University of Cincinnati

Date: 3/22/2013

I, Eileen B Wanamaker, hereby submit this original work as part of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

It is entitled:
Mechanisms of Fluconazole Resistance in <i>Candida parapsilosis</i>
Clinical Isolates

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Mechanisms of Fluconazole Resistance in 
*Candida parapsilosis* Clinical Isolates

A thesis submitted to the 
Graduate School 
of the University of Cincinnati  
in partial fulfillment of the  
requirements for the degree of  

**MASTER OF SCIENCE**

in the Division of Pharmaceutical Sciences  
of the James L. Winkle College of Pharmacy

2013

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ABSTRACT

*Candida parapsilosis* is an emerging fungal pathogen and a leading cause of hospital-acquired bloodstream fungal infections. The prophylactic and long-term use of fluconazole, a widely used antifungal, has resulted in an increased prevalence of resistance in this pathogen. To understand how *C. parapsilosis* escapes the effects of fluconazole, we obtained two sets of matched fluconazole-susceptible and -resistant clinical isolates, Clinical Isolate Set 1 and Clinical Isolate Set 2, and have characterized their differential gene expression using a novel whole genome-wide expression technique, RNA-seq, and real-time RT-PCR. Clinical Isolate Set 1 shows significant upregulation of genes encoding major facilitator transporters that are known to efflux drugs out of the cell, and genes involved in response to chemical stimuli. However, we were unable to identify any mutations that would point to a specific mechanism of resistance. RNA-seq and real-time RT-PCR analyses of Clinical Isolate Set 2 shows upregulation of enzymes in the ergosterol biosynthetic pathway that contains the target of fluconazole. After sequencing genes in this pathway that are involved in fluconazole resistance in other species of *Candida*, we identified a nonsense mutation in *ERG3*. This mutation has been described in other fluconazole-resistant isolates of *Candida* species but never before in *C. parapsilosis*.

Furthermore, we have identified what appear to be two distinct modes of fluconazole resistance in two different sets of clinical isolates. This knowledge will contribute to future pharmacological strategies to maintain the efficacy of fluconazole.
ACKNOWLEDGEMENTS

I would like to thank Dr. Caudle, my advisor and mentor, for all of her encouragement and guidance over the past two years. I’m so grateful that she allowed me to follow my dreams and thoroughly supported me in my decision doing whatever she could to help me along the way. I have truly grown as a person and as a scientist under her guidance. Thank you to Dr. Gregerson for taking me under her wing and allowing us to finish this project. I am very grateful for all of her support and advice. I would also like to thank Dr. Pauletti, Dr. Healy, and Dr. Gudelsky for taking the time to serve on my committee and to guide me through this process. Your patience and counsel are greatly appreciated. Thank you to Tim Stephens for having the patience to teach me everything I know in the lab and for being a great friend. I am also very appreciative of the technical work he provided for this project. Lastly, I would like to thank my family for their unwavering support and confidence in me throughout this endeavor.
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CHAPTER I

Introduction
CHAPTER I- INTRODUCTION

1. Invasive Fungal Infections

Rates of invasive fungal infections have increased dramatically over the past three decades causing fungi to emerge as significant human pathogens and public health concerns. A study supporting this found that fungal sepsis in the United States increased by 207% between 1979 and 2000 (Martin et al., 2003). It is thought that this rise in invasive fungal infections is a result of advances made in medical technologies which have increased the number of patients in an immunocompromised state and, therefore, susceptible to these opportunistic pathogens (Pfaller and Diekema, 2007).

*Candida* species are the leading cause of invasive mycoses and have the highest mortality rates among the fungal pathogens (Pfaller and Diekema, 2007; Perloth et al., 2007; Azie et al., 2012). Additionally, they are the third leading cause of all hospital-acquired bloodstream infections accounting for 8-10% of these infections in the United States (Wisplinghoff et al., 2004; Ostrosky-Zeichner and Pappas, 2006; Perloth et al., 2007). This makes them the most significant human fungal pathogen, and illustrates the importance of studying these organisms.

1.1. *Candida* Species and the Invasive Disease They Cause

*Candida* species are commensal organisms of the human body and are isolated most frequently from vaginal, gastrointestinal, urinary tracts, and the oral cavity in almost all healthy individuals (Silva et al., 2012; Kim and Sudbery, 2011). They grow as budding cells and are known as yeast. *Candida albicans, Candida parapsilosis, Candida dubliniensis, and Candida tropicalis* are capable of growing in a filamentous form called hyphae or pseudohyphae under certain environmental conditions. Hyphae are formed from yeast cells and begin as germ tube
projections that elongate, eventually developing septa that divide hyphae into separate fungal units. Pseudohyphae are formed in the same way but lack septa (Silva et al., 2012).

*Candida* species can cause invasive disease when the healthy immune state of the host is compromised. Under these conditions, the processes that keep the organism’s commensal growth in check are absent and the organism is able to proliferate, adhere and invade through mucosal barriers, and eventually disperse to new tissues and organs establishing systemic disease (Shoham and Levitz, 2005). These organisms can also form biofilms by adhering to a solid surface in the yeast phase and after a period of morphogenesis they form dense layers of cells in different morphological forms fixed in a matrix of glycoproteins. Biofilms can form on the surface of medical devices such as catheters and prosthetic valves, and are a serious clinical concern because they protect the pathogen from the immune response and may prevent the penetration of antifungals. Biofilms also slough off cells into the bloodstream and allow the yeast to disseminate into different organs. The ability to form biofilms on medical devices makes *Candida* species persistent in the hospital setting (Kim and Sudbery, 2011; Silva et al., 2012).

Life-threatening diseases caused by *Candida* species include meningitis, candidemia, peritonitis, endovascular catheter infections, and infective endocarditis (Conti et al., 2009). Mortality rates for these infections are estimated to be 50% by some studies and they do not seem to be decreasing despite the introduction of new classes of antifungals for treatment (Bregenzer et al., 1996; Voss et al., 1997; Blot et al., 2002; Viudes et al., 2002). These invasive diseases caused by *Candida* species also have significant implications on healthcare cost. Patients with candidemia have longer hospital stays compared to control subjects with the same underlying diseases ranging from 10 to 30 days longer and they have higher treatment costs (Pfaller and Diekema, 2007; Pittet et al., 1994; Rentz et al., 1998; Lewis, 2009). One study
showed that the attributable extra cost per patient associated with candidemia was as high as $92,266 and it is estimated that the annual healthcare cost related to treatment of hospital-acquired candidemia in the United States is approaching $1 billion (Zaoutis et al., 2005; Miller et al., 2001).

Predisposing conditions to invasive infections caused by Candida species include cutaneous or mucosal barrier disruptions, dysfunctions in cell-mediated immunity, especially of neutrophils, and geriatric and neonatal patients at the extremes of age (Pfaller, 1995). Risk factors shown to be associated with invasive disease include use of broad spectrum antimicrobial agents that deplete competing flora, indwelling vascular catheters in which Candida species can form biofilms, total parenteral nutrition which supplies the organism with nutrients providing a growth advantage, neutropenia, chemotherapy, prior surgery, hemodialysis, and prior mucosal colonization by Candida species (Kuhn et al., 2002; Kuhn et al., 2004; Ostrosky-Ziechner and Pappas, 2006; Concia et al., 2009). Metabolic diseases and hyperglycemia may also be contributing risk factors because leukocyte function is depressed affecting phagocytosis and clearance of the infecting organisms (Lewis, 2009). Poor attention to hand hygiene by healthcare workers and contamination of catheters or other bioprosthetic materials are also associated risk factors for bloodstream infections cause by Candida species (Al-Tawfig et al., 2012; Pronovost et al., 2006).

1.1.1. The Non-albicans Candida Species

C. albicans is the most frequent of the Candida species isolated from healthy patients as well as from invasive infection and accounts for 45% to 65% of all incidences of invasive candidiasis worldwide (Pfaller and Diekema, 2007; Pappas et al., 2003). Consequently, it is the most widely studied organism of the Candida species. However, the ARTMEIS DISK
Surveillance Program shows that the incidence of Candida infections caused by C. albicans decreased from 73.3% in 1997 to 62.3% in 2003 while other species referred to as non-albicans Candida species increased in incidence during this period (Pfaller et al., 2005). C. parapsilosis is one of these non-albicans Candida species and its incidence over this period increased from 4.2% to 7.3% (Pfaller et al., 2005).

It is thought that the rise in infections caused by non-albicans Candida species is a result of increased use of fluconazole, a widely-used antifungal. Non-albicans Candida species like C. krusei are often intrinsically resistant to fluconazole because of reduced susceptibility of the target enzyme for inhibition by fluconazole (Orozco et al., 1998). C. glabrata can mutate quickly in the human host and acquire fluconazole resistance efficiently as a haploid organism relative to the other diploid Candida species (Chapeland-Leclerc et al., 2010). There has also been an increase in the percentage of C. parapsilosis strains resistant to fluconazole over the last fifteen years (Sarvikivi et al., 2005; Moudgal et al., 2005; Raghuram et al., 2012; Concia et al., 2009; Pfaller et al., 2007). As a result of these decreased susceptibilities to fluconazole, these species have a selective advantage over C. albicans during treatment, and thus, are becoming more prevalent (Pfaller and Diekema, 2004). Price et al. (1994) observed that C. albicans accounted for 87% of bloodstream isolates in one center but only 31% after fluconazole was used frequently for prophylaxis and treatment (Price et al., 1994).

1.1.2. C. parapsilosis: an Emerging Medically Important Fungus

C. parapsilosis is often the third most prevalent cause of candidemia behind C. albicans and C. glabrata. However, in patients under 13 years of age, C. parapsilosis is commonly the second most prevalent with an incidence rate of 30-40% and some studies focused on single-centers have determined C. parapsilosis to be the most significant cause of candidemia in
pediatric intensive care units (Pappas et al., 2003; Lewis, 2009; San Miguel et al., 2005). *C. parapsilosis* also has a propensity to cause fungal endocarditis as it is the main non-*albicans* *Candida* species isolated from patients with this disease (Garzoni et al., 2007; Varghese and Sobel, 2008).

*C. parapsilosis* is more commonly found on skin and under fingernails than on mucosal surfaces. This makes *C. parapsilosis* more of an exogenous organism and allows it to spread easily to patients via the hands of healthcare workers (Kuhn et al., 2004; Pfaller, 1996; Weems, 1992; Waggoner-Fountain et al., 1996; Sarvikivi et al., 2005). Furthermore, this organism’s ability to form biofilms on catheters and other bioprosthetic materials makes it persistent in the hospital setting (Branchini et al., 1994; Clark et al., 2004; Pfaller and Diekema, 2007). Attention to sterile catheter insertion and hand hygiene has shown to reduce bloodstream infections caused by *C. parapsilosis* (Lewis, 2009).

Prematurity is a common underlying factor in infection with *C. parapsilosis* as this organism is prevalent in neonatal intensive care units. Premature infants have immature immune systems with poorly developed protective barriers like the skin and mucosal membranes of the gastrointestinal and respiratory tracts. They also often require broad spectrum antibiotics for long-periods of time, supplementation with total parenteral nutrition, and the use of central venous catheters all of which are significant predisposing factors for candidemia (Levy et al., 1998; Pfaller et al., 2010; Pammi et al., 2013). Furthermore, *C. parapsilosis* is often found on the skin and nails of health care workers in contrast to *C. albicans* and is probably passed to the susceptible neonates this way (Sarvikivi et al., 2005; Weems, 1992; Lupetti et al., 2002). Some work also shows that there might be differences in how neonatal neutrophils confront *C.*
parapsilosis compared to C. albicans and that these differences cause neutrophils to be less efficient at challenging C. parapsilosis (Destin et al., 2009).

Fungal endocarditis only occurs in a small percentage of all infective endocarditis cases, but it is the most severe form of infective endocarditis with the highest mortality rates. C. parapsilosis accounts for 20-30% of all fungal endocarditis cases and is second in etiology behind C. albicans. Neonates and patients with recent prosthetic valve surgery are at the highest risk for these infections as C. parapsilosis has a propensity to grow on bioprosthetic material. These factors combined with the fact that the overall incidence of C. parapsilosis has increased explain why C. parapsilosis has emerged as a prevalent cause of fungal endocarditis (Garbino et al., 2007; Varghese and Sobel, 2008).

1.1.2.1. C. parapsilosis Genetics and Gene Disruption

C. parapsilosis was believed to be a heterogeneous species and was divided into Groups I, II, and III. Recently, these groups have been reclassified into different species. Group I is now defined as C. parapsilosis and Groups II and III are defined as C. orthopsilosis and C. metapsilosis, respectively (Travanti et al., 2005). C. parapsilosis is significantly more homogenous and lacks the sequence diversity that can be found within C. orthopsilosis and C. metapsilosis. Many C. orthopsilosis and C. metapsilosis-specific alleles can be found within C. parapsilosis, but C. parapsilosis-specific alleles are not found within C. orthopsilosis and C. metapsilosis. It is thought, therefore, that C. parapsilosis diverged from these other species as it evolved with the human host (Fundyga et al., 2004). Many key components for mating are also missing from the C. parapsilosis genome. This is in contrast to C. albicans which has a parasexual mating cycle and is capable of mating opposite mating types which are defined at the mating type locus (Hull et al., 2000; Magee and Magee, 2000). One of the two mating type loci
in *C. parapsilosis* is a pseudogene as it has four stop codons. Thus, *C. parapsilosis* cannot mate (Butler et al., 2009; Nosek et al., 2009).

