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# Investigation of Clinically Relevant Fluconazole Resistance Mechanisms in the Fungal Pathogen Candida parapsilosis

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# Investigation of Clinically Relevant Fluconazole Resistance Mechanisms in the Fungal Pathogen Candida parapsilosis


## Abstract

Invasive candidiasis is a severe fungal infection associated with significant morbidity and mortality, particularly among the critically ill and immunocompromised. Candida parapsilosis is the most common non-albicans species causing invasive Candida infections in pediatric and neonatal populations worldwide and is particularly common in the countries of South America, Western Asia, Mediterranean Europe, and Southern Africa. For many of these countries, fluconazole and other triazoles are the first line antifungal agents used for effective treatment of invasive Candida infection. Until recently, rates of fluconazole resistance among C. parapsilosis isolates were relatively low, therefore the determination of clinically relevant resistance mechanisms in C. parapsilosis isolates were primarily presumed from the observations of Candida albicans. Gain-of-function polymorphisms in MRR1 and TAC1 have been shown to elevate the expression of MDR1 and CDR1/CDR2 respectively and directly contribute to fluconazole resistance in Candida albicans. Our lab previously identified three resistant isolates with upregulated CpMDR1 expression that contained CpMRR1 mutations, while CpTAC1 mutations were found in three isolates with upregulated CpCDR1 expression. Deletion of CpMDR1 or CpCDR1 from strains containing the nonsynonymous CpMRR1 or CpTAC1 polymorphisms had little to no impact on fluconazole minimum inhibitory concentrations (MIC), suggesting the presence of uncharacterized resistance effectors. This dissertation reviews the emergence of resistance, presents investigations of three major mediators of fluconazole resistance, and characterizes a collection of clinical isolates to better identify and understand how fluconazole resistance in C. parapsilosis. A recently developed CRISPR-Cas9 system was used to edit CpMRR1 alleles in the clinical isolate backgrounds and allowed for characterization of the single nucleotide polymorphisms (SNPs) leading to the substitutions A854V, I283R, and R479K and gain-of-function in CpMrr1. Antifungal susceptibility testing demonstrated that gain-of-functions (GOF) in CpMrr1 increased fluconazole MIC ⩾128-fold when placed into a susceptible background while correction of CpMRR1 SNPs to the wildtype nucleotides decreased fluconazole MICs ⩾ 32-fold. Transcriptional profiling revealed the previously identified CpMDR1, the novel major facilitator superfamily (MFS) transporter, herein named CpMDR1B, and an ATP-binding cassette (ABC) transporter, herein designated CpCDR1B, were all upregulated by CpMrr1 GOF. Our development of a promotor replacement method for C. parapsilosis and implementation of a barcoded gene disruption system, demonstrated the direct contribution of CpCDR1B and CpMDR1B expression on fluconazole susceptibility and confirmed expression of CpMDR1 was not a primary driver of CpMrr1-mediated resistance. Subsequent investigation of putative GOF mutations in CpTAC1 showed correction of a SNP leading to the G650E substitution in a resistant clinical isolate decreased fluconazole MICs by 32-fold. Transcriptional profiling showed elevated expression for three ABC transporters, CpCDR1, CpCDR1B, and a gene identified here as CpCDR1C. Utilizing the overexpression and disruption systems, we demonstrated the direct effects of CpCDR1, CpCDR1B and CpCDR1C on triazole MICs. The single base editing system was also used to place the SNP leading to the Y132F substitution into the triazole drug target CpErg11 of susceptible isolates. Antifungal susceptibility testing demonstrated this frequently cited driver of resistance was insufficient in-and-of itself to elicit high-level fluconazole resistance in C. parapsilosis. Finally, next generation sequencing was used to genotypically and phenotypically characterize the entire clinical isolate collection to identify key and potentially novel markers of fluconazole resistance in C. parapsilosis. Phylogenic analysis revealed distinctive clusters of isolates with similar resistance mechanisms while implying fluconazole resistant C. parapsilosis both with and without the CpErg11 substitution, Y132F were capable of persisting in healthcare facilities. Additionally, eight isolates with clinical fluconazole resistance demonstrated distinct patterns of upregulated MFS and ABC transporters compared to the susceptible isolates while the presence of wildtype CpMRR1, CpTAC1, CpERG11, CpUPC2 and CpERG3 suggests novel mediators of fluconazole resistance within C. parapsilosis. The absence of meaningful

CpERG11 upregulation among resistant clinical isolates alongside distinct expression patterns for both of MFS and ABC transporters emphasizes the importance of looking beyond CpErg11 when investigating clinical resistance in C. parapsilosis. Understanding how resistance is regulated, develops, and even persists among clinical isolates is fundamental to the preservation of triazoles as effective treatments for invasive C. parapsilosis infection.


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UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTORAL DISSERTATION

# Investigation of Clinically Relevant Fluconazole Resistance Mechanisms in the Fungal Pathogen *Candida parapsilosis*

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*A Dissertation Presented for The Graduate Studies Council of*
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## DEDICATION

To my husband Stephen, my son Landon, and in loving memory of Grandma Angie, Grandma Lu, and Cheyenne Nicole.

