# The Genetics of Candida Infection in the Host Model *Caenorhabditis elegans*

A Major Qualifying Project Report

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# Abstract

Candidiasis is an invasive and potentially life-threatening health condition that can be triggered by *Candida Albicans* infection in immunocompromised individuals. The significant increase in incidence of Candidiasis of recent years and currently ineffective drug treatments have motivated research into the genetics underlying *Candida* infection and host immune responses. To identify gene mutations that may confer resistance to *C. albicans*, we utilized the nematode *C. elegans* as a host model due to its ease of use in lab and its susceptibility to *Candida* infection. Our resistance screens isolated 2 putative mutants that displayed consistently healthy phenotype, movement, and production of offspring when exposed to the yeast over a period of several days.

# Acknowledgements

We would like to thank Professor Mike Buckholt and Professor Sam Politz for advising this project and for all of their guidance and helpful input throughout the process.

We'd also like to thank Professor Gibson for giving us a demo on how to use the fluorescent microscope.

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# Section 1: Background

### 1.1: Introduction

In humans, *Candida albicans* infection has the potential to develop into a serious or even life threatening health condition known as candidiasis. Currently, 10,500-42,000 cases of candidiasis occur every year in the US (Pfaller and Diekema, 2007). The basis behind this shocking number lies in the huge population at risk for *Candida* infection as this population includes every person that can be classified as immunocompromised, including diabetics and HIV patients (Ausubel et al., 2011). In particular, a majority of candidiasis cases actually occur in hospital settings in people undergoing treatment with broad-spectrum antibiotics; in this situation alone, the mortality rate hovers between 45%-49% even with treatment, reflecting the emergence of antifungal-resistant *C. albicans* strains (Ausubel et al., 2011; Pfaller and Diekema, 2007). As such, the widespread occurrence and possibly life threatening nature of candidiasis makes research concerning the genetics underlying *Candida* infection and host response to such infection highly important to the field of medicine. To contribute to this body of research, our project focuses upon utilizing *C. elegans* as a model system to identify gene mutations that can confer resistance to *Candida* infections.

We chose to use *C. elegans,* a soil nematode, as a model system to study *Candida* infection due to its ease of use in the lab and several similarities this organism shares with humans (Gravato-Nobre and Hodgkin, 2005). Like humans, *C. elegans* is susceptible to *C. albicans* and, as such, can function as a working model of candidiasis, with most worms dying rapidly within a few days due to the infection (Ausubel et al., 2011). Therefore, infection of *C. elegans* by *C. albicans* can be used to screen for mutations in worm genes that allow for prolonged survival and, thus, confer resistance to yeast infection (Ewbank and Kurz, 2003). Research in this area has the potential to uncover information that may help in designing new, more effective treatments for *Candida* infection.

### 1.2: Anatomy & Life Cycle of *C. elegans*

*C. elegans* is a free-living nematode that feeds primarily on bacteria and lives in many temperate soil environments around the world (Atlun and Hall, 2009). The worm is covered by a tough exterior, known as the cuticle, to protect it from exposure to various dangers and potential pathogens. Like most nematodes, the body plan of *C. elegans* consists of an inner and outer tube, each of which consists of particular tissues and organs that make up the worm's alimentary, reproductive, excretory, nervous, and muscle systems (Atlun and Hall, 2009). These systems allow the animal to display many different behaviors including, "foraging, feeding, defecation, egg laying,

dauer larva formation, sensory responses to touch, smell, taste, and temperature" (Atlun and Hall, 2009). Additionally, the animal is able to participate in more complex behaviors such as "male mating, social behavior, and learning and memory" (Atlun and Hall, 2009). Thus, although the anatomy and body systems of *C. elegans* appear simple in comparison to other organisms, *C. elegans* is able to display and partake in many complex behaviors, making this nematode a useful model system.

This nematode is primarily hermaphroditic and, through self-fertilization, a single worm is capable of laying approximately 300 eggs, each of which develops into worms genetically identical to the parent (Atlun and Hall, 2009). Male *C. elegans* also exist, and mating between these worms and hermaphrodites can result in anywhere between 1200-1400 progeny that are genetically unlike their parents (Atlun and Hall, 2009). Following hatching, *C. elegans* undergoes four larval stages, denoted L1-L4 (Atlun and Hall, 2009). Upon completion of the L2 stage, it is important to note that *C. elegans* may enter a different, arrested stage during which they are known as dauer larva. Transition to this stage occurs if environmental conditions are not favorable and, during this stage, the animal completely halts feeding behaviors and little to no movement occurs (Atlun and Hall, 2009). Thus, this stage acts as a survival mechanism. Worms will exit this dauer state upon appearance of more favorable conditions, proceeding to L4 (Atlun and Hall, 2009). In favorable conditions at about 25°C, the time frame for normal larval growth is about 12 hours in L1, 7 in L2, 7 in L3, and 9 hours in L4 (Brenner, 1974). Thus, worms reach adulthood in about 3 days and adult worms typically live for about 3 weeks, a lifespan which facilitates ease of maintenance in a laboratory setting.

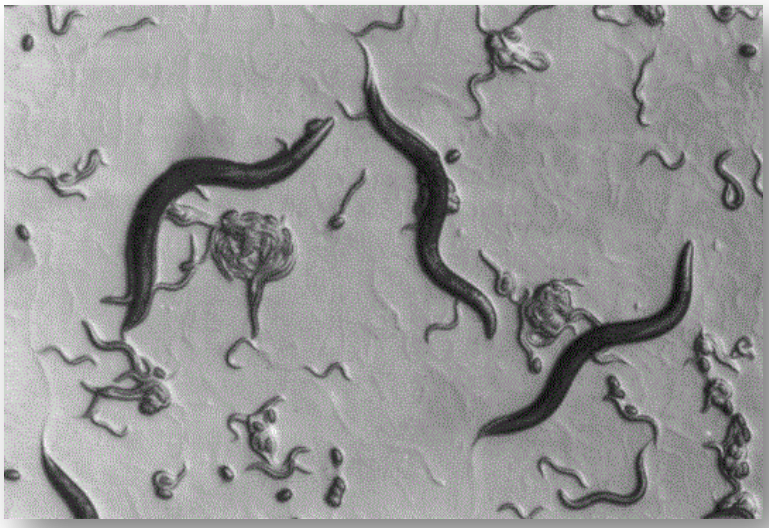


Figure 1. *C. elegans* in a laboratory setting. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Genetics], copyright (2001).

### 1.3: Use of *C. elegans* as a Model System in Research

Study and use of *C. elegans* as a model organism began in the areas of molecular and developmental biology in 1974 by Sydney Brenner (Brenner, 1974). This nematode makes a highly useful model system for reasons that only continue to grow as research on *C. elegans* progresses. Among some of its benefits, this animal is very small in size – approximately 1mm in length (A Short History of C. *elegans* Research). This small size facilitates easy laboratory storage in petri dishes. Additionally, *C. elegans* has a very short life cycle of about 3 days under optimal conditions (Atlun and Hall, 2009). This makes it possible to study several generations of offspring. Furthermore, since each hermaphroditic worm produces about 300 offspring through self fertilization, it is possible to maintain large worm populations in the lab on either solid agar or in liquid cultures. Other attractive qualities of this organism are its transparency, which allows for direct observation of internal structures, its compact genome, which is fully sequenced, and its completely mapped cell lineage (Atlun and Hall, 2009). Genes in *C. elegans* can also be easily mutated either through chemical mutagenesis or exposure to ionizing radiation (Atlun and Hall, 2009). In the succeeding decades since Brenner, use of *C. elegans* as a model to test various hypotheses and to elucidate unknown mechanisms has rapidly extended beyond just the areas of molecular and developmental biology. Other fields that utilize *C. elegans* as a model system include genomics, cell biology, neuroscience, aging, and innate immunity (Atlun and Hall, 2009).

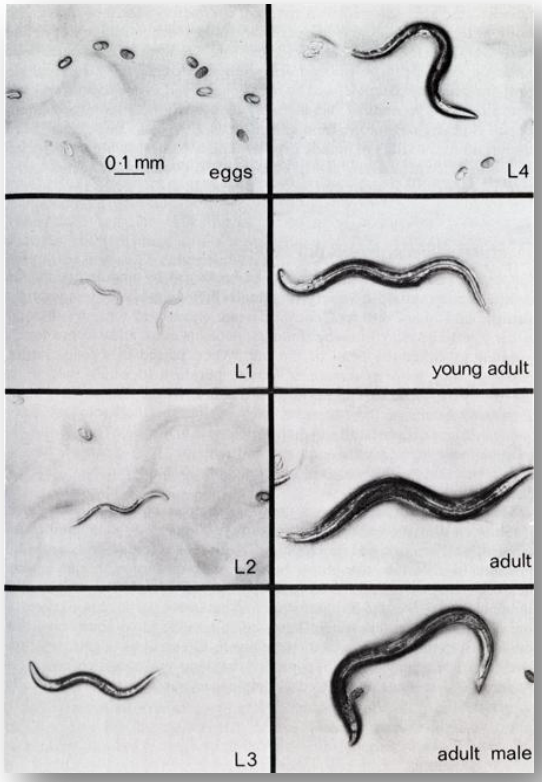



Figure 2. Life Cycle Stages of *C. elegans*
Taken from Kaeberlein & Sutphin, 2009.

### 1.4: The Immune System: Innate and Adaptive Immunity

Due to the constant challenge presented by pathogens in the environment, nearly all living organisms have developed some sort of strategy to defend themselves. This strategy, known in the form of the immune system, functions to recognize, eliminate, and in some cases, prevent reinfection of a particular pathogen. The two primary components of the immune system are innate and adaptive immunity, each of which responds differently to clear the host of infectious agents.

Innate immunity is thought to have evolved first, with the adaptive immune system developing later to augment it (Baish et al., 2003). As such, the innate immune system is present in both vertebrates and invertebrates where it functions to recognize and rapidly raise non-specific defenses in response to an invading pathogen. In contrast, adaptive immunity, only present in vertebrates, responds slowly to infection and utilizes highly specialized cells to rid organisms of pathogens and to retain an immunological memory of a specific pathogen (Murphy et al., 2008).

Although innate immunity developed long before adaptive, most current research in the field of immunology focuses upon adaptive immunity. The immunological memory of specific pathogens in adaptive immunity allows for the prevention of disease if host reinfection occurs and, thus, forms the basis for the development of vaccines. The clear importance of adaptive immunity to medicine is what initially drove researchers to overlook the innate immune system in favor of the adaptive (Ewbank and Kurz, 2003). However, with the discovery that innate immune mechanisms can initiate subsequent adaptive immune responses, immunologists have begun to expand research on innate immunity largely through the use of invertebrate models (Ewbank and Kurz, 2003).

### 1.5: *C. elegans*, a model organism for the study of innate immunity

In recent years, *C. elegans* has become an important invertebrate model in the study of innate immunity. Past research on innate immunity centered mainly on *Drosophila melanogaster*, and current use of *C. elegans* augments these studies. Use of *C. elegans* began with the discovery that this organism does not possess an adaptive immune system and that its innate immune system remains capable of successfully distinguishing between pathogenic attacks and possesses inducible defense systems to protect the host (Gravato-Nobre and Hodgkin, 2005). These defenses are critical as *C. elegans* normally lives freely in the soil, an environment which could expose the worm to various physical, chemical, or biological dangers (Gravato-Nobre and Hodgkin, 2005). Thus, the clearly inducible defense mechanisms in *C. elegans* together with the relative ease of caring for this organism in a laboratory setting make this organism a powerful tool for studying the role of innate immunity in pathogen-host interactions.

### 1.6: *C. elegans* strategies to avoid and prevent infection

Although not considered part of the immune system proper, *C. elegans* possesses an evasion strategy and several physical barriers that allow it to avoid interaction with pathogens in the surrounding environment. The evasive strategy in *C. elegans* utilizes a sophisticated navigation

system to respond to different environmental cues and either move towards a nutritious food source or away from possible noxious factors (Gravato-Nobre and Hodgkin, 2005). Furthermore, worms are able to detect the presence of pathogens even near its food source and can move away or cease ingestion (Andrew and Nicholas, 1976). In addition to this strategy, the worm possesses several physical barriers to prevent entry of pathogens. The major protective barrier between *C. elegans* and the environment is the tough, extracellular cuticle which covers its outer surface (Atlun and Hall, 2009). Although not completely effective against all types of detrimental agents, the cuticle is able to prevent adherence and subsequent entry of many pathogens that the worm comes into contact with on a regular basis. Additionally, *C. elegans* possesses a pharyngeal grinder, an internal barrier against pathogen entry that functions to break up pathogens that enter through the mouth of the worm (Ewbank and Kurz, 2003; Gravato-Nobre and Hodgkin, 2005). The importance of these physical barriers in preventing pathogen entry can be clearly seen in the susceptibility of certain mutants such as *srf-2/-3/-5*, *phm-2*, and *esp*, which have impaired functioning of either the cuticle or the pharyngeal grinder (Hodgkin et al., 2000; Kim et al., 2002). In comparison to wild type worms, these mutants experience decreased survival and poor fitness when exposed to certain pathogens.