*C. parapsilosis* was sequenced in a project at the Sanger Institute and its sequence was first published by Butler et al. (2009). The genome is diploid, estimated to contain about 26.3Mbp, and consists of 14 or 16 chromosomes (7 or 8 pairs) (Nosek et al., 2009). Guida et al. (2011) performed further experiments and analysis to refine this sequence annotation and have made it available on a database on candidagenome.org (Arnaud et al., 2012). This recent annotation consists of 6,229 open reading frames (Guida et al., 2011).

Gene disruption and manipulation in *C. parapsilosis* is accomplished by integrative transformation by homologous recombination since this organism lacks a sexual cycle. A transformation method for gene disruption that relies on a dominant selection marker instead of selection from an auxotrophic strain, which can make mutant phenotypes difficult to interpret, has recently been developed for use in *C. albicans* (Staab and Sundstrom, 2003; Reuβ et al., 2004). This method can be adapted for use in *C. parapsilosis* and consists of transformation by electroporation of a cassette containing the dominant nourseothricin resistant marker (*SAT1*) and the *FLP* recombinase gene under the control of an inducible maltose promoter. Specificity is achieved by the addition of 500bp that are immediately upstream and downstream of the target gene. When expressed, the *FLP* recombinase excises the cassette from the genome by recognition of FLP target sequences that flank the cassette. Once the dominant selection marker is removed, the same cassette is transformed into the organism again to disrupt the second allele. After two rounds of integration and excision of the cassette, a homozygous mutant is generated which only differs from the wild-type strain by the absence of the target gene and presence of the
short FLP target sequences. Reintegration of an intact gene for complementation of a mutant phenotype can be performed using the same method (Reuβ et al., 2004).

2. Antifungals for Treatment of Invasive Candidiasis

Antifungal drug development is challenging because fungi are eukaryotic like human cells and have similar cellular components and processes. However, there are a few key differences between mammalian cells and fungal cells that are exploited as targets for antifungals. One key difference is that ergosterol is the main sterol found in fungal cell membranes whereas cholesterol is the main sterol found in mammalian cell membranes. As the main membrane sterol, ergosterol serves several important cellular and structural functions such as regulating stability, permeability, and protection from environmental stresses. As a constituent of lipid rafts, ergosterol may have important roles in cellular signaling and membrane trafficking (Parks and Casey, 1995). The fungal cell wall is another important antifungal target because it is not a component of human cells and allows for specificity of the antifungal agent. The cell wall functions to protect the organism by maintaining rigidity and resisting osmotic pressure. Disruption of these vital functions makes antifungal agents that target the cell wall effective (Sucher et al., 2009).

2.1. The Pyrimidines

The pyrimidine class of antifungals consists of flucytosine and inhibits RNA and DNA synthesis in Candida species and some other fungi. Flucytosine is taken up into the fungus by cytosine permease in the membrane and once inside the cell cytosine deaminase, an enzyme not found in mammalian cells, converts the drug to 5-fluorouracil. 5-fluorouracil is converted to 5-fluorouridylic, phosphorylated and incorporated into RNA. 5-fluorouracil can also be converted
to 5-fluorodeoxyuridine monophosphate which is an inhibitor of an enzyme in DNA synthesis, thymidylate synthase. Ultimately, the organism dies from the inhibitory effect both end products have on RNA and DNA processes. Mutations or loss of function in any of these enzymes in fungi are known mechanisms of resistance (Polack, 1990; Dodgson et al., 2004; Vanden Bossche et al., 1994).

There is a high prevalence of intrinsic resistance to flucytosine in the United States and acquired resistance, which happens after the pathogen is exposed to the drug, can develop quickly making it a poor choice for monotherapy. It can be used in combination therapies but hematological toxicity is a frequent side effect (White et al., 1998). The Infectious Disease Society of America only recommends flucytosine use in combination with amphotericin B for treatment of invasive disease caused by Candida species (Pappas et al., 2009).

2.2. The Polyenes

The polyene class of antifungals for treatment of invasive Candida infections consists of amphotericin B. Amphotericin B is amphipathic and binds to ergosterol intercalating into the membrane to form a pore. Membrane permeability is disrupted causing ions to leak out and protons to move into the cell destroying the proton and ion gradients (Ermishkin et al., 1976).

Amphotericin B displays fungicidal activity and is highly effective against Candida species. Because of this, amphotericin B is the standard for treatment of severe systemic infections when a rapid response is needed (Pappas et al., 2009). However, this drug is not specific to ergosterol and will bind to sterols within the patient’s cell membranes causing severe side effects such as nephrotoxicity and hepatotoxicity. It is also given intravenously and infusion-related toxicities can result (Gallis et al., 1990).
2.3. The Azoles

The azole class of drugs is comprised of the triazoles (fluconazole, itraconazole, voriconazole, and posaconazole) and the imidazoles (miconazole and ketoconazole) but only the triazoles are used in treatment of invasive candidiasis. These drugs inhibit 14-α-sterol-demethylase (Erg11p), an enzyme in the ergosterol biosynthetic pathway. Erg11p is a cytochrome P-450 enzyme with a heme group in its active site. The azoles bind to the iron in that heme group with an unhindered nitrogen and block enzyme activity. By inhibiting this enzyme, the azoles cause accumulation of toxic methylsterols and prevent cell growth and replication (Hitchcock, 1991).

The azoles, and especially fluconazole, have become a primary choice for antifungal therapy because they can be given orally, are relatively safe, and have good activity against most Candida species (Slavin et al., 1995). However, this class of drugs is more prone to resistance because it is fungistatic. The infecting organism is inhibited from growing and not immediately killed so there is opportunity for the organism to make genetic alterations and begin replicating again if the drug concentration falls below the effective therapeutic window (Perlin, 2007).

Fluconazole is currently recommended for use in less critically ill neutropenic, or patients that have an abnormally low abundance of neutrophils, and non-neutropenic patients with candidemia as well as for fungal prophylaxis in solid-organ transplants, intensive care unit hospitalization, chemotherapy-induced neutropenia, and stem cell transplants (Pappas et al., 2009).

2.4. The Echinocandins

The echinocandins are the newest class of antifungals available for treatment of invasive candidiasis and include caspofungin, micafungin, and anidulafungin. These drugs target the cell
wall by functioning as non-competitive inhibitors of 1,3-β-D-glucan synthase. This enzyme synthesizes 1,3-β-D-glucan, a major component of the fungal cell wall, and is made up of two subunits encoded by \( FKS1 \) and \( FKS2 \). The echinocandins exhibit fungicidal activity because the cell wall becomes destabilized and the organism becomes susceptible to cell lysis as a result of osmotic pressure (Sucher et al., 2009).

The echinocandins display activity against a broad range of \( \text{Candida} \) species including those non-\( \text{albicans Candida} \) species that show higher rates of intrinsic resistance to fluconazole. However, \( \text{C. parapsilosis} \) is naturally less susceptible to the echinocandins as a result of slightly different 1,3-β-D-glucan synthase enzymes and characteristically displays echinocandin minimum inhibitory concentrations 4 to 100 fold greater than those of \( \text{C. albicans} \) (Perlin, 2007).

Caspofungin has been shown to lack efficacy against \( \text{C. parapsilosis} \) in a few studies and several clinical cases of echinocandin resistance have been documented including a bloodstream infection that persisted despite treatment with caspofungin (Garcia-Effron et al., 2008; Barchiesi et al., 2006; Mora-Duarte et al., 2002). In addition, a case of prosthetic valve endocarditis was reported in which the infecting strain of \( \text{C. parapsilosis} \) developed multi-echinocandin and multi-azole resistance (Mougdal et al., 2005). These studies provide evidence that the echinocandins are undesirable for treatment of \( \text{C. parapsilosis} \). Currently, the echinocandins are recommended for treatment in moderately to severely ill patients with candidemia and patients who have had recent azole exposure (Pappas et al., 2009).

The antifungal agents currently available to treat invasive candidiasis and their mechanisms of action are summarized in Table 1.

3. Antifungal Drug Resistance
## Table 1: Antifungal agents used for treatment of invasive candidiasis.

<table>
<thead>
<tr>
<th>Antifungal Agents</th>
<th>Mechanism of Action(^a)</th>
<th>Side Effects(^a)</th>
<th>C. albicans Mechanisms of Resistance(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The Pyrimidines</strong></td>
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</tbody>
</table>
| Flucytosine      | Inhibition of DNA and RNA synthesis | Hematological toxicity, liver dysfunction, acute renal failure | -High prevalence of primary resistance  
-Mutations in or loss of function of cytosine permease, cytosine deaminase, UMP pyrophosphorylase  
-increased production of pyrimidines |
| **The Polyenes**  |                           |                   |                                               |
| Amphotericin B    | Disruption of membrane permeability | Nephrotoxicity, hepatotoxicity, infusion-related reactions | Reduction or absence of ergosterol in cell membrane |
| **The Azoles**    |                           |                   |                                               |
| Fluconazole, itraconazole, voriconazole, and posaconazole | Inhibition of ergosterol biosynthesis by inhibition of Erg11p | Inhibition of CYP-dependent enzymes | -Overexpression of Cdr1p, Cdr2p, and Mdr1p transporters  
-Point mutations in ERG11  
-Overexpression of Erg11p  
-Deactivation of Erg3p |
| **The Echinocandins** | Disruption of cell wall integrity by non-competitive inhibition of 1,3-β-D-glucan synthase | Infusion-related reactions | Point mutations in “hot-spot” regions of FKS1 (encodes catalytic subunit of 1,3-β-D-glucan synthase) |

\(^a\)Thomas, 2009.  
\(^b\)Sanglard et al., 2009
As the incidence of invasive infections caused by *Candida* species increases as a result of the population who survive longer in complex disease states, there is an increasing use of antifungals both in prophylaxis and for treatment. This increases the incidence of resistance which complicates patient therapies and leads to poorer patient outcomes (Rex et al., 1997; Haijeh et al., 2004; Pfaller et al., 2004; Baddley et al., 2008).

Data from the ARTEMIS DISK Surveillance Program shows that the percentage of *C. albicans* isolates resistant to fluconazole increased from 0.8% in 1997 to 1.4% in 2003 while the percentage of *C. parapsilosis* isolates resistant to fluconazole increased from 2.0% in 1997 to 4.2% in 2001 (Pfaller et al., 2005). Additionally, a retrospective study examining invasive fungal infections after liver transplantation in a single center from 2003 to 2007 found that all but one *C. albicans* strain isolated during this time were susceptible to fluconazole while all but one *C. parapsilosis* strain isolated during this time were highly resistant to fluconazole (Raghuram et al., 2012). As these studies show rates of fluconazole-resistant strains of *C. parapsilosis* are emerging faster than fluconazole-resistant strains of *C. albicans*, *C. parapsilosis* resistance mechanisms to fluconazole merit closer investigation.

### 3.1. Definitions of Resistance

Primary resistance signifies that the organism is naturally resistant and occurs without prior exposure to the drug while secondary resistance occurs after exposure. Secondary resistance may be transient and disappear after the removal of the antifungal agent, or it can be permanent as a result of genetic changes (Sanglard and Odds, 2002). Clinical resistance is defined as persistence of an infection despite appropriate treatment and development of clinical resistance is complicated as it is dependent on factors from the pathogen as well as the host. Pathogenic factors include initial drug minimum inhibitory concentration, cell type (yeast vs.
hyphae), stability of the genome, biofilm formation, and organism inoculum. Host factors consist of competence of the immune system, seriousness of the infection, site of the infection, presence of bioprosthetic materials, how well the patient adheres to the drug regime, and drug distribution (White et al., 1998; Hawser et al., 1995).

With the introduction of the National Committee for Clinical Laboratory Standards Subcommittee on Antifungal Susceptibility Testing, in vitro testing of resistance has made vast improvement as minimum inhibitory concentration determinations are now reproducible between different labs. These in vitro determinations are made by culturing the fungus in serial dilutions of drug and visualizing what concentration of drug inhibits growth of the organism. Species-specific clinical breakpoints for antifungal agents have also been standardized based on wild-type minimum inhibitory concentration distributions, minimum inhibitory concentration values correlated with clinical outcomes, and pharmacokinetic and pharmacodynamics parameters of the antifungal agent (Pfaller, 2012). These values are shown in Table 2 for C. albicans and C. parapsilosis.