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# PREFACE

The body of this dissertation is organized in a way that first introduces the research topic through a review of the literature and presents the overarching objectives for the project. Each objective is addressed in individual chapters. The first objective has been previously published and a summary is provided in Chapter 2. The second objective has also been prepared for publication and a summary is provided in Chapter 3. The summaries presented in these chapters include an introduction, a summarized discussion of results, followed by brief concluding remarks. Detailed methods and results for Chapters 2 and 3 can be found in the prepared reprints provided in **Appendices A** and **B**, respectively. The final objective, presented in Chapter 4, includes a brief introduction, materials and methods for described experiments, experimental results, and is followed by a brief discussion of findings. A concluding chapter relates all research elements back to our final thoughts on our findings and their significance to the field.

For readers to have immediate access to the full presentation of our previously published research studies, the articles are presented in the appendices. This mode of presentation allows for Chapters 2 and 3, which use them as their basis, to focus more narrowly on a summary and discussion of those articles in Appendices A and B, and to show specifically how they relate to the dissertation's larger goals. References in the chapters to relevant sections, tables, or figures in these appendices look like the following example. The Chapter 2 callout to **Figure A-1** refers to Figure 1 in **Appendix A.** The blue highlight links to the appendix figure. To return to the Chapter 3 callout page, see the PDF navigation note next.

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## ABSTRACT

Invasive candidiasis is a severe fungal infection associated with significant morbidity and mortality, particularly among the critically ill and immunocompromised. *Candida parapsilosis* is the most common non-*albicans* species causing invasive *Candida* infections in pediatric and neonatal populations worldwide and is particularly common in the countries of South America, Western Asia, Mediterranean Europe, and Southern Africa. For many of these countries, fluconazole and other triazoles are the first line antifungal agents used for effective treatment of invasive *Candida* infection. Until recently, rates of fluconazole resistance among *C. parapsilosis* isolates were relatively low, therefore the determination of clinically relevant resistance mechanisms in *C. parapsilosis* isolates were primarily presumed from the observations of *Candida albicans*.

Gain-of-function polymorphisms in *MRR1* and *TAC1* have been shown to elevate the expression of *MDR1* and *CDR1/CDR2* respectively and directly contribute to fluconazole resistance in *Candida albicans*. Our lab previously identified three resistant isolates with upregulated *CpMDR1* expression that contained *CpMRR1* mutations, while *CpTAC1* mutations were found in three isolates with upregulated *CpCDR1* expression. Deletion of *CpMDR1* or *CpCDR1* from strains containing the nonsynonymous *CpMRR1* or *CpTAC1* polymorphisms had little to no impact on fluconazole minimum inhibitory concentrations (MIC), suggesting the presence of uncharacterized resistance effectors. This dissertation reviews the emergence of resistance, presents investigations of three major mediators of fluconazole resistance, and characterizes a collection of clinical isolates to better identify and understand how fluconazole resistance in *C. parapsilosis*.

A recently developed CRISPR-Cas9 system was used to edit *CpMRR1* alleles in the clinical isolate backgrounds and allowed for characterization of the single nucleotide polymorphisms (SNPs) leading to the substitutions A854V, I283R, and R479K and gain-of-function in CpMrr1. Antifungal susceptibility testing demonstrated that gain-of-functions (GOF) in CpMrr1 increased fluconazole MIC ≥128-fold when placed into a susceptible background while correction of *CpMRR1* SNPs to the wildtype nucleotides decreased fluconazole MICs ≥ 32-fold. Transcriptional profiling revealed the previously identified *CpMDR1*, the novel major facilitator superfamily (MFS) transporter, herein named *CpMDR1B*, and an ATP-binding cassette (ABC) transporter, herein designated *CpCDR1B,* were all upregulated by CpMrr1 GOF. Our development of a promotor replacement method for *C. parapsilosis* and implementation of a barcoded gene disruption system, demonstrated the direct contribution of *CpCDR1B* and *CpMDR1B* expression on fluconazole susceptibility and confirmed expression of *CpMDR1* was not a primary driver of CpMrr1-mediated resistance.

Subsequent investigation of putative GOF mutations in *CpTAC1* showed correction of a SNP leading to the G650E substitution in a resistant clinical isolate decreased fluconazole MICs by 32-fold. Transcriptional profiling showed elevated expression for three ABC transporters, *CpCDR1*, *CpCDR1B*, and a gene identified here

as *CpCDR1C*. Utilizing the overexpression and disruption systems, we demonstrated the direct effects of *CpCDR1*, *CpCDR1B* and *CpCDR1C* on triazole MICs. The single base editing system was also used to place the SNP leading to the Y132F substitution into the triazole drug target CpErg11 of susceptible isolates. Antifungal susceptibility testing demonstrated this frequently cited driver of resistance was insufficient in-and-of itself to elicit high-level fluconazole resistance in *C. parapsilosis*.

Finally, next generation sequencing was used to genotypically and phenotypically characterize the entire clinical isolate collection to identify key and potentially novel markers of fluconazole resistance in *C. parapsilosis.* Phylogenic analysis revealed distinctive clusters of isolates with similar resistance mechanisms while implying fluconazole resistant *C. parapsilosis* both with and without the CpErg11 substitution, Y132F were capable of persisting in healthcare facilities. Additionally, eight isolates with clinical fluconazole resistance demonstrated distinct patterns of upregulated MFS and ABC transporters compared to the susceptible isolates while the presence of wildtype *CpMRR1*, *CpTAC1*, *CpERG11*, *CpUPC2* and *CpERG3* suggests novel mediators of fluconazole resistance within *C. parapsilosis*. The absence of meaningful *CpERG11* upregulation among resistant clinical isolates alongside distinct expression patterns for both of MFS and ABC transporters emphasizes the importance of looking beyond CpErg11 when investigating clinical resistance in *C. parapsilosis*.