**1.7: Characteristics of Innate Immune Response in *C. elegans***

In cases where pathogens manage to penetrate the worms' external defenses, innate immune mechanisms activate to eliminate the challenge. Of these mechanisms, several signaling pathways are known to function in *C. elegans* innate immunity. Currently, at least six different signaling pathways are known to exist in *C. elegans*: the p38 MAP kinase pathway, the programmed cell death (PCD) pathway, the TGF-B-like signaling pathway, the DAF-2/DAF-16 pathway, the ERK pathway, and the Toll pathway (Alegado et al., 2003; Atlun and Hall, 2009). Successful activation of one or more of these pathways leads to the production of effector molecules, which function to either directly destroy or inhibit the growth of a pathogen (Alegado et al., 2003). The genes and pathways that activate in *C. elegans* in response to infection have been found to differ based on the intruding pathogen. Thus, activation of signaling pathways and the genes upregulated in these pathways allow for rapid host defense against different types of pathogens.

The p38 MAP Kinase pathway is one of the major signaling pathways that takes part in the defense mechanisms of *C. elegans* (Kurz and Tan, 2004). Host response to pathogens by this pathway relies heavily upon the phosphorylation of PMK-1, the worm homologue of p38 MAPK, which can lead to the activation of antifungal and bacterial effectors; studies have elucidated 3 of these antifungal immune effectors to be fipr-22/23, cnc-4, and cnc-7 (Ausubel et al., 2011; Kurz and

Tan, 2004). Phosphorylation of PMK-1 has been found to be dependent upon the presence of TIR-1, making this element of the pathway likely to be upstream of PMK-1 (Kurz and Tan, 2004). Studies have also found that the interaction of TIR-1 with its partner RAB-1, another upstream element, is absolutely required for the induction of *nlp* genes when fungal infection occurs; these *nlp* genes code for neuropeptide-like proteins that have antimicrobial functions (Kurz and Tan, 2004). Additionally, the discovery that many members of this pathway are upregulated during *C. albicans* infection make the p38 MAPK pathway a likely candidate for mediating resistance against *C. albicans* and other fungal pathogens (Ausubel et al., 2011). Thus, the p38 MAPK pathway is an inducible signaling pathway whose primarily function is to increase host resistance to fungal pathogens by releasing antifungal effectors.

Two other pathways that function to increase host defenses in *C. elegans* are the DBL-1 and Toll pathways. The DBL-1 pathway is a homologue of the TGF-B signaling cascade in mammals and its primary functions include increasing resistance to bacterial infection as well as regulating worm body size and male tail formation (Kurz and Tan, 2004). The discovery that the DBL-1 pathway participates in resistance to bacterial infection came about due to the fact that mutant worms of the different elements of this signaling cascade were challenged by bacteria and were found to show a much lower resistance compared to WT animals (Kurz and Tan, 2004). Elements of this pathway include the DBL-1 ligand, the SMA-6/DAF-4 receptor kinases, and the SMA-2/SMA-3/SMA-4 cytoplasmic components (Kurz and Tan, 2004). Phosphorylation of these cytoplasmic components activates the transcription of target genes in the nucleus; target genes for increasing bacterial resistance include lys-8 (a lysozyme encoding gene), and genes that encode proteins possessing c-lectin domains which may assist in binding to pathogens (Kurz and Tan, 2004). Another pathway that helps to mediate resistance to pathogens is the Toll pathway. In contrast to the DBL-1 pathway, the Toll pathway does not increase host defenses by upregulating target genes. Rather, the Toll pathway plays a role in *C. elegans*' evasion strategy by keeping the nematode away from potentially harmful pathogens (Alegado et al., 2003). The discovery of the Toll pathway's role in *C. elegans* defense was made by observing a *tol-1* mutant, with the *tol-1* gene normally being part of the functional pathway, and finding that worms with this mutation possessed a behavioral defect that caused them to fail to avoid *S. marcescens*, a pathogenic bacteria that is typically avoided by wild type *C. elegans* (Alegado et al., 2003). Thus, the DBL-1 and Toll pathways both provide defense mechanisms – one with the upregulation of relevant genes and the other with behavioral changes - to protect *C. elegans* from infection.

Another major and well-studied signaling pathway in *C. elegans* is the DAF-2/DAF-16 pathway. This pathway normally regulates dauer formation and lifespan through the DAF-2 gene (Kurz and Tan, 2004). What makes this pathway interesting and the subject of many studies is the fact that loss of function of DAF-2 results in a long-lived phenotype and an increased resistance to gram negative and positive bacteria, suggesting a link between aging and immunity (Kurz and Tan, 2004). Additionally, knockout studies on this pathway have found that all phenotypes associated with the loss of function *daf-2* mutation can be completely suppressed by loss of function mutations in *daf-16*, a transcription factor and member of the pathway (Kurz and Tan, 2004). This seems to indicate that *daf-2* mutants display resistance to pathogens because DAF-16 is normally under negative regulation by DAF-2; thus, loss of function in *daf-2* de-represses DAF-16 (Ausubel et. al, 2003). Analysis of the target genes that are normally regulated by DAF-16 has revealed the following categories: genes coding for stress response (heat shock proteins, antioxidant enzymes, a detoxification enzyme), genes coding for putative antimicrobial proteins (lys-7, lys-8), and a gene encoding a chitinase (Kurz and Tan, 2004). As such, with upregulation of all of these groups of genes, it is not surprising that in *daf-2* mutants (in which DAF-16 is de-repressed) there is an increased resistance of *C. elegans* to pathogens. Thus, *daf-2* mutants present with abnormally long lifespans and increased resistance to infection by pathogens, indicating a link between aging and immunity in the DAF-2/DAF-16 pathway.

It is known that C. elegans mounts a rapid response towards pathogenic bacteria, and some studies have been done to characterize the immune response towards fungal pathogens. These studies use transcriptome profiling of nematodes during a *C. albicans* infection in comparison with transcriptome profiles of control, wild type worms (Ausubel et al., 2011). Such research has found that there is a clear, robust immune response to *C. albicans*, involving approximately 1.6% of the genome: 124 genes were upregulated 2-fold or higher and 189 genes were downregulated at least 2-fold (Ausubel et al., 2011). Of the upregulated genes, several were identified to be putative antifungal immune effectors or antimicrobial peptides, which have been found to have antifungal activity *in vivo* (Ausubel et al., 2011. Additionally, chitinase genes were found to be strongly induced by *C. albicans*; these genes are thought to code for enzymes that act against chitin-containing organisms, such as pathogenic fungi like *C. albicans* (Ausubel et al., 2011). Other genes that were highly upregulated during *C. albicans* infection included genes encoding secreted proteins, detoxifying enzymes, and intestinally-expressed proteins (Ausubel et al., 2011). This is of importance as *C. albicans* enters the nematode through the mouth and establishes infection within the intestine. These more common defense genes were also found to be upregulated in *C. elegans*

response to bacterial infection. However, as a whole, the majority of *C. albicans* induced defense genes did not overlap with genes upregulated in *C. elegans* during bacterial infection – this suggests that *C. elegans* can recognize *C. albicans* as a fungal pathogen and can mount a specific immune response that mainly upregulates antifungal defense genes and, to a smaller extent, common defense genes (Ausubel et al., 2011).

In addition to the upregulation of antifungal defense genes in *C. elegans*, a large number of genes are also downregulated during *C. Albicans* infection. Examination of these genes revealed a majority of them to be involved in binding to sugars or carbohydrates (Ausubel et al., 2011). Since bacterial cell walls contain sugar and carbohydrate components and these same genes were also upregulated in *C. elegans* during infection by different pathogenic bacteria, the downregulated genes were postulated to be "antibacterial defense effectors" (Ausubel et al., 2011). It is possible that this downregulation of unnecessary bacterial resistance genes when challenged by a fungal pathogen may be the result of an evolutionary tradeoff. This tradeoff would allow for a more focused immune response by *C. elegans* in order to produce specific antifungal effectors while inhibiting unneeded antibacterial defenses (Ausubel et al., 2011). Thus, *C. elegans* is able to differentiate between different groups of pathogens in order to mount a more specific and effective immune response to clear an infection.

### 1.8: *Candida Albicans*, an opportunistic fungus

*Candida albicans* is a commensalistic, diploid fungus that can naturally be found on the skin and mucosal surfaces of most humans. Under normal conditions, *C. albicans* exists as part of the natural flora present in the human digestive tract (Ausubel et al., 2011). However, this fungus has the potential to become pathogenic under altered circumstances such as a weakened host immune system or depletion of competing bacterial flora in the gut (Ausubel et al., 2011). In such cases, *C. albicans* can establish a superficial or more systemic infection, in which the yeast invades host tissues. The resulting fungal infection is classified broadly as candidiasis. The extent to which this infection is prevalent in the population can be seen by the fact that approximately 75% of


Figure 3. Morphology of *Candida Albicans*

Taken from Doctorfungus Corporation, 2000.

women will experience at least one case of *Candida vaginitis*, more commonly known as yeast infection; half of these women will experience recurring episodes throughout their lives (Ausubel et al., 2011). *Candida* infection in humans is not normally fatal; however, immunocompromised patients, such as AIDS patients, cannot mount a proper immune response to fend off the opportunistic fungi and these cases can have a 30 to 50% mortality rate (Ausubel et al., 2011; Lazzell et al., 2003). To exacerbate the situation, treatments for *Candida* infection have proven ineffective as this yeast has developed drug resistance to many of the common antifungal drugs currently in use (Anderson et. al, 2002). As such, with the incidence of drug-resistant opportunistic fungal infections on the rise, it is now more important than ever for research to focus upon *C. albicans* pathogenesis and host immune response to such infections (Anderson et. al, 2002).

### 1.9: Pathogenesis of *Candida Albicans* in *C. elegans*

Like most pathogenic fungi, *C. albicans* can exist in several different forms. As such, *C. albicans* possesses the ability to undergo "morphological transformation[s]" in order to exist as either yeast cells or hyphae, each of which contributes to pathogenesis in a host and which are thought to be triggered by different environmental conditions (Brown et al., 2002; Lazzell et al., 2003). Yeast cells are circular fungal cells that allow for dissemination of the fungus throughout the blood stream, thus enabling the pathogen to more easily spread and grow (Mylonakis et al., 2009). Following dissemination, the yeast cells can colonize mucosal surfaces at sites distant from point of entry and begin to multiply (Mylonakis et al., 2009). Hyphae are branchlike filamentous structures that also play an important role in host invasion and tissue destruction (Mylonakis et al., 2009). Studies indicate that in liquid media *C. albicans* undergoes a morphological change from yeast cells to hyphae; this fungal form is capable of piercing and destroying the nematode tissue, leading rapidly to death (Mylonakis et al., 2009). Although studies such as Mylonakis et al. have shown that hyphal formation is necessary for full virulent effect of *C. albicans* in *C. elegans*, yeast cells alone have proven to be sufficient to cause nematode death (Mylonakis et al., 2009).

### 1.10: Interplay Between *C. albicans* and the *C. elegans* Innate Immune System

During an active infection, *C. albicans* and the innate immune system of *C. elegans* interact with one another. This pathogen-host interaction pits a pathogen's virulence (as determined by several factors) against a host's defense mechanisms. In order for this interaction to occur, the host must first recognize that infection has occurred; in *C. elegans*, *Candida* infection has been known to primarily occur via ingestion of the yeast cells. Currently, studies show that *C. elegans* is able to recognize this infection by detecting factors associated with fungal virulence, such as the surface

molecules on a fungus, and by recognizing fungal PAMPs (pathogen-associated molecular patterns) (Alegado et al., 2003). PAMPs are conserved microbial molecules found in the cell wall of *C. albicans* which are usually recognized by mammalian neutrophils, monocytes, and macrophages (Alegado et al., 2003). In *C. elegans*, studies suggest that the majority of the nematode's transcriptional response to infection may be mediated through a Pattern Recognition Receptors system that allows for fungal PAMPs detection through pathogen sensing and signaling (Alegado et al., 2003). Transcriptome profiling experiments and expression analyses of infected nematodes demonstrate that factors involved in fungal virulence also elicit a transcriptional response in *C. elegans* (Ausubel et al., 2011). However, little is known about whether these factors are derived either from the pathogen or from the host (such as factors resulting from injured tissue) (Ausubel et al., 2011). In recent studies, Moyes et al. found that "human epithelial cells integrate *C. albicans* PAMPs via pattern recognition receptors together with ''danger signals'' perceived by the host during invasive fungal growth" (2010). The same integration is thought to occur in the nematode to recognize a ''pattern of pathogenesis'' that may be specific to fungal infection (Ausubel et al., 2011). In this way, *C. elegans* is able to identify a specific type of pathogen and mount an effective immune response.