Correlation between in vivo and in vitro resistance is difficult because clinical resistance may occur despite normal in vitro susceptibility patterns in the infecting organism. Additionally, a patient could respond to treatment despite in vitro resistance of the fungus. It is a complex interaction between the fungal pathogen, antifungal agent in the human host, and the host’s immune system (White et al., 1998; Sanglard and Odds, 2002). Despite this, in vitro resistance can be defined, studied, and may provide insights into future drug targets to reduce the morbidity and mortality associated with invasive fungal disease and to maintain the efficacy of current antifungal agents against the growing prevalence of resistance. However, no antifungal agent is
**Table 2.** Clinical breakpoints established by the Clinical Laboratory Standards Institute.\(^a\)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sensitive (µg/ml)</th>
<th>Susceptible Dose-Dependent (µg/ml)</th>
<th>Resistant (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluconazole</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>≤2</td>
<td>4.0</td>
<td>≥8</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>≤2</td>
<td>4.0</td>
<td>≥8</td>
</tr>
<tr>
<td><strong>Voriconazole</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>≤0.12</td>
<td>0.25-0.5</td>
<td>≥1</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>≤0.12</td>
<td>0.25-0.5</td>
<td>≥1</td>
</tr>
<tr>
<td><strong>Anidulafungin, Caspofungin, Micafungin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>≤0.25</td>
<td>0.5</td>
<td>≥1</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
</tbody>
</table>

\(^a\)(Pfaller et al., 2011)
impervious to the development of resistance.

3.2. Correlation Between Minimum Inhibitory Concentration and Clinical Outcome

A study conducted by Rex et al. (1997) showed that there is a correlation between minimum inhibitory concentration and treatment success rates. In their study, good treatment responses with fluconazole were seen in 92% (370 out of 402) patients infected with susceptible isolates of *C. albicans* (minimum inhibitory concentration less than 2µg/ml), 82% (45 out of 55) patients infected with susceptible-dose dependent isolates (minimum inhibitory concentration of 4µg/ml), and 56% (34 out of 61 patients) patients infected with resistant isolates (minimum inhibitory concentration greater than 8µg/ml) (Rex et al., 1997). Additionally, another study showed that mortality rates were lower among patients with candidemia where the infecting isolate had a minimum inhibitory concentration for fluconazole less than 2µg/ml compared to patients where the infecting isolate minimum inhibitory concentration was greater than 8µg/ml (Baddley et al., 2008). Together these studies show that higher minimum inhibitory concentrations are associated with lower success rates and higher mortality rates compared to lower minimum inhibitory concentrations (Pfaller, 2012).

3.3. Mechanisms of Antifungal Resistance

3.3.1. Drug Efflux-Mediated

ATP-binding cassette transporters (primary active transporters) and major facilitator transporters (secondary active transporters) are families of membrane transporter proteins that are universal to eukaryotic and prokaryotic cells. They are colloquially called efflux pumps and are widely implicated in drug resistance. Upregulation of these efflux pumps causes insufficient accumulation of the azoles within the fungal cell and the organism escapes growth inhibition
ATP-binding cassette transporters have nucleotide binding domains which bind ATP and allow the transporter to use the energy from ATP hydrolysis to drive efflux. Cdr1p and Cdr2p are the ATP-binding cassette transporters in *C. albicans* that have been shown to have a role in azole resistance (Sanglard et al., 1997; Pfaller et al., 2004).

Major facilitator transporters use the proton gradient to drive transport of small molecules out of the cell in exchange for protons entering the cell. Mdr1p is the key major facilitator transporter in fluconazole resistance in *C. albicans* and overexpression of Mdr1p has been characterized in several fluconazole-resistant strains of *C. albicans* (Sanglard et al., 1995). Mdr1p-mediated drug resistance appears to be specific for fluconazole (Pfaller, 2012).

*FLU1* encodes another *C. albicans* major facilitator transporter that has been associated with fluconazole resistance. Disruption of *FLU1* in *C. albicans* strains with inactivating mutations in *CDR1, CDR2*, or *MDR1* caused increased susceptibility to several azole derivatives but disruption of this gene alone only caused a slight increase in fluconazole susceptibility. Overexpression of Flu1p has not been identified as a mechanism of fluconazole resistance in any clinical isolates (Calarese et al., 2000).

The echinocandins are poor substrates for these efflux transporters so drug efflux does not appear to contribute to echinocandin resistance (Niimi et al., 2006; Perlin 2007).

### 3.3.2. Alterations of the Drug Target

Alterations of the drug target are common mechanisms that render *Candida* species less susceptible to the azole and echinocandin classes of antifungals. The azoles target Erg11p and point mutations in *ERG11* which decrease drug affinity for this enzyme have been identified in clinical and laboratory-derived azole-resistant strains of *C. albicans* (White, 1997a; Sanglard et al., 1998; Lamb et al., 1997). Because *Candida* species are diploid (with the exception of *C.*
glabrata), gene conversion or mitotic recombination are important events that need to occur to eliminate variation between alleles and make these point mutations homozygous. A strain with a homozygous point mutation is almost always significantly more resistant than a strain with a heterozygous point mutation (White, 1997a; Coste et al., 2006; Rustad et al., 2001).

Overexpression of Erg11p has also been described in azole-resistant clinical isolates of *C. albicans* (White, 1997a; Flowers et al., 2012). *ERG11* gene amplification as a result of chromosome duplication was identified in one resistant clinical isolate of *C. glabrata* (Marichal et al., 1997).

Point mutations in specific “hot spot” regions of *FKS1*, the gene that encodes the major subunit of the catalytic domain of 1,3-β-D-glucan synthase and is the target of the echinocandins, result in echinocandin resistance (Krutz et al., 1996; Park et al., 2005). *FKS1* “hot-spot” mutations have been identified in *C. albicans, C. glabrata, C. parapsilosis, C. krusei, C. tropicalis,* and *C. dubliniensis,* and result in resistance to all three echinocandins. Point mutations in *FKS2*, another gene that encodes a subunit of the catalytic domain of 1,3-β-D-glucan synthase, have also been identified in a *C. glabrata* resistant strain (Katiyar et al., 2006; Lewis, 2009; Perlin, 2007; Pfaller, 2012).

A study conducted by Park et al. (2005) demonstrates how a single “hot-spot” point mutation in *C. albicans FKS1* can alter the kinetic inhibition properties of 1,3-β-D-glucan synthase by the echinocandins and result in echinocandin resistance. In an *in vitro* assay conducted in this study, a mutant *C. albicans FKS1* (S645P) was 100-fold less sensitive to inhibition by caspofungin compared to wild-type *C. albicans FKS1* and required much higher drug concentrations to achieve the level of inhibition that was seen with the wild-type enzyme (Park et al., 2005; Perlin, 2007).
3.3.3. Alterations in the Ergosterol Biosynthetic Pathway

When Erg11p is inhibited by the activity of the azoles, 14-α-methyl fecosterol and 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol accumulate (see Figure 1) and become the predominant membrane sterols. 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol is toxic to C. albicans and prevents replication. Inhibition of Δ5,6-desaturase, encoded by ERG3, prevents the synthesis of 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol and allows the organism to survive (Kelly et al., 1997; White et al., 1997). This is a mechanism ofazole resistance that has been identified in laboratory-derived strains and a few clinical isolates of C. albicans and C. dubliniensis (Pinjon et al., 2003; Chau et al., 2005). This mutation also causes increases in amphotericin B minimum inhibitory concentrations because of decreased levels of ergosterol found in the membrane (Table 1) (Nolte et al., 1997; Kelly et al., 1997).

The mechanisms of resistance for each class of antifungals that are commonly identified in C. albicans resistant strains are summarized in Table 1.

3.3.4. Transcriptional Control of Drug Resistance

Recent work in antifungal drug resistance in C. albicans has revealed that point mutations in regulatory domains of key transcriptional regulators that result in constitutive activation are often the cause of resistant phenotypes. These mutations are called “gain-of-function” mutations. There are two main classes of fungal transcription factors that are implicated in drug resistance, the zinc cluster and the bzip transcription factors. The zinc cluster transcription factors contain Zn2Cys6 motifs. Tac1p is one such zinc cluster transcription factor and induces upregulation of
Figure 1. Ergosterol biosynthetic pathway in \textit{Candida} species. Intermediates and the necessary enzymes for sterol synthesis are shown with and without inhibition of Erg11p by fluconazole, a widely-used antifungal. In the presence of fluconazole and with the loss of functional Erg3p, 14\(\alpha\)-methyl fecosterol accumulates rather than 14\(\alpha\)-methylergosta-8,24(28)-dien-3\(\beta\),6\(\alpha\)-diol which is toxic to the organism. Dotted arrows indicate multiple enzymatic steps.
the ATP-binding cassette transporters \textit{CDR1} and \textit{CDR2} in response to certain stimuli in \textit{C. albicans}. \textit{TAC1} hyperactive alleles with gain-of-function mutations cause constitutive upregulation of \textit{CDR1} and \textit{CDR2}, result in efflux of the azoles, and ultimately cause azole resistance (Coste et al., 2006; Coste et al., 2004).

\textit{Mrr1p}, another zinc cluster transcription factor, regulates expression of \textit{Mdr1p}, the major facilitator transporter responsible for fluconazole resistance in \textit{C. albicans}. Again, gain-of-function mutations that result in constitutive activation of this protein cause overexpression of \textit{Mdr1p} and fluconazole resistance in \textit{C. albicans} (Dunkel et al., 2008b; Schubert et al., 2008; Morschhauser et al., 2007).

\textit{Upc2p} controls expression of the ergosterol biosynthetic genes in \textit{C. albicans} and also belongs to the zinc cluster family of transcription factors. Constitutive activation of \textit{Upc2p} results in overexpression of the azole drug target, \textit{Erg11p}, and has been characterized in fluconazole-resistant \textit{C. albicans} strains (Silver et al., 2004; MacPherson et al., 2005; Dunkel et al., 2008a; Flowers et al., 2012).

\textit{Ndt80p} is another zinc cluster transcription factor that positively regulates \textit{CDR1} in \textit{C. albicans}. Inactivation of this gene in \textit{C. albicans} caused decrease basal levels of \textit{Cdr1p} and reduced expression of \textit{Cdr1p} in the presence of drug-inducing stimuli (Chen et al., 2004; Sanglard et al., 2009).

\textit{In \textit{C. glabrata}, the zinc cluster transcription factor \textit{Pdr1p} regulates expression of the ATP-binding cassette transporter genes which are \textit{CDR1}, \textit{PDH1}, and \textit{SNQ2} in this organism. Again, point mutations in the regulatory domain of this protein result in constitutive activation and cause overexpression of the ATP-binding cassette transporters. This is a mechanism of azole}
resistance that has been characterized in resistant strains of *C. glabrata* (Vermitsky et al., 2004; Vermitsky et al., 2006; Tsai et al., 2006; Ferrari et al., 2009).

The bZip transcription factors contain basic leucine zipper regions. The transcription factors Mcm1p and Cap1p are in this family and regulate the major facilitator transporter Mdr1p. Loss of regulatory domains, over-activation, or overproduction of these proteins has been shown to increase fluconazole minimum inhibitory concentrations in *C. albicans* (Riggle and Kumamoto, 2006; Alarco and Raymond, 1999; Gulshan and Moye-Rowley, 2007).

### 3.3.5. High-Level Azole Resistance

For a strain to become highly resistant to the azoles it likely needs to accumulate multiple genetic alterations rather than one single mutation. Drug resistance occurs after long periods of exposure to the drug which favor the accumulation of these alterations under the selective pressure of the drug. Alterations in a series of 17 *C. albicans* clinical isolates showing various levels of azole resistance where characterized to include *MDR1*, *CDR1* and *CDR2* overexpression, a homozygous point mutation in *ERG11*, and overexpression of *ERG11*, and support this idea (White, 1997a; White, 1997b; White et al., 1997). A study conducted by MacCallum et al. (2010) showed the additive nature of these resistance mechanisms when they constructed strains of *C. albicans* expressing different levels of Cdr1p and Cdr2p with or without point mutations in *ERG11*. To reach high-level fluconazole resistance both overexpression of these ATP-binding cassette transporters and *ERG11* point mutations needed to occur. Intermediate levels of resistance were observed when one mechanism was at work (MacCallum et al., 2010; Pfaller 2012). Other studies have also found multiple drug resistance mechanisms in single azole-resistant isolates of *C. albicans* (Sanglard et al., 1995; Franz et al., 1998).
4. Study Design and Research Objectives

The aim of this study is to use a novel genome-wide expression technique, RNA-seq, to assess for genetic changes that result in differential gene expression between matched fluconazole-susceptible and -resistant clinical isolates of C. parapsilosis. While this aim is not hypothesis-driven per se, it is a needed antecedent for identification of fluconazole resistance mechanisms employed by C. parapsilosis that is the ultimate goal of this work. We are interested in studying C. parapsilosis because it is a medically important fungus and several studies show that its prevalence in invasive disease is increasing while the prevalence of C. albicans decreases (Pfaller et al., 2008; Pfaller et al., 2005).