Understanding how resistance is regulated, develops, and even persists among clinical isolates is fundamental to the preservation of triazoles as effective treatments for invasive *C. parapsilosis* infection.

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| | |
|---|---|
| ABC | ATP-binding cassette |
| AmB | Amphotericin B |
| ATCC | American type culture collection |
| ATP | Adenosine triphosphate |
| Ca | *Candida albicans* |
| CDC | Centers for Disease Control |
| CDR | *Candida* drug resistance |
| CLSI | Clinical Laboratory and Standards Institute |
| Cp | *Candida parapsilosis* |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CYP | Cytochrome P450 enyme |
| DHA1 | Drug:hydrogen ($H^+$) antiporter family 1 |
| ETEST | Epsilometer test for strip diffusion antifungal susceptibility testing |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| FLU | Fluconazole |
| FDR | False discovery rate |
| GOF | Gain-of-function |
| ICI | Invasive *Candida* infection |
| ICU | Intensive care unit |
| ISAVU | Isavuconazole |
| ITRA | Itraconazole |
| MDR | Multi-drug resistance |
| MFS | Major facilitator superfamily |
| MIC | Minimum inhibitory concentration |
| MRP | Multidrug resistance associated protein |
| MRR | Multidrug resistance regulator |
| MTL | Mating type locus |
| NAC | Non-*albicans Candida* species |
| NICU | Neonatal intensive care unit |
| PCR | Polymerase chain reaction |
| PDR | Pleiotropic drug resistance |
| PICU | Pediatric intensive care unit |
| POSA | posaconazole |
| RNP | Ribo-Nucleo-protein |
| RPMI | Roswell Park Memorial Institute |
| RQ | Relative quantitation |
| spp. | species |
| TAC | Transcriptional activator of CDR transporter genes |
| UPGMA | Unweighted pair-group method with arithmetic mean |
| VORI | Voriconazole |
| WHO | World Health Organization |
| ZCF | Zinc Cluster transcription Factor |

# CHAPTER 1. INTRODUCTION

**NOTE:** When using Adobe Acrobat, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See **Preface** for further details.

## Invasive Candidiasis and Candidemia

Candidiasis is a fungal infection caused by *Candida* yeast species. The severity of candidiasis infection ranges widely depending on the site of infection, age, and immunocompetency of the host. Even though they are are not associated with particularly high mortality, non-invasive *Candida* infections of the skin and mucosa, including thrush and vulvovaginal candidiasis, were still responsible for over 3.6 million outpatient visits in the United States in 2017 (1). Invasive *Candida* infections are life-threatening infections causing significant morbidity and mortality worldwide. Associated with an all-cause mortality rate of up to 60%, there are an estimated 700,000 invasive *Candida* infections each year (2-5). Conservative estimates for the United States placed the total economic burden for invasive *Candida* infections at $1.8 billion for 2019, an estimated $1.2 billion of which was due to the direct medical cost associated with prolonged hospitalizations (6).

Bloodstream *Candida* infections, or candidemia, are the second most common healthcare associated bloodstream infection in the United States with higher incidence reported for the very young and the elderly (7). Crude mortality rates for candidemia in the United States are around 30%, similar to those for all invasive *Candida* infections (8). In terms of mortality, candidemia presents a substantial threat to the critically ill. A 2020 German analysis found a mortality rate of 60% among critically ill patients in the three months following a positive candidemia culture (9). While another study from Germany, found even with implementation of echinocandin antifungal therapy, candidemia attributable mortality rates remain above 25% (10).

Most *Candida* infections in humans are caused by 5 *Candida* spp., *Candida albicans*, *Candida glabrata* (*Nakaseomyces glabratus*), *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (*Pichia kudriavzevii*), however the recent emergence of multi-drug resistant *Candida auris* in health-care facilities worldwide lends to its addition as a *Candida* species of significant concern. While *Candida albicans* is still the predominate causative agent worldwide, the proportion of cases caused by non-*albicans* *Candida* (NAC) species is on the rise (11).

This dissertation focuses on investigating and delineating mechanisms of antifungal resistance in the NAC species, *Candida parapsilosis*. The aim for the remainder of this chapter is to introduce *C. parapsilosis* as a clinically important pathogen, detail the current antifungals available for treatment of *Candida* infections, summarize antifungal resistance mechanisms previously identified among *Candida*

species, and provide an in-depth literature review of the current trends of fluconazole resistance among *C. parapsilosis* isolates worldwide.

## The Fungal Pathogen *Candida parapsilosis*

### *C. parapsilosis* Discovery to Genomic Sequencing

First isolated in Puerto Rico in 1928, *Candida parapsilosis* was originally noted as a distinct species *Monilia parapsilosis* from *Monilia psilosis* (*Candida albicans*), due to its inability to ferment maltose (12). *Candida parapsilosis* has been isolated as the causative agent in both human and veterinary infections and has been isolated from both soil and marine environments (13). *C. parapsilosis* presents as both yeast and pseudohyphal morphologies and as four heritable phenotypic colony types, smooth, concentric, crater, and crepe (14). Smooth colonies, consisting of yeast, grow at accelerated rates compared to other morphologies and phenotypes (14). Concentric and crepe phenotypes consist almost exclusively of pseudohyphae and are most commonly associated with increased biofilm production and increased agar invasion (15, 16). *C. parapsilosis* biofilm formation can occur on a variety of implanted biomedical devices and has been linked to healthcare associated outbreaks. Biofilms also represent an important virulence factor in *C. parapsilosis* infection and have been linked to enhanced antifungal resistance, increased tissue dissemination, and heightened mortality (17, 18).

