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Chapter One
Introduction

Candida albicans is common diploid yeast that can be found in the gastrointestinal tracts of most human beings. (1) It is an opportunistic yeast pathogen that may exist in three forms. Other than the mitochondrial DNA there have been no plasmids found in *Candida*. Since *C. albicans* is a diploid species, it has a greater potential for genetic diversity. In fact, scientists hypothesize that because *C. albicans* does not undergo meiosis, its diploidy, heterozygosity, mitotic recombination, and phenotypic instability, might be its tools to help maintain genetic diversity. (5) Because of its ability to attack the body, scientists strive to find a treatment that is both safe and efficient. Beyond pathogenicity, yeasts are significantly important because of their ability to perform as model organisms in the study of genetics. Many genetic problems have been solved through working with yeast mainly because of the ease in which scientists can culture them, their defined life cycles, and the speed in which generations arise from one another. (3) In fact, much of the progress that has been made thus far in the regulation of eukaryotic gene expression is because of yeast. (4) Therefore, as studies in yeast increase, the ability of scientists to study eukaryotic organisms increases as well. Both the genetics and molecular biological studies of *C. albicans* have increased within the last 10 years. Because of this increase, there is a significant amount of knowledge gained about the genome and the genetic map of *C. albicans*. (5)

Candida albicans generally receives more attention than the other *Candida* species because of its ability to be a human pathogen. *C. albicans* is considered an imperfect yeast. (5) It is a part of the normal flora of the gastrointestinal tract and oral cavity. Normally, *C. albicans* can be found in 80% of humans with no dire effects.

When the body undergoes slight changes (i.e. temperature, or pH) the unicellular yeast form of *C. albicans*, changes to the multicellular filamentous form. This is called Candidiasis and occurs as the common infections; yeast infection, oral thrush, and diaper rash. (1) It is when the body's immune system is under stress; as it is in AIDS/HIV patients, chemotherapy patients, transplant patients, that *Candida* can become a serious medical issue. In some studies, it has even been found that one-third of the cancer patient's after-death autopsies had evidence of *C. albicans* infections. The common vaginal infection accounts for much of the morbidity in women of childbearing age. One of the most frequently seen infections in immunocompromised systems is *Candida* esophagitis.

Scientists are considering two factors that may be important in the pathogenesis of *C. albicans*; it's capability to morph from yeast to hyphal phase, and its differences in colony morphology, cell shape, cell surface and cell permeability. (5) Unfortunately, unlike the bacteria, yeasts do not yet have proven virulence factors. However, science is suggesting that some of the major factors which contribute to the virulence of *Candida* are its ability to form hyphae, its ability to resist phagocytosis, its ability to adhere to epithelial cell surfaces, its ability to grow well at 37 degrees Celsius, and its ability to secrete acid proteinase. (5) Recent studies are looking into an iC3b receptor that may also influence virulence. (5) Unfortunately, factors like nutritional requirements complicate the virulence determinations. (3) Molecular Koch's postulates are now being considered as a way to study virulence in yeasts. (3) Because of the many problems that arise with *C. albicans*, scientists are taking steps to increase the development of its genetics. (5)

Much of the treatments offered for *Candida* infections are those that fight off the common problems caused by slight changes in the body. *C. albicans* has been found to be naturally resistant to many of the antibiotics that scientists have tried to use against it. However, some of the additional antibiotics that are considered unsuitable for treatment against infection, have worked in a laboratory setting. *C. albicans* have been found to be sensitive to mycophenolic acid. (5) Nystatin and amphotericin B make treated *C. albicans* cells more permeable to small molecules. Both of these are widely used in the treatment of surface and systemic infections. (5) Some of the most effective treatments are those that target the cytochrome P450 system. This system is necessary for demethylation among the sterol pathways. These treatments include: miconazole, ketaconazole, fluconazole, itraconazole, and SCH39304. (5) Some of these treatments can be found at general department stores.

