The Importance of the SET2 Gene in the Virulence of *Candida albicans*

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Abstract
Candida albicans, a commensal of digestive and genito-urinary tracts, is a common opportunistic fungal pathogen. This opportunistic pathogen can generate genetic diversity through several mechanisms. Aneuploidy within *Candida albicans* is thought to be the main cause of antifungal drug resistance. Thus, there is an urgent need for new drug targets. SET proteins, which are unique to the *Candida* genome, are a current area of interest and research for potential drug targets, although there is still much research to be done on these unique proteins. The main goal of this experiment was to knock out one allele of SET2 in *Candida albicans* and replace it with a His selectable marker. The results were not completely conclusive, but there was some evidence that the His marker correctly inserted for the SET2 gene. Further research and experimentation is necessary to determine if SET2 contributes to the virulence of *Candida albicans*, and if so, if it would be a good drug target.

I. Introduction

Opportunistic fungal infections have drastically increased over the last two decades, becoming a significant cause of morbidity and mortality. The *Candida* species are ubiquitous fungi that account for the most common fungal pathogens in humans (Lussier 1998). *Candida* is in a competitive balance with several other species of bacteria within our body. Therefore, the combination of these bacteria and the immune system of a healthy individual keep *Candida* growth in check; in most humans, *Candida albicans* is a harmless inhabitant of mucosal surfaces. When the equilibrium of the environment is disturbed, as with immune system suppression and modifications in microbial flora due to antibiotics, the organism is capable of invading and damaging host tissues (Hidalgo 2010).

Candida albicans can cause a range of infections, varying from surface-mucosal infections such as thrush and vaginitis, to more serious systemic infections. Pregnant women, neonates, and immunocompromised individuals are particularly vulnerable to *Candida* infections (Hidalgo 2010). Over 90% of HIV patients who are not receiving

highly active antiretroviral therapy eventually develop oropharyngeal candidasis (Hildago 2010).

Candida albicans' extreme versatility makes it an extraordinarily successful pathogen. A component of this versatility is its ability to survive in several anatomically distinct sites within the body. The fungi can adapt its growth to a range of physiological extremes, such as pH (Calderone 2001). *Candida albicans* can grow within a pH range of 2-10 *in vitro* (Wolf 2009). This allows for a wider spectrum of diseases that can be caused by the *Candida albicans* species (Calderone). In addition, there are several virulence factors that contribute to *Candida's* pathogenesis. These factors include host recognition biomolecules, secretion of aspartyl proteases and phospholipases, and morphogenesis (Calderone 2001).

I. Host recognition biomolecules

It is hypothesized that host recognition plays an important role in the virulence of *Candida albicans*. When the yeast cell binds to either host cells, host cell proteins, or microbial competitors, clearance by the host is prevented or reduced (Calderone 2001).

II. Secretion of phospholipases and aspartyl proteases

Candida albicans has the ability to secrete acid proteases and phospholipases which have the ability to penetrate and damage the cell envelopes of host cells (Hildago 2010). Four phospholipases have been identified thus far: PLA, PLB, PLC, and PLD. Only PLB1 has been shown to be required for virulence in an animal model of candidiasis. When the PLB1 gene was deleted, the gene-deleted strain produced less phospholipase, and displayed only 40% killing, compared to 100% in the wild-type strain (Calderone 2001). At least nine secreted aspartyl proteases have been discovered thus far, and are not

limited to *Candida albicans*. Expression of aspartyl proteases have been linked to invasion of host cell tissue, and are thought to be required for disease development (Calderone 2001).

III. Morphogenesis

Morphogenesis in the *Candida albicans* species can be defined as the transition between unicellular yeast cells and a filamentous growth form. This species has the ability to reversibly convert between unicellular yeast, pseudohyphal, and hyphal forms. Because of this, *Candida albicans* can be classified as a polymorphic species. Numerous investigators have found both unicellular and filamentous forms in infected patients (Calderone 2001). Thus, the ability of *Candida albicans* to switch between these three forms is an important part of its virulence. Still, we have yet to discover the exact relationship between morphological forms and disease progression. While understanding these virulence factors can give insights into development and progression of disease, another important branch of research has focused on treatment of Candiasis and the development of antifungal drugs.