### 1.11: Implications for Future Research

Studies dealing with the pathogen-host interaction between *C. elegans* and *C. albicans* and, in particular, the identification of genes in *C. elegans* that confer resistance to the nematode hold important implications for the fields of medicine and immunology. First of all, research using *C. elegans* as the model system can help to elucidate poorly understood aspects of human innate immunity. This stands to be the case because most research into human immunology focuses on adaptive rather than innate immunity; therefore, discovery of resistance genes that may have analogs in humans is of particular importance since *C. elegans* only possesses innate immunity. Furthermore, this type of research may uncover valuable information necessary in designing new treatments for *Candida* infection. This is critical because *Candida* infection has the possibility to develop into a life threatening condition, particularly in immunocompromised humans. Additionally, current treatments for yeast infection have not proven effective and new therapies are necessary to combat this disease. Thus, genetic approaches and further research into the pathogen-host interaction between *C. elegans* and *C. albicans* can lead to important developments in both the fields of medicine and immunology.

**1.12: Conclusion**

Overall, our project focuses upon identifying gene mutations in *C. elegans* that can confer resistance to infection by *C. albicans*. Identification of such mutations can be accomplished by performing screenings that will kill non-resistant worms while allowing resistant worms to survive. The ease of use of *C. elegans* in these screenings, as well as easy handling, storage, and susceptibility to *Candida* infection make this nematode a powerful tool in studying pathogen-host interactions and innate immune responses. In this case, our project allows us to examine whether mutations to genes involved in *C. elegans* innate immunity can function to make worms more resistant to *Candida* infection or whether any other extraneous genes have a similar effect. Research in this area is important due to the frequency of *Candida* infection in the population, its increasing resistance to current drug treatments, and the high rate of mortality in immunocompromised patients that develop candidiasis.

# Section 2: Methodology

### 2.1: Stock Maintenance

Stock plates of the N2 (wild type) strain were maintained at 20°C on NG agar dishes spotted with OP50 *E. coli* as the food source (Wood, 1988). Every five to six days, 3-4 L4 worms were transferred to fresh feeding dishes. Putative mutants isolated from screens were also kept at 20°C on NG agar feeding dishes.

### 2.2: Mutagenesis Procedure

To stably induce mutations in the *C. elegans* genome, mutagenesis was performed using the mutagen ethyl methanesulfonate (EMS). This mutagen produces mutations through nucleotide substitution at a rate of $5x10^{-4}$ to $5x10^{-2}$ per gene (O'Neil, 2006). Three days before mutagenesis, 3-4 N2 strain hermaphrodites at the L4 stage of the life cycle were transferred to five 60mm feeding dishes. EMS mutagenesis was carried out as described by Wood with minor changes (1988). During each spin down step in the procedure, centrifugation occurred at 20°C for 2 minutes with a speed of 1,600 rpm. Following the four hour wait period and 3-4X washing with M9, EMS-treated worms were resuspended in 800ul M9 and pipetted to four 90mm NG agar recovery plates at 100ul each. These recovery plates were made following the same instructions as for feeding dishes; however, 300ul of OP50 was spread over the plate using aseptic technique. EMS-treated worms were incubated overnight at 20°C.

### 2.3: Preparation of Mutants for Survival Assay

The day after mutagenesis, 3-4 gravid animals at the L4/young adult stage were transferred to ten 60mm feeding dishes. These $P_0$ worms were kept at 20°C for 3 days to allow the worms to produce F1 generation offspring. Worms were allowed to produce the F1 generation because some of these worms, if EMS mutation was successful, should have one copy of the mutation in their genome. Generally, such mutations are recessive (with only a small percentage being dominant) and, as such, a phenotype such as resistance to pathogen infection would not be visible for screening. Thus, all worms – F1 and $P_0$ - were transferred to ten 90mm recovery plates using a small volume (0.8mL per transfer) of M9 to prevent oversoaking the plates. The worms were kept at 20°C for 3-4 days to allow for the production of F2 generation offspring. Some of these F2 worms, the offspring of the F1 generation, should have two copies of a mutation in their genome; two copies of a recessive mutation would allow for a visible phenotype (behavioral change, resistance to yeast, etc). To begin the survival assay, all worms were then transferred to approximately 30 survival plates. Transfer of worms to these yeast plates was accomplished through use of small

volumes of M9 (0.8mL per plate). Survival plates were made by spreading a 1:100 dilution of SC5314 (*C. albicans*) on 90mm YPD plates. The 1:100 dilution was made by adding 100ul of an overnight culture of SC5314 to 900ul M9, and then transferring 100ul of this dilution to a second tube containing another 900ul M9. The overnight culture was kept at 30°C in the shaking incubator for 17 hours. Table 1 shows the weight and OD of the 1:100 dilution of an overnight SC5314 culture.

Table 1. OD & Average Weight of Overnight Culture

| OD at 600nm | Average Weight of Yeast Cells (g) |
|---|---|
| 0.03 AU (absorbance units) | 0.0024 g |

OD was determined through the use of a spectrophotometer at 600nm. This OD was taken for the 1:100 dilution of SC5314 overnight culture. The average weight of the yeast cells was determined by labeling four microfuge tubes 1-4 and weighing them on an analytical balance. These initial weights were recorded and microfuge tubes were then filled with overnight culture. These tubes were centrifuged at 13.2 rpm (x 1000) for 1 minute. The supernatant was then aspirated from each tube and a pipettor was used to take off the supernatant closest to the pellet. Microfuge tubes were reweighed and the average weight of the four pellets was determined.

**2.4: Primary Survival Assay**

The worms present on the survival plates were incubated at 20°C and observed daily via microscope to monitor general worm survival. Worm survival was monitored on a subjective basis by looking at the approximate amount of worms on a plate that could move without stimulus (without being prodded by the worm pick) and the amount of worms that were either dead or dying (did not move without stimulus). Initial selection of putative mutants began at day four when approximately half of the worms plated were dead or dying; this selection continued until the death of approximately all the worms on a certain plate. Worms were selected based on age (L4-adult stage since they have been on the plate longest), phenotypically based on healthy appearance (not-skinny or in the dauer stage), and ability to move non-sluggishly without stimulus as compared to the other worms on the plate. Figure 4 shows a healthy, non-dauer worm at the adult stage.

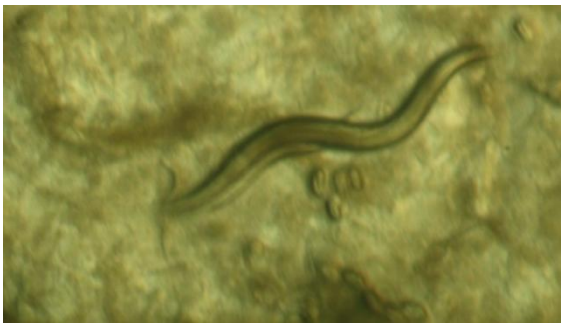


Figure 4. Phenotypic appearance of a selected putative mutant on a survival plate. Magnification Unknown.

In comparison to the healthy appearance displayed by the worm in Figure 4, Figure 5 shows the appearance of a worm that would not be chosen to conduct further putative mutant screenings.

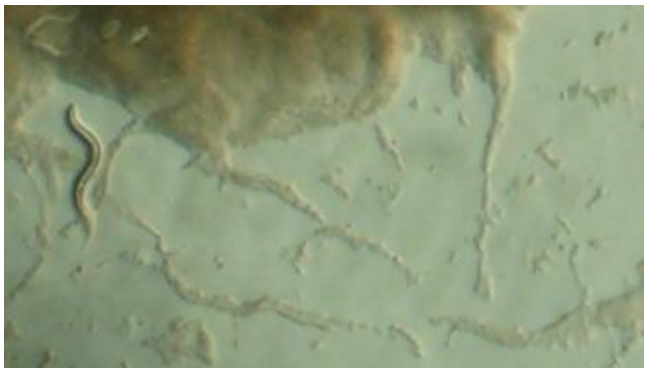


Figure 5. Phenotypic appearance of a non-healthy worm on a survival plate. Magnification unknown.

Figure 5 displays the phenotypic appearance of a worm that would not be selected to test for resistance to *C. albicans*. This worm, in addition to moving sluggishly on the survival plate, is not at the L4-adult stage and appears skinny. Following selection, worms were transferred one to a recovery plate and kept overnight at 20°C; the following day, these worms were transferred one to a feeding dish.

### 2.5: Screening Putative Mutants

#### 2.5.1: Secondary Survival Screens

Following selection of putative mutants, multiple survival screens were run in order to determine whether the putative mutants actually possessed mutations that conferred them with heightened resistance to *C. albicans* pathogenesis. Resistant worms were classified as those which (during exposure to *C. albicans*) produced a larger amount of offspring relative to wildtype,

managed to survive exposure longer than wildtype worms, displayed a relatively healthy appearance (not skinny and in the dauer stage) and exhibited relatively wildtype movement (not sluggish and moving without stimulus) even after several days on survival plates. Repeated screenings were necessary in this case to ensure validity of initial results. A healthy phenotypic appearance for worms in this screen can be seen in Figure 4 and can be compared to the phenotypic appearance of a non-healthy worm in Figure 5.

To accomplish these survival screens, putative mutants, each on an individual plate, were allowed to grow and produce offspring on feeding plates for approximately 3 days so that each plate consisted only of worms with the same genotype. Following this period of growth, 20 L4 worms from each plate were transferred to 60mm survival plates. At the same time, 20 L4 worms from the N2 stock were also transferred to an identical survival plate to act as controls. As such, putative mutants were observed daily via microscope by checking for movement, production of offspring, general appearance, and ability to survive (relative to N2 worms). These putative mutants were compared to the appearance and behavior of N2 worms plated on the survival plate. Resistant worms, as characterized previously, were chosen to repeat the screen and to confirm results.

### 2.5.2: Examining Resistance of Putative Mutants on *C. albicans*/*E. coli* Plates

To further examine resistance in putative mutants, the offspring of putative mutants were observed when plated in the presence of both *Candida* and *E. coli*, their normal food source. To carry out this experiment, a survival/*E. coli* plate was made by spotting *C. albicans* (as done previously for survival plates) and by adding an equal dilution (1:100) of an overnight culture of OP50 *E. coli* to the plate. The overnight culture was kept in the shaking incubator at 30°C for 15 hours. Only one putative mutant was plated on each survival/*E. coli* plate and a plate containing a wild type worm was made as a control. The originally plated worm and its offspring were observed daily under a microscope, checking for both appearance and for amount of growth.

## 2.6: Characterizing Putative Mutants

### 2.6.1: Monitoring Lifespan

The lifespan of putative mutants was also monitored since some characterized mutants, such as *daf-2*, outlive N2 worms and exhibit increased resistance to certain pathogens. Determination of approximate lifespan was accomplished by transferring ten of each putative mutant at the L4 stage to separate feeding plates. At the same time, an identical feeding plate containing N2 worms was also set up to act as the control. Worms were kept at 20°C and every 3

days, the original worms plated were transferred to new plates to prevent overpopulation of young worms and to allow for easy monitoring. The number of original worms still alive was documented for each transfer.

**2.6.2: Dauer Check**

To further attempt characterizing the putative mutants, worms were monitored for the temperature-sensitive dauer-constitutive (*daf-c*) mutation. As noted previously, worms will enter the dauer stage from L2 if placed under stressful environmental conditions; this dauer stage allows worms to resist pathogens and other stressors (Atlun and Hall, 2009). Thus, temperature-sensitive *daf-c* mutants will, at the restrictive temperature, enter the dauer stage regardless of whether stressors are present. To check worms for this mutation, 4-5 of each putative mutant at the L4 stage were transferred to separate feeding plates. At the same time, an identical feeding plate containing N2 worms was also set up to act as the control. Picking 4-5 L4 stage worms ensured the production of a large amount of offspring and plates were kept in an incubator at 25°C, the restrictive temperature, so as to determine whether offspring displayed the dauer phenotype. The dauer phenotype was identified through observation under a dissecting microscope as these worms are very thin, possess a thicker, ridged cuticle, and appear to have a darker coloration; comparison of the dauer phenotype and L1 worms can be seen in Figure 6 (Atlun and Hall, 2009).

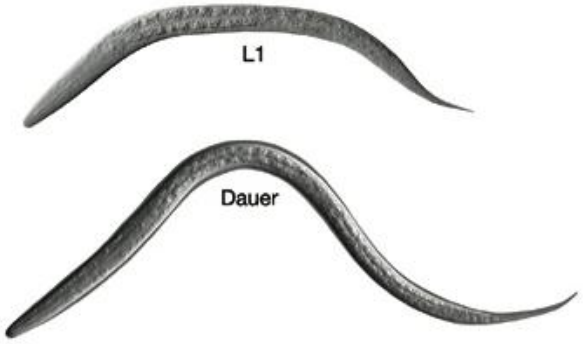



Figure 6. Comparison of Classic Dauer Phenotype and L1-stage *C. elegans*. Adapted from WormAtlas.