Many of the treatments for *C. albicans*, including some of those above, are considerably harmful for the human body. The treatments are considered fungistatic, meaning that they lessen the fungus in question, but do not kill it. These treatments, however, do not just lessen the effects of the *C. albicans*, but any of the natural flora within the body; much of which is necessary for homeostasis. The azoles spoken of above, are becoming less useful because of their widespread use, and the ability of *C. albicans* to form a resistance because of this. Some of the other treatments even result in deleterious side effects. Because of this, treatment for *C. albicans* is in high demand. It is necessary that scientists search for new drug targets. Unfortunately many the proteins and metabolic pathways used with the current drugs, are shared by humans. Scientists must identify products unique to *C. albicans* pathogenesis.

In order to do this, exploratory research is necessary. Using gene reporter systems, we can analyze particular genes by studying their promoters. The objective of my study was to analyze the *CYC1* Promoter using the *Renilla* luciferase reporter system. *CYC1*, cytochrome c, isoform 1, is an electron carrier in cellular respiration. In oxidative phosphorylation, *CYC1* carries electrons from the cytochrome bc1 complex to cytochrome oxidase. (5) The CCAAT binding factor is responsible for activating many of the genes that are used in respiratory metabolism.

In hopes of finding the significance of the *CYC1* promoter regulatory elements in *Candida albicans* we have created six sequential deletions of the *CYC1* promoter to morph that allows it to infect humans. (5) (Five of these deletions were created by a previous undergraduate. One of them was created using the oligonucleotides listed in the table below.) Each deletion is placed in a plasmid that contains the *Renilla* luciferase gene, from *Renilla reniformis*, the sea pansy. Using the *Renilla* luciferase system we are able to detect the expression of the *CYC1* promoter. If the desired promoter is present and effective, a bioluminescence is detected.

This study is limited in that, unfortunately, scientists do not know *C. albicans* to a great extent. Exploratory research is necessary just to understand the workings of *C. albicans*; meaning that treatment may not be found until a considerable time in the future.

Chapter 2
Review of the Literature

Methods of Research

Research of *C. albicans* is an important endeavor that is necessary so that scientists can gain knowledge of its genetics. With this knowledge, proper treatment can be created. Genomics itself is the reason that there are complete or partial genomes for

fungi, which allows for deeper research. (3) Because of the progression of genetics, many of *C. albicans* characteristics have become known. It has also given scientists the opportunity to look back at old information and reinterpret it. (5) Some of the tools most often used to study pathogenic fungi are gene isolation, gene expression analysis, and gene disruption. (3) In these processes, transformation, hybridization, identification, PCR amplification, Northern blotting, and microarrays are all processes used.

Transformation, the ability to introduce new genetic material into an organism was a very important step in *C. albicans* genetics. It occurred in 1986 and utilizes the lithium acetate method to promote the uptake of DNA. *C. albicans* can also undergo electroporation to transform genetic information. (3)

Electrophoretic Karyotyping allows scientists to examine the chromosomes of an organism. *C. albicans* was one of the first species to be examined by pulse field electrophoreses. This process provided the knowledge that *C. albicans* chromosomes are slightly larger than it's "cousins," *Saccharomyces cerevisiae.* (5)

Another important ability concerning genetic methods is that to disrupt or delete genes. It allows the scientist to study the function of these deleted areas. Selectable markers are inserted into the gene and a disruptive mutant is formed. (3)

The cloning of *C. albicans* genes can occur by way of complementation, sequence homology, and *C. albicans* ability to confer new sequences on *S. cerevisiae* strains. The first gene isolated by complementation was URA3. (5)

Microarray technology is now available because of the genome-sequencing project. A great advantage to this is identification of patterns involving multiple genes. Regulatory genes are a main target in this study. (3)

Molecular epidemiology and taxonomy for *Candida* species can be achieved through many methods. One of them is to exam the fluorescence of the sample after a restriction enzyme cleavage with EcoRI. By looking at the background sequence of bands allows the scientist to distinguish which *Candida* species they are dealing with. Specifically in *C. albicans*, it can allow the scientist to distinguish between *C. albicans* isolates in an infected person. (5)

Also physical mapping methods are allowing for a genetic map to be created for *C. albicans*. (5) All of these methods and the new ones arising are allowing for greater knowledge of *C. albicans*. Equally important is the opportunity for scientists to compare *C. albicans* with *S. cerevisiae* and gain more knowledge of its workings.