Most of the structure of fungal and animal cells is highly conserved. This conservation has made it particularly difficult to find pharmaceuticals that selectively discriminate between pathogenic fungi and the human host. Resistance to the existing antifungal drugs is rapidly increasing (Lussier 1998). Azoles are the antifungal drugs of choice in the treatment of *Candida albicans*, since they cause very few side effects. The body builds up a resistance to azoles when used long-term, at low level doses for preventative treatment (Selmecki 2006). Research suggests that azole resistance is due to aneuploidy within *Candida albicans*. Selmecki *et al.* adapted comparative genome

hybridization arrays to analyze 70 azole-resistant and azole-sensitive strains from clinical and laboratory sources (2006). Aneuploidy was seven times as prevalent in fluconazole-resistant strains than in fluconazole-sensitive isolates. Aneuploidy, primarily trisomy, was most prevalent on chromosome 5 (Selmecki 2006). Because of this, there is an urgent need for new strategies and new drug targets. Reagents that are able to reduce chromosome breakage and/or recombination could be potentially useful to aide the current azole antifungal treatments (Selmecki 2006).

Because of *Candida's* ability to mutate in order to evade existing antibiotics, it is crucial to find new drug targets that are unique to *Candida albicans*. A current area of interest and research are the SET-domain proteins.

SET-domain proteins play an important role in catalyzing the methylation of lysine residues (Dehe 2006). Histone methylation is important for the regulation of chromatin and gene expression (Dillon 2005). The SET-domain has a characteristic motif of about 130 residues. Seven families of SET-domain proteins are known: SUV39, SET1, SET2, EZ, RIZ, SMYD, and SUV4-20. Currently, SET1 is the only well studied SET protein in *Candida albicans*. Raman *et al*. showed that disruption of the SET1 protein resulted in complete loss of methylation of histone 3 at lysine residue 4, a hyperfilamentous growth of the fungus, and a less negative cell surface charge which diminished adherence to epithelial cells. These effects were reversed upon gene reinsertion (Raman 2006). Continuing research suggests that SET1 regulates multiple processes important to the pathogenesis of Candidiasis. The N-terminal region of the SET1 protein is not homologous to any human proteins. This means that it may be a good target for the development of antifungal drugs and therapy.

This study opens the door to research on other SET proteins as possible drug targets. We set out to study *Candida albicans'* SET2 gene by deleting it using the homologous recombination method. The lab project also increased understanding of how drug resistance occurs, because histone modifications, specifically methylation, have been shown to be involved in genome stability and therefore may be involved in acquisition of drug resistance. While this study focused on the function of SET2 in *Candida albicans* through deletion analysis, this preliminary research could lead to investigation of the gene as a potential new drug target.

II. Materials and Methods

Plasmid Purification The miniprep method was used to isolate plasmid DNA from the bacterial cells. The plasmids p 1974, p1975, p1886 and p2190 were used. A culture was started from a frozen bacterial stock containing the plasmid. The bacterial cells were isolated using centrifugation. A high pH lysis solution was used to lyse the bacterial cells. Neutralization solution was added to reduce the pH. The reduction in pH caused the bacterial genomic DNA, proteins, and membrane components to precipitate out, while the plasmid DNA remained in the solution. The centrifuge tube was spun in the centrifuge at max rpm for 10 minutes. Resin tubes were made by poking a hole through the bottom of a sterile 1.5mL centrifuge tube, and by placing a small amount of glass wool on the bottom of the tubes. The resin tubes were piggy backed on 2 mL catch tubes. After centrifugation, lysate was added to each resin tube. The tubes were centrifuged at 7,000 rpm for 2 minutes. Liquid from piggy back tube was discarded and miniprep wash was added to the resin tubes. The tubes were centrifuged at 7,000 rpm for 2 minutes. Liquid in the piggy back tubes was discarded and again, miniprep wash was added, and

tubes were centrifuged. Resin tubes were placed on a new labeled 1.5mL tube. Sterile water was added to the tubes, and they were vortexed and spun at 7,000 rpm for 5 minutes to elute the plasmid DNA. The 1.5mL tube containing the purified plasmid was stored in -20° C.

