.[8] Organs can also form abscesses and invasions in the organ walls or even cell death from the presence of the cocci yeast cells alone.[5,9] Candidiasis is difficult to treat due to the widespread infection of various tissues and organs. Also, many treatments for mucosal infections are applied directly to the area of infection. For these reasons, antifungal agents that are currently used to treat common mucosal infections are often ineffective when attempting to treat systemic Candidiasis. The 30%-60% fatality rate of systemic Candidiasis infections is a great concern to the medical community and has helped to fuel the search for a treatment for Candidiasis.[3]

2.1.3 Recent Interest in Candidiasis

Once only considered the cause of aggravating but not dangerous mucosal infections, Candida has recently garnered much more attention due to its potential to cause life threatening systemic infections. Additionally, *C. albicans* has become a common issue in hospitals, especially in intensive care units where close quarters allow for pathogens to spread rapidly.[10]

Fungal infections have always been difficult to treat, especially in patients with weakened or compromised immune systems. Firstly, they show non-specific symptoms, causing difficulties in diagnosing the disease. Effective treatments are complicated by the increase of anti-fungal resistant infections and the need for medications that will not harm the body in the process. When a treatment is administered, it does not always kill the yeast and could lead to later relapses of the disease.[11] Finding new anti-fungal medications has become a major interest to the scientific community.

2.2 *Caenorhabditis elegans* Background

Caenorhabditis elegans are non-parasitic nematodes that are naturally present in the soil around the world. Adult hermaphrodites are approximately 1 mm in length, making them easy to observe with a simple stereo dissecting microscope. In addition, the nematodes are transparent, allowing the details of cell division, as well as physiological and reproductive process to be observed *in vivo* (Figure 4). *C. elegans* are easily cultured in the laboratory and have been the subject of countless research investigations; examples include analysis of the structure and function of the complete nervous system.[12] Thus, these simple worms have become an exceedingly popular experimental model due to their simple genetic makeup, small size, ease of maintenance, and their ability to be stored cryogenically.[13]

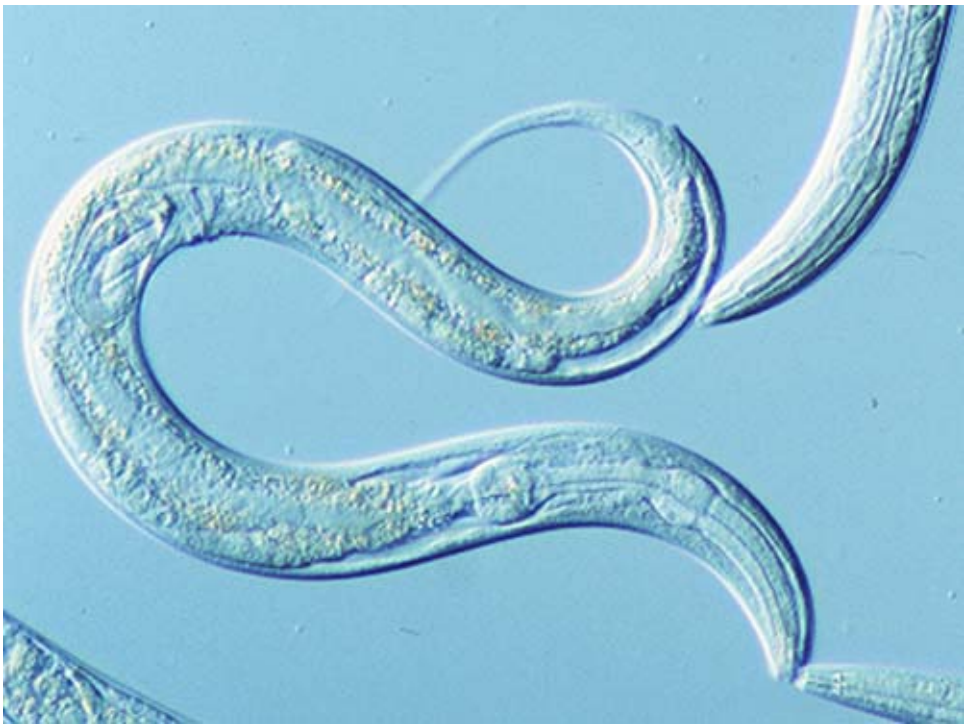

Figure 4: *Caenorhabditis elegans* Adult Hermaphrodite[14]

The popularity of *C. elegans* as a laboratory model organism started in 1963 with Sydney Brenner, who was interested in finding a simple organism with which to investigate animal development.[12] Previously *Drosophila* were used to study genetic patterns; however, the full genome of the fly was not available. Therefore, a new test organism was sought out. *C. elegans* were chosen due to their simplicity and rapid growth cycle. Its simplicity has allowed researchers to sequence the *C. elegans* genome in its entirety, which contains around 100 million base pairs on six chromosomes with an estimated 20,000 genes.[15,16] This makes the *C. elegans* genome much smaller than the human genome, which contains around 3 billion base pairs on 23 chromosomes with an estimated 30,000 genes.[15,17] *C. elegans* can be grown in the lab on agar-filled petri dishes where they feed on *Escherichia coli* bacteria. If a specific worm is desired, they are able to be removed from the plates individually by a process called ‘picking’.

The ability to freeze and thaw worms in a cryostasis cycle, developed by John Sulston in 1969, allows a large number of wild type (N2) and mutant worm strains to exist in a stock center where specific mutants can be requested.[18] The L1 stage survives cryostorage better than other developmental stages After the desired period of storage time, the worms can be thawed and allowed to resume development.[13]

2.2.1 Anatomy of *Caenorhabditis elegans*

C. elegans consist of an unsegmented cylindrical body with tapered ends. Some of the organ systems that will be discussed are shown in Figure 5. Starting from the outside in, the worm is surrounded by a cuticle that is secreted by the epithelial cells (hypodermal stem cells) that reside directly below it. The cuticle surrounds the worm to maintain its shape and protects the worm from environmental conditions. The excretory, nerve and muscle systems all reside below the epithelial cells. The excretory system removes fluid from the worm through a combination of pore, duct, canal and gland cells. This process excretes fluid to help maintain the worm's salt balance. The worm's nerve and muscle systems work together through a process of signals sent by the nerves to the muscles that contract to help move the worm though its environment.[13]

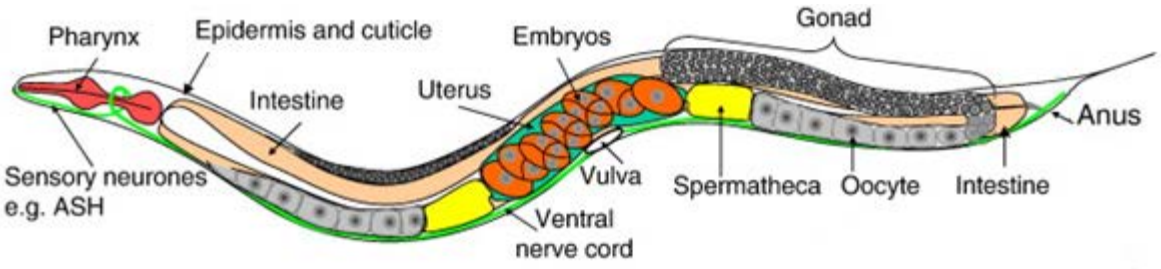

Figure 5: Anatomy of *Caenorhabditis elegans*[19]

Separating these organs from the inner organs is a pseudocoelomic cavity, which is a fluid filled space that helps to separate the organs in the worm, mainly the alimentary and reproductive systems. The alimentary system brings nutrients though a two-lobed pharynx and passes ingested food into the intestine; digestive wastes exit by defecation through the anal opening.[13] The reproductive system varies depending on the gender of the worms, consisting of a male gonad in males and the addition of a female gonad, which produces eggs, in hermaphrodites.

For the most part, *C. elegans* are hermaphrodites and are able to self-fertilize and pass on exact copies of their genes to their offspring. Since one homozygous worm will quickly produce hundreds of exact genetic copies of itself, the worms are very useful in experiments involving genetic mutations. However, a few male worms arise though infrequent spontaneity (0.1%) in the hermaphrodite germ-lines, and when a male and hermaphrodite mate, the male germ-line arises about 50% of the time.[20] The male development is due to a spontaneous X chromosome

nondisjunction – hermaphrodites are (XX) and males are (XO).[13] The physical differences can be seen in Figure 6, comparing the sperm-containing gonads of the male worms with the hermaphrodite gonads that contains both egg and sperm. Additional differences visible in Figure 6 are the embryos that are contained in the body of the hermaphroditic worm and the hook-like tail contained at the end of the male worm.