Saccharomyces cerevisiae Comparison Methods

Saccharomyces cerevisiae is another common yeast that scientists know much about. *S. cerevisiae* is considered a model organism because of the ease that scientists can grow and manipulate it. (4) Its similarities to *C. albicans* have allowed us to make much progress. (2) Because *S. cerevisiae* and *Candida albicans* diverged nearly 300 million years ago, the knowledge that scientists have gained of *S. cerevisiae* cannot be directly applied to *C. albicans*. *S. cervisiae* is an avirulent facultative anaerobe that has two forms: the round yeast form, of the pseudohyphal form. *C. albicans* is a pathogenic obligate aerobe that may live in three forms: the round yeast form, the pseudohyphal form and the true hyphal form. (2) Because there are still some similarities, *S. cerevisiae* is used to make comparisons when attempting to learn more of *C. albicans*. Most of *C. albicans'* cloned genes were isolated through their counterparts in *S. cerevisiae*. (5) Some scientists consider that the beginning of molecular genetics of fungi was the genetic transformation of *S. cerevisiae* in 1977. Since then, transformation methods have increased considerably. (3) The DNA transformation system that

was developed for *C. albicans* is similar to that of *S. cerevisiae*. In fact, its development was possible because of the availability of corresponding genes previously cloned through *S. cerevisiae*. (5) *S. cerevisiae* is one of the reasons that such progress has been made with *C. albicans*, and it is also a model to be considered. For example, before DNA arrays were possible with *C. albicans*, *S. cerevisiae* microarrays were used to prove that *C. albicans* cells induce the principal enzymes MLS1 and ICL1. (3)

Saccharomyces cerevisiae: The Dual Luciferase system

An important system, the dual luciferase system is a reporter system that allows for the quantification of gene expression. (4) In the past, dual reporters have improved the accuracy of transient-reporter assays in mammalian cells. This specific dual luciferase system uses the luminescence of the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase proteins. (4) Because both of them are used, it allows for measurement of both luciferase activities separately in a single protein extract. Firefly and pansy luciferase systems are efficient because of their sensitive, easy, rapid and reproducible assays. (4) This is beneficial in that the sensitivity allows the reporter to be integrated into the genome and provide measurable signals. The control provides a control for experimental variation, like that which can happen in pipetting errors, or cell lysis efficiency. Also, because of the controlled assay system, many assays can be performed at the same time. (4) The reporter system is efficient and requires only one minute to complete each assay. The data yielded accurately reflects the promoter activity. (4) Considering that promoters are an integral part of the gene and the gene processes, this reporter assay is a significant step forward.

Creation of a Luciferase System for *Candida albicans*

Considering the success of the dual luciferase system, it is unfortunate that it cannot work for *C. albicans*. However, a highly sensitive bioluminescent reporter system exists for this purpose. In hopes of functionally characterizing *C. albicans* promoters, this system was created. In the beginning the firefly luciferase gene was used for *C. albicans* as well. However, when scientists noticed a lack of a detectable translation product in certain areas, they came to the realization that this method was not sufficient. (6) *Candida albicans* has adopted an unusual codon strategy where it uses a tRNA with a CAG anticodon to decode the codon CUG as serine, rather than leucine as most other organisms do. (6) Because the firefly luciferase contains 9 in-frame CUG codons, the luciferase system does not function as it should. (6) Scientists then studied the CUG contents of other biolumiscent reporters until they came upon the one that could efficiently analyze promoter action. By using the luciferase gene from *Renilla Reniformis*, the sea pansy, which contains no CUG codons in its reading frame, the problem is solved. This reporter system can be used to detect the strength and developmental regulation of promoters. This system also has the ability to measure gene expression. (6)