Restriction Enzyme Digest After the plasmid DNA was isolated, it was cut with restriction enzymes to make sure that the isolated DNA produced predicted band sizes on an agarose gel. Xho I and Hind III restriction enzymes were used. The restriction enzymes were added to a 10x restriction enzyme buffer and sterile water. This solution was divided up into 3 tubes. 9 μL of miniprep DNA was added to each of the tubes and was repipetted to mix. The tubes were allowed to digest overnight at 37° C.

Agarose Gel Preparation Agarose gel electrophoresis was used several times throughout the experiment to analyze various samples of DNA. A 1% agarose gel was used. 0.3 grams of agarose was added to 30mL of tris-bovate-EDTA (TBE). Contents were swirled and heated in the microwave for 35 seconds. 2 μL of SyberSafe DNA dye was added and the solution was immediately poured into a gel tray. The gel was allowed to solidify for at least 20 minutes.

Gel Electrophoresis TBE buffer was added to the buffer chambers on both sides of the gel, so that the buffer just covered the top of the gel and filled the sample wells created by the comb. 2 μL of loading dye was mixed into each sample to be run. 1 Kb ladder DNA (Promega) was always loaded on the far left well. The samples were loaded and

the order was marked down. The top cover was placed back on the gel box, the wire connections to the electrophoresis power supply were hooked up, and the gel was allowed to run for 45 minutes at 110 volts.

Polymerase Chain Reaction (PCR) PCR was used in order to amplify the bacterial DNA product into thousands to millions of copies, with 5' and 3' ends that are homologous to SET2. A master mix, containing 5x buffer, deoxynucleotide triphosphates (dNTP's), forward primer (GAAATTTCAATTCAATTCAATTCGAT TTAAAAAGATCTTTATATCAAATAATATACTTTAGTATTTCTTATTTAAAAGTT GGTTATTCACGTTTTCCCAGTCACGACGTT), reverse primer (ATCCCTATAA GAAGAAATATTGCATTGAGTAAAACTCATAAAGTCAAATCTTCTAGGTTTTTT AATTTGTTTATTTTTCTTGTATGAACTGTGGAATTGTGAGCGGATA), and template DNA (containing the knockout SET2, and a p1375 His marker), was made. The master mix was split up between 5 tubes, and 1 μL of DNA polymerase was added to each of the 5 tubes. The tubes were placed in the thermal cycler for 30 cycles of PCR. An agarose gel was run to insure that the primers amplified the template DNA correctly.

QIAquick PCR Purification This procedure was used to purify DNA fragments from the PCR reaction, by cleaning out all the unused dNTP's, primers, etc. PBI buffer was added to the PCR sample in a 5:1 buffer to sample ratio. The QIAquick spin column was placed in a collection tube and the sample was added to the column. The column was centrifuged at 13,000 rpm for 1 minute to allow for binding of DNA. The flow-through was discarded and the QIAquick column was placed back in the same tube. The column

was centrifuged for an additional 1 minute. Next, the QIAquick column was placed in a clean 1.5mL micro centrifuge tube. The DNA was eluted by adding 30 μL of sterile water to the center of the QIAquick membrane and by centrifuging for 1 minute. 1 volume of loading dye was added to 5 volumes of sample, and samples were loaded and ran on an agarose gel in order to check for purification and concentration.

Yeast Strain Genotype Conformation The yeast strain, p7617 (from the Berman lab) was struck onto plates in order to obtain bacterial growth. The yeast strain is auxotrophic for histidine (His) and uridine (Uri). 200 μL of Uri was added to the first plate. This plate was used as the control, since the yeast strain requires both Uri and His for growth. 200 μL of Uri and 200 μL of His were added to the second plate. The solutions were allowed to dry on the plates, and they were then swabbed with p7617. The plates were allowed to incubate at 37° C for 1 day. This procedure double checks the genotype of the strain, as well as allows for growth of the strain in preparation for transformation.

