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The University of Southern Mississippi

Characterization of the Function of a Mold Specific Gene, *MS95*, in the Pathogenic
Dimorphic Fungus, *Histoplasma capsulatum*

By

Danielle Jordan Williamson

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
In Partial Fulfillment
Of the Requirements for the Degree of
Bachelor of Science
In the Department of Biological Sciences

May 2013

Approved by

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Abstract

Histoplasma capsulatum is a dimorphic fungus that causes the respiratory disease histoplasmosis. At 25°C, the fungus grows as a multicellular mold in soils contaminated by bird and bat excreta. Once the soil is disturbed, spores are released and inhaled into the lungs. The fungus shifts to a unicellular, pathogenic yeast within the lungs at 37°C. Our laboratory's main objective is to characterize the genes that are involved in the mold-to-yeast dimorphism. This study focuses on the mold-specific gene, *MS95*. According to The Broad Institute *Histoplasma capsulatum* GenBank Blastx search, *MS95* has several homologs, including the well-studied *Saccharomyces cerevisiae*. *MS95* belongs to a gene family of stress proteins known as DDR (DNA Damage Responsive) and is believed to repair DNA damaged by heat stress. To better determine the function of *MS95*, this project explains the creation of a *MS95* knockout by allelic replacement. The *MS95* knockout was confirmed by a Southern blot. Mold and yeast growth curves of the *MS95* knockout did not show any significant changes in the rate of growth when compared to the *Wu27* wild type strain. Comparisons of the yeast and mold morphology in both liquid and solid media showed no significant difference between the *MS95* knockout and the *Wu27* wild type strain. These findings suggest that *MS95* is not involved in dimorphism. Future studies will include observing how *MS95* knockout and *Wu27* wild type strains react to different stressors and overexpressing *MS95* in the yeast phase growing temperature to confirm that it is not involved in dimorphism.

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Chapter 1: Introduction

Parasitic fungi, such as *Histoplasma capsulatum*, have dimorphic capabilities and can grow as either parasitic-unicellular yeast or saprobic-multicellular mold depending on environmental conditions. In soil, *H. capsulatum* exists as a mold at 25°C. If the soil is disturbed, mold cells release spores that can be inhaled into the lungs of passing humans or other mammals. Within the lungs, *H. capsulatum* converts to yeast at 37°C. This morphological transition enables the dimorphic, pathogenic fungi to colonize host tissues including humans (Maresca et al., 1989).

H. capsulatum causes histoplasmosis, a systemic fungal disease. Histoplasmosis is the most common respiratory mycotic infection among humans and other mammals. It is prevalent in temperate, subtropical, and tropical zones such as the Mississippi and Ohio Valleys in the United States and in similar areas in South and Central America, the Mediterranean, Asia, Australia, and Africa. Histoplasmosis has a wide range of symptoms and is an opportunistic fungus in immune deficient hosts. The disease is extensive in areas where patients suffer from acquired immune deficiency syndrome and is a growing concern in nonendemic areas due to the increasing motility of the world's population (Ignatov et al., 2002).

Few studies have been performed on the identification and characterization of the genes and the gene products involved in the morphological transition of *H. capsulatum*. These genes are vital for the early establishment and maintenance of the parasitic yeast phase that invades host cells and causes histoplasmosis. One such gene under study is *MS95*. It is a mold specific and belongs to a class of genes known as DNA damage responsive (*DDR*) genes (Maga et al., 1986). *DDR* genes express increased transcription

in response to DNA lesions or to heat-shock stress (Treger et al., 1990). Scarce studies have been preformed on *MS95* in *H. capsulatum*, but many studies have been conducted on gene *DDR48* in *Saccharomyces cerevisiae*, a homolog of *H. capsulatum. DDR48* has also been studied in another homolog, *Candida albicans*, which is a dimorphic pathogenic fungus that causes mucosal and systemic infections. Dr. Dib et al. (2008) created a knockout of a *DDR48* allele via homologous recombination. His research concluded that *DDR48* is essential for hyphal filament formation in the mold form, stress responses, and viability of *C. albicans*, thus making it a prime target for antifungal drug design. Being a homolog of *H. capsulatum*, *DDR48* is similar in structure and evolutionary origin to *MS95* despite being from a different species. DDR genes may have a critical function in *H. capsulatum* because dimorphic fungi shift between yeast and mold forms in response to environmental changes such as temperature (Maresca et al., 1989).

To better understand the function of *MS95*, this project focuses on characterizing *MS95* in the pathogenic, dimorphic fungus *H. capsulatum*. This goal will be achieved through the creation of a *MS95* knockout via allelic replacement. Knocking out the gene will disrupt its normal function. The knockout will be confirmed through a Polymerase Chain Reaction procedure (PCR) and a Southern Blot. The phenotypic appearance and cellular response of *H. capsulatum* wild type and knockout will be recorded when grown as mold at 25°C and as yeast at 37°C. Also, the wild type and knockout will be grown under stressful conditions to determine if *MS95* is involved in DNA repair. If *MS95* is responsible for initiating the shift between mold and yeast, then the cells should remain as mold and not transform into yeast when the gene is knocked out and nonfunctional.

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Ideally this project's findings will be used to further expand the understanding of the morphological transition mechanism in *H. capsulatum* and other dimorphic fungi and perhaps lead to a prevention or a better treatment procedure for histoplasmosis.

Chapter 2: Literature Review

2.a *Histoplasma capsulatum*: Current Research

Maresca and Kobayashi (1989) have compiled a review article concerning the use of *H. capsulatum* as a model for the study of dimorphism in pathogenic fungi and the biochemical processes believed to be involved in the cell dimorphism. *H. capsulatum* is a pathogenic fungus with the ability to exist either as saprobic-multicellular mold or parasitic-unicellular yeast (Fig. 1). This cellular transition, known as dimorphism, occurs in response to environmental stimuli, such as changes in carbon dioxide levels, temperature, oxidation-reduction potentials, and nutritional factors.
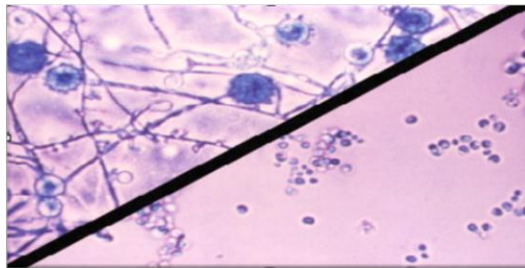

Figure 1. *Histoplasma capsulatum* mold (top left) and yeast (bottom right) stained with lactophenol cotton blue.