The focus of this investigation on resistance to fluconazole is a result of the need to preserve this antifungal because of its safety profile, ease of use, and efficacy against C. parapsilosis. Fluconazole is better tolerated because side effects are limited and not as severe as those caused by amphotericin B (Charlier et al., 2006; Gallis et al., 1990). Furthermore, it can be taken orally which makes treatment more convenient and improves patient compliance. Finally, fluconazole is more preferable to the echinocandins in treatment of C. parapsilosis infections because C. parapsilosis is naturally less susceptible to the echinocandins and treatment has been shown to fail (Garcia-Effron et al., 2008; Barchiesi et al., 2006; Mora-Duarte et al., 2002).

4.1. Matched C. parapsilosis Clinical Isolates

We have acquired two sets of matched fluconazole-resistant and -susceptible C. parapsilosis clinical isolates. Each set contains isolates that were shown through genetic karyotyping to be of the same genetic background, but one isolate was obtained prior to the development of resistance, while the other was obtained after the development of resistance. Theoretically, at least some of the genetic and gene expression differences between the isolates
Table 3. Clinical isolates used in this study and their susceptibilities to fluconazole.

<table>
<thead>
<tr>
<th>Isolate Name</th>
<th>Minimum Inhibitory Concentration (µg/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis Clinical Isolate Set 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20360.053</td>
<td>2</td>
<td>Sarvikivi et al., 2005</td>
</tr>
<tr>
<td>20360.066</td>
<td>&gt;64</td>
<td>Sarvikivi et al., 2005</td>
</tr>
<tr>
<td>C. parapsilosis Clinical Isolate Set 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35177</td>
<td>1</td>
<td>Moudgal et al., 2005</td>
</tr>
<tr>
<td>35176</td>
<td>&gt;64</td>
<td>Moudgal et al., 2005</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isolated from neonates in a neonatal intensive care unit where fluconazole was used routinely in prophylaxis.

<sup>b</sup> Isolated from a patient with prosthetic valve endocarditis in which azole resistance developed during fluconazole therapy.
are related to the development of resistance. Table 3 shows these isolates and their susceptibilities to fluconazole.

Clinical Isolate Set 1 was isolated from the neonatal intensive care unit at Helsinki University Central Hospital in Finland where *C. parapsilosis* had been a continual problem and fluconazole was used routinely for prophylaxis. Over a 12-year period, 26 *C. parapsilosis* isolates causing bloodstream infections were isolated and later characterized. Electrophoretic karyotyping revealed that all 26 isolates were of the same strain and that overtime this strain became progressively less susceptible to fluconazole until high-level resistance was achieved (Sarvikivi et al., 2005). We have acquired all 26 isolates but chose to use only one fluconazole-susceptible isolate (20360.053) and one fluconazole-resistant isolate (20360.066) for further analysis (Table 3). These specific isolates were chosen because they are the most closely related fluconazole-susceptible and high-level fluconazole-resistant isolates, as shown through genetic karyotyping, to minimize genetic changes that occurred overtime in this strain not related to the development of fluconazole resistance.

Clinical Isolate Set 2 was obtained from a patient with prosthetic valve endocarditis. The patient was admitted to the hospital several months after an aortic valve replacement with a high fever. *C. parapsilosis* was isolated from his bloodstream. After combination therapy with intravenous caspofungin and fluconazole his condition improved, he was discharged, and instructed to take fluconazole daily. However, three months later he was readmitted with a fever and *C. parapsilosis* was again isolated from his bloodstream. He was given intravenous caspofungin but his condition did not improve. Minimum inhibitory concentration analysis showed that the infecting organism was resistant to fluconazole (minimum inhibitory concentration >64µg/ml) and caspofungin (minimum inhibitory concentration >16µg/ml), and
had an increased amphotericin B minimum inhibitory concentration (0.25µg/ml to 0.5µg/ml). We have acquired the *C. parapsilosis* isolates from the first admission, 35177, which is fluconazole-susceptible and from the second admission, 35176, which is fluconazole-resistant as shown in Table 3. These isolates were shown to be isogenic through electrophoretic karyotyping (Moudgal et al., 2005).

4.2. RNA-seq as a Tool to Determine Differential Expression

Microarray has traditionally been used to determine what pathways are differentially expressed in *Candida* species under various conditions (Liu et al., 2005; Yan et al., 2008; Cao et al., 2005; Rogers and Barker, 2002). However, there are several limitations with microarray, including high-background levels due to nonspecific hybridization, limitations for quantification due to this high-background and saturation of the probe, variability between different experiments, and the requirement of prior knowledge of the genomic sequence in order to make the probe (Wang et al., 2009). A transcriptome profiling technique called RNA-seq has recently been developed using high-throughput DNA sequencing technology and has advantages over microarray.

In an RNA-seq experiment, RNA is isolated, fractionated in ~200bp pieces, reverse transcribed to cDNA, and adapters are ligated onto the ends of the fractions. The pieces are sometimes amplified depending on the technology used and then sequenced using Next-Generation Sequencing methods. After the fragments are sequenced, the sequence reads are aligned to a reference genome or aligned *de novo*. The final result is a transcriptional map that covers the entire genome with the level of expression for each gene (Wang et al., 2009).

RNA-seq can be used to detect transcripts in the absence of sequence information making it an attractive tool for use in organisms where the genomic sequence has yet to be determined.
RNA-seq also has very low background levels compared to microarray and it does not have an upper limit for quantification. Studies have shown that the range for sensing gene expression levels with RNA-seq is very large whereas microarray lacks sensitivity in detecting transcripts expressed at very low or very high levels (Nagalakshmi et al., 2008; Mortazavi et al., 2008; Wang et al., 2009). Furthermore, experiments have shown that RNA-seq is highly reproducible between different experiments (Nagalakshmi et al., 2008; Wang et al., 2009).

Because of the advantages RNA-seq offer over microarray, we used RNA-seq to determine the differential gene expression between our matched fluconazole-susceptible and -resistant clinical isolates with the goal of characterizing the mechanisms of resistance in each fluconazole-resistant isolate. This work would also importantly ascertain RNA-seq as an effective tool in determining mechanisms of resistance to antifungal agents. Furthermore, our study characterizes mechanisms of fluconazole resistance in _C. parapsilosis_, a medically important pathogen that is on the rise but understudied.
CHAPTER II

Materials and Methods
CHAPTER II- MATERIALS AND METHODS

1. Materials

1.1. Strains and Media

The clinical isolates of *C. parapsilosis* used in this study are listed in Table 3 and were kindly provided by Dr. Daniel Diekema and Dr. Jose A. Vasquez. These strains were routinely grown in liquid yeast extract peptone dextrose (YPD) media (Fischer Scientific, Pittsburgh, Pennsylvania) at 30° C. 1.5% agar was added to the media for growth on solid media plates. Strains were also cultured in liquid Roswell Park Memorial Institute (RPMI) media (10.4 g/L RPMI 1640, 34.53g/L MOPS, Sigma-Aldrich, St. Louis, Missouri) and grown on Difco Sabouraud Dextrose (BD Biosciences, San Jose, California) agar plates prior to drug susceptibility testing.

*Escherichia coli* DH5α cells (Protein Express, Cincinnati, OH) were used for plasmid construction and propagation. These cells were grown in Luria-Bertani (LB) (Fischer) liquid media supplemented with 100 µg/ml ampicillin (Fischer) at 37°C. 1.5% agar was added to the media for growth on solid media plates.

1.2. Antifungal Agents

Fluconazole was obtained from Fischer Scientific. Stock solutions of 5mg/ml were diluted in water and stored at -20°C for up to one month.

2. Methods

2.1. Drug Susceptibility Testing

Drug susceptibility tests to fluconazole were performed in triplicate and according to the standard broth microdilution protocols CLSI M27-A3 (Clinical and Laboratory Standards...
Institute, 2008). Cultures were grown in liquid YPD overnight. The cultures were streaked for isolation on Difco Sabouraud Dextrose agar plates and incubated for 24 hours at 30°C. Individual colonies were picked and added to sterile water to OD$_{600}$ = 0.1 then diluted 1:1000. A microtiter plate with serial dilutions of fluconazole was inoculated with these cells and incubated at 35°C shaking at 110rpm. After incubation for 48 hours, the minimum inhibitory concentration was recorded and defined as 90% growth inhibition relative to a growth control lacking fluconazole.

2.2. Growth Conditions for Gene Expression Analyses

The isolates listed in Table 3 were diluted from overnight cultures to an OD$_{600}$=0.2 and grown in liquid YPD media at 30°C shaking at 250rpm to mid-log phase (OD$_{600}$=0.6-1.0). They were stored at -80°C until RNA preparation. Two independent cultures were grown for each strain and RNA was extracted from each for analysis.

2.3. RNA Extraction

Total RNA was isolated using a hot phenol method (Schmitt et al., 1990). Frozen cells that were stored at -80°C were mixed with 950µl of a buffer consisting of 50mM sodium acetate (Fischer Scientific) and 10mM EDTA (Fischer Scientific), 80µl of 20% sodium dodecyl sulfate (Fischer Scientific), and 950µl phenol (Fischer Scientific), heated at 65°C for 10 minutes with vigorous mixing after each minute, incubated on ice for 5 minutes, and centrifuged at 2,000rpm at 4°C for 15 minutes. The supernatant was collected, spilt between two tubes each containing 950µl chloroform (Fischer Scientific), and centrifuged at 2,000rpm at 4°C for 10 minutes. The aqueous layers were extracted and combined in a new tube. 100µl of 2M sodium acetate and 1ml isopropanol (Fischer Scientific) were added to the tube and the mixture was centrifuged at
12,000 rpms at 4°C for 20 minutes. The supernatant was discarded, the pellet was washed with 500µl of 70% ethanol (Fischer Scientific), and centrifuged at 12,000 rpms at 4°C for 5 minutes. The supernatant was removed, the pellet was air-dried, resuspended in sterile RNase/DNase free water, and stored at -80°C.

2.4. RNA-Seq Preparation and Data Annotation

RNA samples were prepared with the Illumina TruSeq kit and sequenced on a HiSeq 2000 at the Cincinnati Children’s Genetic Variation and Gene Discovery Core Facility (Cincinnati, OH) using single-end 50bp reads. TopHat v1.4.0 was used to align the sequence to the reference genome (Trapnell et al., 2012). Cufflinks v1.3.0 was used to generate the transcriptome and to quantify each transcript (Trapnell et al., 2010).

A twofold increase and a decrease by at least half were arbitrarily set as the threshold fold changes to determine statistical significance of genes that were differentially expressed. P-values and q-values (false discovery rate) were determined using Cufflinks v1.3.0 and genes were considered significantly differentially expressed when these values were less than 0.05. These fold change and statistical cuts-offs were set at these values because they eliminated false positives while also revealing the genes that had the most drastic changes in gene expression between the fluconazole-susceptible isolate and the fluconazole-resistant isolate.

The sequence was aligned using a previously annotated genome of *C. parapsilosis* (Guida et al., 2011). Each gene is designated with a CPAR2 number that was obtained from the *C. parapsilosis* database on candidagenome.org (Arnaud et al., 2012). Because the *C. parapsilosis* genome has yet to be fully characterized, the *C. albicans* orthologs corresponding to the CPAR2 designations were also obtained from candidagenome.org as well as their gene descriptions and ontogenies.
2.5. Real-Time Reverse Transcription (RT)-PCR

The strains were grown and RNA was extracted as described above except that it was performed in triplicate. cDNA was synthesized from 2µg of total RNA using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, California) according to the manufacture’s instructions and stored at -80°C. Real-time RT-PCRs were performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, Foster City, California) with SYBR Green PCR Master Mix (Applied Biosystems). Independent amplifications were performed using the same cDNA for both the gene of interest and the ACT1 endogenous control. Gene-specific real-time RT-PCR primers were designed using Primer3 software (v. 0.4.0) (Rozen and Skaletsky, 2000) and are shown in Table 4. The PCR conditions consisted of polymerase activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. A dissociation curve was generated at the end of each cycle to verify that a single product was amplified. The detection software determined the change in SYBR green fluorescence after each cycle. The threshold cycle (C_T) above the background for each gene of interest was calculated. To calculate a ΔC_T value, the C_T value of the ACT1 reaction was subtracted from each C_T value of the genes of interests. The ΔC_T values from the fluconazole-susceptible isolate were subtracted from the ΔC_T values of the fluconazole-resistant isolate to obtain the ΔΔC_T value. The gene expression level in the fluconazole-resistant isolate relative to the gene expression level in the fluconazole-susceptible isolate was expressed as 2^-ΔΔC_T. The three independent values for each gene of interest were averaged and the standard error of the mean was calculated as well the p-value using a Student t-test in Microsoft Excel. p-values less than 0.05 were considered significant.
Table 4. Gene-specific sequencing and real-time RT-PCR primers used.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequencing Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ERG3</strong></td>
<td>F-5’-CATCACCACCACCACCACTAAGC-3’&lt;br&gt;R-5’-AAATAAAAATAATGTCCTTCATCG-3’&lt;br&gt;Seq1-5’-CAGTGCACTGATCTCTAAAG-3’&lt;br&gt;Seq2-5’-ATGGAAACCAACCCTCTTACC-3’&lt;br&gt;Seq3-5’-GGTAAGAGGTTGGTTTTCAAT-3’&lt;br&gt;Seq4-5’-TTTGTAACCATTCATCTC-3’&lt;br&gt;Seq5-5’-GAGATGCAATGGTGTAACAA-3’&lt;br&gt;Seq6-5’-ACTTCGCATTATCATCAAGCAG-3’</td>
<td>1,496</td>
</tr>
<tr>
<td><strong>MRR1</strong></td>
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<td>GCTGAAACAATAGCAGTAACAGC-3'</td>
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<tr>
<td>Seq</td>
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<td>---------</td>
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| Seq16   | CAAAACATTCCCATTCAACTG | 3'         |
| Seq17   | AGAGTTCAATTGTCCGGTAATCC | 3'         |
| Seq18   | GGATTACCGGACAATTGAACTCT | 3'         |
| Seq19   | GCCATTGGATAGGTTCTTGTC | 3'         |
| Seq20   | GACAAGAACCTATCCAATG | 3'         |
| Seq21   | ATGGGTGCTAGATTGATGTTG | 3'         |
| Seq22   | CAACATCAATCTAGCACCCAT | 3'         |