***Candida albicans* Lithium Acetate Transformation** A transformation involves bacteria taking up exogenous DNA from the environment, and was performed in this experiment in order to replace the SET2 gene with the His marker. Cells were harvested from the plates using a toothpick and were placed into a screw cap conical vial. The cells were then washed with sterile water. The pellet was resuspended in TELiAc solution. 10mg/ml single stranded salmon sperm DNA was added to 2 empty microfuge tubes. Transforming DNA was added to one tube, while the other was used as a negative control. 0.1 mL of cells were added into the 2 tubes and the tubes were incubated at

room temperature for 30 minutes. PLATE mix was added to each tube, and the tubes were vortexed and incubated overnight. After overnight incubation period, cells were heat shocked in a hot water bath at 42° C for 1 hour. The *Candida* cells do not easily transform, so a temperature shock forces them to take up DNA from the environment. Samples were centrifuged at 5,000 rpm for 3 minutes to pellet the cells. The supernatant was decanted, and the pellet was resuspended in sterile water. The cells were then plated on 4 selective plates, each containing 200 µL of uridine (4mg/ml). Because the bacterial cells require both uridine and histidine in order to grow, only bacteria that have successfully taken up the PCR product with the selectable marker (His1) will survive. The plates were allowed to incubate at 30° C for 3 days.

Preserving Samples 500 µL of 15% glycerol was added to 16 different centrifuge tubes. A toothpick swab of cells from the selective plates was added to each tube. The tubes were then vortexed and then stored at -70°C. This procedure was done in order to save the transformed yeast indefinitely. When the strains were needed to be grown up for DNA isolation, the samples were thawed and struck out on 4 selective plates, again containing 200 µL of Uri.

Yeast Genomic DNA Preparation This procedure was done in order to isolate the genomic DNA of the *Candida albicans* cells. A swab of cells was scraped off the plate and added to a mix containing lysis buffer and glass beads. Next, phenol chloroform was added. The lysis buffer and glass beads act to disrupt the bacterial cell wall, while the phenol chloroform extracts protein. The mixture was vortexed for 20 minutes, and then

centrifuged for 5 minutes. The aqueous layer was transferred to a fresh tube containing 1 mL of 95% ethanol, and mixed well. Again, the tube was centrifuged for 5 minutes, this time to pellet the DNA/RNA. The supernatant was aspirated and the pellet was washed with 70% ethanol. The pellet was allowed to dry for 20 minutes at room temperature, then was resuspended in sterile water.

PCR Transformation Conformation 6 PCR primers were used in this experiment:

1. WT 5' Forward GGATGTGCCAAAGAATTAG
2. WT 3' Reverse CTACTAGAGCCATTTCTTG
3. His 5' Forward CCCAGAAATGGTCAATATAATCGTCC
4. His 3' Reverse CTCGAGTACCAATATATCGGTTGC
5. SET2 5' Forward GAATGGCAAACTGATGAAG
6. SET2 3 Reverse CTACTAGAGCCATTTCTTG

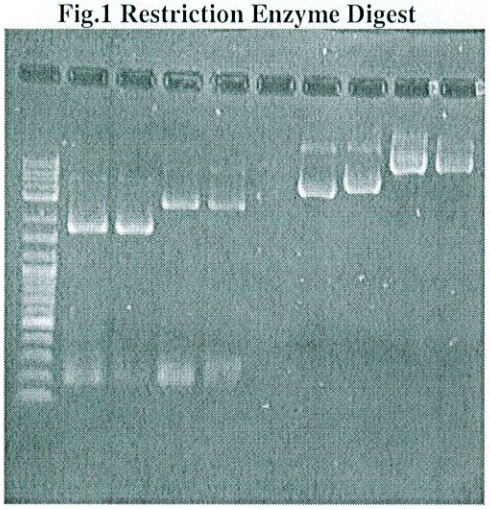
The primers were diluted into a 100 μM stock. The wild-type primers were added to 12 tubes containing the transformation DNA. The WT 5' forward primer and the His 3' reverse primer were added to 12 more tubes containing transformation DNA. The last 12 tubes contained the WT 3' reverse and His 5' forward primers. The first 12 tubes contained the WT reaction, the second set of tubes contained the forward reaction, and the last set of tubes contained the reverse reaction. The tubes were placed in the thermal cycler for 30 cycles of PCR. An argrose gel was run to test the transformation.