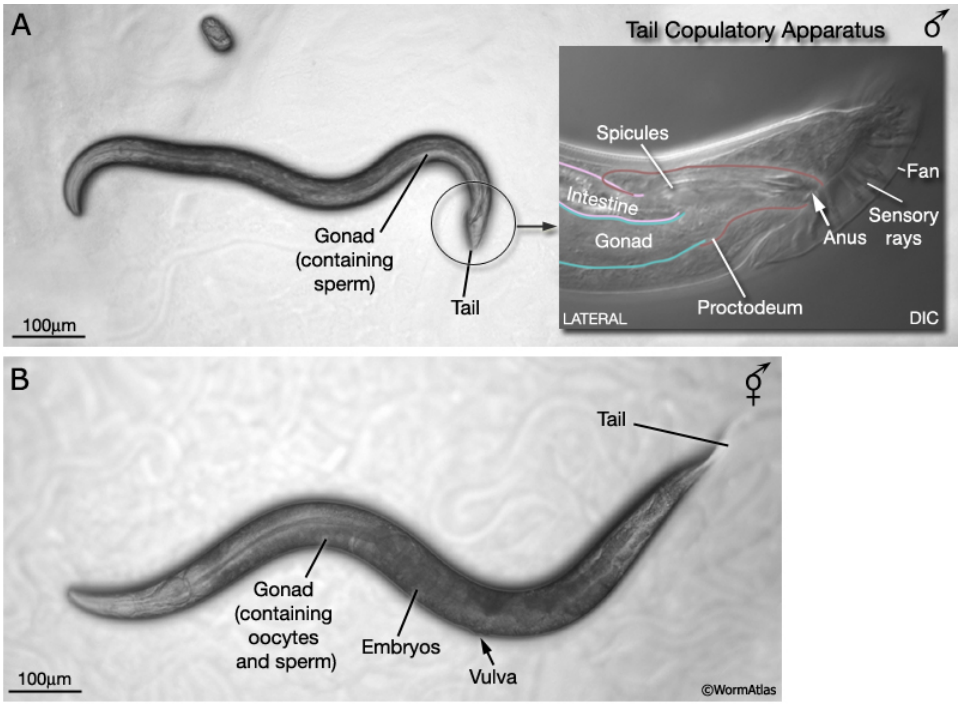

Figure 6: Differences between Male (A) and Hermaphrodite (B) *Caenorhabditis elegans*[21]

For males to fertilize hermaphrodite eggs, they use their tail, which houses the 'fan' and aids the male worm in hooking onto the hermaphroditic worm to position itself and fertilize the eggs. In the hermaphrodites, the eggs are simply fertilized by the gonad containing sperm located next to the eggs. Once the eggs are fertilized, either though self-fertilization or mating, the wild-type hermaphrodite is able to lay about 300 eggs.[13]

2.2.2 Lifecycle of *Caenorhabditis elegans*

The worm life cycle has a generation time of 3 days at 20°C and falls into 6 stages. These stages are embryos, first stage larva (L1), L2, L3, L4, and adult, which can be seen in Figure 7 below.[12] The four larval stages are separated from each other and from the adult by the process of molting and synthesizing new cuticles at each stage. Each cuticle has a different physical and chemical structure from the one before. An additional option for the worms is to enter into the dauer larval stage when there are unfavorable conditions such as lack of food or decrease in temperature. This stage allows the worms to be dormant for long periods of time.[13]

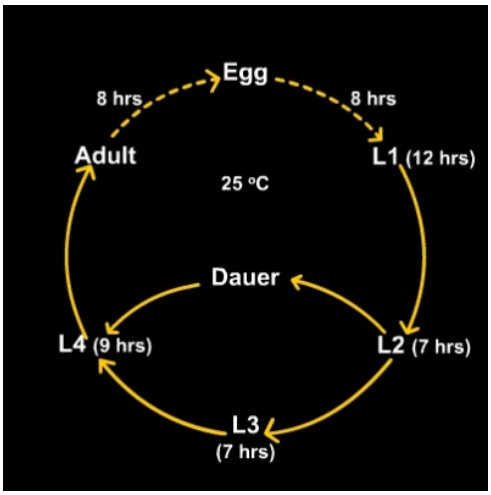

Figure 7: Life Cycle of *Caenorhabditis elegans*[13]

The size of the worms is used to roughly distinguish the stages. However, the only clear-cut distinction visible in the dissecting microscope is the presence of the hermaphrodite vulva in adults. In Figure 8, the relative sizes of the different stages are shown in comparison to each other.

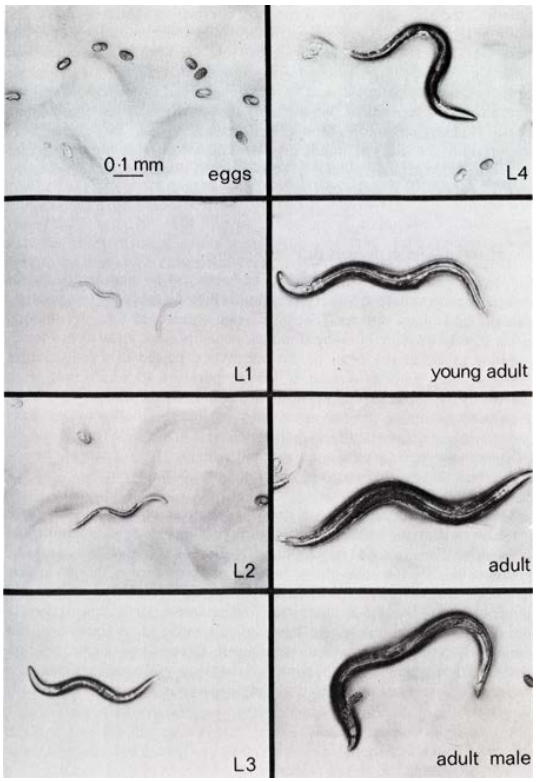

Figure 8: Relative Sizes of the Different Stages of *Caenorhabditis elegans* Development[13]

The different sizes of these varying stages provide a rough guide to identify the appropriate stages to use for specific experiments.

2.2.3 *Candida albicans* Effect on *Caenorhabditis elegans*

C. elegans have been used as a model for this infectious pathogen due to the way that *C. elegans* are able to model key aspects of the mammalian pathogenesis. It has already been determined by many researchers including that *C. albicans* have a negative effect on the health of *C. elegans* when exposed. Figure 9 shows the survival rate of *C. elegans* when exposed to heat-killed (HK) *E. coli* and *C. albicans* in relation to live *C. albicans* over a span of 150 hours in an experiment performed by Pukkila-Worley et al. From the graph, it is seen that 100% of the worms survived in the presence of the heat-killed *E. coli* and *C. albicans*, while less than 20% survived in the presence of the live *C. albicans*.[22] This demonstrates that it is the presence of live *C. albicans* that causes the disease and not just the presence of the yeast cells alone.

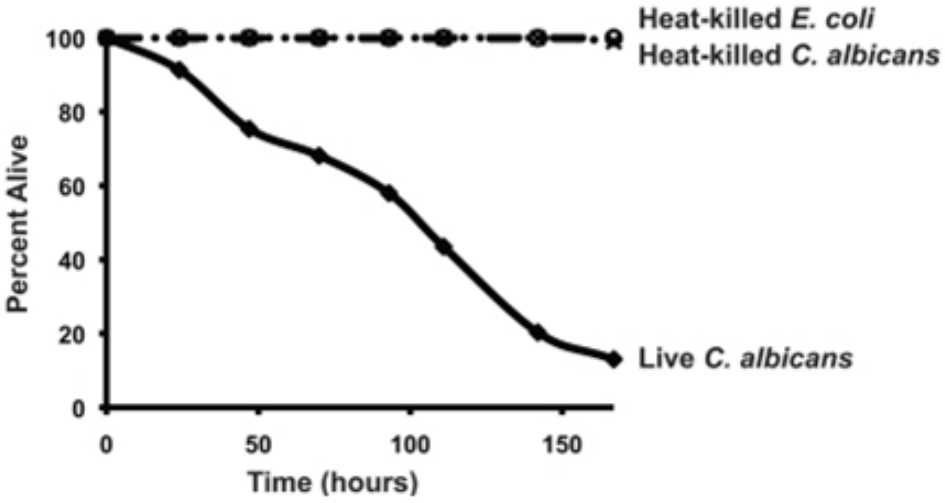

Figure 9: Survival Rate of *Caenorhabditis elegans* Exposed to *Candida albicans*[22]

The physical effects of the live *C. albicans* versus the heat-killed *C. albicans* are shown in Figure 10 below. After 16 hours at 25°C, the heat-killed *E. coli* showed no distention or expansion of the intestine in the proximal (left) or distal (right) sections of the worm body. The lumen or outer edge of the intestine is marked with the black arrows. HK *C. albicans* showed slight distention of the intestine from the collection of a small amount of the dead yeast. However, the live *C. albicans* yeasts caused a large distention of the intestine, illustrating the harmful effects the yeast can have on the worms.[22]

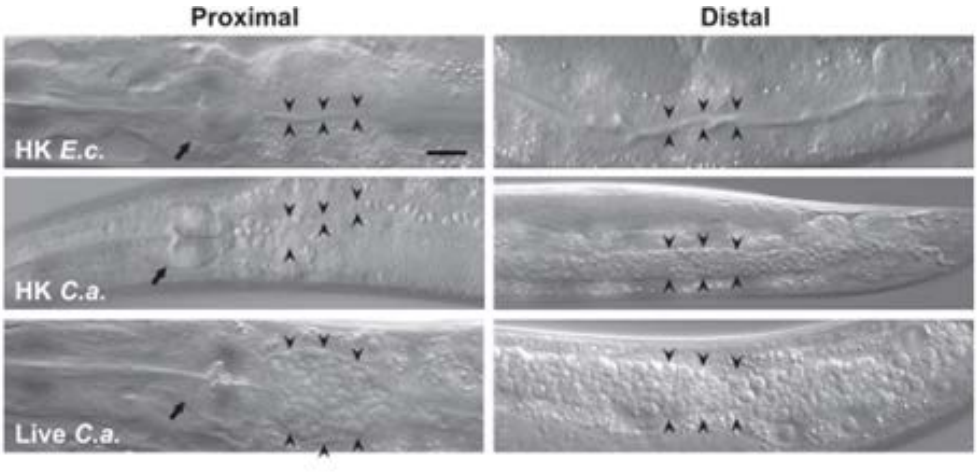

Figure 10: Images of *Caenorhabditis elegans* Exposed to Heat-Killed *E. coli*, Heat-Killed *Candida albicans*, and Live *Candida albicans*[22]

Similar to the pathology of the yeast infection in humans, once the *C. albicans* is in the intestine of the *C. elegans* the yeast converts into its hyphal form, usually seen in liquid media cultures, and fatally destroys the surrounding tissues as shown in Figure 11.