Worms being tested for the *daf-c* mutation were kept at 25°C for 3-4 days and observed for the dauer-specific phenotype.

# Section 3: Results

In order to begin the process of isolating putative mutants that display an increased resistance to *C. albicans*, several EMS mutagenesis procedures were run to increase the probability of inducing random mutations in the *C. elegans* genome. EMS-treated worms were then screened in order to isolate putative mutants and to determine whether these worms displayed consistent resistance characteristics to *C. albicans* over several trials. Characterization of possible mutations in putative mutants then began by determining lifespan and using differential interference contrast microscopy to observe for any visible phenotypic differences from wild type.

### 3.1: Survival Assay for Initial Isolation of Putative Mutants

#### 3.1.1: Probability of Generating Mutation in Desired Gene

To generate mutations in the *C. elegans* genome, worms were treated with the mutagen ethyl methanesulfonate. Ethyl methanesulfonate generates mutations at a rate of about 1 in 2000 per gene and fails to alter a gene at a frequency of 1999 in 2000; as such, the probability of generating one mutation in a desired gene can be derived by the following calculation:

$(1/2 \times 10^3)(1999/2 \times 10^3) + (1/2 \times 10^3)(1999/2 \times 10^3) + (1/2 \times 10^3)(1/2 \times 10^3)$

| Probability of hitting gene on one homologue | Probability of hitting gene on other homologue | Probability of hitting both homologues |
|---|---|---|

Thus, the probability of generating a desired mutation on either homologous chromosome can be seen to be 2 in $2 \times 10^3$, or 1 in $10^3$ worms. The number of F1 worms was counted to determine whether enough worms were being screened to isolate putative mutants displaying resistance to *C. albicans*. Ten F1 progeny plates were made for each mutagenesis performed and it was found that approximately 720 F1 worms were on each plate. Thus, 720 F1 worms x 10 plates = 7200 worms. This indicates that approximately 7 times as many worms needed to isolate at least one putative mutant were being screened during each survival assay.

#### 3.1.2: Several putative mutants were isolated from four mutagenesis procedures.

Four mutagenesis procedures were performed to generate putative mutants. Selection of putative mutants began on day 4 of the screen and ended at day 9, when approximately all plated worms were dead or dying. Table 2 shows the putative mutants isolated from each survival assay, and the mutagenesis procedure from which they were derived.

Table 2. Putative Mutants isolated from each mutagenesis

| Mutagenesis | Putative Mutants |
|---|---|
| #1 | 10A, 10C, 3B, 9A, 9B, 4A, 4B, 2A, 2B, 8A, 8B |
| #2 | 4, 5, 6, 7, 8 |
| #3 | 2-1, 7-1 |
| #4 | 1b, 7a, 3b, 10b |

Putative mutants were selected based on a phenotypically healthy appearance compared to other worms on the plate (as seen in Figures 4 & 5) and the ability to move non-sluggishly. Each putative mutant was placed on individual plates and labeled according to the plate they were isolated from as seen in Table 2.

### 3.2: Secondary Screenings of Putative Mutants

#### 3.2.1: Isolated putative mutants were rescreened to identify true *C. albicans* resistance.

To determine whether selected putative mutants displayed resistance to *Candida albicans* in a consistent and reproducible manner, we screened worms by exposing them to the yeast as described in Section 2.5.1: Secondary Survival Screens. Over the course of each screen, worms were monitored on a daily basis and observations concerning their appearance, movement, and offspring were recorded. To best compare putative mutants to N2, or wild type, worms, a scale was set up to rate putative mutants on each of the observations as seen in Table 3.

Table 3. Rating Scale for Appearance, Movement, and Amount of Offspring

| Observation | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Appearance | Scrawny/skinny | Unhealthy | Relatively healthy | Moderately healthy | Healthy like WT |
| Movement | Will not move without stimulus | Sluggish | Slightly sluggish | Moderately normal | WT-like movement |
| Offspring | None | Few/small amount | Some | Many | Abundant |

As seen in Table 3, ratings closer to 5 indicate that the putative mutants are displaying appearance, movement, and ability to produce offspring that is similar to N2 worms on a feeding dish and, thus, display increased *Candida* resistance, whereas ratings closer to 1 are indicative of poor survival on *Candida* plates. At the end of each screen, plates that were found to have high ratings on the scale were considered for rescreening.

### 3.2.2: Secondary Screens 1-4 for Putative Mutants Isolated from Mutagenesis 1 & 2

Screen 1 was run with the eleven mutants isolated from mutagenesis 1 & 2 and an N2 control. The rating for each plate can be seen in Table 4.

Table 4. Screen 1 of Isolated Putative Mutants from Mutagenesis 1 + 2

| Days | Observations | **8A** | **3B** | **4A** | **9A** | **10A** | **10C** | **4** | **5** | **6** | **7** | **8** | **WT** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Appearance | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | Movement | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | Offspring | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | Appearance | 5 | 2 | 4 | 4 | 5 | 4 | 3 | 3 | 2 | 4 | 2 | 2 |
| | Movement | 5 | 3 | 4 | 4 | 5 | 4 | 3 | 3 | 3 | 4 | 3 | 3 |
| | Offspring | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | Appearance | 4 | 4 | 3 | 4 | 4 | 4 | 4 | 4 | 2 | 4 | 2 | 1 |
| | Movement | 4 | 4 | 2 | 4 | 4 | 4 | 4 | 3 | 3 | 4 | 3 | 2 |
| | Offspring | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| 4 | Appearance | 4 | 3 | 1 | 3 | 4 | 3 | 2 | 3 | 2 | 3 | 3 | 1 |
| | Movement | 4 | 2 | 3 | 3 | 4 | 2 | 1 | 4 | 3 | 4 | 4 | 2 |
| | Offspring | 2 | 1 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 5 | Appearance | 4 | 2 | 1 | 1 | 4 | 3 | 1 | 3 | 1 | 3 | 3 | 1 |
| | Movement | 3 | 2 | 2 | 2 | 4 | 3 | 2 | 4 | 2 | 4 | 4 | 2 |
| | Offspring | 2 | 1 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 6 | Appearance | 3 | 2 | 1 | 1 | 4 | 2 | 1 | 3 | 1 | 2 | 3 | 1 |
| | Movement | 4 | 2 | 2 | 2 | 4 | 2 | 2 | 3 | 2 | 2 | 4 | 2 |
| | Offspring | 3 | 1 | 2 | 1 | 3 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 7 | Appearance | 3 | 3 | 1 | 1 | 4 | 2 | 1 | 3 | 1 | 1 | 3 | 1 |
| | Movement | 4 | 2 | 2 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Offspring | 3 | 1 | 2 | 1 | 4 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 8 | Appearance | 3 | 2 | 1 | 1 | 3 | 2 | 1 | 2 | 1 | 1 | 2 | 1 |
| | Movement | 3 | 2 | 2 | 2 | 4 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Offspring | 4 | 1 | 2 | 3 | 4 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 9 | Appearance | 3 | 1 | 1 | 1 | 3 | 2 | 1 | 2 | 1 | 1 | 1 | 1 |
| | Movement | 3 | 2 | 3 | 3 | 4 | 3 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Offspring | 4 | 1 | 3 | 3 | 4 | 3 | 2 | 3 | 1 | 2 | 3 | 2 |
| 10 | Appearance | 3 | 1 | 1 | 1 | 3 | 2 | 1 | 2 | 1 | 1 | 1 | 1 |
| | Movement | 3 | 2 | 2 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 1 |
| | Offspring | 4 | 1 | 3 | 3 | 4 | 3 | 2 | 3 | 2 | 2 | 3 | 2 |
| 11 | Appearance | 3 | - | 1 | - | 3 | - | - | 2 | - | - | 1 | 1 |
| | Movement | 3 | - | 3 | - | 3 | - | - | 3 | - | - | 2 | 1 |
| | Offspring | 4 | - | 3 | - | 5 | - | - | 4 | - | - | 3 | 2 |

As seen in this table, Screen 1 was run for a longer time frame than necessary in order to determine when future screens could be halted while still allowing for a fairly accurate estimate of resistivity in comparison to N2 worms. Days 6-7 were found to be reasonable days to halt the screen as, by this point, plates with N2 worms were rated lowly and more than half of the N2 worms were dead.

A second screen was run with the putative mutants from screen 1 that seemed to display the most resistivity to *C. albicans*. Thus, putative mutants 8A, 10A, 5, 8, and N2 worms (as a control) were included in the second screen as can be seen in Table 5.

Table 5. Screen 2 of Putative Mutants Isolated from Mutagenesis 1 + 2

| Days | Observations | **8A** | **10A** | **5** | **8** | **WT** |
|---|---|---|---|---|---|---|
| 1 | Appearance | 4 | 4 | 4 | 4 | 4 |
| | Movement | 4 | 4 | 4 | 4 | 3 |
| | Offspring | 1 | 1 | 1 | 1 | 1 |
| 2 | Appearance | 4 | 4 | 4 | 4 | 3 |
| | Movement | 4 | 4 | 3 | 3 | 3 |
| | Offspring | 2 | 2 | 1 | 1 | 1 |
| 3 | Appearance | 4 | 4 | 3 | 3 | 2 |
| | Movement | 4 | 4 | 3 | 3 | 3 |
| | Offspring | 3 | 3 | 2 | 2 | 2 |
| 4 | Appearance | 4 | 4 | 2 | 2 | 1 |
| | Movement | 4 | 4 | 3 | 3 | 2 |
| | Offspring | 4 | 4 | 2 | 2 | 2 |
| 5 | Appearance | 4 | 4 | 2 | 1 | 1 |
| | Movement | 4 | 4 | 2 | 3 | 2 |
| | Offspring | 5 | 5 | 3 | 2 | 2 |
| 6 | Appearance | 4 | 4 | 1 | 1 | 1 |
| | Movement | 4 | 4 | 2 | 2 | 2 |
| | Offspring | 5 | 5 | 3 | 2 | 2 |

Table 6. Screen 3 of Putative Mutants Isolated from Mutagenesis 1 + 2

| Days | Observations | **8A** | **10A** | **WT** |
|---|---|---|---|---|
| 1 | Appearance | 4 | 4 | 4 |
| | Movement | 4 | 4 | 4 |
| | Offspring | 2 | 2 | 2 |
| 2 | Appearance | 4 | 4 | 4 |
| | Movement | 4 | 4 | 3 |
| | Offspring | 3 | 3 | 2 |
| 3 | Appearance | 4 | 4 | 3 |
| | Movement | 3 | 4 | 3 |
| | Offspring | 3 | 3 | 2 |
| 4 | Appearance | 4 | 3 | 2 |
| | Movement | 4 | 4 | 2 |
| | Offspring | 4 | 4 | 2 |
| 5 | Appearance | 4 | 4 | 1 |
| | Movement | 4 | 4 | 2 |
| | Offspring | 4 | 4 | 2 |
| 6 | Appearance | 4 | 4 | 1 |
| | Movement | 4 | 4 | 1 |
| | Offspring | 5 | 5 | 2 |

By day 4 of Screen 2, the appearance, movement, and production of offspring of wild type worms were all rated two times lower than putative mutants 10A and 8A. At this same time point in the screen, putative mutants 5 and 8 were given only slightly increased ratings compared to wild type. By the final day of the screen, day 6, putative mutants 5, 8, and wild type worms received very similar low ratings whereas putative mutants 10A and 8A were given a rating of 4 for appearance/movement and a rating of 5 for offspring.

The secondary screen was repeated, as seen in Table 6, to determine whether putative mutants were able to display resistance characteristics in a consistent manner over several trials. Once again, putative mutants 10A and 8A received ratings two times better than wild type worms by day 4 of the screen. Additionally, by day 6 of the screen putative mutants were given a rating of 4 for appearance/movement, indicating moderately healthy phenotype/movement, and a rating of 5

for offspring, indicating the production of a large amount of offspring. A comparison between N2, 8A, and 10A worms on day 4 of the screen can be seen in Figure 7.