III. Results

The plasmids p1374 and p1375 were used in this experiment, because p1374 contains the selectable marker Uri, and p1375 contains the selectable marker His. The plasmids were cut with restriction enzymes (Hind III and Xho I) and the products were

run on a gel. The products produced strong bands within the DNA ladder. This suggests that in fact, the correct plasmid is being used.

Fig.1 Restriction Enzyme Digest



Lane 1: Ladder
Lane 2: 1374 Uncut
Lane 3: 1374 Hind III
Lane 4: 1374 Xho I
Lane 5: 1374 Hind III & Xho I
Lane 7: 1375 Uncut
Lane 8: 1375 Hind III
Lane 9: 1375 Xho I
Lane 10: 1375 Hind III & Xho I

It appears that the Hind III restriction site is absent from the plasmids, as the Hind III bands in lanes 3 and 8 are in line with the uncut bands in lanes 2 and 7. However, the Xho I restriction enzyme appeared to cut in the correct spot, as we see a shift in the bands in lanes 4 and 9. Lanes 5 and 10, which contain both restriction enzymes, appear in the same spot as the Xho I bands, because again, there was no Hind III site on the plasmids.

Another PCR was done in order to add 5' and 3' ends to the His plasmid that were homologous to SET2, so that it would be recognized and inserted during transformation. The success of the PCR reaction was checked using gel electrophoresis and there were no bands present, suggesting that the experiment didn't work. The experiment was repeated, using half the amount of template DNA. Because the template DNA is not completely purified, the various constituents of the plasmid DNA could have inhibited the reaction. After decreasing the amount of template DNA, the PCR worked, as the agarose gel showed the PCR products.

Fig. 2 Hybrid Plasmid The blue strands represent primers homologous to the His gene, while the red strands represent primers homologous to the SET2 gene.

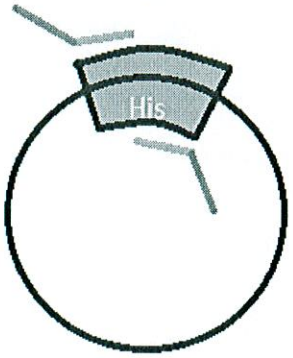
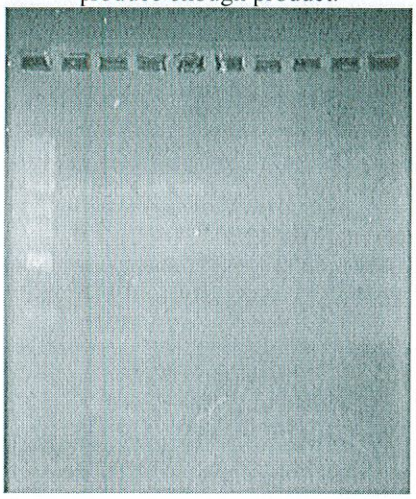
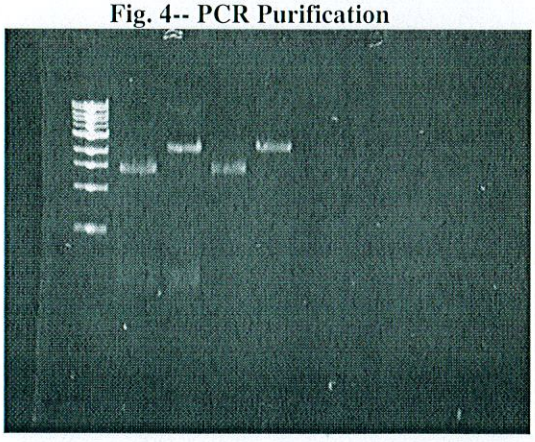


Fig. 3 PCR Product Transformation requires greater than a microgram of DNA, therefore multiple PCR reactions (5) were required to produce enough product.



After the PCR, the reactions from the 5 tubes were mixed together into 1 tube. Next, the DNA was purified to clean out all the unused products, using the QIAquick purification method. My sample was loaded onto an agarose gel, along with samples from other students performing the same experiment. We conducted gel electrophoresis, and the agarose gel showed that the products were purified, as they displayed strong, clean breaks.