**RT-PCR Primers**

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<td><strong>ERG2</strong></td>
<td>F-5’-ACTGGTCGCTAGATGGTTGGA</td>
<td>3’</td>
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<tr>
<td><strong>ERG3</strong></td>
<td>F-5’-TCCTTCATAGATGGTGTTGGC</td>
<td>3’</td>
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<tr>
<td><strong>ERG6</strong></td>
<td>F-5’-GAAACAAGAGATTGGACGC</td>
<td>3’</td>
</tr>
<tr>
<td><strong>ERG11</strong></td>
<td>F-5’-GGTTTACTTTGTGTTTGCTCT</td>
<td>3’</td>
</tr>
<tr>
<td><strong>ERG28</strong></td>
<td>F-5’-TTTACCTTTGGCGTGCTGATT</td>
<td>3’</td>
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<td><strong>TAC1</strong></td>
<td>F-5’-ACCCTACCTTCCAATCAGCAG</td>
<td>3’</td>
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<tr>
<td><strong>CDR1</strong></td>
<td>F-5’-CATAACAAAGACCAACTGCTG</td>
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5,801 71 144 108 93 127 87 50 79
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<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Tm</th>
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<td>R-5’-TCACTTCTCGGATCCAACA-3’</td>
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<td>MRR1</td>
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<td>R-5’-GGCAATACCTGGTAGGGA-3’</td>
<td>109</td>
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<tr>
<td>MDR1</td>
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<td>R-5’-TGAACCTGGAGTACCTTG-3’</td>
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<td>R-5’-TTGGTAGCTGAAACACCTGG-3’</td>
<td>96</td>
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<tr>
<td>HAP43</td>
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<td>R-5’-TCGATCTAGCTCCGCAACACAT-3’</td>
<td>172</td>
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<tr>
<td>NDT80</td>
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<td>R-5’-ATGTTGAGGAGGAGTAAGA-3’</td>
<td>100</td>
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<tr>
<td>GZF3</td>
<td>F-5’-TGGAAGTGGCAATGCAACTGAC-3’</td>
<td>R-5’-TATGCAAAGGTGGCAATGATG-3’</td>
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<td>R-5’-TTGACGCTCTGCTTGCTTCGGA-3’</td>
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<td>FLU1</td>
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<td>R-5’-TTCTATCTGCCGATCATGCGGA-3’</td>
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<tr>
<td>HGT2</td>
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<td>R-5’-TATCCACCCGATTCTGGCCTGGC-3’</td>
<td>171</td>
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<tr>
<td>HGT8</td>
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<td>R-5’-CTGCTATGCAGCTTGTTGCTCA-3’</td>
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<tr>
<td>PHO89</td>
<td>F-5’-ATGGGCCATTCCCTTGTTCCACCAC-3’</td>
<td>R-5’-GTAAACCCGCAATGCAAACAGGCA-3’</td>
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<tr>
<td>OPT5</td>
<td>F-5’-AGCTGTTTACGAAATCAGGCAAC-3’</td>
<td>R-5’-AAATCTGGAATTGCTCCGGTACGCT-3’</td>
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<tr>
<td>GIT2</td>
<td>F-5’-TACATCCAGGTCATCCCCCTGAT-3’</td>
<td>R-5’-CTGCAACACACACCAAGCAGC-3’</td>
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<td>CRH11</td>
<td>F-5’-AGCCTCGCTCATACTAGCGAAGA-3’</td>
<td>R-5’-AGCGGATTGTTGGCGACAGATGGA-3’</td>
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<td>MKC1</td>
<td>F-5’-CTTTGGGCCTGACTTTGGGAC-3’</td>
<td>R-5’-AACCTGCGATTGCGTATGG-3’</td>
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<tr>
<td>CPAR2_2</td>
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<td>R-5’-ACACATCCACCCAGACCTCAGA-3’</td>
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<td>CPAR2_4</td>
<td>F-5’-AGTTGGCTCCGTCGTTGCAATTACAT-3’</td>
<td>R-5’-TGAAGCCACAAACCTTGCGCAGA-3’</td>
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<td>SEO1</td>
<td>F-5’-TCCCTGAAACGTCCAAATGTTTGAGAC-3’</td>
<td>R-5’-AAACCAATGTCGCAATTCAGGCGG-3’</td>
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<tr>
<td>ACT1</td>
<td>F-5’-ACTACCTGAAAGAATTGTTAGAGAC-3’</td>
<td>R-5’-ATGATAGAGTGGAAAGTATTTGGAATA-3’</td>
<td>240</td>
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</table>

F=Forward primer, R=Reverse primer.
2.6. Genomic DNA Extraction

To extract genomic DNA for sequencing, the isolates were grown overnight in 5ml YPD. The cells were collected by centrifugation and washed in sterile RNase/DNase-free water. The cells were lysed by vortexing with 0.5mm acid-washed beads (BioSpec Products, Inc., Bartlesville, Oklahoma), and equal volumes of Triton-SDS buffer (2% Triton X-100, 1% sodium-dodecyl-sulfate, 100mM NaCl, 1mM Na2EDTA, at pH 8.0, Fischer Scientific), and phenol:chloroform:isoamyl alcohol (25:24:1) (Fischer Scientific). The aqueous layer was extracted with 1X TE buffer (100mM Tris-HCl, 10mM EDTA, Fischer Scientific). DNA and RNA were precipitated with 100% ethanol. The ethanol was removed, the nucleic acid material was dissolved in 1X TE buffer, and incubated at 37°C with RNase A (Invitrogen). DNA was precipitated with 4M ammonium acetate (Fischer Scientific) and 100% ethanol. The DNA pellet was stored in sterile water at -20°C until needed.

2.7. Gene Sequencing

Genomic DNA was used to amplify the genes of interest by PCR using the forward and reverse primers listed in Table 4 with iProof, a high fidelity polymerase (BioRad, Hercules, California). For sequencing of ERG11, UPC2, and TAC1, PCR products were cloned into pCR-BLUNTII-TOPO® using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) and transformed into E. coli DH5α cells with selection on LB agar plates containing 100 µg/ml ampicillin. Plasmid DNA was purified (QIAprep®; Qiagen; Germantown, MD) and sequenced at Cincinnati Children’s Genetic Variation and Gene Discovery Core Facility on the ABI Prism® 3730xl DNA Analyzer using the universal primers M13R and T7 and a set of gene-specific sequencing primers (Table 4). Three sets of clones derived from three different PCR products were sequenced from each isolate for each gene. For sequencing of all other genes, the PCR
products were gel-cleaned and 5ng for each 100bp to be sequenced were mixed with 7pmol of sequencing primer in a total volume of 12µl. These samples were also sequenced using the ABI Prism® 3730xl DNA Analyzer and the sequencing primers are listed in Table 4. At least 3 different PCR products were used to sequence each gene. ERG3 was sequenced in 35176 and 35177 6 times and cloned into a plasmid as described above to confirm the mutation. DNASTAR Lasergene® 10.1 was used to analyze the sequence data.
CHAPTER III

Results
CHAPTER III- RESULTS

1. Clinical Isolate Set 1

1.1. RNA-seq Analysis

To characterize the mechanisms of fluconazole resistance employed by 20360.066, we determined which genes were up significantly more than two-fold or down significantly by at least half in 20360.066 compared to its matched fluconazole-susceptible isolate, 20360.053, by RNA-seq. By determining which genes were differentially expressed between these isolates, the pathways that are involved in the mechanisms of fluconazole resistance in 20360.066 could theoretically be elucidated.

The genes that had significantly higher or lower expression in the fluconazole-resistant isolate 20360.066 compared to the fluconazole-susceptible isolate 20360.053 are shown in Table 5. There was a significant upregulation of 21 genes and a significant downregulation of 11 genes. Genes that were upregulated in 20360.066 are mostly involved in transport (orthologs of C. albicans PHO89, OPT5, GIT2, OPT1, SEO1, and KAR2) and response to drug stimuli (orthologs of C. albicans OPT1, orf19.6586, orf19.4116, and RBT4). Genes that were downregulated fall under a variety of gene ontogenies (Table 5). RNA-seq analysis was inconclusive for 2,431 of the total 6,229 genes (see Chapter IV Discussion). For these genes, we analyzed selected genes (orthologs of C. albicans genes involved in azole resistance) by real-time reverse transcription (RT)-PCR (TAC1, MRR1, MDRI, CAP1, HAP43, MCM1, HGT8, and FLU1) (Figure 2).

1.2. Real-Time RT-PCR analysis

As our study represents the first application of RNA-seq in clinical isolates of C. parapsilosis, it was important to validate the results by independent methods. Thus, real-
Table 5. Genes upregulated or downregulated in 20360.066 (fluconazole-resistant isolate) relative to 20360.053 (fluconazole-susceptible isolate) based on RNA-seq analysis.