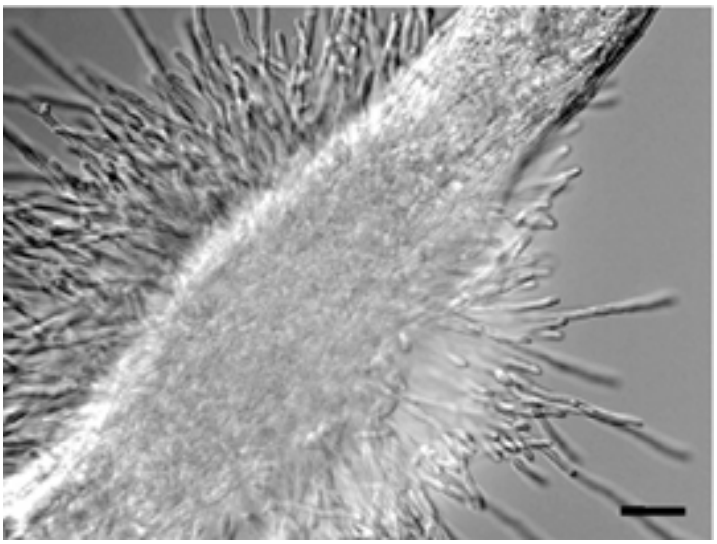

Figure 11: *Candida albicans* Hyphae Protruding from a *Caenorhabditis elegans*[23]

2.3 Mutations Overview

Mutations are changes to the genome of an organism. These are normally caused at low frequencies by spontaneous errors in DNA replication. The frequency of mutation is increased by mutagens, which include environmental and chemical agents such as ionizing radiation, ultraviolet light, nitrous acid and many other natural and artificial chemicals. Induced mutations can include changes not only to the nucleotide sequence, but also the structure and function of the DNA such as the creation of pyrimidine dimers and hydroxyl radicals. In chemical mutagenesis, there are generally two major types of DNA alterations: point mutations and insertion/deletion mutations. In point mutations, a single base pair is replaced with another different base pair. In this category, there are two subclasses: transitions, where one type of nucleotide (purines or pyrimidines) is replaced by a nucleotide from the same class, and transversions, where one type of nucleotide is replaced by another nucleotide from a different class. With insertion/deletion mutations, one or more base pairs are either removed or added to the DNA, both resulting in the disruption of the DNA sequence.[24]

These changes, when found in the coding sequence of a gene, may then affect the translated protein sequence, showing the results of missense, nonsense, silent, or frame-shift mutations. Missense mutations occur when a mutation in the codon for the protein results in the change of the original amino acid into another amino acid. Nonsense mutations cause the codon that was originally coded for an amino acid to now code for a stop codon and terminate the protein sequence prematurely. Silent mutations are mutations that have no effect on the protein sequence. Frame-shift mutations occur from an insertion or deletion of a nucleotide where the entire sequence is shifted over, resulting in an almost entirely different protein sequence.[24] Finally, insertions and deletions can also delete portions of a protein-coding sequence or insert unrelated sequences, resulting in alterations of a whole section of a protein.

In this research, the desired mutations are point mutations, where one of the bases in the DNA is changed to another base pair. This single mutation can cause a change in the amino acid that it codes and, as seen in the example in Figure 12, can have a significant effect like causing normal hemoglobin to become sickle-cell hemoglobin. A frame-shift mutation in the same gene would likely to cause a complete absence of hemoglobin instead of just a function change.

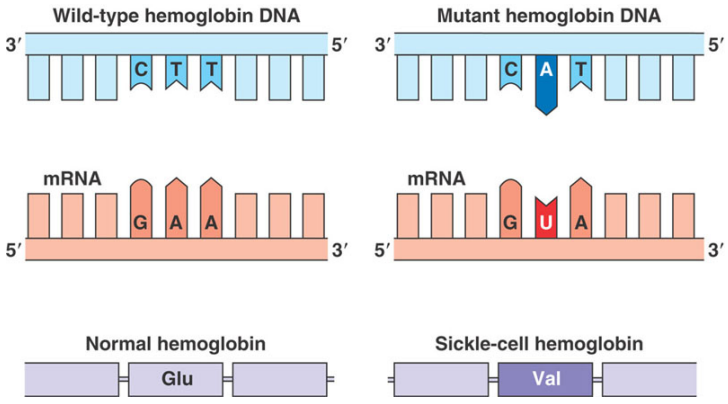

Figure 12: Example of Point Mutations in Normal versus Sickle-Cell Hemoglobins[25]

Mutations can occur in any type of cells, either somatic (normal cells) or germ-line (reproductive) cells. Only changes to germ-line cells can be heritable and passed down from parent to offspring while mutations in somatic cells only affect the individual.[24] The geneticist is primarily interested in heritable mutations, which can be maintained in mutant stocks for the study of mutant phenotypes and identification of the mutated genes.

2.3.1 Mutagens used on *Caenorhabditis elegans*

When introducing mutations into *C. elegans* or any organism, the goal is to create mutations in the genome of the germ-line cells that will eventually be passed on to its progeny. This creates the ability to test the progeny for both the retention of the mutations and its effects on normal functions. Mutagenesis of the *C. elegans* is focused on N2 L4 worms that are in the process of becoming adults and are forming germ line cells inside the gonad lumen. The germ line cells will be actively dividing and multiplying mitotically, and will easily be affected by the chemicals creating the mutations. The resulting first generation progeny (F1) will contain mutations that were generated independently of each other and in effect, are different from each other even though they came from the same parent *C. elegans*. In the F1 generation, mutations will be present in heterozygotes. However, by self-fertilization of such F1 hermaphrodites, the F2 will include an average of ¼ homozygous individuals for each F1 carrying a mutation. Thus, hermaphrodite reproduction allows homozygous mutation to be easily obtained.

One the most common chemicals used for mutagenesis of *C. elegans* is ethyl methanesulfonate (EMS). EMS is an alkylating agent that induces point mutations by chemically modifying the nucleotide guanine (G) to form O^6-ethylguanine that would later base pair with thymine (T) and not with cytosine (C). After subsequent DNA repair and division, the original G/C pair would then be substituted into an A/T pair.[26] What makes EMS such a popular chemical to use for mutagenesis is its ability to create mutations at high frequencies of $3x10^{-4}$ per gene per genome compared to $1x10^{-6}$ for spontaneous mutations, with relatively small amounts of the chemical and minimal toxicity to the rest of the organism.[27] Along with the high mutability, EMS is also relatively inexpensive, easily storable, and can be safely neutralized inexpensively with salts such as sodium hydroxide (NaOH).

Another chemical that is used for mutagenesis is N-ethyl-N-nitrosourea (ENU), which creates a greater chance of an A/T base pair being altered to a G/C base pair. However, the ENU mutagen is much more toxic to the worms than EMS. This creates the necessity to determine a balance between concentrations that are too toxic versus concentrations that are too low to induce mutations.[27]

Even though the mutations can occur on any gene and in multiple locations, there are generally three types of second generation progeny (F2) that can result from the mutated first generation: F2 without any mutations on both homologs, F2 with mutations on only one of the homologs, or F2 with mutations on both homologs. Amongst the F2 progeny of an F1 hermaphrodite carrying a mutation, the F2 will include 50% homozygous wild type and 25% each for the offspring with the homozygous or heterozygous mutations.

Although the F2s may all have different genotypes, the phenotypic differences between them may not be significantly noticeable. Therefore, they must be tested and screened to distinguish F2s with mutations and F2s without mutations. In our case, the screening of mutated *C. elegans* will consist of exposing the F2s to *C. albicans* for an extensive amount of time. Since *C. albicans* is toxic to the worms, the F2s that survive should include homozygous recessive mutants and homozygous or heterozygous dominant mutants.

2.3.2 Screening for Mutated *Caenorhabditis elegans*

In order to screen for resistance to infection by *C. albicans*, F2 progeny of mutated worms would be transferred onto a plate containing yeast and any that survive would be transferred back onto recovery *E. coli* plates. After initial screening, only one worm would be placed on each recovery plate to segregate mutant offspring. This allows for easier testing of one mutant strain at a time since hermaphrodite *C. elegans* self-fertilize. Surviving worms would later be transferred back onto yeast plates for a second screening, to ensure that they are in fact resistant to the infection and have retained that resistance throughout multiple generations. Once a successfully mutated *C. elegans* able to survive in the presence of *C. albicans* has been isolated, the mutant phenotype can be characterized, the mutation mapped to a region on a chromosome, and the gene identified by genome sequencing.

2.4 Conclusion

In this research, *Caenorhabditis elegans* were mutated with either EMS or ENU mutagens and exposed to *Candida albicans* to determine if any mutations are able to confer resistance to the fatal yeast infection. Clones of the mutated worms were then retested to confirm their resistant properties to the *C. albicans* infection before they were studied further. Over 40 mutants were isolated and 13 putative resistant mutants were identified by additional survival screening. Investigation of what mutations cause these putative mutants to be resistant will allow us to further understand the mechanism of resistance to this yeast infection.

3. Materials and Methods

The overall research procedures are summarized below. The details on how each material was made are described in Appendix A.

3.1 Labeling and Naming of *Caenorhabditis elegans*

All of the plates with worms were labeled using the following system. First was the mutation number or wild type (WT) name. The WT genome is referred to as N2, while the mutation batches are labeled as M1, M2, etc. Next is the strain number. If some worms from that mutation batch survived a test on the yeast plates and were recovered, they received their own strain number. The numbers follow as S1, S2, S3, etc. Next is the test number or the number of times that specific strain has been on yeast (T1, T2, T3, etc.), starting with T0 if they have not been exposed to yeast. Finally, the date on which the worms were plated on the plate was recorded to keep track of their life cycle. An example would be the first mutant found on mutation batch 2 would have the label M2S1 T1 since it was only exposed to the yeast once.

3.2 Maintenance of *Caenorhabditis elegans*

Three N2 *C. elegans* were placed onto a 60 mm x 15 mm *E. coli* spotted NG agar plate with a worm pick, which consisted of a platinum wire attached to the end of a glass Pasteur pipette, that allows individual worms to be picked up and transferred. The plates were then placed into a 22°C incubator and observed. The process was repeated once the *E. coli* spotting was close to being depleted or after multiple generations of the N2 *C. elegans* progeny (3-5 days).