Fig. 4-- PCR Purification



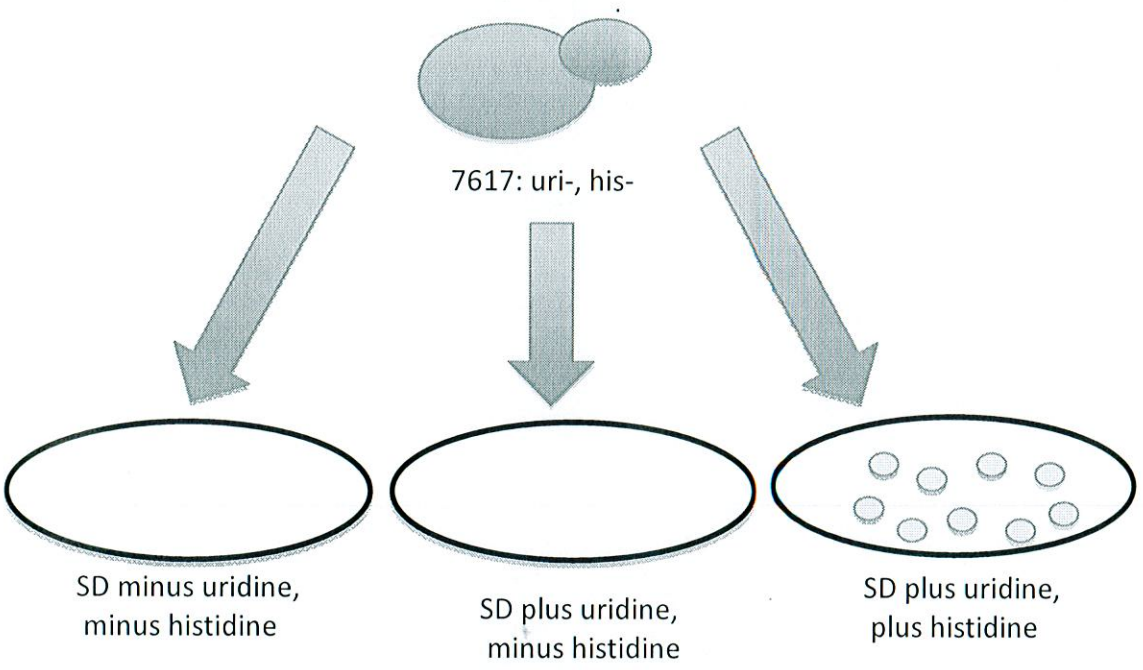
Lane 1: Ladder
Lanes 2 & 4: Uri reactions
Lanes 3 & 5: His reactions

*Lane 3 contained my His reaction

The *Candida albicans* strain p7617 from the Berman Lab (University of Minnesota) was used in the transformation because its genotype is his-, uri-. After the

incubation period, the negative controls had no growth, and the selective plate had a lot of bacterial cell growth, suggesting that the yeast strain is in fact, auxotrophic for both Uri and His.

Fig. 5—Before Transformation *Candida albicans* cells will only grow in the presence of both uridine and histidine



After the lithium acetate transformation and heat shock, the His marker should have inserted in place of the SET2 gene. This strain should now be only auxotrophic for Uri, since it now contains His in its genome. There was abundant cell growth on the selective plate containing Uri and the transformed product. This means that the transformation worked. However, there was also growth on the negative control which suggests that the experiment could have possibly been contaminated. Also, the media could have been incorrect, and not selective for the particular strain.

Fig. 6—Gene Transformation The His gene, which contains 5' and 3' ends homologous to SET2, replaces the SET2 gene.