The cellular dimorphism characteristic of *H. capsulatum* is not essential to the cell's life cycle, is reversible, and can grant the cells the ability to infect host tissues (Maresca et al., 1989).

H. capsulatum is the causative agent of histoplasmosis, a systemic fungal disease. It has been diagnosed worldwide and is the most common respiratory mycotic infection found in humans and animals. In the United States alone, about 500,000 infections are diagnosed each year (Nosanchuk et al., 2008). Histoplasmosis has high prevalence in temperate, subtropical and tropical climate, including the Mississippi and Ohio Valleys, portions of South and Central America, the Mediterranean area, Asia, Australia, and Africa (Ignatov et al., 2002). It is a highly opportunistic pathogen that seriously affects humans with acquired immune deficiency syndrome (Weidenheim et al. 1992). In a study by Brilhante et al. (2012), of patient cases reviewed, 38.9% had histoplasmosis as the first indicator of AIDS. Histoplasmosis is becoming a greater concern due to the increasing mobility of the world's populations.

H. capsulatum yeast cells are ovals with 1 to 3 μm diameters, grow at 37°C, and reproduce by budding (Maresca et al., 1989 (31)). The mold cells measure about 1.2 to 1.5 μm in diameter, grow at 25°C, and reproduce by macroconidia spores and microconidia spores. It is believed that the microconidia are the favored infectious form of *H. capsulatum* due to their small size (Maresca et. al 1989 (23, 57, 32, 63)). Yeast cells are the only form of *H. capsulatum* discovered in infected host tissues, but little is known about the transition from the microconidia mold spores to the yeast cells at 37°C.

Researchers have currently tried to understand the chromosomal characteristics of *H. capsulatum*. They isolated twenty-three strains of *H. capsulatum* from infected human

tissue using restriction fragment patterns of mitochondrial DNA and ribosomal DNA. Common strains studied in humans are G184AS, G186AS, G217B, and Downs (Maresca et al., 1989 (167)). Researchers have also identified seven chromosome-sized bands all exceeding one megabase (one million base pairs), except for a five hundred thousand base pair band in one of the strains. Each band also has a unique pattern depending on the strain (Maresca et al., 1989).

Currently, it is known that *H. capsulatum* yeast cells transition into mold at 22°C to 25°C. This transition does not occur at the same time in all cells, but does begin with the formation of a budlike structure containing large quantities of storage material. After twenty-four to forty-eight hours, the transition is complete. (Maresca et al., 1989 (38, 44, 73)). When transitioning back into yeast, mold cells require more time. This transition is believed to occur through an "enlarged cell" described as an oidial yeast cell. Pine and Webster studied the transition of mold to yeast cells in *H. capsulatum* and discovered that contiguous mold cells enlarge to form chains of yeastlike cells or undergo budding of unswollen mold cells to form yeast cells (Maresca et. al 1989 (130)).

Environmental requirements that are needed to complete a successful transition have also been studied. Salvin has shown that nutritional factors such as cysteine levels, which contain thiol groups (sulfur-hydrogen groups), may affect the transition from mold to yeast. He found that the addition of cysteine was necessary for the maintenance of the yeast phase because it is required to complete the mold to yeast transition and acts as a nutrient for the yeast cells (Maresca et al., 1989 (148)). It is clear from other research that thiol containing compounds must be present in the culture medium to initiate the mold to yeast transition (Maresca et al., 1989 (154)). Research conducted by Zarnowski et al.

(2007) demonstrates how *H. capsulatum* can degrade collagen and digest albumin and casein. By degrading large proteins, *H. capsulatum* can acquire the nutrients, such as thiol groups from proteins, essential for fungal growth and metabolism. Once other conditions such as vitamins along with thiol groups are satisfied, the transition is directly mediated by temperature changes (Maresca et al., 1989 (125, 129, 147, 154)). Transition occurs at about 30°C, with the optimal temperature reaching 37°C. *H. capsulatum* can grow as mold regardless of the incubation temperature; however, the cells require 37°C to transform to and persist as yeast. This leads researchers to believe that mold-specific genes express at any temperature, while yeast-specific genes can only be expressed at higher temperatures and in the presence of thiol groups (Maresca et al., 1989). Therefore, in order to understand the phase transition of dimorphic fungi, it is necessary to characterize the genes and their functions in mold and yeast cells within *H. capsulatum*.

2.b Gene *MS95*: Overview

To begin to understand the role certain genes play in the transition from mold to yeast cells in *H. capsulatum*, the genes must be characterized and identified as either mold-specific or yeast-specific. Due to the lack of research on *H. capsulatum*, some information pertaining to the function of certain genes must be taken from research conducted on homologous organisms including *Saccharomyces cerevisiae* and *Candida glabrata*.

Temperature changes within the environment are the driving force of the dimorphic transition of *H. capsulatum* cells; therefore, genes that respond to heat-shock are at the forefront of research. A class of genes known as DNA damage response genes (DDR) respond to heat-shock and show an increased transcription in response to heat-

shock stress or to treatments that produce DNA lesions (Treger et al., 1990). Within *H. capsulatum*, the mold-specific gene *MS95* belongs to the class of DDR genes. Expression of certain mold-specific genes is required for mold formation and maintenance, and lack of expression allows the cells the transition into yeast (Tian et al. 2001). Therefore, it is speculated that *MS95* may be involved in initiating the transition from mold to yeast cells in a human host. A Broad Institute gene search concluded that *MS95* consists of 1499 nucleotides, codes for a protein consisting of 315 amino acids, and occurs once within the genome. However, there is scarce research published on *MS95* in *H. capsulatum*, so current information must be taken from research done on the *DDR48* gene of *S. cerevisiae* and *C. glabrata.*

Chapter 3: Methods

This project focuses on expanding the knowledge of the mechanisms involved in the transition between cellular forms of dimorphic fungus, specifically *H. capsulatum*. Specifically, this project will try to determine the function of the gene *MS95* by creating a knockout.