Prior to 2005, *C. parapsilosis* was comprised of three major groups, based on isoenzymatic profiling. Group I was the most predominant among clinical *Candida* isolates, the most virulent, and included the type strain ATCC22019, also known as CLIB214 (19, 20). An attempt to define multilocus sequence typing for *C. parapsilosis* revealed fixed DNA sequence differences within each group, thus groups I, II, and III were separated into *Candia parapsilosis sensu stricto, Candida orthopsilosis* and *Candida metapsilosis*, respectively, however they are still commonly referred to as species within the *C. parapsilosis* complex (21, 22). Some surveillance studies and publications report isolates of the *C. parapsilosis* complex as simply '*C. parapsilosis*' without designation between members of the complex. For the purpose of clarification, *C. parapsilosis* as used in this body of work refers to *C. parapsilosis sensu stricto*. Publication of the genome for the *C. parapsilosis* clinical isolate strain CDC317 (23) in 2009 revealed a remarkable lack of heterozygosity for a diploid *Candida* species (24). Additional genomic distinctions between the species complex were identified following the genomic sequencing of multiple *C. parapsilosis, C. orthopsilosis*, and *C. metapsilosis*, clinical and environmental isolates. These studies confirmed the presence of early stop codons in MTLa1, the absence of a compatible alpha Mating Type Locus (MTL$\alpha$) in *C. parapsilosis* isolates, and corroborated the lack of observed mating and meiosis observed within *C. parapsilosis* (24-27).

Genotyping is an important tool in the investigation of nosocomial candidemia outbreaks (28-30). *C. parapsilosis* collected from the environment and even from the

hands of health care workers display increased genotypic heterogeneity (31, 32) however, *C. parapsilosis* clinical isolates with shared genotypes have been identified in multiple countries (33-35). Comparisons of clinical and environmental *C. parapsilosis* genotypes have also identified clustering patterns among isolates from the same medical facility, (36, 37), among those causing bloodstream infection (33), and among fluconazole resistant isolates (38).

### *C. parapsilosis* Candidemia

Due to several challenges associated with the data collection between various countries, calculation of global candidemia trends can be difficult. Only a select number of countries currently implement rigorous fungal surveillance programs, therefore incorporation of smaller case studies is sometimes necessary (39). Variation in regional or institutional reporting standards can lead to the lack of common criteria required to make proper statistical comparisons. Candidemia incidence and resulting medical outcomes are disproportionately affected by patient age and underlying health conditions, making it difficult to generalize smaller studies across entire populations (40). Overall mortality associated with *C. parapsilosis* etiologic candidemia is lower than that for other *Candida* species (41-43). However, candidemia by *C. parapsilosis* has been associated with decreased survivability among solid organ transplant recipients (44-46). For developing countries where species such as *Candida tropicalis* and *Candida parapsilosis* are common, crude mortality rates for candidemia remain well above 50% (3).

Incidence of *C. parapsilosis* candidemia varies considerably by both patient population and geographical region. *Candida parapsilosis* represents the most common non-*albicans* species causing candidemia in South America, Africa, Southern Europe, and parts of Western Asia and is second to either *C. glabrata* or *C. tropicalis* in North America, Europe, East Asia, and Northern Africa (**Figure 1-1**) (8, 47). *Candida parapsilosis* is the most common non-*albicans* agent causing candidemia in neonatal and pediatric populations (48). Associated risk factors for *C. parapsilosis* candidemia include immunosuppression, prior antibiotic therapy, recent chemotherapy, pre-term gestation for neonates, central venous catheter placement, organ transplant, prolonged hospitalizations, and hospitalization in pediatric and neonatal intensive care units (NICUs, PICUs) (11, 49-52). Additional surveys have attributed increased proliferation within high glucose-containing solutions used for parenteral nutrition, horizontal transmission from the hands of healthcare workers, and increased adherence to synthetic biomedical materials as key determinants of *C. parapsilosis* infection and transmission in healthcare settings (23, 53-57).

## Antifungal Armament for Invasive Candidiasis

Currently, there are three major classes of pharmaceuticals approved for the treatment of invasive *Candida* infection and candidemia, polyenes, azoles, and echinocandins. A fourth class of antifungal drug, 5-flucytosine, can also be given for