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<th>Gene Ontogeny</th>
<th>C. parapsilosis Designation</th>
<th>C. albicans Ortholog</th>
<th>Gene Description</th>
<th>Fold Change&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>PHO89</td>
<td>Sodium:inorganic phosphate symporter activity</td>
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<td>Role in transmembrane transport</td>
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<td>GIT2</td>
<td>Transmembrane transporter activity</td>
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<td>CPAR2_800720</td>
<td>OPT1</td>
<td>Proton-dependent secondary active transmembrane transporter activity</td>
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<td>CPAR2_203100</td>
<td>SEO1</td>
<td>Role in transmembrane transport</td>
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<td>KAR2</td>
<td>ATPase activity, unfolded protein binding activity</td>
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<td>-3.89</td>
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<tr>
<td>Response to Stress</td>
<td>CPAR2_213780</td>
<td>KAR2</td>
<td>ATPase activity, unfolded protein binding activity</td>
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<td>Response to Drug Stimuli</td>
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<td>RBT5</td>
<td>Putative membrane protein, member of the CFEM family</td>
<td>-3.89</td>
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<tr>
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<td>OPT1</td>
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<td>CPAR2_702640</td>
<td>orf19.6586</td>
<td>Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains</td>
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<td>orf19.4116</td>
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<td>RBT4</td>
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<td>MKC1</td>
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<td>MKC1</td>
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<td>ATPase activity, unfolded protein binding activity</td>
<td>2.04</td>
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<td>CPAR2_800090</td>
<td>MKC1</td>
<td>MAP kinase activity</td>
<td>-2.40</td>
</tr>
<tr>
<td><strong>Organelle Organization</strong></td>
<td>CPAR2_400510</td>
<td>PHO89</td>
<td>Sodium:inorganic phosphate symporter activity</td>
<td>35.14</td>
</tr>
<tr>
<td></td>
<td>CPAR2_213780</td>
<td>KAR2</td>
<td>ATPase activity, unfolded protein binding activity</td>
<td>2.04</td>
</tr>
<tr>
<td><strong>Interspecies Interaction</strong></td>
<td>CPAR2_404800</td>
<td>ALS7</td>
<td>Cell adhesion involved in multi-species biofilm formation</td>
<td>6.13</td>
</tr>
<tr>
<td><strong>Conjugation</strong></td>
<td>CPAR2_800720</td>
<td>OPT1</td>
<td>Proton-dependent oligopeptide secondary active transmembrane transporter activity and role in cellular response to drug</td>
<td>3.47</td>
</tr>
<tr>
<td></td>
<td>CPAR2_213780</td>
<td>KAR2</td>
<td>ATPase activity, unfolded protein binding activity</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td>CPAR2_213080</td>
<td>SOD4</td>
<td>Superoxide dismutase activity</td>
<td>-2.93</td>
</tr>
<tr>
<td><strong>Cell Adhesion</strong></td>
<td>CPAR2_404800</td>
<td>ALS7</td>
<td>Cell adhesion involved in multi-species biofilm formation</td>
<td>6.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALS1</td>
<td>Putative adhesin</td>
<td>3.99</td>
</tr>
<tr>
<td><strong>Carbohydrate Metabolism</strong></td>
<td>CPAR2_502120</td>
<td>CHT2</td>
<td>Role in carbohydrate metabolic process</td>
<td>7.60</td>
</tr>
<tr>
<td></td>
<td>CPAR2_402090</td>
<td>PYC2</td>
<td>Role in gluconeogenesis</td>
<td>3.15</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td>CPAR2_802660</td>
<td>CHO2</td>
<td>Phosphatidylethanolamine N-methyltransferase activity</td>
<td>-2.64</td>
</tr>
<tr>
<td><strong>Biofilm Formation</strong></td>
<td>CPAR2_800090</td>
<td>MKC1</td>
<td>MAP kinase activity</td>
<td>-2.40</td>
</tr>
<tr>
<td></td>
<td>CPAR2_402920</td>
<td>RBT5</td>
<td>Putative membrane protein, member of the CFEM family</td>
<td>-3.89</td>
</tr>
<tr>
<td><strong>Biological Process Unknown</strong></td>
<td>CPAR2_404780</td>
<td>ALS1</td>
<td>Uncharacterized</td>
<td>20.09</td>
</tr>
<tr>
<td></td>
<td>CPAR2_500490</td>
<td>orf19.1239</td>
<td>Uncharacterized</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>CPAR2_805710</td>
<td>PHM7</td>
<td>Ortholog(s) have Golgi apparatus, fungal-type vacuole, plasma</td>
<td>8.43</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene ID</td>
<td>Description</td>
<td>Fold Change</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
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<td>-------------</td>
<td></td>
</tr>
<tr>
<td>CPAR2_602040</td>
<td>orf19.5513</td>
<td>Uncharacterized</td>
<td>3.24</td>
<td></td>
</tr>
<tr>
<td>CPAR2_102480</td>
<td>orf19.278</td>
<td>Uncharacterized</td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td>CPAR2_701570</td>
<td>orf19.4116</td>
<td>18S ribosomal RNA</td>
<td>2.75</td>
<td></td>
</tr>
<tr>
<td>CPAR2_808730</td>
<td>orf19.6805</td>
<td>Uncharacterized</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>CPAR2_502060</td>
<td>orf19.2638</td>
<td>Uncharacterized</td>
<td>2.30</td>
<td></td>
</tr>
<tr>
<td>CPAR2_213270</td>
<td>orf19.2076</td>
<td>Ortholog(s) have cytosol, nucleus localization</td>
<td>-2.16</td>
<td></td>
</tr>
<tr>
<td>CPAR2_502090</td>
<td>orf19.6654</td>
<td>Uncharacterized</td>
<td>-2.34</td>
<td></td>
</tr>
<tr>
<td>CPAR2_501370</td>
<td>SOU1</td>
<td>Uncharacterized</td>
<td>-2.73</td>
<td></td>
</tr>
<tr>
<td>CPAR2_800070</td>
<td>PGA28</td>
<td>Uncharacterized</td>
<td>-2.75</td>
<td></td>
</tr>
<tr>
<td>CPAR2_108330</td>
<td>BRG1</td>
<td>Uncharacterized</td>
<td>-2.85</td>
<td></td>
</tr>
<tr>
<td>CPAR2_105370</td>
<td>orf19.6852</td>
<td>Uncharacterized</td>
<td>-3.32</td>
<td></td>
</tr>
<tr>
<td>CPAR2_805920</td>
<td>orf19.4066</td>
<td>Uncharacterized</td>
<td>-4.62</td>
<td></td>
</tr>
<tr>
<td>CPAR2_304110</td>
<td>orf19.951</td>
<td>Uncharacterized</td>
<td>-14.26</td>
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</tr>
</tbody>
</table>

**Other**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene ID</th>
<th>Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPAR2_102560</td>
<td>FAA21</td>
<td>Catalytic activity and role in metabolic process</td>
<td>3.48</td>
</tr>
<tr>
<td>CPAR2_203350</td>
<td>GLN1</td>
<td>Glutamate-ammonia ligase activity</td>
<td>2.69</td>
</tr>
<tr>
<td>CPAR2_601950</td>
<td>orf19.5517</td>
<td>Alcohol metabolism; expression increased in fluconazole and voriconazole resistant strains</td>
<td>2.17</td>
</tr>
<tr>
<td>CPAR2_601840</td>
<td>orf19.5517</td>
<td>Putative alcohol dehydrogenase; expression increased in fluconazole and voriconazole resistant strains</td>
<td>2.13</td>
</tr>
<tr>
<td>CPAR2_500880</td>
<td>ALD6</td>
<td>Methylmalonate-semialdehyde dehydrogenase (acylating) activity</td>
<td>2.08</td>
</tr>
<tr>
<td>CPAR2_102630</td>
<td>MET13</td>
<td>Methylene tetrahydrofolate reductase (NADPH) activity</td>
<td>-2.17</td>
</tr>
<tr>
<td>CPAR2_101480</td>
<td>PUT2</td>
<td>1-pyrroline-5-carboxylate dehydrogenase activity</td>
<td>-2.32</td>
</tr>
<tr>
<td>CPAR2_102500</td>
<td>DTD2</td>
<td>D-tyrosyl-tRNA(Tyr) deacylase activity</td>
<td>-2.48</td>
</tr>
<tr>
<td>CPAR2_400880</td>
<td>YIM1</td>
<td>Golgi apparatus, endoplasmic reticulum localization</td>
<td>-4.64</td>
</tr>
</tbody>
</table>

*Fold change consider significant when the p-value and q-value are less than 0.05.*
time RT-PCR was used to examine the expression of selected genes identified as upregulated in the RNA-seq analysis. Real time RT-PCR confirmed 20360.066 upregulation relative to the parent 20360.053 isolate for all of these tested genes (*PHO89, OPT5, SEO1, GIT2, CPAR2_400160, CPAR2_200970, and GZF3*) (Figure 2). The *C. albicans* ortholog of MKC1 was downregulated significantly in the RNA-seq data and appeared to be trending this way in the real-time RT-PCR analysis but was not significant (p-value is greater than 0.05) (Figure 2, Table 5).

We next analyzed gene expression in selected genes that were found to be “inconclusive” in the RNA-seq analysis (see Chapter IV Discussion) but are orthologs of *C. albicans* genes involved in resistance (Figure 2). *TAC1, MRR1, MDR1, CAP1, HAP43, MCM1*, and *FLU1* are orthologs of *C. albicans* genes encoding transcriptional regulators and were upregulated in 20360.066 compared to 20360.053. Mrr1p, Cap1p, and Mcm1p have been shown in *C. albicans* to induce expression of the major facilitator transporter Mdr1p (Morschhauser et al., 2007; Alarco and Raymond, 1999; Riggle and Kumamato, 2006). The other genes that were significantly overexpressed in this isolate, orthologs of *C. albicans MDR1, FLU1, PHO89, OPT5, GIT2*, and SEO1, and *CPAR2_200970, and CPAR2_400160*, are putative transporters (Arnaud et al., 2012). Of the genes tested, the ortholog of *C. albicans HGT8* was the only gene that showed decreased expression in 20360.066. This gene encodes a glucose transporter of the major facilitator superfamily in *C. albicans* (Fan et al., 2002). There was no difference in genes that have involvement in the ergosterol biosynthetic pathway in *C. albicans* (*UPC2, ERG2, ERG3, ERG6, ERG11*, and *ERG28*), or in orthologs of *C. albicans* ATP-binding cassette transporters that are involved in fluconazole resistance, *CDR1* and *CDR2*, even though their transcriptional regulator, *TAC1*, was significantly upregulated in 20360.066.
Figure 2. Differential expression of 20360.066 (fluconazole-resistant isolate) relative to 20360.053 (fluconazole-susceptible isolate) by real-time RT-PCR. RNA was extracted and expression of the indicated genes was measured by real-time RT-PCR. The data represent three independent experiments and gene expression values marked with an asterisk are significantly upregulated or downregulated by more than 2-fold in the fluconazole-resistant isolate 20360.066 (p-value <0.05). Errors bars represent the SE.
1.3. Sequencing of Key Genes

To identify mutations that potentially cause fluconazole resistance in 20360.066, we sequenced genes implicated in the mechanism of resistance from gene expression analyses in both 20360.066 and 20360.053 and compared their genetic sequences. Recent work has elucidated that gain-of-function mutations in transcriptional regulators that control pathways involved in azole resistance cause constitutive activation of these transcriptional regulators, overexpression of the pathways that they regulate, and result in azole resistance (Coste et al., 2006; Dunkel et al., 2008b; Schubert et al., 2008; Morschhauser et al., 2007; Silver et al., 2004; MacPherson et al., 2005; Dunkel et al., 2008a; Flowers et al., 2012). *C. parapsilosis* orthologs of *C. albicans* *MRR1, HAP43, CAP1*, and *GZF3*, were sequenced because they are transcriptional regulators that were upregulated in the real-time RT-PCR data, but there were no mutations in these genes in 20360.066 (Figure 2). The orthologs of genes involved in the ergosterol biosynthetic pathway that frequently have mutations in fluconazole-resistant strains of *C. albicans*, *UPC2, ERG11*, and *ERG3*, were also sequenced in these isolates, but there were no mutations.

2. Clinical Isolate Set 2

2.1. RNA-seq analysis

RNA-seq analysis of the genes that had significant differential expression between 35176, the fluconazole-resistant isolate, and 35177, the fluconazole-susceptible isolate, are shown in Table 6. There were 6 genes that were upregulated more than two-fold in 35176 compared to 35177 and 4 genes that were downregulated more than two-fold. There were no genes involved in transport that are upregulated, but the ortholog of *C. albicans ERG2* which encodes an enzyme in the ergosterol biosynthetic pathway was upregulated. Several genes were
also “inconclusive” in this data set and were not considered in further analysis. ERG6 was one of these genes.

2.2. Real-Time RT-PCR Analysis

Real-time RT-PCR was used to confirm RNA-seq data and to determine if other genes that are commonly associated with fluconazole resistance in C. albicans were differentially expressed in 35176 compared to 35177. Real-time RT-PCR was also used to determine if any transporters that were significantly upregulated in the other fluconazole-resistant isolate, 20360.066, were also upregulated in 35176 (Figure 3). Of the genes tested, orthologs of C. albicans ERG2 and ERG6 were the only genes upregulated in 35176 compared to 35177. This suggests the ergosterol biosynthetic pathway is involved in the mechanism of fluconazole resistance in 35176. Additionally, there were no genes that were upregulated in both Clinical Isolate Set 1 and Clinical Isolate Set 2 implying different mechanisms of resistance in the fluconazole-resistant isolates.

2.3. Sequencing of Key Genes

The RNA-seq and real-time RT-PCR data suggested involvement of the ergosterol biosynthetic pathway in fluconazole resistance in 35176 (Table 6, Figure 3). In C. albicans, UPC2 is a transcriptional regulator that induces the expression of enzymes needed to produce ergosterol. Gain-of-function mutations in UPC2 cause fluconazole resistance by overexpression of the ergosterol biosynthetic genes which contain the target of fluconazole (Silver et al., 2004; MacPherson et al., 2005; Dunkel et al., 2008a; Flowers et al., 2012). Thus, the ortholog of C. albicans UPC2 was sequenced in both 35176 and 35177. However, there was no mutation in this
Table 6. Genes upregulated or downregulated in 35176 (fluconazole-resistant isolate) relative to 35177 (fluconazole-susceptible isolate) based on RNA-seq analysis.