3.a Overview of Common Procedures

Polymerase Chain Reaction (PCR) is a biochemical procedure used to generate thousands to millions of copies of a specific DNA sequence from a few fragments of DNA. This procedure involves three phases that are typically repeated thirty times in a thermal cycler. Before the first phase, there is an optional step used to heat the lid of the thermal cycler to 95°C for 60 seconds. The first phase is denaturing, or separation of the two DNA strands. Denaturing occurs around 95°C for 10 seconds and causes the

hydrogen bonds between the DNA strands to break. Once the two strands of DNA separate, the annealing phase begins. Annealing occurs around 50°C to 60°C for 30 seconds and allows the primers to adhere to the now single strand DNA. Forward and reverse primers are designed and ordered to bind at the beginning and end respectively of the DNA sequence that is being amplified. Due to the uniqueness of the DNA strand, the annealing temperature depends on the primers. The final phase is elongation of the DNA sequence. This phase requires the heat-tolerant enzyme Taq Polymerase to bind to the primers and begin replicating the DNA strands. Taq Polymerase has an optimal temperature around 72°C and can polymerize about a thousand base pairs per minute. Therefore, the extension time for this cycle depends on the length of the DNA sequence that is being replicated. Each sample to be amplified is prepared with 18μL of water, 2.5μL of Taq Polymerase buffer, 0.5μL of Taq polymerase, 0.5μL of 0.2mM dNTPs, 1.3μL of the 0.2μM forward primer, 1.3μL of the 0.2μM reverse primer, and 2–3μL of the sample DNA. The buffer and polymerase are prepackaged in the advantage polymerase kit from Clontech®. To ensure that the correct DNA sequence is amplified, the DNA fragments are separated via gel electrophoresis and compared to standard measurements.

Gel electrophoresis separates fragments of DNA based on their size. The fragments of DNA are loaded into a 0.7% agarose gel in 1X Tris-acetate-EDTA (1X TAE) buffer solution with pH 7.6 (48.8g/L Tris base, 11.4mL/L glacial acetic acid, 20 mL/L 0.5 M Na EDTA), and an electrical current is applied to the gel. 1X TAE is made by mixing 50mL of 20X TAE and 950mL of water. The electric field causes the negatively charged DNA fragments to move through the porous gel toward the positive

leads. The smaller fragments move faster and farther than larger ones, thus separating the fragments based on size. DNA ladders with standard fragment sizes are also run with the DNA samples in order to provide a "ruler" to measure the unknown fragments. The 0.7% agarose gels range from small, medium, and large sizes. Small agarose gels contain 0.21g of agarose in 30mL of 1X TAE. Medium gels contain 0.35g of agarose in 50mL of 1X TAE. Large gels contain 0.42g of agarose in 60mL of 1X TAE. The solution is heated for about one minute before adding 0.5µL of 1mg/mL ethidium bromide and being poured into the gel mold to solidify. The ethidium bromide binds to the DNA and causes the fragments to fluoresce under UV light. Loading dye is also added to the DNA samples so that they are easier to see when being loaded into the gel wells.

In order for yeast cells to uptake the DNA amplified via PCR from the environment, the cells must undergo electroporation. Electroporation increases the permeability of a cell's plasma membrane via an external electrical field. This process allows the *H. capsulatum* yeast cells to uptake the DNA construct that will replace and knockout the *MS95* gene. To begin, 5mL of cells is placed in 15mL tubes and spun down in a centrifuge at -25°C and 200 RCF for 2 minutes. After the supernatant is poured out, the pellet is resuspended in 5mL of 10% mannitol. The solution is pulse vortexed to resuspend the cells. The cells are centrifuged again at -25°C and 200 RCF for 2 minutes, and all of the supernatant is removed with a sterile pipette. Next, 200µL of fresh 10% mannitol is added to the tubes, and a sterile pipette is used to resuspend the cells. Once suspended, the cells and DNA are added to a cuvette. One cuvette has cells without DNA and is labeled as a blank. The cuvettes are placed into a BIO RAD GenePulser Xcell™, with the following settings: V = 750; µF = 25; Ω = 600; and mm = 2. When ready, the

cells are pulsed with an electrical force to increase the permeability of the plasma membrane. Once electroporated, the cells are removed from the cuvettes and plated onto *Histoplasma*-macrophage media (HMM) ura (-) plates. Using plates that lack uracil will ensure that only the cells that took up the DNA fragments will grow.

 H. capsulatum cells are grown on solid or in liquid HMM. HMM is prepared by mixing 20mL of Type 1 water and 25mL of 2X HMM stock [100ml of 2X stock is 2.14g of F-12 HAMS powder (Sigma Chemical Co.), 3.64g of glucose (purchased from American Type Culture Collection in Manassas, Virginia), 0.2g glutamic acid, 2mL cysteine stock, 1.2g HEPES, pH 7.5, and filter sterilized]. The broth was supplemented with 50μg/ml of ampicillin and 100μg/ml of streptomycin, to prevent contamination. The solution can be added to 1.5% w/v agarose to make plates or kept in its liquid state at 4°C for broth. Two different types of plates can be made: ones with uracil [ura (+)] and ones without uracil [ura (-)]. Ura (+) plates are made by autoclaving 3.75g of agarose in 217mL of water. When cooled, 250mL of HMM, 33mL of 15x uracil (100μg/mL), and 500μL of amp/strep antibiotic are added to the agarose solution, and the solution is poured into plates to solidify. Ura (-) plates are made by autoclaving 3.75g of agarose in 250mL of water. When cooled, 250mL of HMM and 500μL of amp/strep antibiotic is added, and the solution is poured into plates to solidify. Cells that may contain a knockout are grown on 5-FOA plates. 5-FOA plates contain 10.7g F-12 Nutrient Mix, 18.2g glucose, 1.0g glutamic acid, 0.084g cysteine, 7.5g sodium citrate, 3g 5-FOA, and 0.1g uracil. The solution is heated to 55°C, cooled, (pH) to 7.5, and filter sterilized. Before being mixed with agarose, 2.5mL of hygromycin (150μg/mL) and 2.0mL of amp/strep antibiotic are added to the solution.

Although cells may grow on the 5-FOA plates, two procedures are used to ensure that the *H. capsulatum* cells underwent allelic replacement and contain a knockout: PCR and Southern Blot. The knockout DNA sequence is larger than the wild type DNA and can be separated and identified using PCR. However, a more inclusive procedure is a Southern Blot. The Southern Blot involves extracting DNA from the potential knockout colonies, cutting the DNA with specific restriction enzymes, subjecting the cut DNA to gel electrophoresis, and then transferring the DNA bands to a Nylon N+ membrane (Millipore). The blot is probed with P^{32} labeled fragment containing the *MS95* open reading frame, stripped, and re-probed with the hygromycin (*Hyg*) antibiotic resistant marker. Images are taken using a Typhoon 9400 phosphoimager. For a knockout confirmation, DNA bands must appear with the *Hyg* marker probe, but not with the *MS95* open reading frame probe. This confirms that the *Hyg* antibiotic resistant marker replaced the *MS95* open reading frame in the DNA sequences, thus knocking out the gene. The wild type DNA should have a band with the *MS95* open reading frame probe, but not the *Hyg* antibiotic resistant marker probe because this DNA has not been knocked out.