The CCAAT-Binding Factor in *Candida albicans*

In *Saccharomyces cerevisiae* the CCAAT-binding factor is an integral transcription activator that stimulates the expression of genes that encode for proteins necessary in respiration. (2) Because of its importance in *S. cerevisiae*, scientists hope to discover that if it plays a similar in *C. albicans*. With exploratory research such as this, knowledge of the organism grows and the possibility for treatment is nearer. Because of the differences between the two species, scientists cannot guarantee that the CCAAT

binding factor has maintained the same function or even the same import between the organisms. *C. albicans*, is generally a pathogen, surviving upon a warm-blooded host. *S. cerevisiae* can be found on sugary, fresh or decaying fruits. (2) *S. cerevisiae* is a respirofermentative yeast that gets its energy through fermentation, whether in the presence of oxygen or not. (2) *C. albicans* gets its energy through respiratory metabolism when in the presence of oxygen, although it can survive without oxygen. Research proved that CCAAT-binding factor was important in the repression of *COX 5* and *CYC1* promoters. (2) More research is necessary to detect precisely how, however scientists now have reason to believe that the CCAAT-binding factor is important even in facultative anaerobe. They also know that although the CCAAT binding factor is a necessity in *S. cerevisiae*, it does not necessitate that it be the same in *C. albicans*.

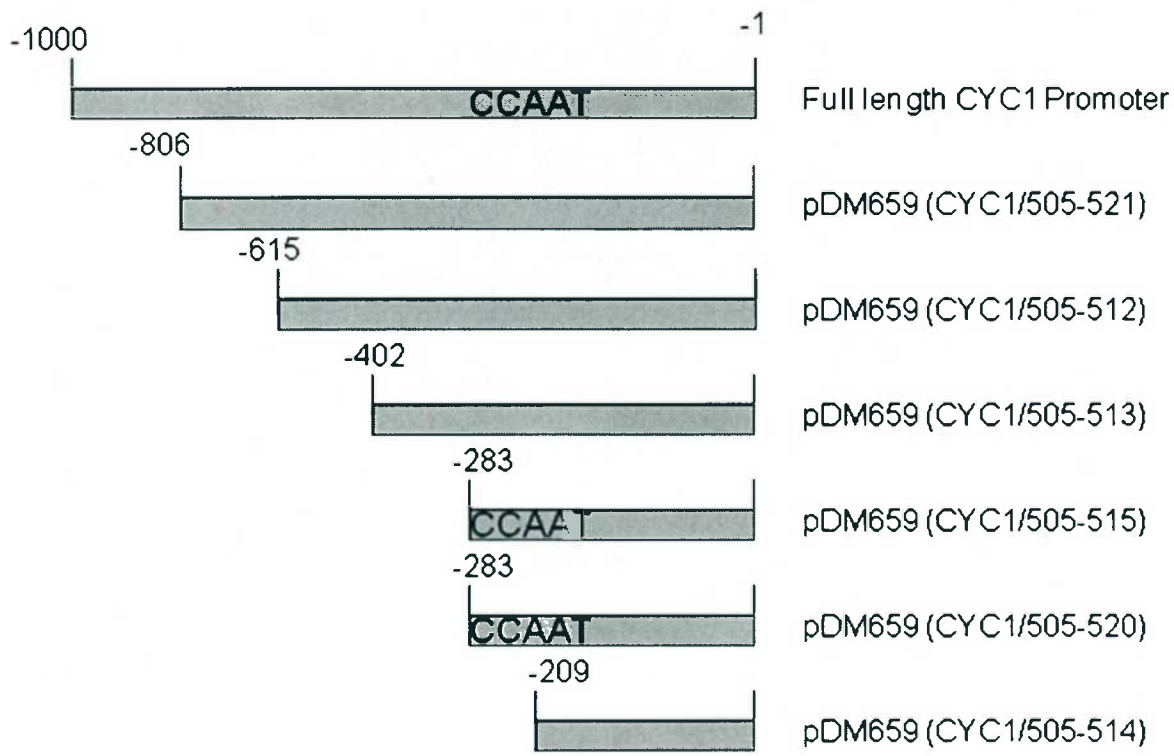
Chapter 3
Methodology

- Oligonucleotides: The sequential deletions of the *CYC1* promoter region used pDM659 (*CYC1*/505-504) as the template. The 5' primer used was oDM505 while the 3' primers used were oDM0512, oDM0513, oDM0514, oDM0515, oDM0520, and oDM0521. While the oDM505 provided the unique BAMHI site, the successive 3' primers added a unique HindIII site. A PCR reaction was performed for pdM659(*CYC1*/505-521) using the program : 1. 95°C for 2 minutes; 2. 95°C for 1 minutes; 3. 48°C for 1 minute; 4. 62°C for 3 minutes; 5. 72°C for 8 minutes. Steps 1-3 would repeat 30 times before proceeding to steps 4 and 5.