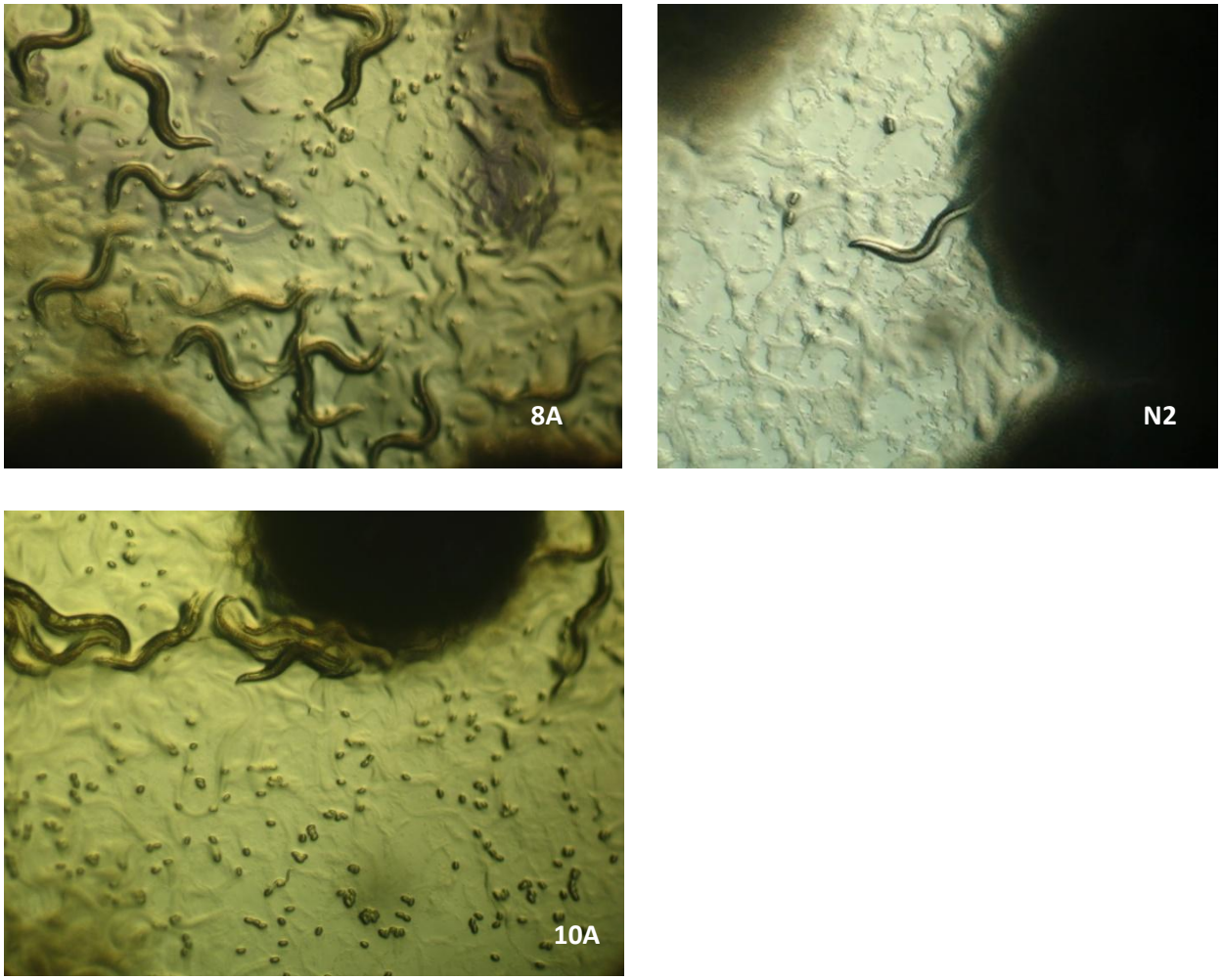



Figure 7. Comparison of 8A, 10A, and N2 growth at Day 4 on Survival Plates

As seen in Figure 7, N2 worms display the scrawny/skinny phenotype by day 4 of the screen due to ingestion of *C. albicans*. In comparison, both putative mutants 10A and 8A continue to display the healthy phenotype, as previously characterized, even after exposure to the yeast for 4 days. As Figure 7 shows, 10A and 8A worms also produced a large quantity of offspring in comparison to the small amount of offspring produced by N2 worms.

A fourth screen was run with the same putative mutants as Screen 3 to further determine whether 10A and 8A worms were consistently displaying increased resistance to *C. albicans*. The daily ratings assigned to each survival plate can be seen in Table 7.

Table 7. Screen 4 of Putative Mutants Isolated from Mutagenesis 1 + 2

| Days | Observations | **8A** | **10A** | **WT** |
|---|---|---|---|---|
| 1 | Appearance | 5 | 5 | 4 |
| | Movement | 5 | 4 | 4 |
| | Offspring | 1 | 1 | 1 |
| 2 | Appearance | 5 | 5 | 4 |
| | Movement | 4 | 5 | 3 |
| | Offspring | 2 | 2 | 2 |
| 3 | Appearance | 4 | 5 | 3 |
| | Movement | 4 | 5 | 2 |
| | Offspring | 2 | 3 | 2 |
| 4 | Appearance | 4 | 5 | 3 |
| | Movement | 4 | 4 | 2 |
| | Offspring | 3 | 3 | 2 |
| 5 | Appearance | 4 | 4 | 2 |
| | Movement | 4 | 4 | 2 |
| | Offspring | 4 | 4 | 2 |
| 6 | Appearance | 4 | 4 | 2 |
| | Movement | 4 | 4 | 2 |
| | Offspring | 5 | 5 | 2 |

As in previous screens, by day 4, wild type worms were given a rating two times less than 10A and 8A worms. By day 6, putative mutants continued to be rated more highly than N2 worms, which displayed poor appearance, movement, and much less production of offspring. Thus, putative mutants 8A and 10A were found to consistently display a healthy phenotype, movement, and the ability to produce many offspring following extended periods of exposure to *C. albicans* on survival plates.

### 3.2.3: Secondary Screen for Putative Mutants Isolated from Mutagenesis 3 & 4

A screen for the putative mutants isolated from mutagenesis 3 and 4 was also set up in the same manner as previous screens. As such, Table 8 displays the rating for each plate during the days the screen was run.

Table 8. Screen 1 of Putative Mutants Isolated from Mutagenesis 3 + 4

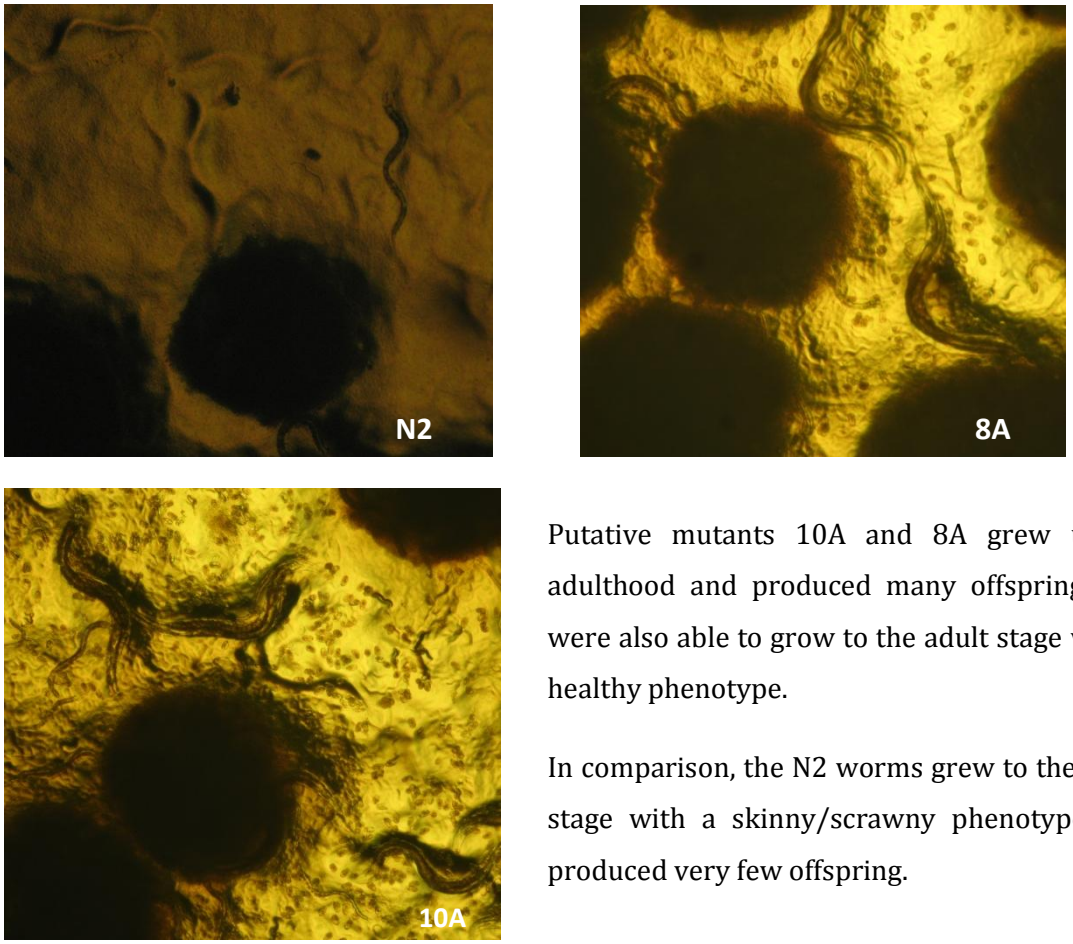
| Days | Observations | **1b** | **3b** | **7a** | **10b** | **7-1** | **2-1** | **WT** |
|---|---|---|---|---|---|---|---|---|
| 1 | Appearance | 4 | 4 | 4 | 4 | 5 | 4 | 4 |
| | Movement | 5 | 5 | 5 | 4 | 4 | 5 | 4 |
| | Offspring | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | Appearance | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| | Movement | 3 | 3 | 4 | 5 | 4 | 5 | 4 |
| | Offspring | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 3 | Appearance | 3 | 4 | 3 | 3 | 2 | 3 | 3 |
| | Movement | 2 | 2 | 3 | 3 | 3 | 3 | 2 |
| | Offspring | 2 | 2 | 1 | 1 | 1 | 2 | 2 |
| 4 | Appearance | 3 | 3 | 3 | 2 | 2 | 1 | 2 |
| | Movement | 3 | 2 | 2 | 2 | 2 | 2 | 2 |
| | Offspring | 2 | 2 | 1 | 1 | 2 | 2 | 2 |
| 5 | Appearance | 2 | 2 | 2 | 2 | 2 | 1 | 1 |
| | Movement | 3 | 3 | 2 | 2 | 2 | 2 | 2 |
| | Offspring | 2 | 2 | 1 | 2 | 2 | 2 | 2 |
| 6 | Appearance | 2 | 2 | 2 | 2 | 2 | 1 | 1 |
| | Movement | 2 | 3 | 3 | 2 | 2 | 2 | 2 |
| | Offspring | 2 | 2 | 1 | 2 | 2 | 2 | 2 |

As seen in Table 8, by day 4, the putative mutants used in this screen were rated similarly to N2 worms. The movement of putative mutants was found to be sluggish and their appearance ranging from moderately healthy to unhealthy, like wild type worms. Production of offspring was found to be low for all worms screened, with most plates receiving ratings of 1-2. By the final day of the screen, day 6, putative mutants continued to be given low ratings similar to N2 worms. Thus, putative mutants isolated from mutagenesis 3 and 4 were determined to not display resistivity to *C. albicans*.

### 3.2.4: Resistance of Putative Mutants on *E. coli*/Survival Plates

To further examine the increased resistance to *Candida* observed in putative mutants 10A and 8A, we plated one L4-stage worm on a survival plate that also contained an equal dilution of *E. coli* OP50, the normal food source for *C. elegans.* If the putative mutants display true resistance to the yeast, then the originally plated worm should be able to grow up to the adult stage with a healthy phenotype and produce offspring of its own. In comparison, worms that do not display resistance to *Candida* will also grow to adulthood, but their phenotype will appear skinny/scrawny and offspring production will be limited. *E. coli* was included on the survival plates because smaller worms are unable to ingest yeast cells (due to the large size of the yeast cells and the comparatively smaller size of the worm mouth). As such, by adding *E. coli* to the plate, the offspring of the initially plated worm will be able to ingest the *E. coli* until they are large enough to ingest the yeast cells.

This experiment is a good test for resistance as the offspring of truly resistant worms should be able to grow to adulthood and begin producing their own offspring even in the presence of *Candida*. Figure 8 shows N2, 10A, and 8A worms after 9 days on survival/*E. coli* plates.




Putative mutants 10A and 8A grew up to adulthood and produced many offspring that were also able to grow to the adult stage with a healthy phenotype.

In comparison, the N2 worms grew to the adult stage with a skinny/scrawny phenotype and produced very few offspring.

Figure 8. Putative Mutants 10A and 8A on *E. coli/C. albicans* plates after 9 Days
Magnification unknown.

### 3.3: Initial Characterization of Putative Mutants

#### 3.3.1: Differential Interference Contrast Microscopy at 400X Magnification

To determine whether any visible phenotypic differences existed in putative mutants 10A and 8A compared to N2 worms, we examined all worms using differential interference contrast microscopy. As seen in Figure 9, these worms were examined following 3 days on survival plates and the intestinal lumen can be seen for all three groups of worms.