3.b Characterization of *MS95*

The characterization of *MS95* will focus on determining the specific function of the gene. Through unpublished research, *MS95* has already been classified as a single copy gene and mold-specific. This project will focus on determining if the cells can still transition from mold into yeast if the gene is made inactive, which is known as a knockout.

The *MS95* knockout was made via fusion PCR in which a large portion of the open reading frame was replaced with a *Hyg* antibiotic resistant marker and ligated into

the telomeric vector pRPU1 to create pDWU1. The vector has a Ura5 marker that is used

for selection after electroporation. The vector was cut with the restriction enzyme PmeI to

expose the telomeric repeats. The exposed telomeres allow the vector to mimic a

chromosome, thereby enhancing the chances of a successful electroporation into *H.*

capsulatum cells. This new DNA combination was electroporated into a *MS95* expressing

strain, 186AS *Wu27* ura –, which did not contain a Ura5 marker. The cells containing the

plasmid that harbored the Ura5 marker were streaked onto HMM ura – plates and grown

for seven days. Colonies were chosen and inoculated to 1mL of HMM and grown for

seven days under stressful conditions at 37°C to induce allelic replacement (Fig 2).

Native locus

Mutant locus
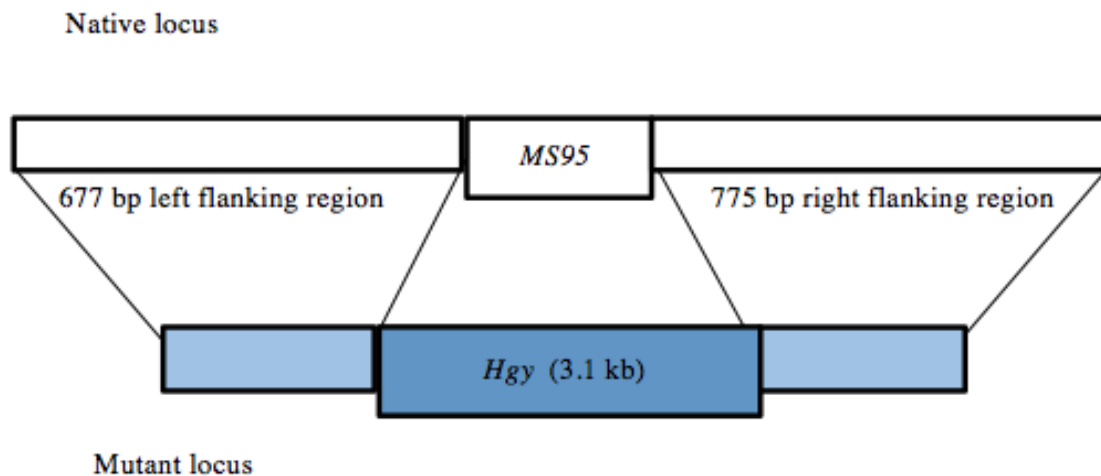
Figure 2. Genomic Replacement Map of *MS95* knockout. The 677 base pair left flanking region and the 775 base pair right flanking region were fused with the *Hyg* marker via fusion PCR. During allelic replacement, the *Hyg* marker replaced part of the *MS95* open reading frame, which created a knockout.

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Stressful growing conditions encouraged recombination to occur, which causes the cells to undergo genetic recombination and to take up the *Hyg* antibiotic resistant marker into their genetic code. This process replaces a portion of the *MS95* open reading frame with the *Hyg* antibiotic resistant marker. After the seven days, the cells were streaked onto 5-FOA plates that were supplemented with the antibiotic hygromycin and grown for ten to fourteen days. This process allowed for selection against the Ura5 marker and for the *Hyg* antibiotic resistant marker. Of the colonies that grew, twenty-five were chosen at random and had their DNA extracted. The DNA was analyzed with PCR and Southern Blot to scan for a knockout.

The *MS95* knockout was confirmed with PCR and Southern Blot procedures. The PCR denaturing phase occurred at 98°C for 30 seconds, and the annealing temperature was set to 60°C for 60 seconds. The extension time was set for 6 minutes at 72°C because the knockout DNA fragment is 5.5 kilobases, while the wild type DNA fragment is 3.5 kilobases. The primers for the PCR were designed to amplify 500 bp up and down stream on the chromosome that is not present in the vector. For the Southern Blot, *MS95* knockout and *Wu27* wild type strain genomic DNA was digested with restriction enzymes EcoRI and EcoRV. EcoRI and EcorV are non-cutters and do not cut within the *MS95* open reading frame. The blot was probed with P^{32} labeled fragment containing the *MS95* open reading frame, stripped, and re-probed with the *Hyg* antibiotic resistant marker.

Once conformed, the mold and yeast *MS95* knockout cells were compared to the *Wu27* wild type cells on various characterization studies including: phenotypic

comparisons, mold and yeast growth curves, and a mold and yeast stressor test with methyl methanesulfonate (MMS).

The physical appearance of the yeast and mold cells of both *MS95* and *Wu27* wild type strains were compared in liquid and solid HMM. Both strains were grown as yeast at 37°C in 50mL of HMM broth for three days. Each day, Pictures of the cells were taken with a 150 confocal microscope (Zeiss) at 63X. *MS95* knockout and *Wu27* wild type strains were also grown as mold at 25°C on HMM plates for twelve days. Pictures were taken with an EOS 20D Canon camera for seven days.

The growth rates of *MS95* knockout and *Wu27* wild type strains were compared in yeast and mold growth curves. For the yeast growth curve, *MS95* knockout and *Wu27* wild type cells were grown as yeast at 37°C in HMM liquid broth. The klett of each strain was measured and recorded in triplicates every six hours for a total of thirty hours using a Klett Colorimeter (Scienceware). For the mold growth curve, *MS95* knockout and *Wu27* wild type cells were grown as mold at 25°C on HMM plates. The hyphal extensions of each sample were viewed and measured using a 10X ocular micrometer on a C2M4 Labomed microscope once a day for ten days.