Oligonucleotides used:

Primer	Sequence
oDM505	5'-ggccaagctttgtaatgatatgtatatagattaaattaaattaaattaaattgg-3
oDM521	5'-ggccggatgccgcttggacgtattcccaaatgtacac-3'

- Plasmid Construction: The plasmid pDM659 was digested with the enzymes BamH I and Hind III, resolved by agarose gel electrophoresis and purified using the GeneClean Kit BIO 101, according to the manufacturer's instructions. The plasmid pDM659 was then ligated, with the six PCR products, successively, using T4 DNA ligase under standard conditions. They were then introduced to Ca□Cl□ competent *E. coli* DH5alpha by transformation. Transformants containing the correct plasmids were identified by alkaline lysis mini-preparations of the DNA, restriction enzyme digestion, and agarose gel electrophoresis.

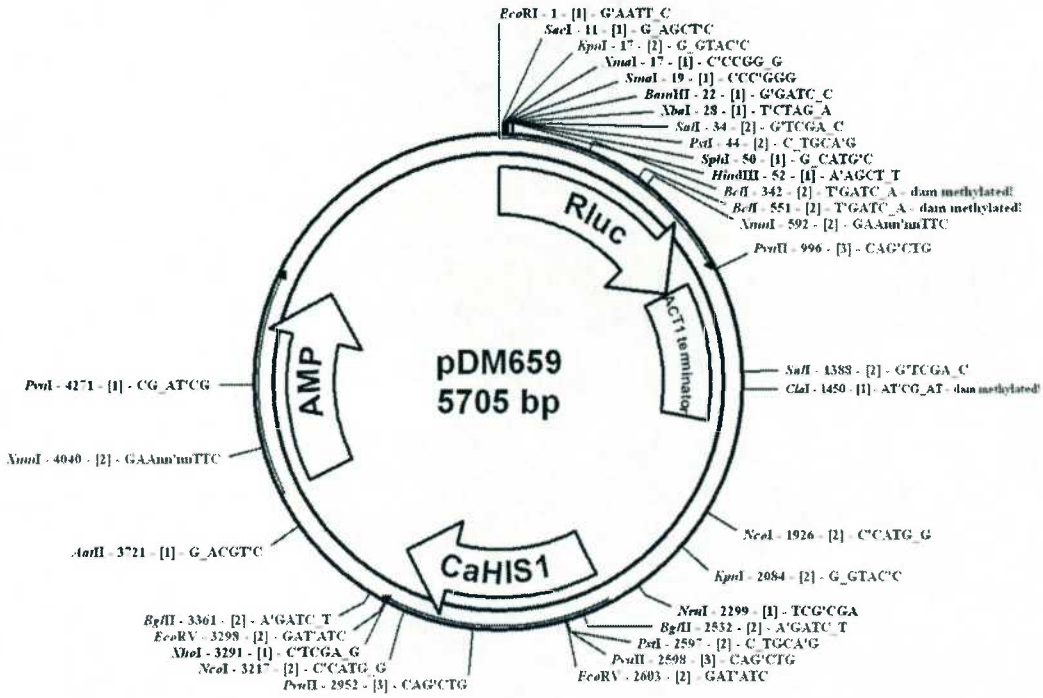


- Construction of luciferase reporter yeast strains: All yeast transformations were performed using the lithium acetate transformation protocol. The created plasmids were linearized with Nru1. The linearized plasmids were subsequently introduced to the BWP17pDM596 strain and were selected on medium lacking histidine. Upon completing the transformation of pDM659 (*CYC1*/505-512) into *Candida albicans*, independent colonies were chosen and assayed for *Renilla* luciferase activity.
- *Renilla* luciferase assay: Renilla luciferase assays were performed with the Renilla luciferase reporter assay system, Promega Corp., Madison, WI. The reagents were prepared according to the manufacturer's instructions. The cells were grown to exponential phase in appropriate media at 30°C. The cells were quantified using a spectrophotometer and 1mL of cells was pelleted in a centrifuge for 1 minute. After discarding the supernatant, 100µL of 1X passive lysis buffer was added to resuspend the cells before adding approximately 100µL of sterile glass beads. The sample was vortexed for 1 minute, placed on ice for 30 seconds, and vortexed again for 1 minute. 10µL of the sample was placed in a sample tube and 100µL aliquot of the substrate was added. Using the Turner Designs model TD-20/20 luminometer, the luminescence was measured. The Renilla *luciferase* activity was measured using the formula:

$$RLA = \frac{RLU}{OD \times \frac{Va \times Vc}{Vb}}$$

-Where: RLA-*Renilla* luciferase activity; RLU-*Renilla* luciferase luminescence reading; OD-optical density A600nm; Va-volume of sample placed in luminometer tube, 0.01mL; Vb-volume of lysis buffer, 0.1mL; Vc-volume of cells taken from original culture, 1mL. Agarose gel electrophoresis: DNA samples were analyzed on .8-1% agarose gel, containing 1XTAE according to standard procedures. The DNA samples were loaded with 6X loading dye and 1kbDNA ladder (Promega) was the standard.

Figure 2: the plasmid used: pDM659:



Chapter 4
Analysis of Results

Plasmid construction results: I was able to successfully create seven plasmids. These plasmids contained the full length *CYC1* Promoter and truncated portions of it, each smaller than the first, successively.

Transformation results: I was unable to transform plasmids: pDM(0521), pDM(0520), pDM(0515), pDM(014), pDM(0513). I was successful in transforming plasmid pDM(0512).

Renilla Luciferase results: I was successful in testing the yeast with the pDM(0512) transformant.