3.3 Mutagenesis of *Caenorhabditis elegans*

Mutagenesis was performed on worms from at least five NG agar plates, each containing around 300 unsynchronized L4 stage worms as well as a mixture of other developmental stages. The worms were washed off the media plates with M9 buffer and placed in a 15 mL conical tube. The tube was then placed into a centrifuge and spun at 2,000 x g relative centrifugal field (RCF) for 1 minute before the supernatant was removed and the resulting pellet of worms was rinsed with more M9. The centrifuge process was then repeated and the supernatant was removed. The total volume inside the tube was then brought up to 2 mL using M9. Once 20 µL of 50 mM mutagen (either the EMS or the ENU) was added, another 2 mL of M9 was added, giving a total volume of 4 mL of M9 in the tube. The tube was then placed on a nutator to rotate for 4 hours at 20ºC to allow for the even distribution of EMS or ENU with the *C. elegans*. Afterwards, the solution was centrifuged again, the supernatant removed, and the pellet rinsed with M9. This process was repeated 3-4 times. Once the worms were sufficiently washed to remove the mutagen, they were placed onto NG agar plates containing *E. coli* and placed in a

22ºC incubator to recover. Due to the toxicity and carcinogenicity of these chemicals great care was taken when working with EMS or ENU. All work was done in a hood while wearing gloves and protective eyewear. All liquids or materials that were in contact with the mutagen were properly neutralized and discarded. EMS solution was neutralized using NaOH pellets and the chemical left to degrade for 4 days, while ENU was degraded at room temperature over 24 hours.

3.4 Testing Process for *Caenorhabditis elegans*

Two to three days of recovery after mutagenesis occurred on *E. coli* plates, the worms were tested by transferring about 300 *C. elegans* Po's plus F1's onto each 90 mm *C. albicans, E. coli,* and streptomycin spotted plates. The function of the *E. coli* was to provide the worms with some nutrition and the streptomycin prevents growth of bacterial contamination of the plates and the overgrowth of the *E. coli.* The worms were transferred to the yeast plates by washing the original plates with M9 buffer. All of the worms were transferred onto yeast plates regardless of generation and size. Plates containing N2 worms were also plated to act as a negative control to determine how long a non-resistant worm can survive on the yeast. Any worms that significantly survived longer than the number of days the N2 worms survived were considered successfully mutated and resistant to *C. albicans*. If a worm is able to survive past this bench mark, it was taken off of the yeast plate and placed onto its own *E. coli* plate and given a strain number.

After a few days when the strains have a chance to repopulate, 3 or 20 of the L4 stage *C. elegans* were re-plated onto 60 mm X 15 mm plates containing *C. albicans, E. coli,* and streptomycin for a second test. This process ensured that the worms that were thought to have a mutation resulting in resistance to *C. albicans* were not false positives.

3.4.1 Troubleshooting Problems with Overgrown *Candida albicans*

There were issues with the overgrowth of *C. albicans* on the plates. When testing the *C. elegans,* it was difficult to determine if the worms were alive due to the difficulty of seeing the worms. This problem was avoided by taking a sample of the yeast mixture from the plate using a worm pick and spreading it out on an empty plate. If the worms were motionless even when stimulated by the worm pick as illustrated in Figure 13 left image, then that plate can be searched for possible survivors. The arrow in the left image indicates a dead worm. However, if most worms end up moving around as illustrated in the right image of Figure 13, then the majority of the worms are still alive. An indication that they are moving around is a telltale trail that the worms leave when moving though the *E. coli,* as indicated by the arrow in the right image.

Figure 13: Troubleshooting Problems with Overgrown Yeast
Comparing Dead to Live *Caenorhabditis elegans* (32x)

The large amounts of yeast on the *C. albicans*-containing plates can be difficult to work with when looking for survivors. However, typically the worms that were alive were found moving around on top of the yeast and were visible under the microscope.

3.5 How to Determine if *Caenorhabditis elegans* are Healthy

A worm was considered healthy if it grew to a normal adult size, was able to reproduce, and was constantly moving about in its environment. The unhealthy worms were seen as scrawny and sluggish moving individuals. The dead worms did not move or respond to stimulation by a worm pick. Images of the three different classifications of worms are seen in Figure 14 below.

Figure 14: Images of Healthy, Unhealthy, and Dead N2 *Caenorhabditis elegans* Respectively (264x)

3.6 Methods for Retesting Candida Resistance

The retests were performed by taking 3 or 20 of the healthiest looking L4 worms from the maintained recovery plates. The worms were counted every few days using a grid system. A lid containing 24 drawn out squares, as shown in Figure 15, was placed over the plate and the number of worms in one of the squares was counted and then multiplied by 24 to get an estimate of the number of healthy worms on the entire plate.

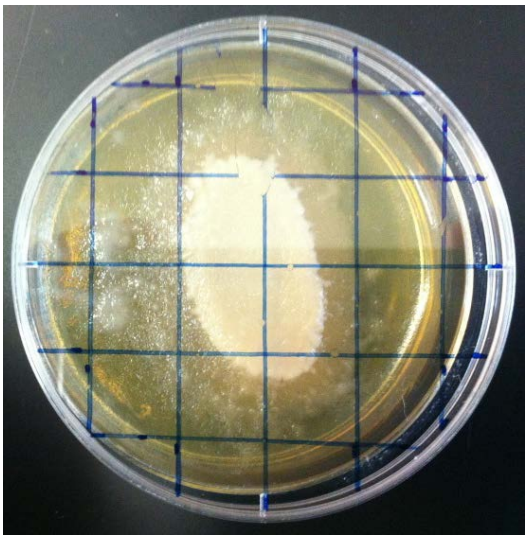

Figure 15: Grid Counting System used to Determine Approximate Number of *Caenorhabditis elegans*

4. Results

4.1 First Survival Screens

Nine different batches of *C. elegans* were mutated using either EMS or ENU, allowed to recover for 2-3 days on *E. coli* containing plates, then tested for survival on *C. albicans, E .coli,* and streptomycin-containing media plates. When most of the worms had died and only few healthy survivors remained, those survivors were allowed to recover and multiply on NG agar plates before retesting to ensure the worms were resistant to the *C. albicans*. All generations of *C. elegans* were transferred from the recovery plates to the yeast test plates.

After the first survival test (T1) of the nine mutation batches, survivors were identified, isolated, and labeled. Table 1 shows the mutation number along with the mutagen used, number of worms tested, and the isolated strains after survival test 1. All of the raw and normalized data throughout the results are located in Appendix B.

Table 1: *Candida albicans* Survivor Information for Mutated *Caenorhabditis elegans* in Survival Assay Test 1

Mutation Number	Mutagen Used	Approximate Number of *C. elegans* Tested	Survivors after Test 1
1	EMS	3,300	M1S1, M1S2, M1S3, M1S4, M1S5, M1S6, M1S7, M1S8
2	EMS	84,700	M2S1, M2S2, M2S3, M2S4
3	EMS	40,000	M3S1, M3S2
4	EMS	62,500	M4S1, M4S2, M4S3, M4S4
5	EMS	57,600	NONE
6	ENU	45,000	M6S1, M6S2, M6S3, M6S4, M6S5, M6S6
7	ENU	61,000	M7S1, M7S2, M7S3, M7S4, M7S5, M7S6, M7S7
8	EMS	31,500	M8S1, M8S2, M8S3, M8S4, M8S5, M8S6, M8S7, M8S8
9	ENU	46,700	M9S1, M9S2, M9S3, M9S4

Using the number of *C. elegans* that were mutated and screened along with the total number of T1 survivors, the percent survival was calculated for each mutagenesis. The average percent survival for all mutations is compared to the average for EMS mutations and the average for ENU mutations. Standard deviations were also calculated for easy comparison of the averages; all this information is shown below in Table 2.

Table 2: Average Percent Survival and Standard Deviation of EMS and ENU Mutations Compared to All of the Mutations

Mutagen	EMS	ENU	All Mutations
Average Percent Survival	0.0473%	0.0111%	0.0099%
Standard Deviation	0.0960%	0.0024%	0.0740%

The percent survival for each individual mutagenesis batch is graphed below in Figure 16, showing the difference in survival rate between mutations. While mutagenesis 1 is a clear outlier, the rest of the survival rates are similar. With the exception of mutagenesis 1, all survival rates fall within the error bars of the other points.

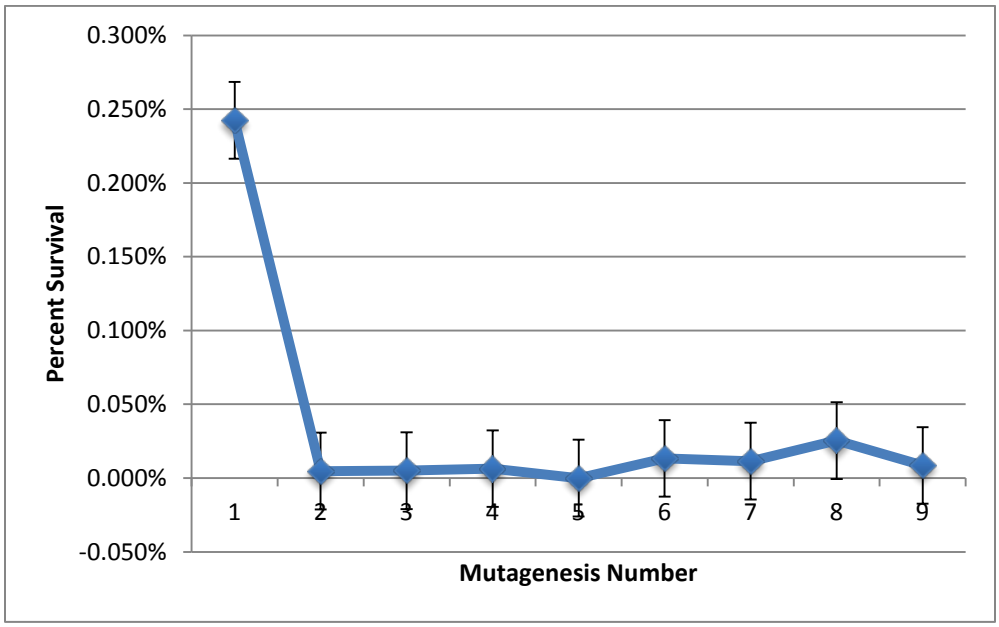

Figure 16: Percent Survival Rates for Each Mutagenesis
Error bars show 1 Standard Deviation

4.2 Second Survival Screens

After the first survival screens, the 43 identified survivors were allowed to recover on *E. coli* plates and multiply before being tested again on *C. albicans*, *E. coli*, and streptomycin–containing media. They were tested for a second time (T2) to ensure that the identified survivors were truly resistant mutants to *C. albicans*. When performing the second survival test, L4 *C. elegans* were selected for retesting and the results are shown in the graphs below. After the second survival assay, there were 13 mutant *C. elegans* strains that stood out as being likely resistant mutants.