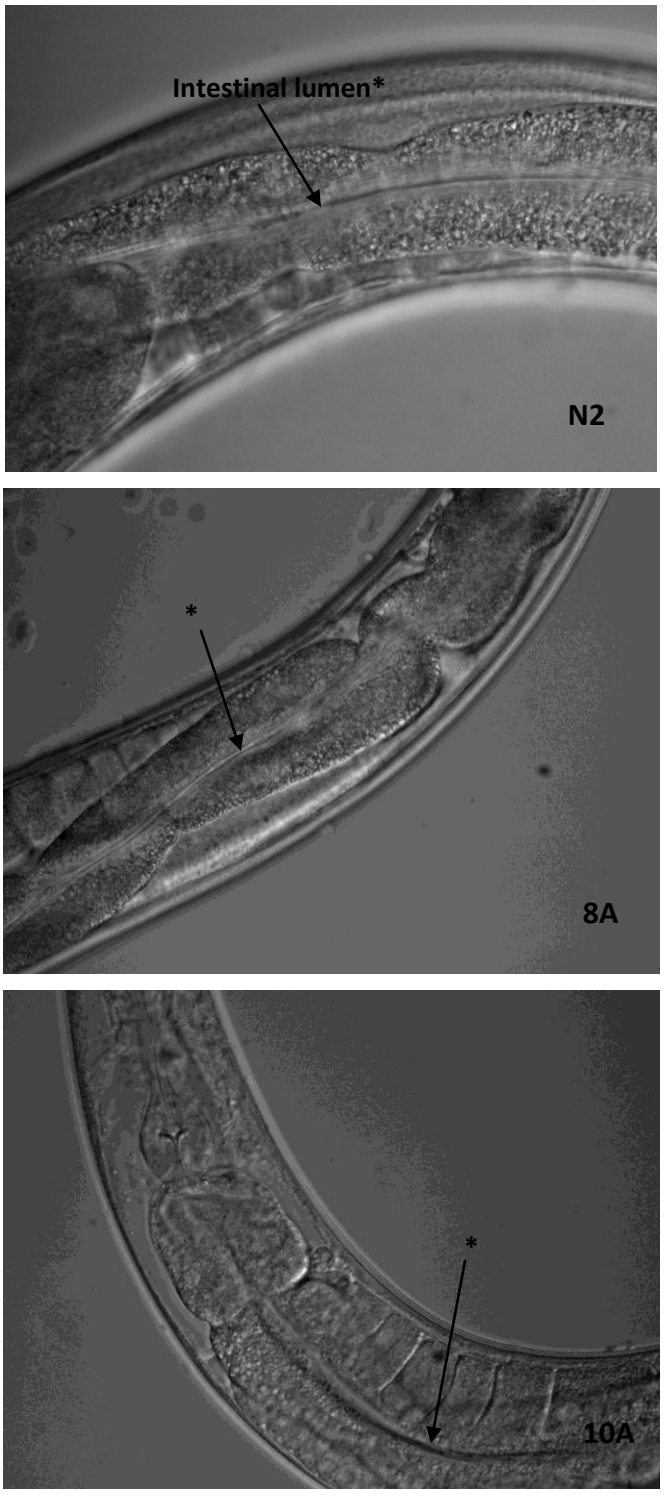



Figure 9. Differential Interference Contrast Images of the *C. elegans* Intestine at 400X

The intestine was examined in particular because it is the site for digestion and absorption of the yeast cells that the worms have ingested while on the survival plates. Examination of this structure

does not reveal any visible phenotypic difference that may account for the increased resistance to *C. albicans* noted in putative mutants 10A and 8A.

### 3.3.2: Putative mutants do not display the daf-2 phenotype.

The lifespan of putative mutants isolated from mutagenesis 1 and 2 was monitored in order to determine whether any of the worms displayed an extended lifespan, a phenotype associated with the *daf-2* mutation. A lifespan check was conducted in order to check worms for this phenotype because *daf-2* mutations have been known to result in increased survival in the presence of pathogens and other environmental stresses. As such, results of this experiment will reveal whether any of the putative mutants are *daf-2* mutants. Lifespan monitoring was accomplished by plating 10 of each putative mutant to a feeding dish and transferring these worms to new dishes every 3-4 days. This experiment was conducted once due to its length. The lifespan for putative mutants 8A and 10A can be seen in relation to N2 worms in Figure 10.

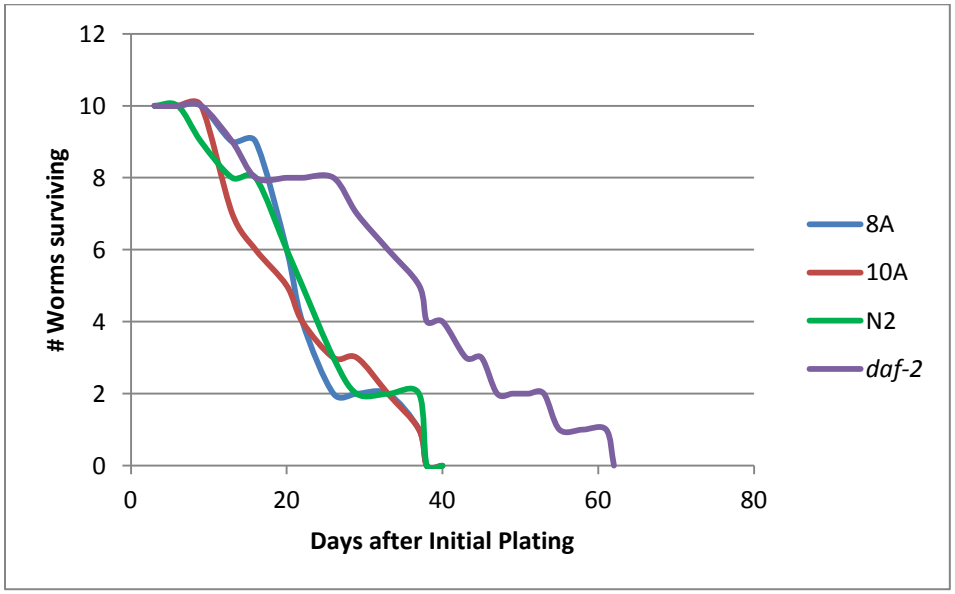



Figure 10. Average Lifespan for Putative Mutants 10A and 8A

As seen in Figure 10, the putative mutants were found to have a lifespan almost identical to N2 worms, which do not possess the *daf-2* mutation. To statistically check this observation, a single factor ANOVA was run on N2 worms and putative mutants 10A and 8A as can be seen in Table 9.

Table 9. Single Factor ANOVA on
10A, 8A, and N2 worms.

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| 10A | 23 | 61 | 2.652174 | 13.05534 |
| 8A | 23 | 65 | 2.826087 | 15.60474 |
| N2 | 23 | 65 | 2.826087 | 13.96838 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 0.463768 | 2 | 0.231884 | 0.016319 | 0.983817 | 3.135918 |
| Within Groups | 937.8261 | 66 | 14.20949 | | | |
| | | | | | | |
| Total | 938.2899 | 68 | | | | |

As seen in Table 9, the p-value found for N2 worms and putative mutants was 0.984. This is larger than a p-value of 0.05 and, thus, indicates that there is no significant difference between the lifespans found for the groups.

Although a *daf-2* mutant was not available to act as a control in this lifespan screen, Figure 10 shows the lifespan of such a mutant as determined by Kaberlein & Sutphin (2009). As such, the lifespan for *daf-2* mutants can be seen to extend to day 62. In comparison, by day 20 after plating, less than half of the worms initially plated for 8A, 10A, and N2 worms were still alive. Thus, putative mutants 8A and 10A were concluded to not possess the daf-2 phenotype.

The other putative mutants isolated from mutagenesis 1 and 2 were also examined for length of lifespan and can be seen in Figure 11.

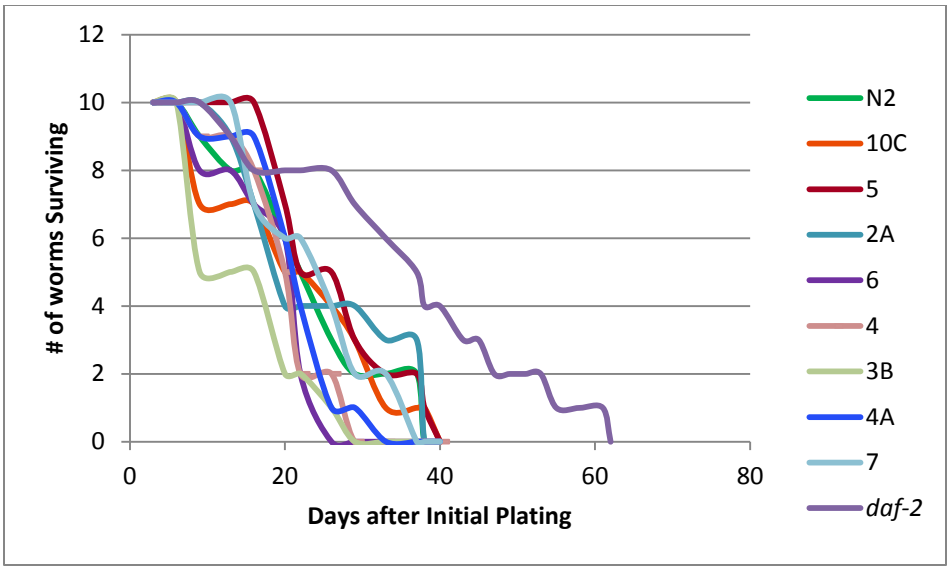



Figure 11. Average Lifespan for Putative Mutants Derived from Mutagenesis 1 & 2

As with putative mutants 10A and 8A, a majority of the other putative mutants appear to have a lifespan similar to N2 worms. However, three putative mutants (3B, 6, and 4) seem to have shorter lifespans than the other groups tested. To determine whether a significant difference between the putative mutants and N2 worms was present, a single factor ANOVA was run as seen in Table 10.

Table 10. Single Factor ANOVA on putative mutants and N2 worms.

SUMMARY

| *Groups* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| 5 | 13 | 75 | 5.769231 | 15.35897 |
| 2A | 13 | 68 | 5.230769 | 13.02564 |
| 9A | 13 | 58 | 4.461538 | 17.60256 |
| 10 | 11 | 23 | 2.090909 | 16.09091 |
| 8B | 13 | 27 | 2.076923 | 14.24359 |
| 6 | 13 | 51 | 3.923077 | 18.07692 |
| 4 | 13 | 55 | 4.230769 | 18.85897 |
| 3B | 13 | 40 | 3.076923 | 13.41026 |
| 4A | 13 | 59 | 4.538462 | 19.10256 |
| 7 | 13 | 67 | 5.153846 | 16.64103 |
| WT | 13 | 65 | 5 | 13.83333 |
| 10C | 13 | 61 | 4.692308 | 11.5641 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 196.4041 | 11 | 17.85492 | 1.141288 | 0.333939 | 1.856652 |
| Within Groups | 2221.524 | 142 | 15.64454 | | | |
| Total | 2417.929 | 153 | | | | |

The ANOVA found a p-value of 0.334, which is larger than a p-value of 0.05 and, as such, indicates that there is no significant difference between the groups tested. Thus, these putative mutants were determined to possess a lifespan not significantly different from N2 worms and, thus, putative mutants were determined to not possess the *daf-2* phenotype.

### 3.3.3: Putative mutants 8A and 10A do not display the *daf-constitutive* phenotype.

Another type of *daf-2* mutation, known as *daf-constitutive*, allows worms to enter the dauer state at the restrictive temperature of 25°C. In N2 worms, entry into the dauer state is normally triggered by exposure to pathogens or environmental stresses and, thus, acts as a survival mechanism. However, *daf-constitutive* worms are able to enter the dauer state even in the absence of environmental stresses when plated at the restrictive temperature. As such, to determine whether isolated putative mutants possessed the *daf-constitutive* phenotype, worms were plated on new feeding dishes and incubated at 25°C. The worms were observed daily to determine whether their offspring displayed the dauer phenotype in response to incubation at 25°C. After 5 days, none

of the offspring of the putative mutants displayed the dauer phenotype and appeared similar to the offspring of N2 worms.

# Section 4: Discussion

The main goals of this project were to isolate *C. elegans* putative mutants that display consistent resistance to *Candida albicans* infection and to begin the process of characterization of the possible mutations. These goals were accomplished by screening a significant number of EMS-treated worms to increase the probability of finding relevant mutations and by defining *Candida albicans* resistance in terms of *C. elegans* characteristics such as phenotype, movement, and production of offspring. The isolation and later characterization of putative mutants that are resistant to *C. albicans* has the potential to uncover information that may help in designing new, more effective treatments for *Candida* infection in humans.

### 4.1: Primary Screening of EMS-treated Worms

**Seven times the number of worms necessary to have a statistical chance of isolating at least one *Candida* resistant mutant were screened during each survival assay.**

To ensure that enough worms were being screened that we had a statistical chance of isolating a putative mutant displaying resistance to *C. albicans*, the probability of generating a mutation in a relevant gene was calculated. This calculation found that statistically 1 in $10^3$ worms would possess such a mutation. Thus, each primary screen needed to include $10^3$ or more worms in order to increase the chance of isolating a relevant mutant. By counting the number of F1 worms, it was estimated that 7200 worms were being screened during each trial. As such, primary screenings were found to have a high probability of isolating relevant mutants since seven times the minimum number of worms were being screened with each trial.