To determine if the *MS95* functioned in DNA repair, *MS95* knockout and *Wu27* wild type strains were grown on ura (+) plates on top of filters as mold at 25°C. After one week, the filters were transferred to plates containing different concentrations of MMS: 0.005%, 0.1%, and 0.3%. The cells were grown as mold at 25°C for 12 days. Pictures were taken with the AM4113T Dino-Lite Pro USB Digital Microscope on days 0, 4, 7, 10, and 12.

Chapter 4: Results

  According to the Broad Institute Database, *MS95* consists of 1499 nucleotides with 3 introns. *MS95* codes for a protein containing 316 amino acids (Fig. 3).

  Figure 3. *MS95* gene sequence. Translated protein sequence of 316 amino acids shown top. DNA coding sequence of 1499 nucleotides with introns in grey shown bottom.

After the *MS95* knockout was created with fusion PCR, it had to be confirmed with a PCR and a Southern Blot. The *Wu27* wild type gDNA is 3.5 kilobase pairs and is represented by the 3.5 kb band. The *MS95* knockout is 5.5 kilobase pairs because the open reading frame has been deleted and replaced with a larger fragment that contains the *Hgy* antibiotic resistant marker. Lanes 1, 3, and 4 represent the *Wu27* wild type gDNA, while lane 2 represents the *MS95* knockout gDNA (Fig. 4).
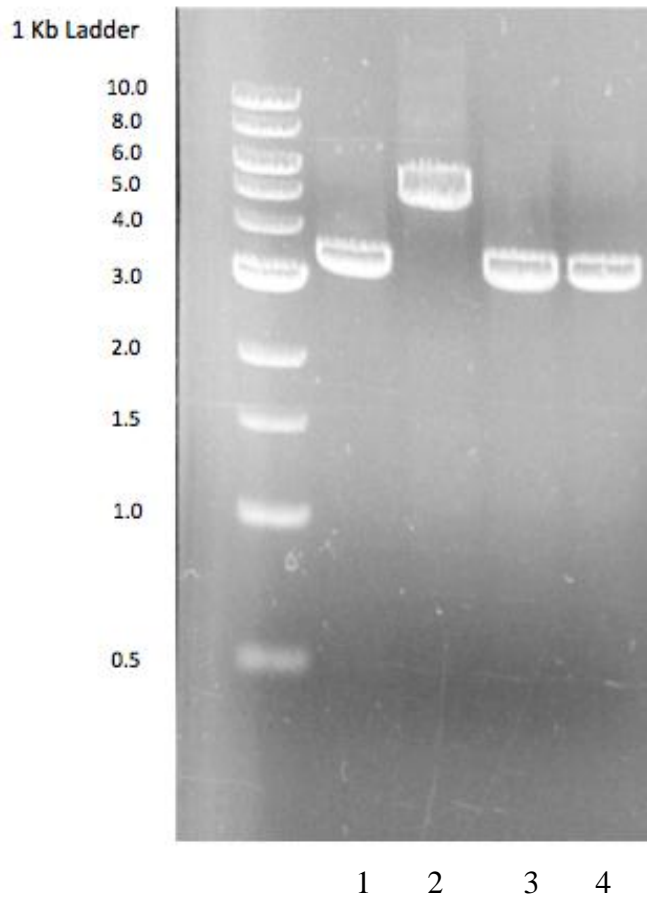
Figure 4. PCR confirmation of *MS95* knockout. The knockout is represented by the 5.5 kb band in lane 2. The *Wu27* wild type is represented by the 3.5 kb band in lanes 1, 3, and 4.

The *MS95* knockout was also confirmed with a Southern Blot. After the gDNA was digested with restriction enzymes EcoRI (A) and EcoRV (B), the blot was probed with P^{32} labeled fragment containing the *MS95* open reading frame, stripped, and re-probed with the *Hyg* antibiotic resistant marker. The results show that the *MS95* open reading frame is present in the *Wu27* wild type strain, but not the *MS95* knockout. They also show that the *Hyg* antibiotic resistant marker is present in the *MS95* knockout, but not in

the *Wu27* wild type strain. These results were expected because the open reading frame in the *MS95* knockout was replaced with the *Hyg* antibiotic resistant marker, but still remains intact in the *Wu27* wild type strain (Fig. 5).
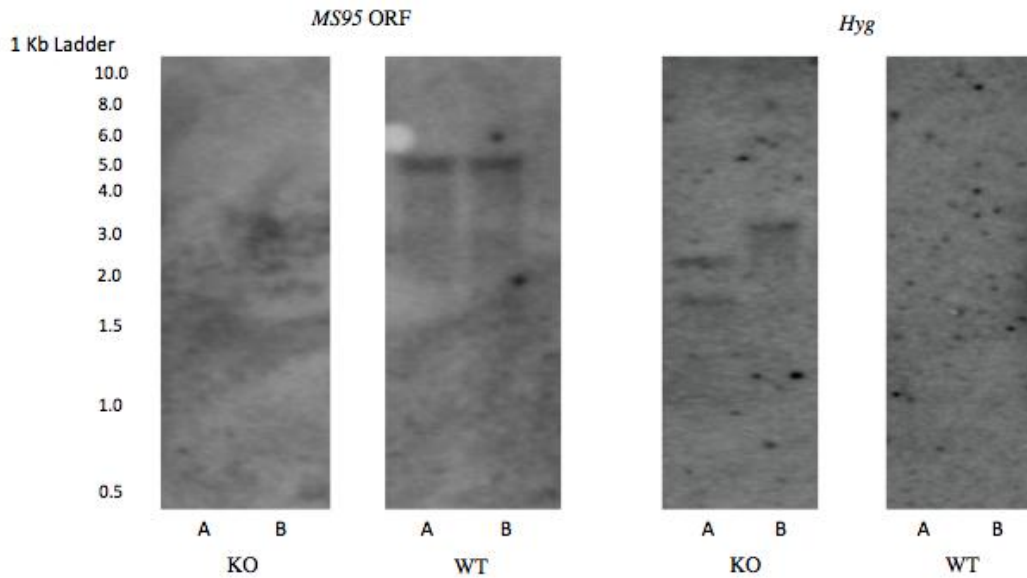
Figure 5. Southern Blot confirmation of *MS95* knockout. *MS95* knockout and *Wu27* wild type strain genomic DNA was digested with restriction enzymes: EcoRI (A) and EcoRV (B). The blot was probed with P^{32} labeled fragment containing the *MS95* ORF, stripped, and re-probed with the *Hyg* marker.