Worm counts for test 2 were normalized because different T2 tests of different strains started with varying numbers of *C. elegans*. Due to the differences in timing between mutagenizing different batches, secondary tests could not be conducted at the same time. By normalizing the data all of the graphs can easily be compared to each other. This was done by taking the number of worms that were counted each day and dividing it by the number of worms that started out on the plate.

The average number of offspring of N2 *C. elegans* was determined by plating 20 L4 *C. elegans* on *C. albicans*, *E. coli*, and streptomycin-containing media. The average number of healthy worms, classified by their movement, size, and eventually their ability to grow, was counted every few days. The graph showing the survival counts of the N2 worms is illustrated in Figure 17 below. The data was normalized by dividing the number of worms counted each day by the initial number of worms on the plate.

Figure 17: Number of N2 *Caenorhabditis elegans* on *Candida albicans* Plates over Time

The peaking of the number of worms is seen when new worms were born and looked healthy before they were starved from the lack of *E. coli* and the inability to ingest the *C. albicans*. Overall, the infection and dying process of N2 worms on the yeast plates took about 17 days.

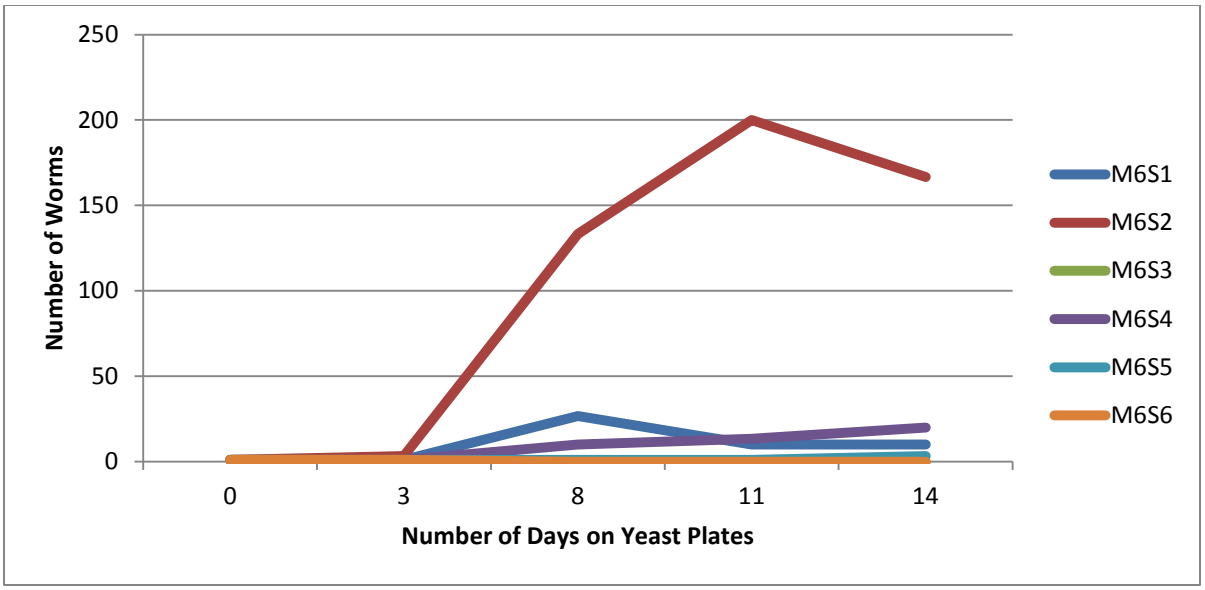

Figure 18: Number of *Caenorhabditis elegans* from Mutation 6 on *Candida albicans* Plates over Time

The graph in Figure 18 shows the number of original *C. elegans* and the offspring that remained healthy in mutation batch 6. Out of the six strains collected from test 1 only one strain, M6S2, looked to be a probable resistant mutant due to the larger number of worms that remained healthy.

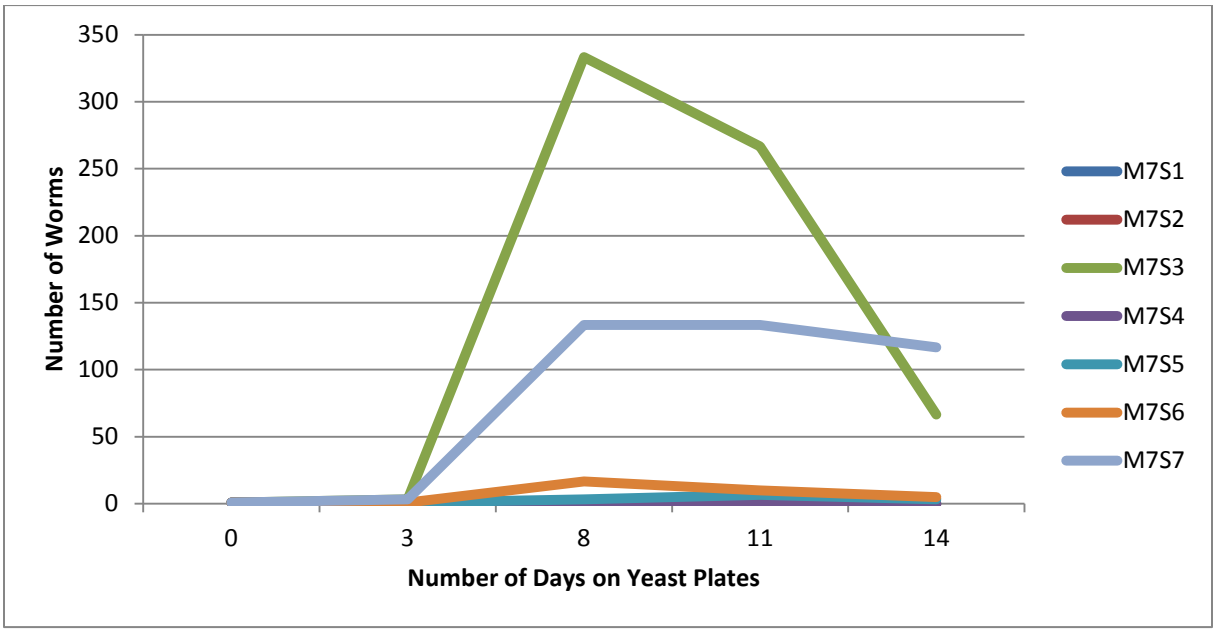

Figure 19: Number of *Caenorhabditis elegans* from Mutation 7 on *Candida albicans* Plates over Time

The graph in Figure 19 shows the number of original *C. elegans* and the offspring that remained healthy in mutation batch 7. Out of the seven strains collected from test 1 only two strains, M7S3 and M7S7, looked to be probable resistant strains.

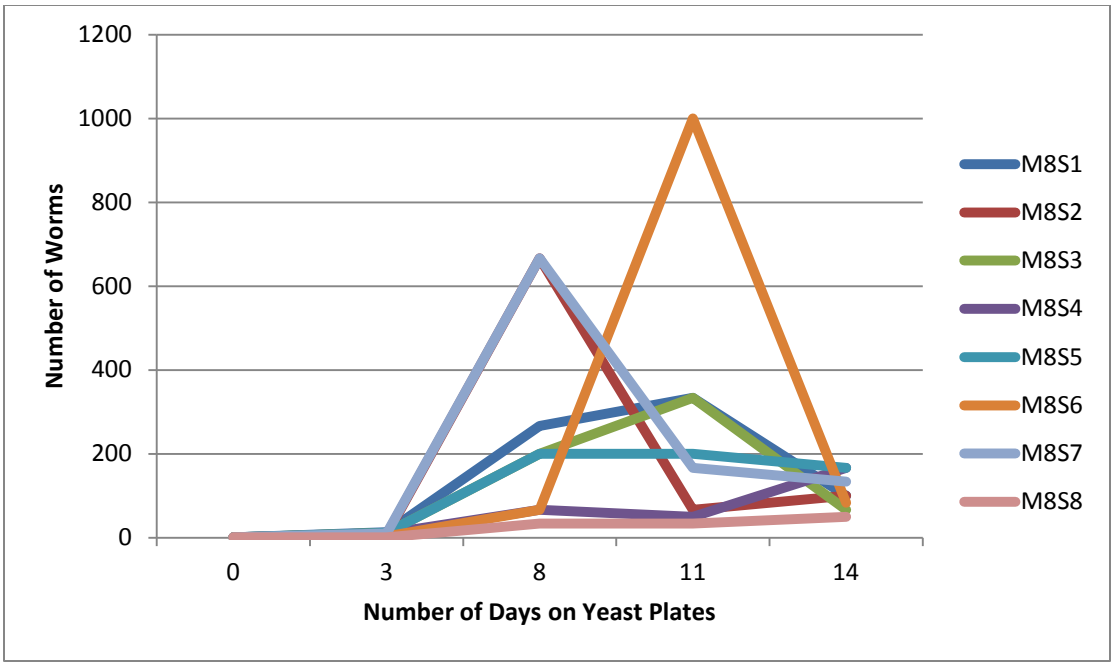

Figure 20: Number of *Caenorhabditis elegans* from Mutation 8 on *Candida albicans* Plates over Time

The graph in Figure 20 shows the number of original *C. elegans* and the offspring that remained healthy in mutation batch 8. The results gathered were inconclusive. Therefore, the strains were considered survivors were based off of their morphology seen on the plates on the last day of testing with the focus on healthy L4 worms. Using this classification, the strains that were considered survivors are M8S1, M8S3, M8S5, M8S6, and M8S8.