**Following four days on survival plates, primary screening isolated several putative mutants based on healthy phenotype and movement.**

After each mutagenesis procedure, primary screening began by plating all chemically treated worms on survival plates. By day 4 of this screen approximately half of the worms on each survival plate were dead or dying. Dying or unhealthy worms were noted to have a scrawny/skinny appearance and either moved sluggishly or did not move without stimulus. As such, selection of putative mutants began after day 4 of the screen and continued until day 9. Putative mutants were selected based on two criteria: healthy appearance and good movement. Additionally, the selected putative mutants were at the L4 to adult stage because worms at younger larval stages are not able to ingest large yeast cells and, thus, these worms enter the dauer stage due to their inability to ingest the yeast as a food source. Because it was estimated that approximately seven relevant mutants were being generated per mutagenesis procedure, several candidates were isolated from

each primary screening as seen in Table 2. In particular, mutagenesis 1 seemed to result in the isolation of the most putative mutants. This most likely occurred due to inexperience in identifying worms that were the most likely candidates to display resistance to *Candida*.

### 4.2: Secondary Screening of Initially Isolated Putative Mutants

**Putative mutants 10A and 8A were found to display consistently healthy phenotypes, movement, and production of offspring after extended periods on survival plates.**

We determined whether initially isolated putative mutants displayed attributes of resistance against *Candida albicans*, by plating the worms on survival plates. This allowed the phenotype, movement, and production of offspring to be more easily observed in many worms of the same genotype. Wild type, N2, worms were plated under the same conditions as putative mutants to act as controls. As such, the different characteristics of *Candida* resistance were rated on a scale from 1-5 as seen in Table 3, with 5 indicating phenotype, movement or production of offspring similar to N2 worms plated on normal feeding dishes. Thus, at the end of each screen, worms with ratings lower or very similar to the N2 worms on the survival plates were not considered for further screenings.

The results of Screen 1 of putative mutants isolated from mutagenesis procedures 1 and 2 can be seen in Table 4. By day 6 of the screen, four isolated putative mutants were given a rating of 3 or higher in all three categories of phenotype, movement, and production of offspring as compared to the wild type worms, which were given a rating of 2 or lower. These ratings indicate that putative mutants 8A, 10A, 5, and 8 were observed to possess a range of appearance, movement, and production of offspring from relatively healthy to completely healthy at day 6 of the screen, whereas wild type worms were observed to display unhealthy appearance, sluggish movement, and few offspring. Additionally, putative mutants 8A and 10A can be seen to have slightly increased ratings for resistance characteristics as compared to putative mutants 8 and 5. A second screen was run under the same conditions, with only putative mutants 8A, 10A, 5, and 8. Ratings for each day of screen 2 can be seen in Table 5. At the end of this screen (day 6), putative mutants 10A and 8A continued to display higher ratings than wild type worms, whereas putative mutants 5 and 8 were observed to display ratings that were more comparable to wild type.

Two further screens were run with putative mutants 10A, 8A, and wild type worms under the same conditions as seen in Table 6 and Table 7; these screens yielded the same observations. Thus, putative mutants 10A and 8A were found to display characteristics of resistance to *C. albicans*

in a consistent manner throughout the screening process. As seen in Figure 7, the putative mutants displayed a healthy phenotype as compared to the scrawny/skinny phenotype observed in wild type worms. Additionally, 8A and 10A worms consistently produced a large number of offspring, indicative of health, whereas wild type worms produced many fewer offspring. Both putative mutants 8A and 10A also displayed consistently non-sluggish movement, resembling wild-type movement on a feeding dish.

An additional screen was run with putative mutants isolated from mutagenesis procedures 3 and 4 as seen in Table 8. However, by day 6 of the screen, none of these putative mutants were observed to display increased resistance to *C. albicans* as indicated by ratings that were comparable to the wild type control. Thus, none of the putative mutants isolated from mutagenesis procedures 3 and 4 were considered for further rescreening.

In examining the limitations of these screens, it is important to note that ratings for the putative mutants were primarily qualitative, as each daily rating was based on observations of each plate by use of a microscope. Thus, although some amount of subjectivity was involved in taking observations, this subjectivity was limited by defining and looking specifically for 3 main characteristics of *C. elegans* behavior and phenotype that could be indicative of resistance to *Candida*. Additionally, running the screens several times helped to ensure that the observations being made were consistent over several trials.

### 4.3: Survival of Putative Mutants on *C. albicans*/*E. coli* Plates

**When plated on survival/*E.coli* plates, putative mutants 10A and 8A produced many offspring that were able to grow up to the adult stage with a healthy phenotype.**

To further examine the resistance of putative mutants, one 10A worm and one 8A worm was plated on separate survival plates that also contained an equal dilution of OP50, the normal food source for *C. elegans* in the lab. The offspring of these worms were able to feed on the *E. coli* until they reached a large enough size to ingest the yeast cells. As such, the offspring of worms that display an actual resistance to *Candida* should be able to grow to adulthood on these plates, display a healthy phenotype, and produce their own offspring. In contrast, the offspring of non-resistant worms should be able to grow (due to the presence of OP50 on the plate); however, there will be a comparatively small amount of offspring, and these offspring will appear scrawny/skinny. As seen in Figure 8, 10A and 8A worms produced a large amount of offspring and were able to grow up to the adult stage with a healthy phenotype. In comparison, N2 worms display a skinny/scrawny

phenotype and had little production of offspring. This indicates that although N2 worms were plated with their normal food source, OP50, the presence of *Candida* on the plate still restricted their growth. Putative mutants, in contrast, were able to grow well even in the presence of the yeast. Thus, these results further support the observation that putative mutants 10A and 8A display an increased resistance to *Candida.*

### 4.4: Differential Interference Contrast Microscopy

**DIC images of the intestinal lumen of 10A and 8A worms do not reveal any visible phenotypic differences from wild type.**

The intestinal lumen of 10A and 8A worms was observed under 400X magnification using differential interference contrast (DIC) microscopy. This technique enhances the contrast of unstained samples and allows for visualization of the internal structures of *C. elegans*. As seen in Figure 9, the intestine of wild type worms appears as a thin tube that extends down the body cavity of the worm. The intestine is the location for the digestion of the food, normally bacteria, consumed by *C. elegans*. In this case, worms primarily fed on *C. albicans* present on the survival plates used for both primary and secondary screenings. Thus, the appearance of the intestinal lumen, where digested food is absorbed, was examined in order to determine whether the resistance to yeast noted in putative mutants 10A and 8A resulted from visible, phenotypic abnormalities. As seen in Figure 9, no major physical differences can be noted between the intestine of wild type worms and putative mutants 10A and 8A. This suggests that a difference in visible physical structure is not the means by which 10A and 8A worms are better able to survive *C. albicans* relative to wild type. However, this observation does not necessarily indicate that no differences in digestion or absorption of yeast cells are present.

Further experiments should be conducted in order to monitor intake of yeast cells over several days in 10A and 8A worms. These types of experiments could be accomplished by use of a fluorescently labeled *C. albicans* strain. In this way, 10A and 8A worms could be kept on separate plates streaked with the fluorescently labeled strain and, after each 24 hour period, these worms could be observed under a fluorescent microscope to view accumulation of the fluorescent yeast cells in the intestinal lumen. Ingestion of yeast by putative mutants could be compared to N2 worms by keeping the N2 worms on *C. albicans* streaked plates as well. Additionally, the intestine of putative mutants and N2 worms on the yeast plates should be compared to the intestine of putative mutants and N2 worms on feeding dishes with their normal food source. This would allow for the determination of whether putative mutants ingest *C. albicans* differently than wild type, which

could be the means by which 10A and 8A worms display increased resistance to the yeast. Furthermore, experiments with a fluorescent *C. albicans* strain could allow for the determination of when worms are large enough to ingest the yeast cells. As mentioned previously, smaller worms are unable to ingest yeast cells due to the size of yeast cells and the comparatively much smaller size of the worms' mouth. Thus, worms at different life stages could be kept on fluorescent yeast plates and observed under a fluorescent microscope to determine at what size/life stage worms are large enough to ingest the yeast cells.

### 4.5: Checking Putative Mutants for the *daf-2* phenotype.

#### Putative mutants 10A and 8A do not possess the *daf-2* phenotype.

The *C. elegans* gene, *daf*-2, is involved in a well-characterized signaling pathway that is thought to play a role in both aging and immunity through the regulation of worm dauer formation. In response to a lack of food, the presence of pathogens, or other environmental stresses, wild type worms are able to enter an alternative, arrested state known as dauer, which gives the worms an increased ability to survive the stressful conditions. Thus, mutations in the *daf-2* gene often result in changes to dauer formation, aging, or response to pathogens. In particular, one well-known *daf-2* mutation results in both increased lifespan and ability to fend off pathogens. To determine whether isolated putative mutants 8A and 10A possessed this particular mutation, ten worms were plated on separate feeding dishes and transferred every three days to prevent overpopulation. In this way, the lifespan for 10A and 8A could be determined by how long worms persisted on feeding dishes. N2 worms were plated under the same conditions to act as controls. Following development into adults, N2 worms have been found to live approximately 3 weeks under normal conditions on agar plates with OP50 as a food source. As seen in Figure 10, more than half of the ten N2 worms originally plated were dead by the three week mark. This same pattern was observed for 8A and 10A worms. To statistically determine whether a significant difference existed between putative mutants and N2 worms, a single factor ANOVA was run on the groups. As seen in Table 9, the groups were given a p-value of 0.984, which is larger than the 0.05 needed to indicate significance and, as such, there was no significant difference between the lifespan of 10A/8A worms and N2 worms. Thus, initial tests suggest that putative mutants 8A and 10A do not possess the *daf-2* phenotype of increased longevity, although further DNA sequencing could verify this finding.

Another *daf-2* mutation, known as *daf-constitutive* or *daf-c*, is a temperature sensitive mutation that results in dauer formation at 25°C even in the absence of stressful environmental

stimuli. This constitutive dauer formation results in an increased ability of these worms to survive pathogens and other stress factors. To determine whether putative mutants 8A and 10A possess this mutation, each putative mutant was plated on separate feeding dishes and incubated at the restrictive temperature of 25°C where they were allowed to produce offspring for three days. N2 worms were also plated under the same conditions. Offspring of 8A, 10A, and N2 worms were then observed under the microscope and were not found to have entered the arrested dauer stage, which is characterized by a darker coloration, thin appearance, and a thicker, more ridged cuticle (Atlun and Hall, 2009). This experiment was performed only once since the offspring of putative mutants will either enter the dauer stage or will fail to enter this stage when incubated at the restrictive temperature. Thus, putative mutants 10A and 8A were concluded to not possess the *daf-c* phenotype.

### 4.6: Techniques for Characterization of Putative Mutants

To further characterize the possible mutations present in the putative mutants, complementation assays and other genetic mapping techniques may be utilized to identify the chromosome and specific gene in which the mutation resides.

#### 4.6.1: Complementation Assays

A complementation assay is a genetic test utilized to assign genes to one of the six chromosomes in *C. elegans.* To perform a complementation test, both the isolated mutation of interest and the test mutation must be recessive (Yook, 2005). The test mutation is a mutation that has already been mapped to a particular genetic locus and whose resulting phenotype is already known (Yook, 2005). As such, during the complementation test, if combining the test mutation in trans with the mutation of interest produces a worm with wild type phenotype, then the two mutations are alleles of different genes (Yook, 2005). The wild type phenotype results from the fact that a wild type copy of each mutated gene is present. In contrast, if combining the test mutation in trans with the mutation of interest does not produce the wild type phenotype, then the mutations must be alleles of the same gene as they cannot restore the wild type phenotype (Yook, 2005). Thus, no wild type copy of the mutated gene is present to rescue the phenotype. A diagram of this concept can be seen in Figure 12.

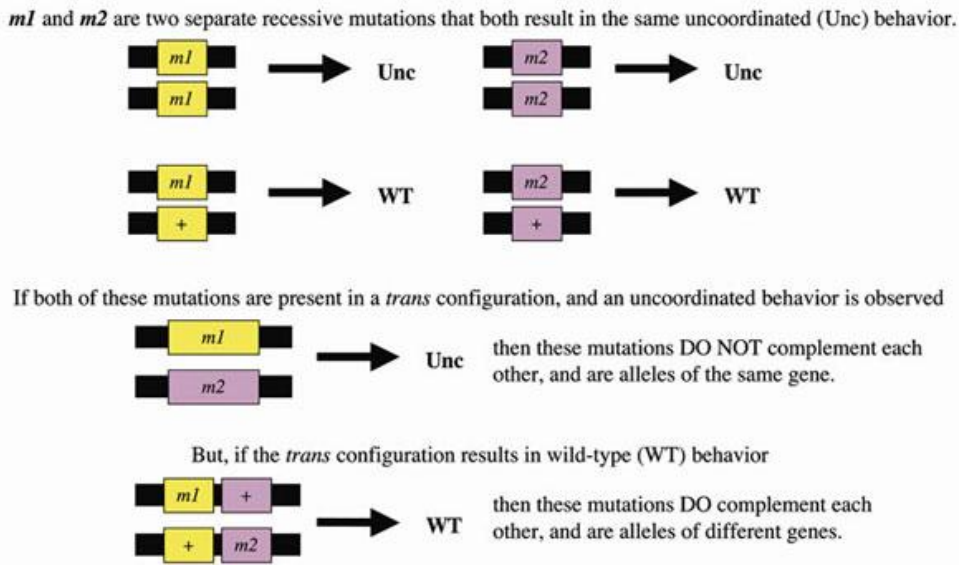



Figure 12. Examples of Complementation and Non-Complementation
Taken from Yook, 2005.