With the *MS95* knockout created and cultured regularly in liquid HMM at 37°C, different phenotypic, growth, and stressor tests were used to determine if *MS95* is involved in mold to yeast dimorphism. The first phenotypic comparison was between yeast and mold morphological forms of *MS95* knockout and *Wu27* wild type strains grown on solid HMM (Fig. 6).
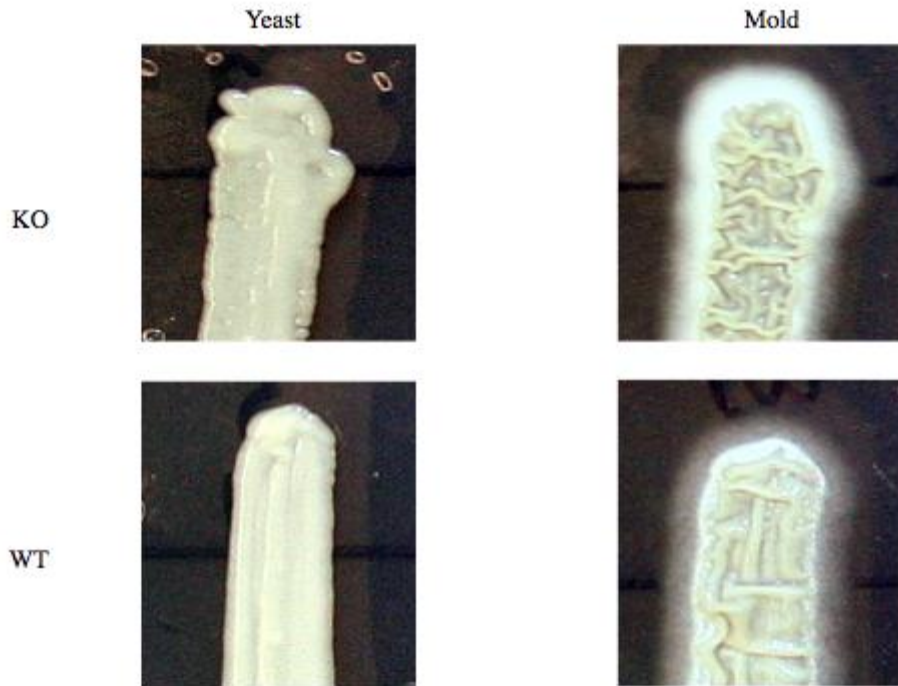
Figure 6. Comparison of Yeast and Mold phenotypes of *MS95* knockout and *Wu27* wild type strains on solid HMM. Pictures taken on day 1 (left) and 6 (right).

The second phenotypic comparison was between yeast and mold forms of *MS95* knockout and *Wu27* wild type strains grown in liquid HMM (Fig. 7). Both phenotypic tests were used to see if *MS95* functioned in controlling the phenotypical expression of cells and regulating cellular dimorphism between mold and yeast forms.

The next analytical tests were mold and yeast growth curves. The growth curves determined if *MS95* functioned in regulating cellular growth under normal conditions. All of the growth curves were performed in triplicates and averaged together. For the yeast growth curve, yeast cells were grown in liquid HMM at 37°C and were measured with a Klett Colorimeter every six hours for a total of thirty hours (Fig 8).
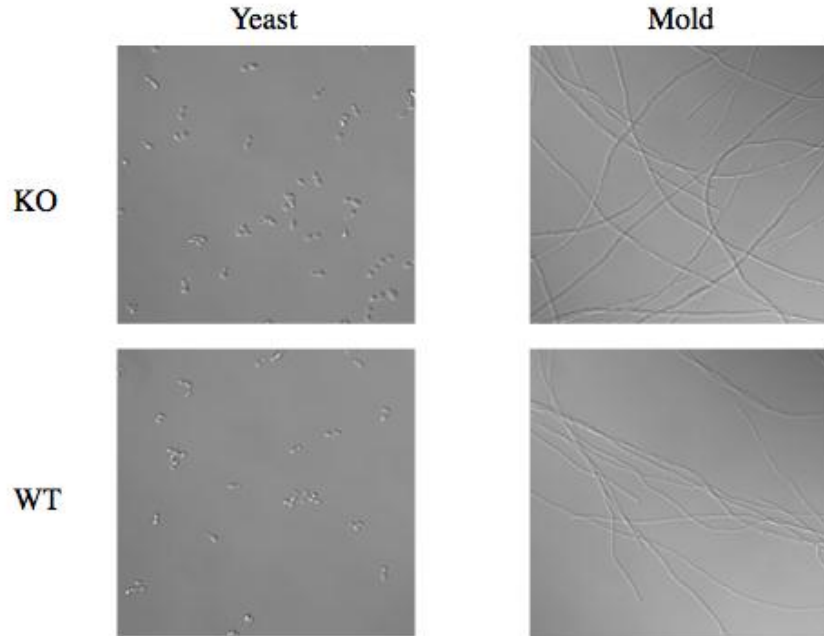
Figure 7. Comparison of Yeast and Mold phenotypes of *MS95* knockout and *Wu27* wild type strains in liquid HMM.
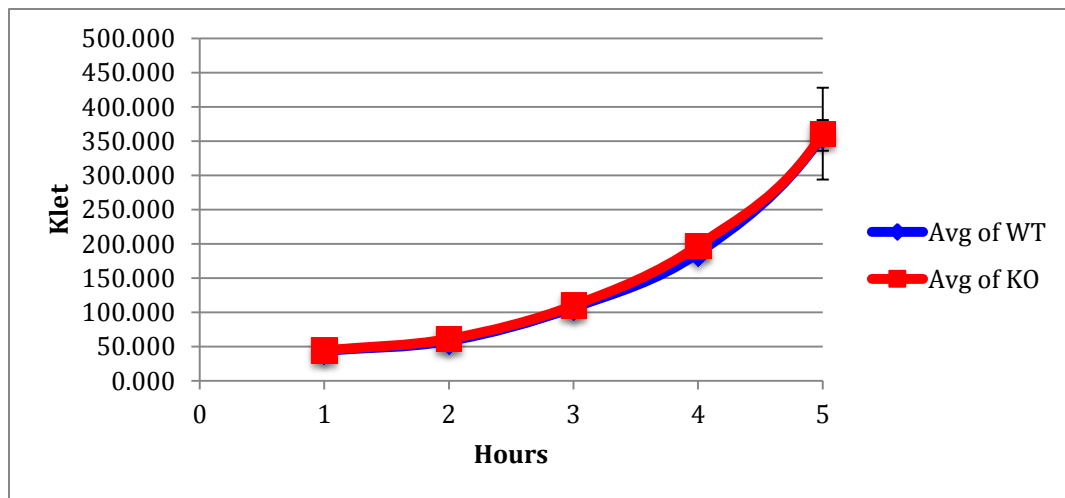

Figure 8. Growth Curve Comparisons of *MS95* knockout (KO) and *Wu27* wild type (WT) strains grown as yeast at 37°C in liquid HMM.