<table>
<thead>
<tr>
<th>Gene Ontogeny</th>
<th>C. parapsilosis Designation</th>
<th>C. albicans Ortholog</th>
<th>Gene Description</th>
<th>Fold Change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td>CPAR2_201490</td>
<td>ERG2</td>
<td>C-8 sterol isomerase activity</td>
<td>3.42</td>
</tr>
<tr>
<td><strong>Pathogenesis</strong></td>
<td>CPAR2_107940</td>
<td>BRG1</td>
<td>Sequence-specific DNA binding activity</td>
<td>2.58</td>
</tr>
<tr>
<td><strong>Cell Wall Organization</strong></td>
<td>CPAR2_403180</td>
<td>PGA62</td>
<td>Cell wall organization and hyphal cell wall localization</td>
<td>-5.43</td>
</tr>
<tr>
<td><strong>Filamentous Growth</strong></td>
<td>CPAR2_107940</td>
<td>BRG1</td>
<td>Sequence-specific DNA binding activity</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>CPAR2_209130</td>
<td>orf19.2299</td>
<td>Protein tag activity and role in cell budding</td>
<td>2.14</td>
</tr>
<tr>
<td><strong>Response to Stress</strong></td>
<td>CPAR2_107940</td>
<td>BRG1</td>
<td>Sequence-specific DNA binding activity</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>CPAR2_209130</td>
<td>orf19.2299</td>
<td>Protein tag activity and role in cell budding</td>
<td>2.14</td>
</tr>
<tr>
<td><strong>Cell Budding</strong></td>
<td>CPAR2_209130</td>
<td>orf19.2299</td>
<td>Protein tag activity and role in cell budding</td>
<td>2.14</td>
</tr>
<tr>
<td><strong>Response to Chemical Stimuli</strong></td>
<td>CPAR2_209130</td>
<td>orf19.2299</td>
<td>Protein tag activity and role in cell budding</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>CPAR2_403180</td>
<td>PGA62</td>
<td>Cell wall organization and hyphal cell wall localization</td>
<td>-2.34</td>
</tr>
<tr>
<td><strong>RNA Metabolism</strong></td>
<td>CPAR2_209130</td>
<td>orf19.2299</td>
<td>Protein tag activity and role in cell budding</td>
<td>2.14</td>
</tr>
<tr>
<td><strong>Transport</strong></td>
<td>CPAR2_106560</td>
<td>orf19.2959.1</td>
<td>Cation transport, regulation of membrane potential</td>
<td>-5.43</td>
</tr>
<tr>
<td><strong>Cellular Homeostasis</strong></td>
<td>CPAR2_106560</td>
<td>orf19.2959.1</td>
<td>Cation transport, regulation of membrane potential</td>
<td>-5.43</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>CPAR2_500880</td>
<td>ALD6</td>
<td>Methylmalonate-semialdehyde</td>
<td>3.85</td>
</tr>
<tr>
<td>Biological Process</td>
<td>Accession</td>
<td>Gene Symbol</td>
<td>Description</td>
<td>Fold Change</td>
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<tr>
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<td>------------</td>
<td>-------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>CPAR2_700020</td>
<td>GCY1</td>
<td></td>
<td>dehydrogenase activity and role in oxidation-reduction process</td>
<td>-2.34</td>
</tr>
<tr>
<td>CGAR2_800070</td>
<td>PGA28</td>
<td></td>
<td>Oxidoreductase activity and role in oxidation-reduction process</td>
<td>2.47</td>
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<td>CPAR2_602420</td>
<td>orf19.3439</td>
<td></td>
<td>Uncharacterized</td>
<td>-2.29</td>
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</table>

Fold change considered significant when the p-value and q-value are less than 0.05.
Figure 3. Differential expression of 35176 (fluconazole-resistant isolate) relative to 35177 (fluconazole-susceptible isolate) by real-time RT-PCR. RNA was extracted and expression of the indicated genes was measured by real-time RT-PCR. The data represent three independent experiments and gene expression values marked with an asterisk are significantly upregulated or downregulated by more than 2-fold in resistant isolate 35176 (p-value <0.05). Errors bars represent the SE.
gene in 35176 (Table 7). In *C. albicans, ERG11* encodes the target of fluconazole and point mutations that result in a lowered affinity of fluconazole for this enzyme cause fluconazole resistance (White, 1997a; Sanglard et al., 1998; Lamb et al., 1997). This gene was sequenced but there were no mutations in 35176 (Table 7). Because no mutations were identified in the orthologs of *UPC2* or *ERG11*, the *C. parapsilosis* ortholog of *C. albicans* ERG3 was sequenced which is another enzyme in the ergosterol biosynthetic pathway that has mutations in fluconazole-resistant strains of *C. albicans*. Deactivation of Erg3p in *C. albicans* prevents the accumulation of toxic sterols that accumulate in the presence of fluconazole and allows the organism to replicate (Kelly et al., 1997; White et al., 1997). Sequencing of this gene revealed a homozygous nonsense mutation that results in a premature stop codon at amino acid 110 in 35176 (Table 7).

Point mutations in specific regions of *FKS1* in *C. albicans* result in echinocandin resistance (Kratz et al., 1996; Park et al., 2005). Because 35176 also exhibits resistance to this class of antifungals, the *C. parapsilosis* ortholog of this gene was sequenced in these isolates (Moudgal et al., 2005). No mutation was identified (Table 7).
Table 7. Genes sequenced and mutations identified in 35176 (fluconazole-resistant isolate).

<table>
<thead>
<tr>
<th>C. albicans Ortholog</th>
<th>Mutation</th>
</tr>
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<tbody>
<tr>
<td>FKS1</td>
<td>None</td>
</tr>
<tr>
<td>TAC1</td>
<td>None</td>
</tr>
<tr>
<td>MRR1</td>
<td>None</td>
</tr>
<tr>
<td>UPC2</td>
<td>None</td>
</tr>
<tr>
<td>ERG11</td>
<td>None</td>
</tr>
<tr>
<td>ERG3</td>
<td>Homozygous Nonsense Mutation in 35176 (W110Ter)</td>
</tr>
</tbody>
</table>
CHAPTER IV

Discussion
CHAPTER IV- DISCUSSION

4.1. Clinical Isolate Set 1

The isolates used in this study consist of a fluconazole-susceptible isolate, 20360.053, and a high-level fluconazole-resistant isolate, 20360.066, that are of the same genetic background, and thus, originated from the same *C. parapsilosis* strain (Table 3). The genetic similarity of these isolates makes them ideal for further study employing differential expression to identify potential mechanisms of fluconazole resistance in 20360.066. Furthermore, these isolates were taken from bloodstream infections that occurred in neonates in a neonatal intensive care unit and represent the particular problem *C. parapsilosis* poses in this susceptible population (Sarvikivi et al., 2005).

RNA-seq analysis of these isolates revealed a significant upregulation of genes involved in transport (orthologs of *C. albicans* PHO89, OPT5, GIT3, OPT1, SEO1, and KAR2) (Table 5). Efflux-mediated transport is a known mechanism of drug resistance in *Candida* species so it is notable that multiple genes with transmembrane transport activity are upregulated in the fluconazole-resistant isolate 20360.066. In *C. albicans*, Cdr1p, Cdr2p, and Mdr1p are the transporters that efflux fluconazole out of the organism (Sanglard and Odds 2002; Cannon et al., 2009; Coste et al., 2006; Coste et al., 2004; Dunkel et al., 2008b; Schubert et al., 2008; Morschhauser et al., 2007). However, none of these transporters were upregulated in the RNA-seq data. Of the transporters that were upregulated, little is known about them and it remains to be determined if fluconazole could be a substrate for them. *SEO1*, however, is characterized as a major facilitator small molecule transporter and one study found that this gene was upregulated in a microarray experiment in *C. albicans* in response to ketoconazole, another member of the azole class of antifungals (Liu et al., 2005). This same study found that *RBT5* expression was
repressed in response to ketoconazole (Liu et al., 2005). This supports our RNA-seq data in that
the ortholog of *C. albicans RBT5* was the only gene with transport activity that is downregulated
in 20360.066, but it remains to be seen if fluconazole could be a substrate for either of these
transporters.

Real-time RT-PCR data also suggested the involvement of transporters in 20360.066 with
increased expression of the orthologs of *C. albicans TAC1* (transcriptional regulator of the ATP-
binding cassette transporters, Cdr1p and Cdr2p), *MRR1* (transcriptional regulator of the major
facilitator transporter Mdr1p), *MDR1, CAP1* (transcriptional regulator known to upregulate
Mdr1p under certain conditions), *MCM1* (transcriptional regulator known to upregulate Mdr1p
under certain condition), *FLU1* (major facilitator transporter), *PHO89, OPT5, GIT3, and SEO1,*
and *CPAR2_200970* (major facilitator transporter), and *CPAR2_400160* (major facilitator
transporter) (Figure 2) (Schubert et al., 2011; Coste et al., 2006; Coste et al., 2004; Dunkel et al.,
2008b; Schubert et al., 2008; Morschhauser et al., 2007; Riggle and Kumanmoto, 2006; Alarco
and Raymond, 1999; Gulshan and Moye-Rowley, 2007; Arnaud et al., 2012). Sequencing of
some of these transcriptional regulators that control expression of transporters in *C. albicans*
showed that there were no mutations in any of these genes in 20360.066. Upregulation of several
transporters does suggests that efflux of fluconazole could be one mechanism of resistance
employed by 20360.066, but the mechanism of upregulation remains unclear.

Two other genes upregulated in 20360.066 in the RNA-seq data, orthologs of *C. albicans
orf19.5517* and *PYC2*, additionally suggest the involvement of transporters in fluconazole
resistance. In a study conducted by Karababa et al. (2004), transcripts of these genes were
elevated when *C. albicans* strains were subjected to benomyl which is well-known substrate and
activator of Mdr1p. This study also showed that *PYC2* and *orf19.5517* were upregulated in azole-
resistant clinical isolates of *C. albicans* that overexpress Mdr1p (Karababa et al., 2004). The exact function of these genes is unknown but it is interesting that they were upregulated in our azole-resistant isolate that overexpresses transcripts of several putative transporters and other azole-resistant strains of *C. albicans* with Mdr1p overexpression.

Further analysis of the genes differentially expressed in the RNA-seq analysis imply involvement of genes regulated by Hap43p in the mechanism of fluconazole resistance in 20360.066. Hap43p is a transcriptional regulator that regulates iron homeostasis and iron acquisition in *C. albicans* which are important for pathogenesis and survival of *Candida* species in the human host where iron is sequestered (Singh et al., 2011; Hsu et al., 2011; Lan et al., 2004). Singh et al. (2011) conducted a study to characterize the network of genes regulated by Hap43p in *C. albicans*. In this study, CHO2, orf19.6589, RBT4, and PHM7 were found to be induced by Hap43p under iron-depriving conditions. The *C. parapsilosis* orthologs of these genes were significantly upregulated in 20360.066 in our study. Orf19.951, orf19.2076, and BRG1 are genes that Singh et al. (2011) found to be repressed by Hap43p and the orthologs of these genes were significantly downregulated in 20360.066 in our study (Table 5). Because upregulation/downregulation of these genes in our study agree with Hap43p-mediated induction/repression determined by Singh et al. (2011), it is possible that the Hap43p regulatory network has become over-activated in 20360.066. Furthermore, the *C. parapsilosis* ortholog of HAP43 was significantly upregulated in the real-time RT-PCR data in 20360.066 (it was “inconclusive” in RNA-seq analysis) (Figure 2). There was no mutation in 20360.066 in this gene however. It is possible that there is an activating or deactivating mutation in an inducer or suppressor of Hap43p that has yet to be characterized, or that mutations exist in a homolog of Hap43 that has yet to be identified in *C. parapsilosis*. 
Many of these genes controlled by Hap43p have yet to be carefully characterized so it is difficult to infer if they have a direct role in fluconazole resistance in 20360.066. There is work though that shows that iron deprivation enhances *Candida* species’ drug susceptibilities to many different antifungals including fluconazole (Prasad et al., 2006). One study also found that a combination of iron-free lactoferrin and fluconazole had a cooperative fungistatic effect and significantly lowered fluconazole minimum inhibitory concentrations (Kuiper et al., 1999). Doxycycline, an iron chelator, and fluconazole were also shown to have synergistic effect in reducing fluconazole minimum inhibitory concentrations even in resistant strains of *C. albicans*. The observed reduction in fluconazole minimum inhibitory concentration was reversed with the addition of free iron (Fiori and Van Dijck, 2012). These studies show that there is a possible link between intracellular iron concentrations and drug sensitivities. It is possible that 20360.066 has altered mechanisms of iron acquisition as suggested by the *C. albicans* orthologs controlled by Hap43p that were upregulated or downregulated in 20360.066 and implicated in iron homeostasis. Furthermore, the hypothetical increase in intracellular iron in this isolate could recruit more heme groups for Erg11p and compete with the heme-dependent inhibition of fluconazole.

### 4.2. Clinical Isolate Set 2

35176 and 35177, fluconazole-resistant and -susceptible *C. parapsilosis* isolates, respectively, were obtained from a patient suffering from prosthetic valve endocarditis and serve as testimony to the tropism *C. parapsilosis* has for bioprosthetic materials. These isolates were also shown to be of the same genetic background by electrophoretic karyotyping which makes them ideal to study mechanisms of fluconazole resistance by differential gene expression (Moudgal et al., 2005).
RNA-seq analysis showed few genes were differentially expressed between 35176 and 35177. The ortholog of *C. albicans* ERG2 was notable because of its role in the ergosterol biosynthetic pathway (Table 6). Real-time RT-PCR also showed significant upregulation of this gene as well as *ERG6*, another gene that encodes an enzyme in the ergosterol biosynthetic pathway (Figure 3). After sequencing genes in the ergosterol biosynthetic pathway known to be involved in the acquisition of fluconazole resistance in *C. albicans*, we discovered a homozygous nonsense mutation in 35176 in the ortholog of *C. albicans* ERG3 which encodes Δ5,6-desaturase (Erg3p) (Table 7).

Mutations that cause the inactivation of Erg3p have been characterized in isolates of *C. albicans*, *C. glabrata*, *C. dubliniensis*, and *C. lusitaniae* but never before in *C. parapsilosis* (Kelly et al., 1997; Nolte et al., 1997; Chau et al., 2005; Morio et al., 2012; Vale-Silva et al., 2012, Gerber et al., 1995; Pinjon et al., 2003; Young et al., 2003). In *C. albicans*, ERG3 nonsense mutations protect the organism by preventing the accumulation of the toxic 14α-methylergosta-8,24-dien-3β,6α-diol sterol which accumulates when the ergosterol biosynthetic pathway is altered in the presence of the azoles (Figure 1). The non-toxic product 14α-methylfecosterol accumulates in the membrane instead and the organism is able to replicate (Kelly et al., 1997; White et al., 1998). Presumably, this is what occurs in the *C. parapsilosis* clinical isolate 35176 but more work is underway to confirm this.