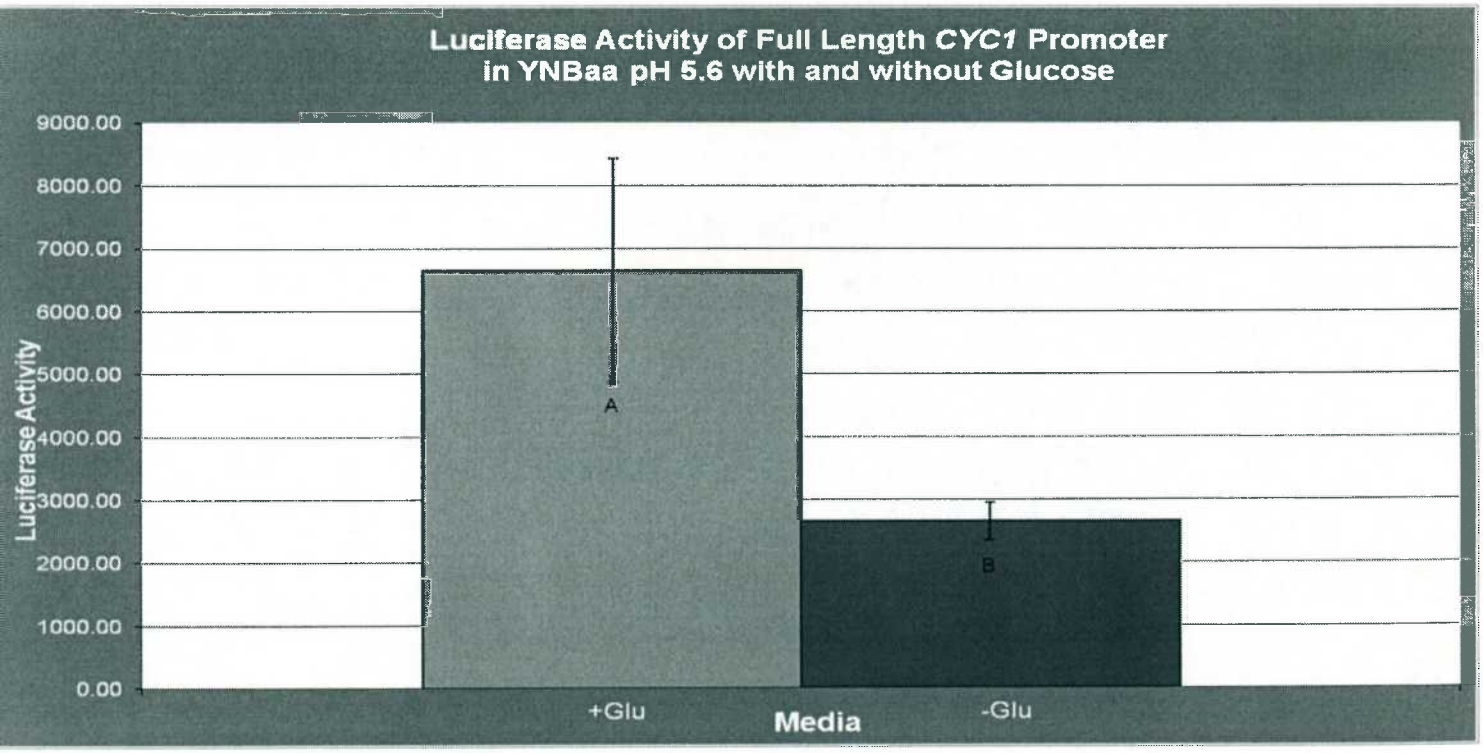


Figure 3: The luciferase activity of a full length *CYC1* Promoter in +/-Glucose solution.