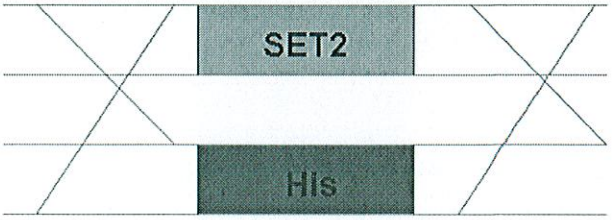
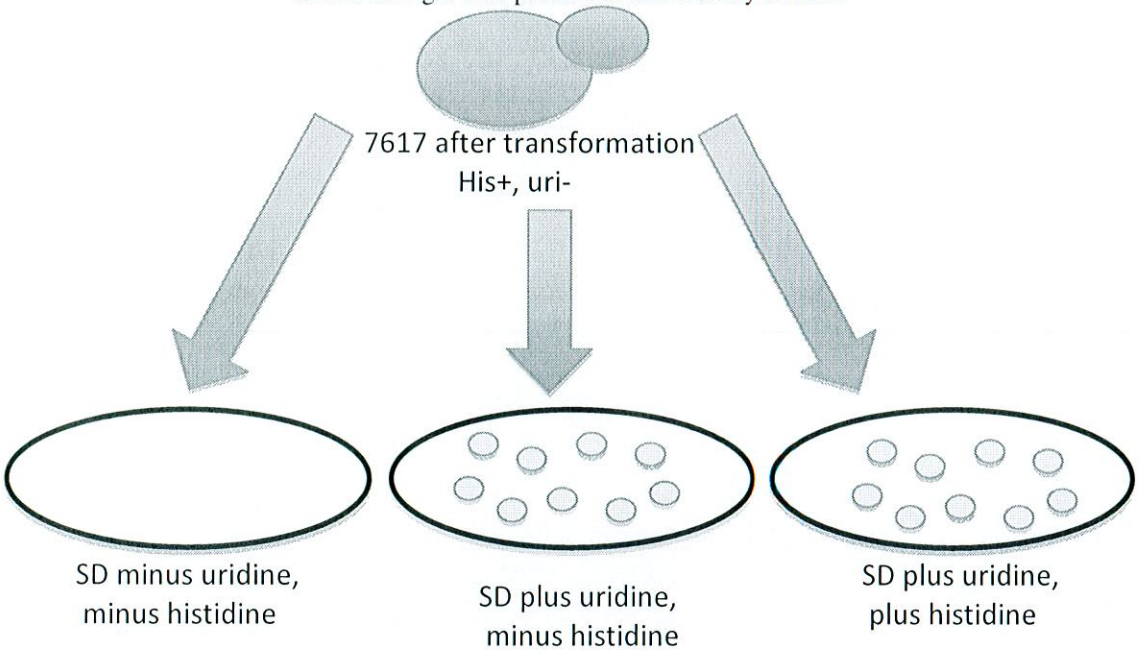
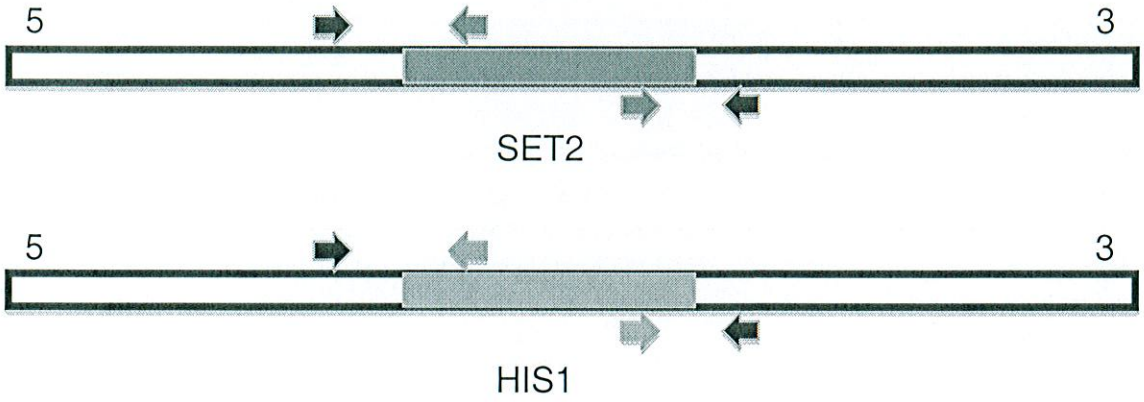


Fig. 7—After Transformation After the His gene has inserted into the *Candida albicans* allele, the strain should now grow on plates that contain only uridine.



Although we knew that the His marker inserted into the *Candida albicans* genome, it is important to check that it inserted in the desired location in the genome (SET2).