For the mold growth curve, mold cells were grown on solid HMM plates at 25°C, and the hyphal extension were measured with a 10X ocular micrometer on a C2M4 Labomed microscope once a day for ten days and averaged together (Fig. 9).
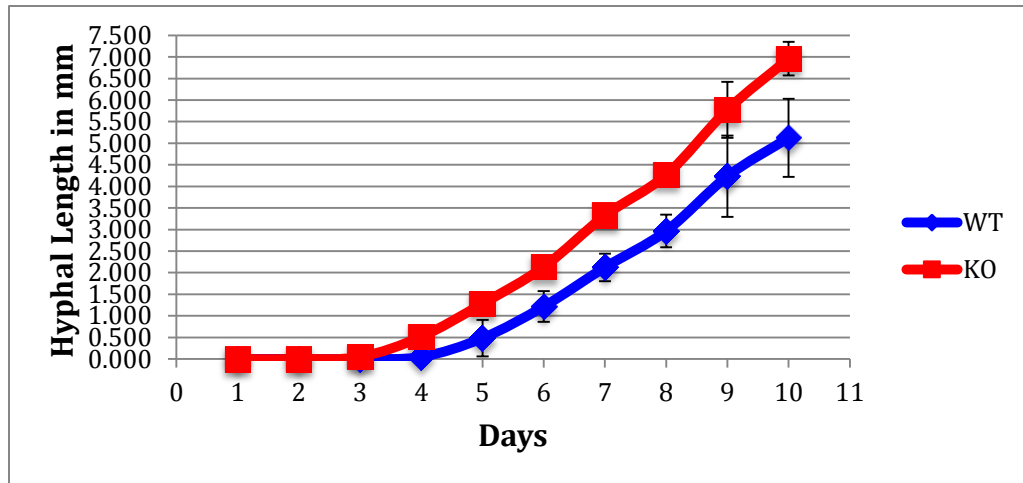

Figure 9. Growth Curve Comparisons of *MS95* knockout (KO) and *Wu27* wild type (WT) strains grown as mold at 25°C on solid HMM

The final test was a stress response test to determine if *MS95* functions in DNA repair. *MS95* knockout and *Wu27* wild type strains were grown on solid HMM plates for twelve days with three different concentrations of MMS: 0.005%, 0.1%, and 0.3%. (Figs. 10, 11, 12 respectively). Pictures were taken with the AM4113T Dino-Lite Pro USB Digital Microscope on days 0, 4, 7, 10, and 12.
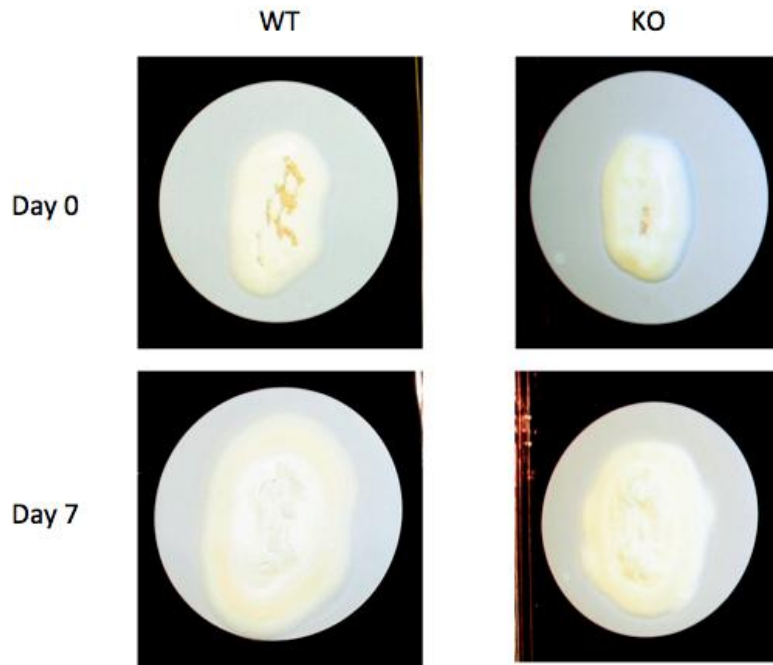
Figure 10. *MS95* knockout and *Wu27* wild type strains on 0.005% MMS stressor plates.
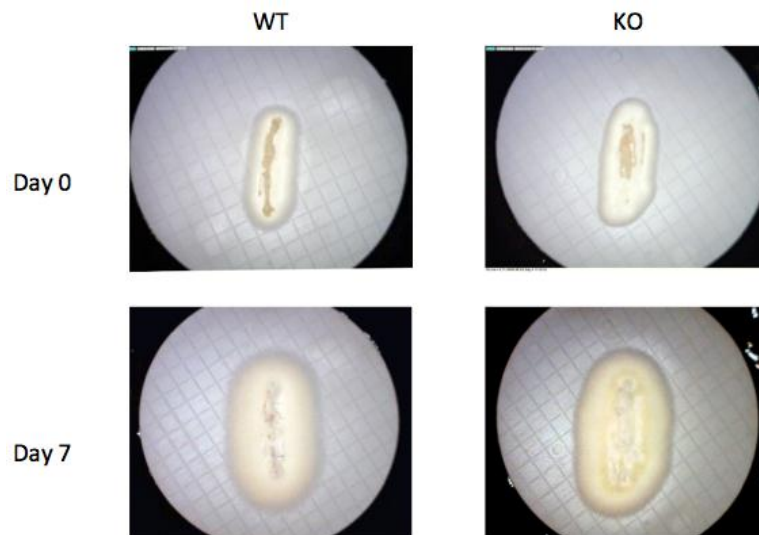