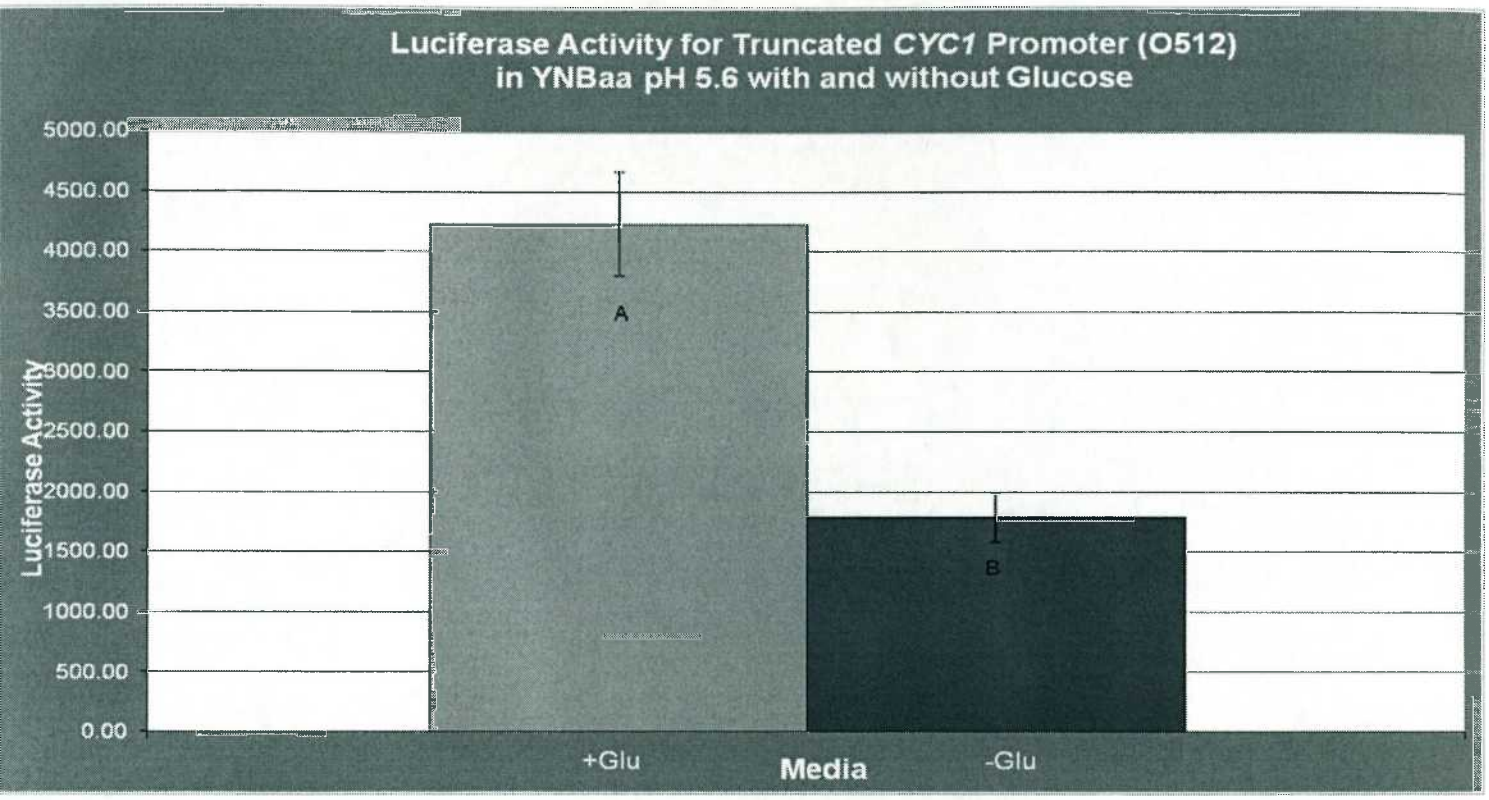


Figure 4: The Luciferase activity for the truncated *CYC1* Promoter (0512) in +/-Glucose solution.

Graphs: These graphs, as said above, express the activity of the full *CYC1* Promoter and the truncated *CYC1* Promoter (0512). They were tested in glucose, not necessarily for the quantitative results, but more to ensure that the *C. albicans* is functioning as it normally would. *C. albicans* is naturally more active in +Glu than in –Glu. Had *C. albicans* not reacted as such, we would have known that our experiment was faulty; in other words the +/- Glu is a method of control.

Discussion:

From these results there is one very important detail to take. Although the *CYC1* Promoter is missing nearly four hundred base pairs, it is still an effective promoter. This

is proven by the fact that it still emits luciferase activity although some of it has been truncated. Not only does it show luciferase activity, it reacts correctly in + and – Glucose.

Chapter 5
Summary, Conclusion and Further Research

Candida albicans is an opportunistic pathogen that is found in the gastrointestinal tracts of most humans. It generally takes on simple infections, however there are an increasing number of serious problem from *C. albicans* with those whose immune systems have been compromised. The virulence of *C. albicans* is yet unknown however, scientists speculate on the factors. *C. albicans* is not only being studied because of its pathogenicity, but because of the strides that can be made in all genetics through the study of yeasts. The research that is being done with *C. albicans* is only increasing our knowledge of the genetics of the organisms and so the genetics of other eukaryotic organisms. Scientists increase their knowledge of *C. albicans* by