Combining the test mutation in trans with the mutation of interest thus means that the test mutation should be present on one homologous chromosome and the mutation of interest should be present on the other. This can be accomplished by mating a homozygous hermaphrodite that possesses the recessive mutation of interest with a homozygous male worm that possesses the test mutation. As such, complementation can be a more simple and efficient means by which to assign a mutation to a certain genetic locus.

### 4.6.2: Genetic Mapping

Genetic manipulation in *C. elegans* can be utilized to precisely determine the location of isolated mutations and to identify the gene products that have been affected by the mutation (Fay[1], 2006). An overview of this process can be seen in Figure 13.

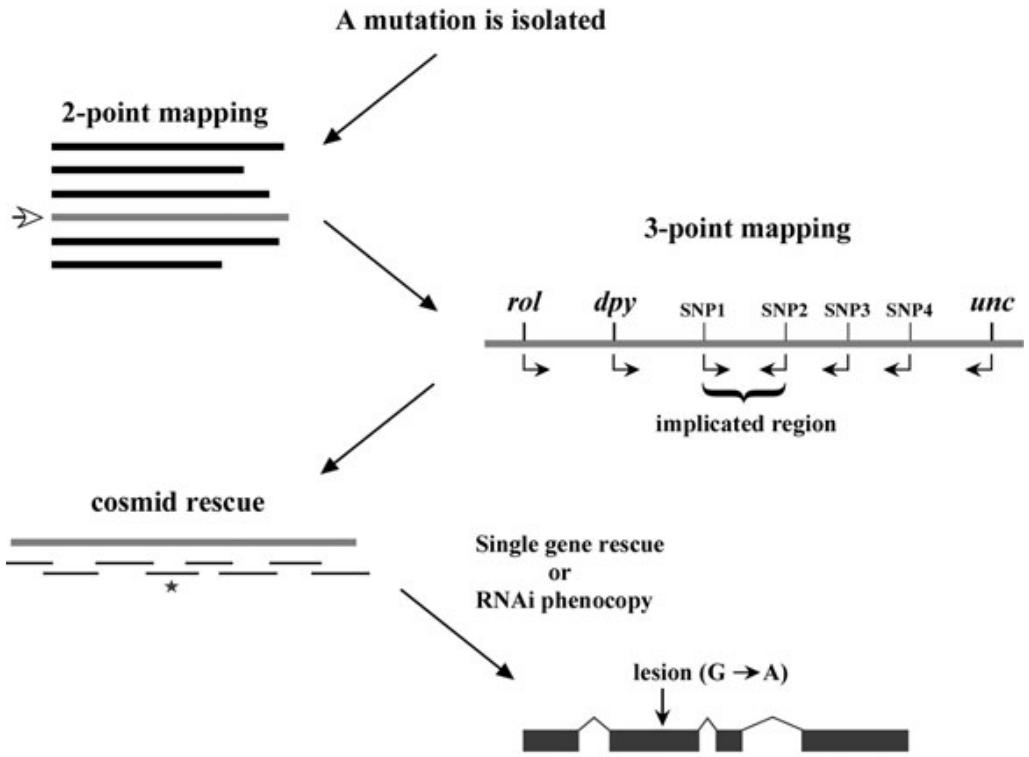



Figure 13. Genetic Mapping in *C. elegans*
Taken from Fay[1], 2006.

Following the isolation of a possible mutation, two-point mapping using standard genetic markers can be used to determine which of the six *C. elegans* chromosomes the mutation of interest is located on (Fay[1], 2006). Three-point mapping can then be used to narrow the region in which the mutation must be located and then transgene rescue or RNA phenocopy can implicate a particular gene (Fay[1], 2006). Gene sequencing can then identify the specific molecular defect that is responsible for the mutation and its resulting phenotype.

Two-point mapping is a process in which the mutation of interest is mapped to a specific chromosome by utilizing two marker mutations which have known locations on a chromosome and which have known phenotypes (Fay[2], 2006). Thus, a hermaphrodite worm possessing the mutation of interest is crossed to a male worm that possesses the marker mutations. The progeny of this cross can then be examined to determine whether the mutation of interest is present on the same chromosome as the marker mutations or if it is located on another chromosome (Fay[2], 2006). A diagram of two-point mapping can be seen in Figure 14.

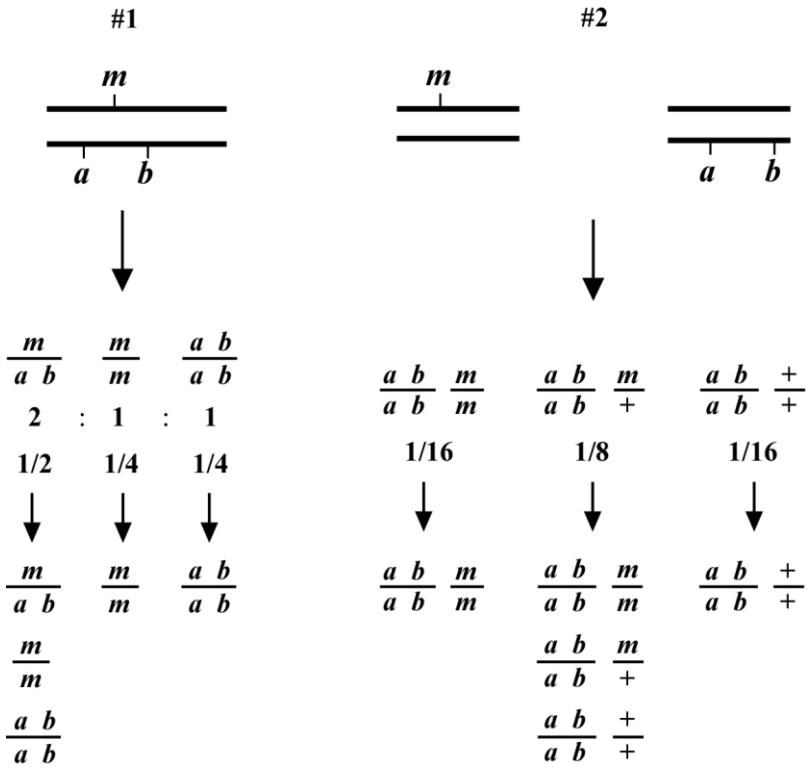



Figure 14. Two-point mapping with marker mutations *a* and *b* and the mutation of interest, *m*
Taken from Fay[2], 2006.

The marker mutations are denoted by *a* and *b* whereas the mutation of interest is denoted by *m*. As such, Figure 14 shows the predicted genotypes of the progeny resulting from a cross in which the mutation of interest is located on the same chromosome as the marker mutations (outcome #1) and the progeny resulting from a cross in which the mutation of interest is located on a different chromosome from marker mutations *a* and *b* (outcome #2) (Fay[2], 2006). When *m* is present on a different chromosome, there will be a much higher chance for recombination to occur between *m* and *a* and *b* such that offspring with the genotype *mab* will be produced (Fay[2], 2006). In contrast, if *m* is present on the same chromosome as *a* and *b*, the production of offspring with the genotype *mab* would be very rare as this would require a double recombination event to occur (Fay[2], 2006).

Following two-point mapping, three-point mapping can narrow down the area containing the mutation. In this process, the mutation of interest, *m*, is crossed into a strain that possesses the marker mutations *a* and *b* which are present on the same chromosome as *m* and which possess known phenotypes (Fay[3], 2006). The genotypes of the progeny produced are then examined to determine whether the mutation is located to the left, to the right, or between the markers (Fay[3], 2006). A diagram of three-point mapping can be seen in Figure 15.

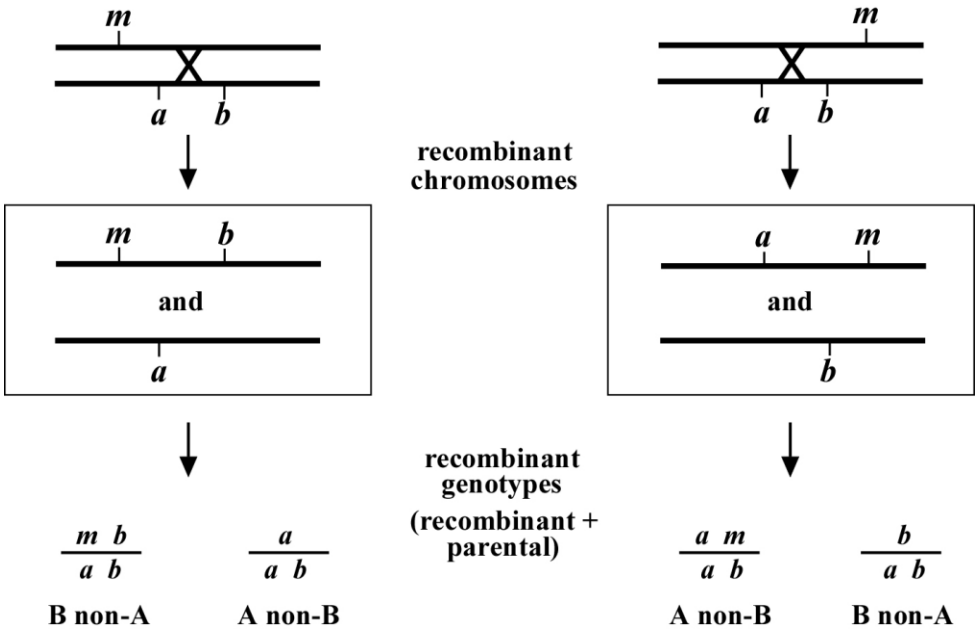



Figure 15. Three-Point mapping with marker mutations *a* and *b* and mutation of interest, *m*

Taken from Fay[3], 2006.

Figure 15 shows the outcome of recombination between *m* and markers *a* and *b* if the mutation of interest is located to the left or right of the markers. When *m* is located to the left, all progeny that only display the *a* phenotype will produce only progeny with the indicated genotypes (Fay[3], 2006). In comparison, all progeny that only display the *b* phenotype will produce progeny with the listed genotypes (Fay[3], 2006). If *m* resides to the right of the markers, then the opposite situation will exist for the genotypes of the progeny (Fay[3], 2006). In this way, the region in which the mutation of interest resides can be narrowed down considerably.

Once the location of the mutation of interest has been narrowed down sufficiently, transgene rescue or RNAi methods can be used to implicate a specific gene (Yook, 2005). Transgene rescue can be accomplished by creating a transgenic strain that contains the wild type version of the implicated gene; bacterial vectors that contain the wild type copy of the gene are then injected into the mutant to see if a wild type copy of the gene reverts the phenotype caused by the mutation of interest (Yook, 2005). If transgene insertion is successful in reverting the mutant phenotype back to wild type, then the gene coded on the bacterial vector is most likely the affected gene in the mutant. To further implicate a specific gene, double stranded RNA that is complementary to the gene of interest can be used to trigger the RNAi pathway which will result in knockdown of the gene in wild type worms (Yook, 2005). If these worms then express the known

mutant phenotype, it is likely that the mutation of interest is located in this gene. Following both transgene rescue and RNAi knockdown, DNA sequencing can then be performed to find the exact molecular lesion that causes the defect (Yook, 2005).

**4.7: Summary & Future Directions**

The main purpose behind this project was to isolate *C. albicans* resistant worms for use in future projects and to begin the process of characterization of the possible mutations. This was accomplished by means of EMS mutagenesis which increases the chance of random mutation in the *C. elegans* genome. Worms were screened multiple times in order to determine whether they displayed consistent resistance to the yeast over several trials. In this way, putative mutants 8A and 10A were found to display consistent resistance to *C. albicans*. These putative mutants were found to not possess the *daf-2* phenotype, the result of a mutation in the *daf-2* gene which has been known to confer increased resistance in *C. elegans* to environmental stresses and pathogens. Additionally, DIC microscopy found that putative mutants 10A and 8A did not possess any visible phenotypic differences from wild type that may have conferred resistance to *C. albicans*. Complementation tests, fluorescent microscopy, and DNA sequencing will be of use in future characterization attempts for these putative mutants.

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