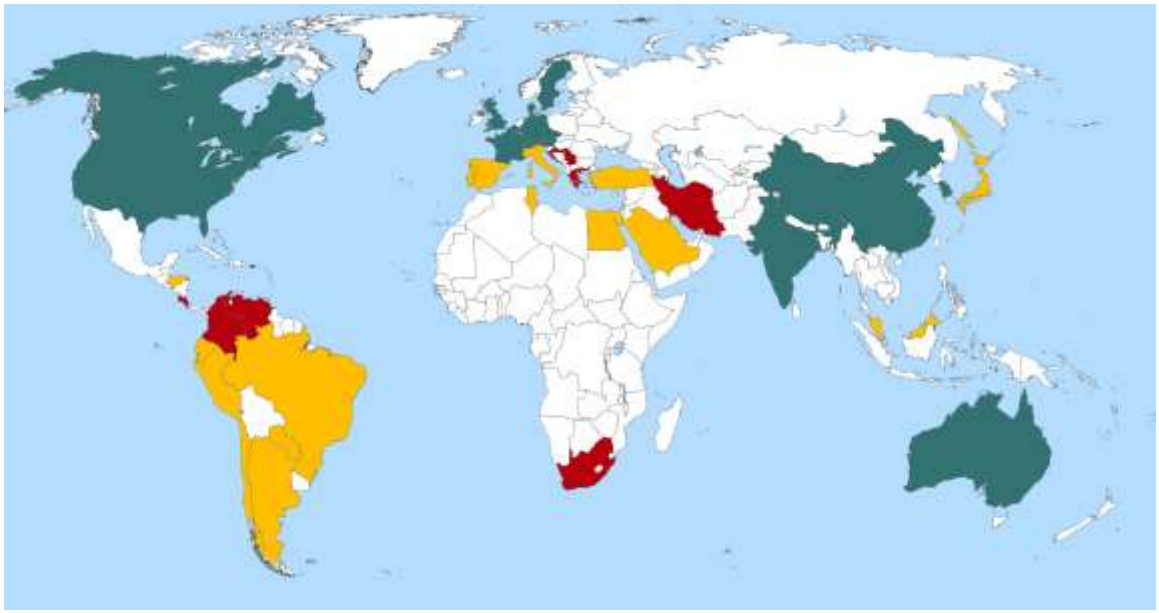

**Figure 1-1. Worldwide *Candida parapsilosis* prevalence in invasive candidiasis.**
Note: Red: represents countries where *C. parapsilosis* is the most common *Candida* species overall based on multiple regional reports or nationwide surveillance; Yellow: represents countries where *C. parapsilosis* is the most common NAC species based on multiple regional reports or nationwide surveillance; Teal: represents countries where *C. parapsilosis* is the second most common NAC species based on multiple regional reports or nationwide surveillance.

select treatment of refractory *Candida* infections however it is recommended that this pyrimidine analog be given in combination with other antifungals such as amphotericin B or triazoles due to rapid resistance development when used as monotherapy (58). Polyene and azole antifungals target the presence and biosynthesis of the fungal specific membrane sterol, ergosterol. Ergosterol is critical in the maintenance, stability, permeability, and organization of fungal cell membrane, thus its presence and biosynthesis are prime targets in the development of antifungal drugs (59). The newest antifungal class, echinocandins, target the synthesis of β-1,3-glucan, a key component of the fungal wall. The complex glucan-rich fungal cell wall protects against environmental stressors and is involved in many aspects of *Candida* pathogenicity.

**Polyenes**

Currently, amphotericin B, in various formulations, is the only polyene antifungal used in the treatment of invasive *Candida* infection. Amphotericin B (AmB) is a fungicidal drug capable of forming structures both atop and within the fungal cell membrane. The primary fungicidal activity of AmB depends on its binding to the surface of the cell (60) and the subsequent formation homodimer aggregates (61). These extra-membranous aggregates act as sterol sponges, extracting the vital ergosterol from the lipid bilayer (62). The physical removal of ergosterol from the lipid bilayer rapidly results in membrane depolarization, cell component leakage, and cell lysis (60). The complimentary formation of ergosterol-containing ion channels within lipid bilayer increases the rate of fungal cell death (60). A recent study performed on synthetic lipid bilayer models demonstrated that AmB heptamers and ergosterol would form relatively stable ion channels, however it has not been confirmed if these structures are present in real fungal cell membranes under biological conditions (63). Although these functions allow for potent fungicidal activity, the use of AmB is limited due to associated renal toxicity and its poor bioavailability in oral formulations (58, 64).

**Azoles**

The azole antifungals are fungistatic drugs composed of two categories; the imidazoles, which contain two nitrogen atoms in the heterocyclic ring, and the triazoles, which contain three nitrogen atoms in the heterocyclic ring. Imidazoles are associated with significant toxicity during systemic administration and are mainly used in the treatment of topical fungal infection while the triazoles are key therapeutics used in both the prophylaxis and treatment of superficial and invasive *Candida* infections (64).

Triazole antifungals are fungistatic drugs that target the, Cyp51 enzyme, 14α-lanosterol demethylase, encoded by *ERG11* in *C. albicans*. The removal of the 14α-methyl group from lanosterol occurs in a three-step reaction and represents a key rate limiting step in the ergosterol biosynthesis pathway (65). While there is a strong preference for the natural substrate, lanosterol, azoles competitively bind the heme iron within the Erg11 active site (66). Triazoles that feature long side chains, such as

posaconazole and itraconazole, also block the substrate channel entrance allowing for more potent inhibition (66). Inhibition of Erg11 activity leads to ergosterol depletion, a buildup of ergosterol precursors, and the subsequent accumulation of the toxic sterol, 14α-methylergosta-8,24(28)-dien-3β, 6α-diol (14α-methyl-3,6-diol) (67-69). Depletion of ergosterol in the cell membrane arrests normal cell cycle progression and slows cell growth (70), while the increased production of the toxic 14α-methyl-3,6-diol by C-5 sterol desaturase, encoded by *ERG3* in *C. albicans*, leads to defects in membrane plasticity, membrane heterogeneity osmotic regulation, and lipid raft formation (69, 71-73).