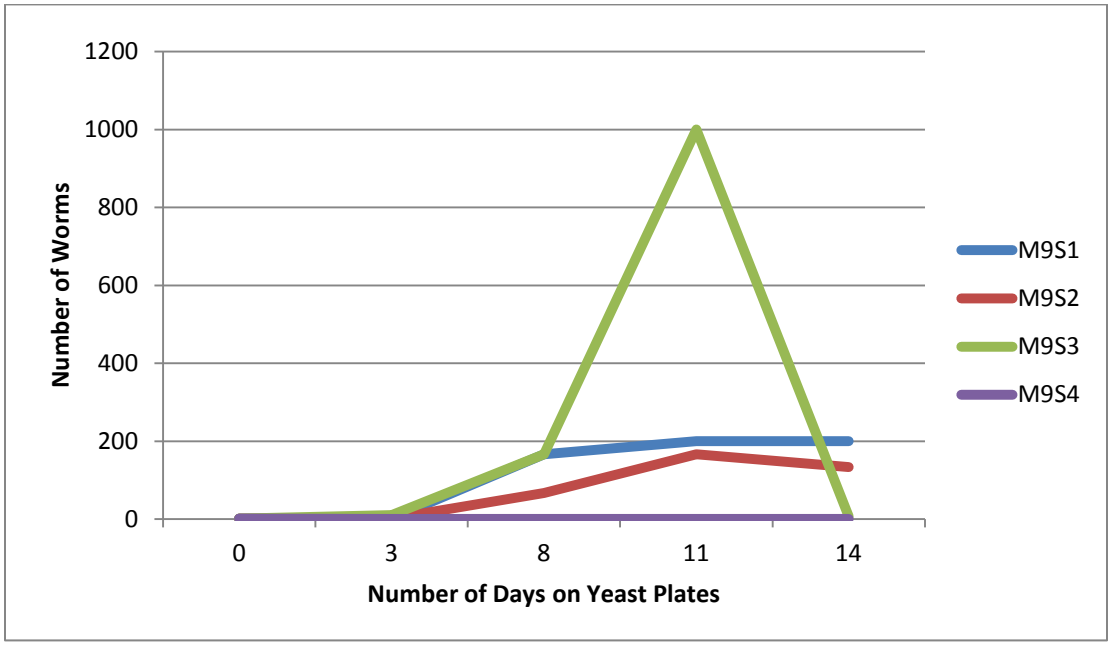

Figure 21: Number of *Caenorhabditis elegans* from Mutation 9 on *Candida albicans* Plates over Time

The graph in Figure 21 shows the number of original *C. elegans* and the offspring that remained healthy in mutation batch 9. Out of the four strains collected from test 1 only two strains, M9S1 and M9S2, looked to be probable resistant mutants. All likely survivor strains after test 2 are listed in Table 3 below.

Table 3: Survivor Information for Mutated *Caenorhabditis elegans* from Survival Assay Test 2

Mutation Number	Mutagen Used	Survivors after Test 2
1	EMS	M1S2, M1S3
2	EMS	NONE
3	EMS	NONE
4	EMS	M4S1
5	EMS	NONE
6	ENU	M6S2
7	ENU	M7S3, M7S7
8	EMS	M8S1, M8S3, M8S5, M8S6, M8S8
9	ENU	M9S1, M9S2

4.3 Third Survival Screen on Three Putative Mutants

Due to timing, out of the 13 mutant *C. elegans* strains, only three were able to undergo a third survival assay: M1S2, M1S3, and M4S1. These three mutant worms underwent testing along with a N2 control. Figure 22 shows the three putative mutants compared to N2 *C. elegans*. The arrows indicate changes in the morphology, specifically the extended or distended lumen. These morphological changes were seen in the majority of the worms in that strain.

Figure 22: Three Putative Mutants Compared to N2 *Caenorhabditis elegans* (*264x*)

The putative mutants that survived the T2 were taken off their respective plates and allowed to recover. The number of healthy worms in one grid section for each plate was counted every few days, multiplied by the number of grid sections on the plate, normalized for easy comparison, and graphed in Figure 23. The graph shows the number of original *C. elegans* and the offspring that remained healthy over time.

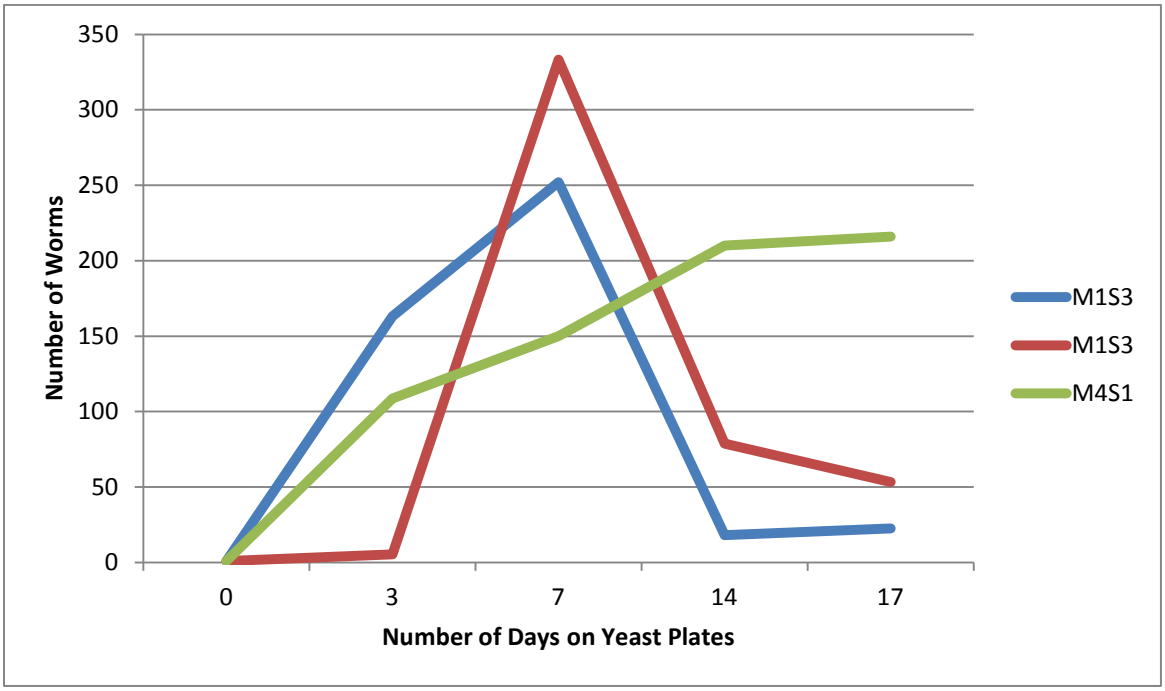

Figure 23: Number of *Caenorhabditis elegans* from T3 on *Candida albicans* Plates over Time

As the graph illustrates, most of the worm strains reproduced and peaked around day 7 before starting a steady decline of healthy worms. However, M4S1 continued a steady growth in the number of healthy worms on the plates.

5. Discussion

5.1 Mutagenesis of *Caenorhabditis elegans*

After mutagenizing *C. elegans* using either EMS or ENU, the mutants were exposed to *C. albicans, E. coli,* and streptomycin-containing media in multiple survival assays. Both types of mutagens created point mutations by switching purine base pairs to pyrimidines or vice versa. These mutagens were selected because point mutations are much less likely to be severely detrimental to the organism than frame-shift mutations, which causes insertions or deletions of nucleotides. This can completely disrupt the transcription of the entire gene by prematurely stopping the transcription of the gene or altering the entire amino acid sequence of the protein because all the codons after the mutation are disrupted. EMS and ENU induce point mutations that change the identity of a single nucleotide that can either alter a single amino acid used when translating the gene or alter regulatory protein and RNA binding affinity to the gene. Both mutagens will change a base to pair with the opposite type of base – for example nucleotide A would be chemically altered to pair with nucleotide G instead of nucleotide T. The DNA replication that takes place during cell division would insert a different base opposite the altered one. Effectively, an A/T base pair has been changed to a G/C base pair. EMS changes G/C pairs to A/T, while ENU does the opposite, changing A/T pairs to G/C using the same mechanism. Due to the stress that mutagenesis causes, worms were allowed to recover on *E. coli* plates after mutagenesis before testing on yeast plates.

Due to the uncertainty of what gene, when mutated, can confer resistance to *C. albicans* infections, mutation was not directed. The overall frequency of mutation for EMS is $3x10^{-4}$ per gene per genome. We cannot compare this frequency directly to the apparent frequencies we obtained because the only relevant counts were the numbers of F1s. This is because the F1 generation contains each mutation in a single individual; thus the number of mutant F1s most directly reflects the observed mutation frequency. In our mutagenesis experiments, we tested mixed generations of offspring of mutagenized worms, so they were not all from the F1 or F2 generation.

5.2 First Survival Screens Analysis

Once the *C. elegans* were mutated, the primary test was done to serve two purposes. The first purpose was to eliminate the non-mutant *C. elegans* from the mutant ones and this was visible when most of the worms were dying at around the same time as the control N2. The second purpose was to isolate the mutants that displayed a resistance to the *C. albicans*. This was determined when the worms were alive and healthy on the yeast plates for an extended period of time, sometimes greater than one month. This would ensure that the supply of *E. coli* would be completely depleted in a short period of time due to the streptomycin, forcing the worms to rely on ingesting the *C. albicans*. The mutated, non-resistant *C. elegans* would be eliminated, thereby leaving the *C. albicans* resistant *C. elegans* for isolation.