Figure 11. *MS95* knockout and *Wu27* wild type strains on 0.1% MMS stressor plates.
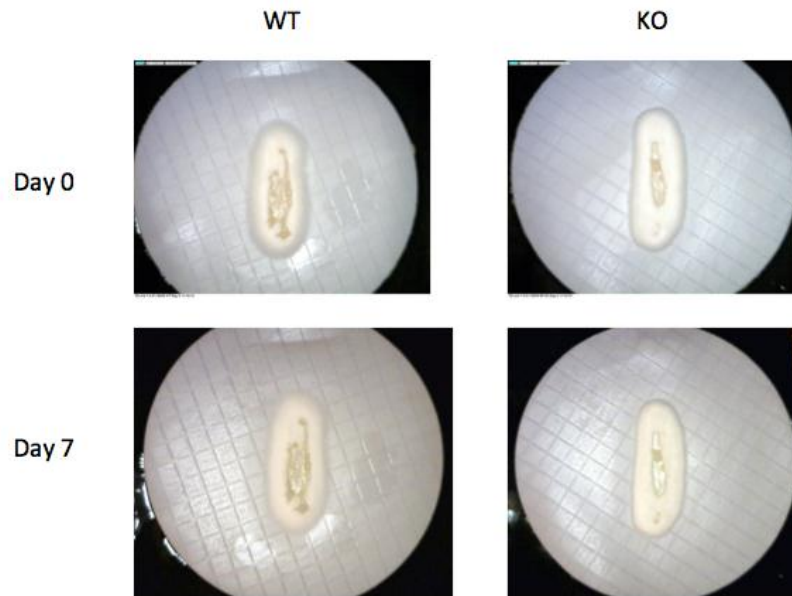
Figure 12. *MS95* knockout and *Wu27* wild type strains on 0.3% MMS stressor plates.

Chapter 5: Discussion

Histoplasma capsulatum, the dimorphic fungus, causes the respiratory disease histoplasmosis. This dimorphic fungus grows as mold at 25°C in soil contaminated by bird or bat excreta. If the soil is disturbed, mold spores can enter animal lungs and shift to the pathogenic yeast at 37°C. Dramatic changes in gene regulation must occur for the shift to be successful, which is evident in the numerous genes that are only expressed in either the yeast or mold forms.

In order to determine which genes are involved in the dimorphic shift, differently expressed genes are chosen and characterized. *MS95* is a mold specific gene that belongs to a gene family of stress proteins known as DDR (DNA Damage Responsive) and is believed to repair DNA damaged by heat stress. To determine *MS95* function, a knockout

was created via fusion PCR and tested under different conditions. The *MS95* knockout was confirmed with a PCR and Southern Blot. The PCR shows the knockout gene at 5.5 kb. This is 2 kb larger than the *Wu27* wild type because the *MS95* knockout contains the *Hgy* antibiotic resistant marker (Fig. 4). The Southern Blot further confirms the knockout by showing that the *Hgy* antibiotic resistant marker is present in the *MS95* knockout strain, but not in the *Wu27* wild type strain. The blot also shows that the *MS95* open reading frame is present in the *Wu27* wild type strain, but has been replaced in the knockout strain.

With the *MS95* knockout confirmed, two phenotypic tests were performed to determine if *MS95* plays a role in determining the phenotypic appearance of either mold or yeast cells. *MS95* knockout and *Wu27* wild type strains were grown as yeast at 37°C in liquid HMM (Fig. 6) and as mold at 25°C on solid HMM plates (Fig. 7). The phenotypic comparisons of the yeast and mold cells do not indicate any significant differences in morphology. The *MS95* knockout is similar to the *Wu27* wild type in size, shape, texture, and color in both yeast and mold forms. These findings suggest that *MS95* does not play a significant role in determining phenotype. Next, two growth curves of *MS95* knockout and *Wu27* wild type strains grown as yeast in liquid HMM at 37°C (Fig. 8) and as mold on solid HMM at 25°C (Fig. 9) were compared. The klett of the yeast was measured over thirty-six hours and averaged, while the mold hyphal extensions were measured once a day for ten days and averaged. The two graphs show similar growth rates for both *MS95* knockout and *Wu27* wild type strains in both mold and yeast forms. These results suggest that *MS95* does not play a significant role in maintaining normal growth rates. Also, hyphal extensions appeared at the same rate for both the *MS95* knockout and *Wu27* wild

types strains, suggesting that *MS95* does not directly control the dimorphic shift from yeast to mold. Finally, to determine if *MS95* functions in DNA repair, *MS95* knockout and *Wu27* wild type strains were grown on stressor plates containing 0.005%, 0.1%, and 0.3% concentrations of MMS for twelve days (Fig. 10, 11, 12 respectively). From the results, it is evident that the *MS95* knockout strains were not affected by the MMS due to their similarity in color, size, and concentration of hyphal extensions to the *Wu27* wild type strains. It may be possible that the concentrations of MMS were too low and that another experiment will need to be conducted. Nevertheless, these preliminarily results suggest that *MS95* does not play a significant role in DNA repair because the knockout cells were able to compensate for the gene loss, remain healthy, shift from yeast to mold, and prosper as well as the *Wu27* wild type cells.

All of these results suggest that *MS95* is not directly involved in the dimorphic shift form mold to yeast. If it did play a direct role, then it would be expected that the knockout cells could not shift from mold to yeast during the temperate change or that some physical, structural, or growth difference would be evident between the *MS95* knockout and *Wu27* wild type strains. Future plans for *MS95* will be forcing overexpression of *MS95* in the yeast phase growing temperature in order to confirm that it is not involved in dimorphism.

Primers

Special note: there are 87 bp left of the open reading frame from the knockout that have not been deleted. The open reading frame starts at base pair 530-1762. The *MS95* knockout primers for the PCR confirmation are as follows:

MS95 forward chromosome
GGGACTTAACAAAGTGAGCGCCAATCTTTCC

MS95 reverse chromosome
CCCTATTTATATGTTTTTTCCTTGTCTTCTTTCTATTTTC

These primers are designed to amplify 500 bp up and down stream on the chromosome that is not present in the vector.

The primers used for the Southern Blot confirmation are as follows:

MS95 open reading frame forward
CAATCTCATCTCCCTTGCTGGCCAGCCCATTCCAGTTC

MS95 open reading frame reversed
GAATAATTAGTATGCAACTTGTAGAAATGAATGGATAGTC

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