Fluconazole and voriconazole demonstrate high therapeutic selectivity for the *Candida* Cyp51 enzyme over human Cyp51 (74) and represent the most commonly used azoles in the treatment of invasive candidiasis, while itraconazole, isavuconazole, and posaconazole are usually reserved for select instances (58). Triazoles, and fluconazole specifically, are available in oral and parental formulations and are generally less expensive than the echinocandins. Consequently, fluconazole is one of the most common therapeutics used for prophylaxis and treatment of systemic and superficial *Candida* infection worldwide (58, 75, 76).

**Echinocandins**

Fungal cell walls are mainly made up of chitin, glycoprotein, and glucan moieties. In *Candida*, an inner cell wall, composed primarily of chitin and β-1,3-glucans, surrounds the cell membrane and attaches the various mannans and proteins of the outer cell wall through variable length β-1,6-glucans (77). The inner cell wall is responsible for providing structure and strength to the *Candida* cell while the more fluid outer wall interacts with the environment and works to mask the immune responsive β-1,3-glucans from the host immune system (78). The biosynthesis of the structurally important β-1,3-glucans are catalyzed by β-(1,3)-D-glucan synthase. Echinocandins non-competitively bind to the catalytic site of the Fks1p subunit, encoded by FKS1 in *C. albicans*, halting production of β-1,3-glucans (79) which results in inhibition of cell growth, loss of cell wall integrity, and culminates in cell death via dysregulated osmotic pressure (80, 81).

Studies examining echinocandin killing activity and post-antifungal effects have demonstrated concentration dependent fungicidal activity for these drugs against multiple *Candida* species (81, 82). Three echinocandins, caspofungin, micafungin, and anidulafungin, are currently approved in the United States for treatment of invasive *Candida* infections. While caspofungin, micafungin and anidulafungin all share a similar, large lipopeptide structure, they differ in the composition of the acyl lipid side chains involved in anchoring the antifungal to the cell membrane (83).

In the United States, echinocandins are the first line antifungal recommended for invasive candidiasis while amphotericin B and triazoles antifungals are considered second lines of defense (58). Recent meta-analysis studies have found echinocandins to be associated with improved rates of candidiasis treatment success (84) and when used

initially, echinocandins were associated with reduced mortality when the causative *Candida* species was not considered (85). Major caveats to this recommendation include *Candida* infections involving the central nervous system, intraocular *Candida* infection, and infections in pre-term and full-term neonates ≤ 2 months (86, 87). Echinocandin usage is also limited by its intravenous-only preparation due to low bioavailability in oral formulations (58).

## Antifungal Resistance

Antifungal fungal susceptibility testing provides an in-vitro measurement of resistance to help predict patient outcomes associated with a preferred therapy (88, 89). In general, antimicrobial resistance is the ability of a pathogen to defeat a drug that is normally capable of killing or inhibiting growth that organism. A resistant *Candida* strain, as we denote here, is a strain with a minimum inhibitory concentration, as measured by standardized antifungal susceptibility testing, greater than that of the clinical breakpoint as set by either the Clinical and Laboratory Standards Institute (CLSI) (88, 90) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (91). The clinical breakpoint for resistance to an antifungal drug is the threshold for which treatment failure is more likely than treatment success (89). Mechanisms by which *Candida* have adapted resistance to antifungals have been identified for multiple species however the most well studied mechanisms are those in *C. albicans*. Antifungal resistance within *Candida* species is generally achieved through alterations to the drug target, increased expression of the drug target, alternative sterol synthesis, increased drug efflux, alterations in the cell stress response, or a combination thereof (92).

### Amphotericin B Resistance

Resistance to amphotericin B is exceedingly uncommon, 97%-99.9% of *Candida* spp. clinical isolates have MICs at or below the epidemiological cutoff value (2, 93). However the emergence of multi-drug resistant *Candida auris*, 30% of which display MICs greater than 2 μg/mL, is a cause for concern (94). For non-*auris Candida* species, the low levels of resistance have primarily been attributed to the high fitness cost associated with these mutations (95). *C. albicans* isolates with defective C-5 sterol desaturase due to deleterious mutations in *ERG3* have exhibited decreased susceptibility to both amphotericin B and the triazole antifungals (96, 97). *C. albicans* strains with defective C-5 sterol desaturase lack detectable levels of ergosterol, and membrane composition analysis shows increased proportions of ergosta 7,22-dienol, episterol, and ergosta 7-enol (98). The absence of ergosterol in the membrane leads to increased resistance to amphotericin B treatment while the absence of the toxic 14α-methyl-3,6-diol produced by Erg3 lends to reduced triazole susceptibility (92).

*Candida albicans* isolates with mutations in *ERG3* have also been shown to have diminished virulence primarily due to defective hyphal formation (98-101). Similarly, mutations in the sterol-methyltransferase gene, *ERG6*, abrogates normal ergosterol

biosynthesis, altering membrane sterol composition and resulting in increased amphotericin B resistance for *C. auris* (102). Mutations in the *ERG6* and *ERG2* genes also confer amphotericin B resistance in *C. glabrata* although to a lesser degree than was observed for *C. auris* (102, 103).