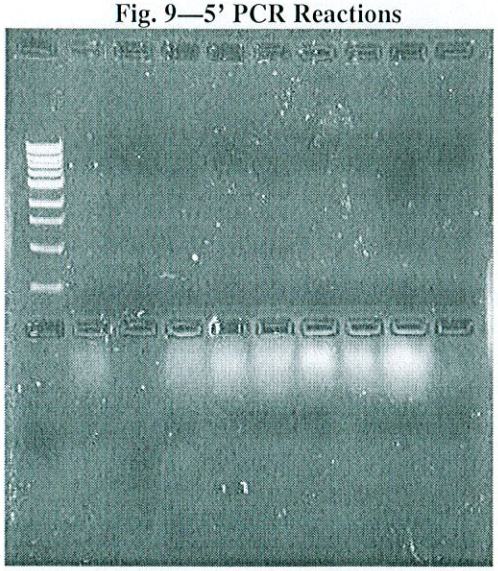
Fig. 8—Checking Transformation Products Black arrows indicate universal primers for the 5' and 3' ends of SET2. The red arrows indicate the SET2 wildtype primers. The blue arrows indicate the His 1 primers



Only the correct transformants will give PCR products with the His1 primers and the universal primers, but all reactions should give the WT products.

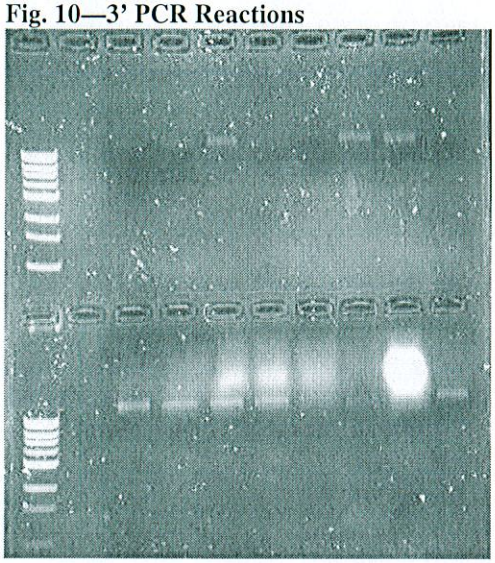
As displayed in the figures below, the wild type reactions did not work well in either of the gels, although you can see light bands appearing in some of the wild type lanes in figure 10. The 5' forward reaction didn't work well either, as all you can see are the byproducts of the reaction. The 3' reaction worked extremely well, as noted by the strong bands on the 3' reaction wells at the bottom of figure 9.

Fig. 9—5' PCR Reactions



Lane 1: Ladder
Lanes 2-13: WT reactions
Lanes 14-20: 5' reactions

Fig. 10—3' PCR Reactions



Lanes 1 & 11: Ladder
Lanes 2-10 &12: WT reactions
Lanes 13-20: 3' reactions

Because the 3' PCR reactions were successful, we can deduce that the His gene did insert correctly into the *Candida albicans* allele. The most likely reason for the weak wild-type reactions and failed 5' PCR reactions is the use of faulty primers, which failed to attach in the correct areas. Like any other scientific research, duplication of the experiment is central to further understanding of the SET2 gene and it's virulence in *Candida albicans*.

IV Discussion/Conclusion

The results are inconclusive, but suggest that SET2 was indeed replaced by His. While the wild type SET2 PCR failed in all samples, the 3' Reverse PCR reactions suggest that the His marker did insert correctly, taking the place of SET2. At this point in the project, only one allele of the SET2 gene was knocked out. To create a complete SET2 knockout, the second SET2 allele would need to be knocked out, using the Uri selectable marker. Once both alleles have been deleted and confirmed through PCR and Southern blots, the homozygous deletion strains can be tested for growth under various conditions, including: temperature, pH, and osmotic ranges. Histone methylation in the

mutant strains can also be tested in order to see if it disappeared. Testing these various conditions can give insight into the potential decreased virulence of the knockout strain, and help us determine if SET2 is a good drug target. Replication of the experiment is needed in order to obtain more information on SET2. Continuing research is critical, as an increase in knowledge of SET2 function may also increase understanding of chromosome structure, centromere function and mechanisms leading to aneuploidy. Ultimately, understanding how aneuploidy occurs is key to understanding drug resistance in *Candida albican*s and cancer in humans. Although this experiment was only a small piece of the puzzle, it paves the way for other research in this area.

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