One study showed that introducing *ERG3* mutant alleles into a wild-type, fluconazole-susceptible strain of *C. albicans* resulted in a fluconazole minimum inhibitory concentration greater than 128 µg/ml (Vale-Silva et al., 2012). Therefore, it is possible that this single mutation in 35176 resulted in a fluconazole minimum inhibitory concentration greater than 64 µg/ml and clinical failure of fluconazole in the patient.
Other studies have observed increases in amphotericin B minimum inhibitory concentrations in strains with deactivation of Erg3p (Nolte et al., 1997; Kelly et al., 1997). This is likely a result of a decrease in ergosterol in the membrane since 14α-methylfecosterol accumulates instead (Figure 1, Table 1). Amphotericin B requires ergosterol in the membrane to be effective as it specificity binds to ergosterol to form a pore in the membrane to enact its mechanism of action (Ermishkin et al., 1976). As expected, 35176 displays an elevated amphotericin B minimum inhibitory concentration. Moudgal et al. (2005) found that the amphotericin B minimum inhibitory concentration of the infecting organism on the patient’s first admission, corresponding to 35177, was 0.25µg/ml and 0.5µg/ml on the patient’s second admission, corresponding to 35176 (Moudgal et al., 2005). This is disconcerting because, despite its toxicity, amphotericin B is an effective drug against invasive Candida infections, is often used in the most severe cases of candidemia, and has a very low incidence of resistance (Gallis et al., 1990, Pfaller et al., 2010).

35176 is also resistant to caspofungin (minimum inhibitory concentration greater than 16 µg/ml) and micafungin (minimum inhibitory concentration greater than 16 µg/ml), however, there were no mutations in FKS1 (Table 7), which are commonly seen in echinocandin-resistant strains of Candida species (Moudgal et al., 2005; Kurtz et al., 1996; Park et al., 2005). It is possible that point mutations in FKS2 could also result in echinocandin resistance which has been described in C. glabrata echinocandin-resistant strains (Katiyar et al., 2006; Lewis, 2009; Pfaller 2012).

4.3. Clinical Isolate Set 1 vs. Clinical Isolate Set 2

Clinical Isolate Set 1 and Clinical Isolate Set 2 appear to exhibit different mechanisms of resistance as the mechanism of fluconazole resistance employed by 20360.066 (Clinical Isolate
Set 1) seems to be transport-mediated while the mechanism of resistance employed by 35176 (Clinical Isolate Set 2) is potential inactivation of Erg3p. Furthermore, there were no overlaps between these isolate sets for any genes in differential expression analyses. The isolate sets did developed in different patient populations and resistance was achieved over differing amounts of time. The isolates in Clinical Isolate Set 1 were obtained from different neonates and there may have been years between when they were isolated as they are from a set of 26 bloodstream isolates that were obtained over a period of 12 years (Sarvikivi et al., 2005). The isolates in Clinical Isolate Set 2 were taken from a single patient with a period of 3 months between their isolation (Moudgal et al., 2005). It is possible that Clinical Isolate Set 1 slowly acquired genetic changes that resulted in subtle changes in fluconazole susceptibility overtime. For these reasons, it may be more difficult to pinpoint an exact mechanism of resistance in 20360.066. In contrast, it appears that 35176 of Clinical Isolate Set 2 acquired one single point mutation in \(ERG3\) that resulted in high-level fluconazole resistance. This change was less subtle and easier to detect in our analysis. Ultimately, the clinical isolate sets display two different modes of fluconazole resistance employed by \(C.\) \textit{parapsilosis} and they also illustrate that fluconazole resistance can develop slowly overtime or happen quickly with a single genetic alteration.

4.4. **Challenges of RNA-seq in Clinical Isolates of \(C.\) \textit{parapsilosis}**

Because RNA-seq is a novel technique, this study was conducted in part to determine if it is a viable experimental method in determining differential expression in clinical isolates of \(C.\) \textit{parapsilosis}. Real-time RT-PCR analysis confirmed genes that were shown to either be differentially expressed or had no change in expression in the RNA-seq data (Clinical Isolate Set 1: orthologs of \(C.\) \textit{albicans} \textit{PHO89, OPT5, SEO1, GIT2, GZF3, CPAR2_400160, CPAR2_200970, UPC2, ERG2, ERG6, ERG11, ERG28, CDR1, CDR2, HGT2}, and \textit{CRH11}).
Clinical Isolate Set 2: orthologs of *C. albicans* ERG2, UPC2, ERG3, ERG11, ERG28, TAC1, CDR1, CDR2, MRR1, MDR1, CAP1, HAP43, GZF3, MCM1, FLU1, HGT2, HGT8, PHO89, OPT5, GIT2, CRH11, NDT80, MKC1, SEO1, CPAR2_200970, and CPAR2_400160), but it also revealed differential expression of genes that were not identified in the RNA-seq data as a result of being “inconclusive” (Clinical Isolate Set 1: orthologs of *C. albicans* TAC1, MRR1, MDR1, CAP1, HGT8, HAP43, MCM1, and FLU1. Clinical Isolate Set 2: ortholog of *C. albicans* ERG6).

The software had difficulty in aligning these genes to the reference genome and they could not be analyzed further for differential expression. Accurate mapping of the short reads generated from transcripts in RNA-seq to a reference genome is essential for a quality estimation of differential gene expression. The reference genome never completely matches the biological source of the RNA, and single nucleotide polymorphisms and insertion or deletions can cause alignment errors. The short reads can also align to more than one place in the reference genome and cause errors (Wang et al., 2009; Nookaew et al., 2012; Oshlack et al., 2010). As an understudied organism, *C. parapsilosis* has recently been sequenced and the short reads generated in this study were aligned to this recent sequence annotation. High quality genomic data and good annotation of gene structures is needed to map high proportions of genes to the reference genome and to generate a quality estimation of gene expression levels. The newly annotated genome of *C. parapsilosis* probably lacks these features and caused several genes to be “inconclusive” in this study. Furthermore, the isolates used in this study were obtained from infections in the human host while the reference genome was generated from a strain routinely grown in a laboratory setting. This could be a source of genetic variation as these isolates have evolved in different environments and encountered different stresses (Wang et al., 2009; Nookaew et al., 2012; Oshlack et al., 2010; Guida et al., 2011). Further study of *C. parapsilosis*
will result in a more in depth understanding of this organism’s genome and will help improve results obtained from RNA-seq. Development of the analysis software will also continue to evolve and help improve the efficacy of RNA-seq.

The short reads generated in RNA-seq do not have to be aligned to a reference genome and can be assembled *de novo*. However, this process is substantially more complicated requiring more time and computation resources. It also increases the cost, as more resources are needed to house, process, and analyze the data (Oshlack et al., 2010). For these reasons, *de novo* assembly was not feasible in this study.

Upregulation or downregulation of transcripts does not necessary reflect increased or decreased levels of the corresponding proteins which ultimately determine the resistant phenotype. There are many processes between transcription and translation that could cause transcript and protein levels to differ as well as changes in protein stability and half-life. There are studies that show transcript and protein levels do not always correlate and it can be highly variable depending on the system (Vogel and Marcotte, 2012). A study conducted in *Saccharomyces cerevisiae*, a close but nonpathogenic relative of *Candida* species, determined a significant relationship between mRNA and protein levels with a Spearman rank correlation coefficient of 0.57. Generally, abundant mRNA correlated to abundant protein but variability was observed for some individual proteins (Ghaemmaghami et al., 2002). It would be better to directly measure differential protein levels but proteomics cannot be performed with the same efficiency and specificity as transcriptomics and lacks the high-throughput technology that has been developed recently for genomic sequencing (Mann, 2009). Thus, transcriptomic studies with methods like RNA-seq are the best option currently to do organism-wide studies and
Ghaemmaghami et al. (2003) suggest that transcript levels can serve as suitable proxies for protein levels in yeast.

4.5. Conclusion

We have identified two different mechanisms of fluconazole resistance in two different clinical isolates of *C. parapsilosis*. The fluconazole-resistant isolate of Clinical Isolate Set 1, 20360.066, appears to efflux fluconazole out of the cell as differential gene expression analyses show upregulation of many putative transporters (orthologs of *C. albicans* PHO89, OPT5, GIT2, OPT1, SEO1, KAR2, CPAR2_200970, and CPAR2_400160) as well as transporters and transcriptional regulators that control transporters that efflux fluconazole out of the cell in *C. albicans* (orthologs of *C. albicans* MRR1, MDR1, CAP1, MCM1, and FLU1) (Table 5, Figure 2). The fluconazole-resistant isolate of Clinical Isolate Set 2, 35176, appears to inactivate Erg3p with a homozygous nonsense mutation (Table 7). This potentially inhibits accumulation of a toxic sterol that would otherwise be synthesized (Figure 1). This is the first *ERG3* nonsense mutation identified in *C. parapsilosis*. This work also validated RNA-seq as a useful tool in differential gene expression in *C. parapsilosis* as results were supported by real-time RT-PCR experiments.
CHAPTER V

Future Directions
CHAPTER V- FUTURE DIRECTIONS

5.1. Clinical Isolate Set 1

We hypothesize that fluconazole resistance in 20360.066 of Clinical Isolate Set 1 is a result of transport-mediated drug efflux as many transporters and genes associated with transport were upregulated in this isolate in differential gene expression analyses (orthologs of C. albicans PHO89, OPT5, GIT3, OPT1, SEO1, KAR2, TAC1, MRR1, MDR1, CAP1, MCM1, FLU1, orf19.5517 PYC2, CPAR2_200970, and CPAR2_400160) (Table 5, Figure 2). However, no mutations were identified in the genes we sequenced. Therefore, whole genome sequencing should be performed to identify single nucleotide polymorphisms and insertions or deletions that occur between 20360.053 and 20360.066 to identify new targets that could be involved in the mechanism of upregulation of these transporters since we were unable to find one in this study.

Because several putative Hap43p-regulated genes were differentially expressed in 20360.066 (orthologs of C. albicans orf19.6589, RBT4, PHM7, CHO2, orf19.951, orf19.2076, and BRG1) and intracellular iron concentrations are associated with fluconazole susceptibility in C. albicans (Prasad et al., 2006; Kuiper et al., 1999; Fiori and Van Dijck, 2012), we also hypothesize that differences in intracellular iron concentration and processes of iron acquisition are involved in the mechanism of fluconazole resistance in 20360.066. Studies should be preformed to determine if there are in fact differences in intracellular iron concentration. This can be accomplished by directly visualizing and measuring cytoplasmic iron with calcein and phen-green fluorescent dyes (Esposito et al., 2002).

5.2. Clinical Isolate Set 2
We hypothesize that the nonsense mutation identified in \textit{ERG3} in 35176, the fluconazole-resistant isolate of this set, results in inactivation of Erg3p and high-level azole resistance as characterized in fluconazole-resistant strains of \textit{C. albicans} and \textit{C. dubliniensis} with \textit{ERG3} nonsense mutations (Kelly et al., 1997; Pinjon et al., 2003; Chau et al., 2005; Nolte et al., 1997). To determine if the \textit{ERG3} nonsense mutation in 35176 is indeed the cause of high-level azole resistance in this isolate, the SAT1-Flipper method, a \textit{Candida} specific gene disruption technique, should be employed to construct knockout and complement cassettes to introduce this mutation into the susceptible isolate, 35177 (Reuβ et al., 2004). Once both wild-type alleles are replaced with the mutant allele using this technique, fluconazole susceptibility tests should be performed to determine the level of fluconazole resistance this mutation causes. Biochemical studies should also be performed to determine how the membrane sterol composition is altered. This will help characterize the consequences of Erg3p inactivation in \textit{C. parapsilosis} and further describe the phenotype of this mutation. Gas chromatography-mass spectrometry could be used to accomplish this (Martel et al., 2010).

5.3. Future of Antifungal Therapy

The azoles, and especially, fluconazole, are a critical class of antifungals for treatment of invasive candidiasis because of their ease of use, safety profile, and effectiveness against \textit{C. parapsilosis} relative to the other antifungal agents currently available, and need to be maintained. Therefore, this study and the studies to follow will allow for a better understanding of fluconazole resistance in \textit{C. parapsilosis}, a significant, yet understudied pathogen, and will elucidate strategies for future antifungal therapy to circumvent the problem of azole antifungal resistance and to preserve the efficacy of this class of antifungals. However, the best strategy to prevent resistance to fluconazole in \textit{C. parapsilosis} is to prevent the establishment of an
infection. This can be achieved with careful attention to hand hygiene and sterility of bioprosthetic material.
CHAPTER VI- REFERENCES