**Echinocandin Resistance**

Resistance to the echinocandin antifungals currently on the market remains low among *Candida albicans* and *Candida parapsilosis* clinical isolates (104-106). Echinocandins are not substrates for the drug transporters in *Candida albicans* as elevated expression does not significantly change susceptibility (107) and tolerance to echinocandins has been linked to the cell wall remodeling and repair mechanisms (108). The only mechanism of echinocandin resistance associated with therapeutic failure involves mutations in the one of the hot spot regions of β-(1,3)-D-glucan synthase, encoded by *FKS1* in *C. albicans* (108, 109). There are two major hot spot regions in *C. albicans FKS1* (110) and topological studies of *S. cerevisiae FKS1* demonstrated the externality of these regions for echinocandin binding (111). Mutations in *FKS1* alter the ability of echinocandins to bind to Fks1 within the cell membrane (110) and hinder the overall catalytic capacity for the Fks1 protein (112, 113).

Historically, there has been hesitation in implementing echinocandin therapy for *C. parapsilosis* etiologic infections. Breakthrough infection caused by *C. parapsilosis* has been associated with prior micafungin use (114, 115) while increased use of caspofungin and echinocandin prophylaxis have been associated with increased incidence in *C. parapsilosis* candidemia (44, 116). Additionally, echinocandins have been found to be less effective against *C. parapsilosis* biofilms (117) and *C. parapsilosis* isolates generally exhibit reduced susceptibility all to three echinocandins in-vitro (114, 118, 119). Although higher doses of echinocandins may be required for the treatment of *C. parapsilosis* infection (120), the use of echinocandin therapy over fluconazole or amphotericin B has not been correlated with significant differences in *C. parapsilosis* candidemia mortality rates (121-124). Reduced echinocandin susceptibility in *C. parapsilosis* has been attributed to the presence of the naturally occurring A660P within the hot-spot 1 region of CpFks1 (125).

Recently, a novel *CpFKS1* mutation leading a R658G substitution has been associated with in-vitro micafungin resistance and in-vivo caspofungin therapeutic failure among *C. parapsilosis* isolates (126). Additionally, the F652S and S656P substitutions have been identified in a pan-echinocandin resistant *C. parapsilosis* strain isolated from patients receiving prolonged echinocandin therapy (127, 128). The introduction of the mutation leading to CpFks1$^{S656P}$ into a susceptible isolate of *C. parapsilosis* demonstrated a 64-fold increase in resistance to all echinocandins (128).

## Triazole Resistance

### Modification of *ERG11*

Resistance to triazoles among *Candida* species is usually the result of multiple mechanisms working in concert. In *Candida albicans*, multiple mutations in *ERG11* have been associated with increased resistance to fluconazole however these mutations demonstrated variable effects on voriconazole and itraconazole susceptibility (129). Moreover, the expression of select mutant *C. albicans ERG11* alleles (G129A, S405F, Y132H, G464S, R467K and combinations thereof) in the model organism *S. cerevisiae* showed fluconazole MICs to be the most affected by these substitutions in Erg11 followed by voriconazole, isavuconazole, and itraconazole, respectively (130). However, for all Erg11 substitutions tested, posaconazole MIC remained unchanged (130).

Mutations leading to amino acid substitutions in Erg11 tend to cluster into 3 hot spot regions, 105 to 165, 266 to 287, and 405 to 488, however not all mutations in these regions affect triazole resistance (131). Substitutions such as Y132F/H and N136Y (132) are predicted to interfere with the hydrogen bonding between the triazoles and the heme ring within the active site. The K143R substitution potentially interferes with the conformation of the heme bulge (133). Additionally, the R467K and I471T mutations potentially interfere with the environment and binding involved with the K143 side chain in the active site while substitutions Y118A, T123I, F126S, G307S and S405F potentially interfere with overall active-site cavity volume (66, 133, 134). Non-active site mutations such as A61V and F380S are located in the entry and putative exit channels whereas substitutions Y447H, G448E, G448V, G450E, and V456I are predicted to be located on external loops, potentially interfering with the interaction between NADPH-cytochrome P450 reductase and 14α-lanosterol demethylase (66, 133). Among *C. parapsilosis* clinical isolates, mutations leading to theY132F and K143R substitutions appear to be predominant mutations, with MICs ranging from 2μg/mL to 128μg/mL (104, 135).

### Increased *ERG11* expression

Increased expression of *ERG11*, through either gene amplification or by modified transcriptional regulation, has been correlated with increased triazole resistance (136, 137). In *C. albicans*, the expression of ergosterol biosynthesis pathway genes, including triazole drug target Erg11, is regulated by the zinc cluster transcription factor Upc2 (138-140). Multiple gain-of-function *UPC2* mutations have been identified as causing increased expression of *ERG11* and triazole resistance in *C. albicans* clinical isolates (139, 141, 142). The disruption of *CpUPC2* in a posaconazole evolved *C. parapsilosis* strain abrogated all triazole resistance, however no GOF mutations in *CpUPC2* have been directly correlated with fluconazole resistance among clinical isolates (143).