the comparison of it to *Saccharomyces cerevisiae*, which scientists know much of. However, because of the divergence of the two species, scientists cannot make assumptions that they are exactly alike. They have proven often that because of their differences, they are not. For example, the dual luciferase system used to detect gene expression in *S. cerevisiae* cannot be used in *C. albicans* because of its unusual codon strategy. Also, although the CCAAT binding factor is a necessity in *S. cerevisiae,* scientists could not assume that it would be the same in *C. albicans*, and because of this have gained new knowledge of its purpose.

The success of the creation of the reporter plasmids provides a learning tool that will give an understanding of the transcriptional regulation of the genetic programs of *C.*

albicans. In researching the regulatory pathways of *C. albicans*, scientists may be able to find treatments that are not toxic to humans. With the help of the *Renilla* luciferase bioluminescent reporter system, analysis of uncharacterized promoters is achievable. Through the successful transformation and assaying of pDM0512 we know that the *CYC1* Promoter is still effective although a portion of it has been deleted.

Further research includes:

- The successful transformation of the following plasmids into the BWP17 strain of *C. albians*.
  - -pDM0513
  - -pDM0514
  - -pDM0515
  - -pDM0520
  - -pDM0521
- The testing of each of the plasmids using the *Renilla* luciferase system.
- Then, scientists can determine the point at which the *CYC1* Promoter becomes ineffective.
- The relevance of the CCAAT binding factor in *C. albicans* may then also be observed; which would lead into another realm of research.

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