After survival test 1, 43 resistant mutants were isolated. Some of the survivors selected from mutagenesis 1 were not resistant to the yeast infection, contributing to the apparent high survival frequency from that mutation batch. The survival frequency of mutation 1 was calculated to be 0.2424% and when compared to the survival frequency for the rest of the mutations shown in Figure 16, it is the only outlier. However, this data was still included in the calculation of the overall percent survival 0.0099%, shown in Table 2. This major difference is what caused the large standard deviation of 0.0740% for the overall survival rate and for the EMS survival rate. The reason for this discrepancy is likely that in the first mutagenesis, the mutant worms were selected and isolated too soon so they could have been sick worms instead of resistant mutant *C. elegans*. After secondary testing, it was observed that most of the mutagenesis 1 survivors were not truly resistant and the duration of the first test was increased for future mutation batches. As shown in Figure 16, subsequent mutations 2 through 9 had consistent survival rates.

5.3 Second Survival Screens Analysis

There were several reasons why a strain selected after test 1 may not be a resistant mutant. Young worms are too small to ingest the *C. albicans*, and therefore can be in the presence of the yeast without being infected. A mutant may not have ingested a significant amount of yeast on the first test plate, thereby allowing it to survive even though it was not resistant to the infection in the first place. Additionally, a worm that was infected and had not begun to show significant symptoms could have been selected as a survivor. A worm could also be slightly resistant to the infection, allowing it to survive longer than the others but not enabling it to endure prolonged exposure to the *C. albicans*. Some mutations may confer resistance but also inhibit other functions. Therefore, secondary tests were performed to ensure that the worms were resistant to the *C. albicans* and could survive and reproduce in the presence of yeast for an extended period of time. After test 1, the 43 initial survivors were allowed to recover on NG agar plates before secondary testing.

The basis of how putative mutant worms were selected from the second survival assay to be further deemed as potentially having resistant genes to the yeast was based on the number of healthy worms on the plates. The health of the *C. elegans* was based on their movement and stage of life. After a certain number of days, if the *C. elegans* are at the L4 or higher life stage, has good movement that is comparable to N2, appears healthy, and is fairly thick in diameter the worms are deemed as further possible candidates that may possess a *C. albicans* resistant gene. However, in mutagenesis 7, shown in Figure 19, there were two *C. elegans* that seemed to be good mutants: M7S3 and M7S7. Because the yeast plates still contained a lot of *E. coli*, these worms may not truly be mutants and will require further testing. The large amounts of *E. coli* potentially allowed the worms to live longer on the plates and to avoid contact with *C. albicans*. For mutagenesis 8, shown in Figure 20, the numerical data were inconclusive, so the strains that were selected as putative mutants were based on their morphology and behavior. After secondary testing, only 13 of the 43 original mutants survived. These mutants were allowed to recover

before a third and final test. As a note, mutation batches 1-5 were not graphed due to the lack of data. For strains that appeared to be dying early in the test, the number of worms on the plates was not recorded.

5.4 Third Survival Screens on Three Putative Mutants Analysis

Due to time constraints, at one point in the testing only three mutant strains survived test 2 and were able to be tested a third time. The three putative mutants and N2 worms were plated on yeast plates at the same time and were counted every few days. Figure 23 shows that N2 control along with M1S2 and M1S3 reproduced and their progeny numbers reached a peak around day 7 before steadily declining afterwards. However, the total number of worms on the M4S1 plate steadily increased over the 17 days of testing, ending with a total of over 4,000 worms. The other two mutants were under 1,000 and the N2 control had a total of 192 living worms after 17 days of yeast exposure. Therefore, the M4S1 mutant was identified as the mutant with the greatest ability to reproduce in the presence of yeast. Upon examining the photos in Figure 22 comparing the three mutants to a N2 worm, the morphological symptoms of infection are more evident in the M1S2 and M1S3 mutants than in the M4S1. *C. elegans* infected with *C. albicans* often exhibit an enlarged intestinal lumen, indicated by swelling throughout the center of the worm. While the M1S2 and M1S3 mutants have this characteristic swelling of the infection, the M4S1 mutant does not show this symptom. In fact, it more closely resembles the N2 worm shown in Figure 22 than the other two mutants. This lack of swelling combined with its superior survival numbers indicates that the M4S1 mutant has a strong resistance to infection and is decidedly the best mutant from all three of the putative mutants that underwent test 3. For mutant M4S1, there was no data analysis for the second survival test because the data collected was lost. However, the mutant strain was still tested on three survival assays and the data collected for the third screening was still significant in considering the yeast infection resistant ability of the mutant strain.

5.5 Conclusion

The goal of this research was to isolate mutant strains of *C. elegans* that may contain a mutation that confers resistance against *C. albicans* infection. The generation of the *C. elegans* progeny was disregarded for the second and third assays since the *C. elegans* are hermaphrodites and each progeny has a genetically identical genome as the parent worm. Instead, the main consideration was ensuring that the initial *C. elegans* plated for test 2 and test 3 were L4 or higher life stages where they are able to reproduce and ingest and be infected by *C. albicans.*

After performing three rounds of tests on over 432,300 *C. elegans,* 13 different resistant strains were isolated. Out of those 13 mutants, specifically the three that underwent test 3, one particular mutant, M4S1, was considered the most probable resistant mutant strain due to its lack of physical symptoms of infection and high survival rates.

5.6 Recommendations and Future Applications

For future research on determining more possible mutant *C. elegans* that can confer resistance to *C. albicans*, liquid media could be used that will convert the yeast into it hyphal form. By using this form of the yeast, a more accurate study can be done in *C. elegans* that is more reflective of the physiological and morphological changes that occur in systemic patients.

We further suggest that more testing be performed on the remaining strains that survived test 2 to ensure resistance and to identify the most probable resistant mutant strains. These tests should be true survival tests in which survival of the original L4 worms put on the test plate is distinguished from their reproduction, and the growth of their offspring.

Subsequently through complementation tests and genome sequencing, the specific mutation that confers resistance can be identified. The identification of the mutated gene will show where and possibly how *C. albicans* normally affects *C. elegans*. Moving forward from this point, the function of the gene related to the resistance can be investigated and similar genes in other organisms, eventually humans, could be identified. The knowledge gained from this and other studies can potentially lead to the development of a drug to treat systemic Candidiasis.

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Appendix A: Recipes and Specifics for Materials and Methods

NG Agar Plates

Materials:

- 1L glass bottle
- Stir Bar & Plate
- 1000 mL graduated cylinder
- Repeating pipettor
- Aluminum foil
- Autoclave tape
- Sterile 1 mL pipettes
- Sterile 25 mL pipettes
- Pipetman

Chemicals:

- 3.0 g NaCl
- 2.5 g Peptone
- 17.0 g Agar
- Sterile 1 mL 1M $CaCl_2$
- Sterile 1M $MgSO_4$
- Sterile 25 mL 1M KPO_4
- Sterile 1 mL 0.5% cholesterol

Procedure:

1. Fill 1L glass bottle with 500 mL of dH_2O
2. Add a stir bar and then the NaCl and peptone
3. Pour the solution back into the graduated cylinder and add more dH_2O until the 975 mL mark is reached
    a. Note: ensure not to measure with the stir bar in the graduated cylinder
4. Pour solution back into the glass bottle
5. Add agar and mix well
6. Autoclave the mixture and the repeating pipettor that is wrapped in aluminum foil for 15 minutes at 121°C
    a. Note: Be sure to leave the stir bar in the bottle while autoclaving
    b. Note: When placing the autoclave tape on to the bottle, loosen the tops of the bottle to prevent explosions
7. Cool mixture down to 50°C and aseptically add the remaining chemicals
8. Using the repeating pipettor, 10 mL of the solution was dispensed into 60 mm X 15 mm Petri dishes
9. Rinse bottle and repeat pipettor with hot water

Yeast Peptone Dextrose (YPD) Agar Plates

Materials:

- 1L glass bottle
- Stir Bar & Plate
- 1000 mL graduated cylinder
- Sterile 30 mL pipet
- Pipetman
- Autoclave tape

Chemicals:

- 10.0 g Yeast extract
- 20.0 g Peptone
- 20.0 g Dextrose
- 15.0 g Agar

Procedure:

1. Fill 1L glass bottle with 1L of dH_2O
2. Add a stir bar and then all of the ingredients
3. Autoclave the mixture for 15 minutes at 121°C
    a. Note: Be sure to leave the stir bar in the bottle while autoclaving
    b. Note: When placing the autoclave tape on to the bottle, loosen the tops of the bottle to prevent explosions
4. When the solution is cooled down, dispense 30 mL of the solution into 90 mm Petri dishes using the pipet and the pipetman
5. Rinse bottle with hot water

LB Media

Materials:

- 500 mL glass bottle
- 100 mL glass bottles
- Stir Bar & Plate

Chemicals:

- 2.0 g Tryptone
- 1.0 g Yeast extract
- 1.0 g NaCl
- Sterile 80 µL 5N NaOH

Procedure:

1. Add 150 mL dH_2O and all the ingredients to 500 mL glass bottle and stir until dissolved
2. Bring up to a final volume of 200 mL and dispense 50 mL into 100 mL glass bottles
3. Autoclave immediately

YPD Media

Materials:

- 1L glass bottle
- 100 mL glass bottles
- Stir Bar & Plate
- 1000 mL graduated cylinder

Chemicals:

- 10.0 g Yeast extract
- 20.0 g Peptone
- 20.0 g Dextrose

Procedure:

1. Add 1000 mL dH_2O and all the ingredients to 1L glass bottle and stir until dissolved
2. Dispense 50 mL into 100 mL glass bottles
3. Autoclave immediately

OP50 *E. coli* Spotting and Streaking

Materials:

- OP50 *E. coli* streaked plate
- Sterile metal loop
- NG agar plates
- Pipette bulb
- Bunsen burner
- Sterile Pasteur pipette
- LB agar plates
- Parafilm

Chemicals:

- Sterile 50 mL LB media

Procedure:

1. Aseptically removed one colony from an OP50 *E. coli* streaked plate and place in a bottle containing 50 mL LB media whose bottle opening was flamed
2. Place the bottle into a shaker incubator overnight at 37°C to allow the bacteria to grow
3. After 24 hours, 2-3 drops of the liquid *E. coli* solution was placed into each of the N2 agar plates using a sterile Pasteur pipette
4. To maintain a fresh stock of OP50 *E. coli*, a new LB agar plate was streaked by removing a single colony from the old OP50 *E. coli* streaked plate with a flamed loop and zig-zagged over an area of the plate at room temperature for isolation
5. The zig-zagged plate was placed into a 37°C incubator overnight to allow a lawn to grow before it was removed and parafilmed for storage

***Candida albicans* Streaking and Spotting with OP50 *E. coli* and Streptomycin**

Materials:

- *Candida albicans* streaked plate
- *E. coli* streaked plate
- Sterile metal loop
- YPD agar plates
- Sterile conical tubes
- Bunsen burner
- Sterile Pasteur pipette
- Pipette bulb
- Parafilm

Chemicals:

- Sterile 50 mL YPD media
- Sterile 50 mg/mL Streptomycin
- Sterile 50 mL LB media
- Sterile M9 solution

Procedure:

1. Aseptically removed one colony from a *Candida albicans* streaked plate and place it in a bottle containing 50 mL YPD media whose bottle opening was flamed
2. Aseptically removed one colony from an *E. coli* streaked plate and place it in a bottle containing 50 mL LB media whose bottle opening was flamed
3. Place both bottles into a shaker incubator overnight at 37°C to allow the yeast and the bacteria to grow
4. After 24 hours, the inoculated media was removed from the incubator and 1 mL of the yeast solution was placed into a sterile conical tube
5. 9 mL M9 was then added to the conical tube to perform a 1:10 dilution and the mixture mixed
6. Afterwards, 3.3 mL *E. coli* inoculated LB media and 13.3 mL streptomycin was placed into the conical tube and the solution mixed
7. 3-5 drops of the liquid mixture was dropped onto the center of each YPD agar plates
8. To maintain a fresh stock of *Candida albicans*, a new YPD plate was streaked by removing a single colony from the old *Candida albicans* streaked plate with a flamed loop and zig-zagged over an area of the plate at room temperature for isolation
9. The zig-zagged plate was placed into a 37°C incubator overnight to allow a lawn to grow before it was removed and parafilmed for storage

1M $CaCl_2$, 500mL
73.5 g $CaCl_2 \cdot 2H_2O$
Add 450 mL dH_2O to $CaCl_2$ and stir until dissolved. Bring up to a final volume of 500 mL. Autoclave.

1M KPO_4 Buffer 1L
108.3 g KH_2PO_4 (monobasic, anhydrous)
35.6 g K_2HPO_4 (dibasic, anhydrous)
Add 800 mL dH_2O, pH to 6, and then bring to 1L. Autoclave.

1M $MgSO_4$, 500mL
123.2 g $MgSO_4 \cdot 7H_2O$
Add 450 mL dH_2O to $MgSO_4$ and stir until dissolved. Bring up to a final volume of 500 mL. Autoclave.

M9 Salt
10.5 g $Na_2HPO_4 \cdot 7H_2O$
3.0 g KH_2PO_4
0.5 g NaCl
1.0 g NH_4Cl
Add 800 mL dH_2O, pH to 7.2, and then bring to 1L. Autoclave.

0.5% Cholesterol in Ethanol
2.5 g cholesterol in 500 mL ethanol
Stir until dissolved and filter sterilize. Do not autoclave.

***Caenorhabditis elegans* Mutagenesis**

Materials:

- At least 5 plates of ~300 L4 worms on each plate
- Glass Pipettes and Bulb
- Sterile 2 15 mL conical
- Sterile 50 mL conical

Chemicals:

- Sterile M9 Solution
- 20 µL 50 mM EMS or ENU solution
- NaOH Pellets

Procedure:

1. Fill 50 mL conical with NaOH pellets to the 5 mL mark, any item or liquid that comes in contact with EMS must be placed into this container and discarded 4 days later
    a. This is only for EMS, for ENU, the chemical will degrade at room temperature over 24 hours.
2. Wash worms off of media plates with M9 and place in 15 mL conical tube
3. Centrifuge tubes at 2,000 RCF (3,000 rpm) for 1 minute, remove supernatant and rinse with M9, repeat centrifuge process
4. Remove supernatant and bring total volume up to 2mL
5. Add either the EMS or ENU mutagenic, making the final concentration 25 mM in a total volume of 4 mL
    a. $C_1V_1 = C_2V_2\ (2mL)x(50mM) = (4mL)x(X)\ \ X = 25mM$
6. Allow to rotate on a nutator for 4 hours at 20ºC
7. After 4 hours centrifuge down the sample, remove the supernatant and rinse with M9, repeat this step 3-4 times
    a. Ensure to dispose of any materials or liquids that could possibly contain the chemicals correctly
8. Once rinsed correctly, plate onto white plates containing *E. coli* and place in 22ºC incubator

Appendix B: Results Data

Table B 1: Number of *Caenorhabditis elegans* Tested and Number of Survivors and Survival Rate for Each Mutagenesis

Mutation Number	Mutagen Used	Approximate Number of Worms Tested	Number of Survivors	Percent Survivors
1	EMS	3300	8	0.2424%
2	EMS	84700	4	0.0047%
3	EMS	40000	2	0.0050%
4	EMS	62500	4	0.0064%
5	EMS	57600	0	0.0000%
6	ENU	45000	6	0.0133%
7	ENU	61000	7	0.0115%
8	EMS	31500	8	0.0254%
9	ENU	46700	4	0.0086%
Total		432300	43	0.0099%

Table B 2: Raw Data for Number of Wild Type N2 *Caenorhabditis elegans* on *Candida albicans* Plates over Time

Plate Name	Number of Worms: Day 0	Number of Worms: Day 3	Number of Worms: Day 7	Number of Worms: Day 14	Number of Worms: Day 17
N2 T1-1	20	3024	6000	312	192
N2 T1-2	20	3120	5928	240	72

Table B 3: Averaged and Normalized Data for Number of Wild Type N2 *Caenorhabditis elegans* on *Candida albicans* Plates over Time

Strain	0	3	7	14	17
N2 Control	1	153.6	372	13.2	6.6

Table B 4: Raw Data for Number of *Caenorhabditis elegans* from each Strain for T2 of Mutations 6-9 on *Candida albicans* Plates over Time

Strain	Day 0	Day 3	Day 8	Day 11	Day 14
M6S1	3	3	80	30	30
M6S2	3	10	400	600	500
M6S3	3	3	0	0	0
M6S4	3	3	30	40	60
M6S5	3	3	3	3	10
M6S6	3	3	0	0	0
M7S1	3	3	0	0	0
M7S2	3	3	1	1	0
M7S3	3	10	1000	800	200
M7S4	3	3	0	0	0
M7S5	3	3	10	20	10
M7S6	3	3	50	30	15
M7S7	3	10	400	400	350
M8S1	3	40	800	1000	300
M8S2	3	20	2000	200	300
M8S3	3	35	600	1000	200
M8S4	3	30	200	150	500
M8S5	3	35	600	600	500
M8S6	3	10	200	3000	250
M8S7	3	30	2000	500	400
M8S8	3	3	100	100	150
M9S1	3	3	500	600	600
M9S2	3	3	200	500	400
M9S3	3	30	500	3000	20
M9S4	3	1	1	1	1

Table B 5: Normalized Data for Number of *Caenorhabditis elegans* from Each Strain for T2 of Mutations 6-9 on Containing *Candida albicans* Plates over Time

Strain	0	3	8	11	14
M6S1	1.00	1.00	26.67	10.00	10.00
M6S2	1.00	3.33	133.33	200.00	166.67
M6S3	1.00	1.00	0.00	0.00	0.00
M6S4	1.00	1.00	10.00	13.33	20.00
M6S5	1.00	1.00	1.00	1.00	3.33
M6S6	1.00	1.00	0.00	0.00	0.00
M7S1	1.00	1.00	0.00	0.00	0.00
M7S2	1.00	1.00	0.33	0.33	0.00
M7S3	1.00	3.33	333.33	266.67	66.67
M7S4	1.00	1.00	0.00	0.00	0.00
M7S5	1.00	1.00	3.33	6.67	3.33
M7S6	1.00	1.00	16.67	10.00	5.00
M7S7	1.00	3.33	133.33	133.33	116.67
M8S1	1.00	13.33	266.67	333.33	100.00
M8S2	1.00	6.67	666.67	66.67	100.00
M8S3	1.00	11.67	200.00	333.33	66.67
M8S4	1.00	10.00	66.67	50.00	166.67
M8S5	1.00	11.67	200.00	200.00	166.67
M8S6	1.00	3.33	66.67	1000.00	83.33
M8S7	1.00	10.00	666.67	166.67	133.33
M8S8	1.00	1.00	33.33	33.33	50.00
M9S1	1.00	1.00	166.67	200.00	200.00
M9S2	1.00	1.00	66.67	166.67	133.33
M9S3	1.00	10.00	166.67	1000.00	6.67
M9S4	1.00	0.33	0.33	0.33	0.33

Table B 6: Raw Data for Number of *Caenorhabditis elegans* from Each Strain for T3 of Three Putative Strain on *Candida albicans* Plates over Time

Plate Name	Number of Worms: Day 0	Number of Worms: Day 3	Number of Worms: Day 7	Number of Worms: Day 14	Number of Worms: Day 17
M1S2 T3-1	20	3000	4800	480	480
M1S2 T3-2	20	3552	5280	240	432
M1S3 T3-1	18	96	6000	1296	960
M4S1 T3-1	20	2832	3600	2400	3000
M4S1 T3-2	20	1512	2400	3600	2664

Table B 7: Averaged and Normalized Data for Number of *Caenorhabditis elegans* from each Strain for T3 of Three Putative Strain on *Candida albicans* Plates over Time

	0	3	7	14	17
M1S3	1	163.2	252	18	22.5
M1S3	1	5.33333333	333.333333	78.6666667	53.3333333
M4S1	1	108.6	150	210	216