### Alternative sterol biosynthesis

Other members of the ergosterol biosynthesis pathway can also affect fluconazole susceptibility. Upon fluconazole treatment, Erg3 produces an accumulation of a toxic 14-methyl-3,6-diol (69, 97). Deleterious mutations in *ERG3* prevent this accumulation and have been shown to decrease fluconazole susceptibility in *C. albicans* (98, 100, 144). Mutations leading to D14Y and R135I amino acid substitutions in CpErg3 have been identified in posaconazole evolved *C. parapsilosis* strains (143, 145) which also displayed increased expression of *CpUPC2* and other ergosterol biosynthesis genes, decreased susceptibility to all triazoles, and, interestingly, slightly higher echinocandin MICs. However, the posaconazole evolved strain also displayed a less virulent phenotype in a mouse model of invasive candidiasis (145). Accordingly, to date only one instance of *CpERG3* mutation has been identified among azole resistant *C. parapsilosis* clinical isolates (146).

### Drug efflux

***ATP-binding cassette (ABC) transporters.*** Multiple investigations into the genomic inventory of ABC proteins transporters have been performed in *S. cerevisiae* and *C. albicans*. A total of 22 ABC transporter proteins have been identified in *S. cerevisiae* (147) while 26 ABC transporters have been identified in *C. albicans* (148, 149). Assessments of the ABC transporter groups, ABCG and ABCC which are the two groups primarily associated with xenobiotic resistance, have identified eight ABCG transporters and six ABCC transporters encoded within the *C. albican*s genome. The sequestering of fluconazole in the vacuole by the ABCC transporter *MLT1* has been shown to affect fluconazole susceptibility in *C. albicans* (150). However, only the ABCG transporters *CDR1* and *CDR2* (*Candida* drug resistance) have been shown to mediate fluconazole resistance in clinical *C. albicans* isolates through the physical removal of drug from the cell cytoplasm (151).

Constitutive expression of *C. albicans* ABC transporters *CDR1* and *CDR2* have been shown to increase resistance to all triazoles with *CDR1* reporting higher MIC fold changes than *CDR2* (130, 152). Conversely, deletion of *CDR1* from *C. albicans* strains with *CDR1* and *CDR2* overexpression causes significant drops in triazole MICs, while the deletion of *CDR2* causes weaker drops in MICs for the same triazole (153, 154). The deletion of *CDR1* and *CDR2* from already susceptible *C. albicans* strains results in the creation of a hypersusceptible phenotype (130).

Induced expression of *CDR1* and *CDR2* in response to azole stress in *C. albicans* is regulated by the zinc cluster transcription factor, Tac1 (155, 156). DNA binding assays confirmed the constitutive binding of Tac1 to the cis-acting drug response elements (DREs) present in the *CDR1* and *CDR2* promoters (157). Activated Tac1 facilitates the recruitment of the mediator coactivation complex (Med3 and Med15) to the promoter of *CDR1*, thus inducing expression (158). Resistance phenotypes driven by gain-of-function mutations in *TAC1* involves the recruitment and interaction of the transcriptional activation domain of Tac1 with the tail module of the mediator complex (158).

***Major facilitator superfamily (MFS) transporters.*** The major facilitator superfamily represents a large group of membrane carriers involved in the passage of everything from ions to nutrients to large complex biomolecules across the plasma membrane. However, only a fraction of MFS transporters are members of the Drug/H+ Antiporter 1 (DHA1) family of transporters and capable of multidrug efflux. Only one DHA1 transporters, *MDR1*, has been shown to affect fluconazole resistance in clinical *Candida albicans* isolates (157). Phylogenetic studies of MFS transporters have shown the *C. parapsilosis* reference genome encodes 140 MFS transporters, 47% more than the *C. albicans* genome and 18% more than the *S. cerevisiae* genome, which only encode 95 and 119, respectively (159, 160).

Eight of the 28 full length DHA1 proteins identified in *C. parapsilosis* are genes homologous to *C. albicans FLU1* and *TPO1* in *S. cerevisiae* (161). Investigations of the *C. albicans* Flu1 transporter found that expression within the *S. cerevisiae* model increased resistance to fluconazole and cycloheximide however deletion from the native *C. albicans* resulted in a minimal effect on fluconazole susceptibility (162). Additionally, the efflux pump proteins encoded by *QDR2*, *AQR1* and *TPO3* have been shown to contribute to azole drug resistance in *C. glabrata*, however *MDR1* is the only DHA1 transporter specifically related to fluconazole resistance within this species (163).

Deletion of the major facilitator superfamily (MFS) transporter *MDR1* (Multi-drug resistance) from two *MDR1* overexpressing isolates resulted in increased susceptibility to fluconazole and other chemicals (164). Constitutive expression of *C. albicans MDR1* in the model organism *S. cerevisiae* conferred moderate resistance to fluconazole and voriconazole along with several non-azole drugs including benomyl, brefeldin-A and methotrexate (165). No effects were observed for itraconazole, isavuconazole or posaconazole MICs upon *MDR1* expression (130). Deletion of *MDR1* from a susceptible *C. albicans* strain failed to yield a hypersusceptible phenotype (153) and overexpression of *MDR1* by the *ADH1* promoter had little to no effect on fluconazole MIC, indicating fluconazole resistance is dependent on the degree of *MDR1* expression (166).

Transcriptional profiling and *in vivo* ChIP-chip experiments have confirmed the *C. albicans* zinc cluster transcription factor Mrr1 can induce high level *MDR1* expression when constitutively activated by the P683S substitution (167, 168). Numerous additional *MRR1* gain-of-function mutations have been identified in clinical isolates since its role in fluconazole resistance was discovered in 2007 (92). Interestingly, while deletion of the *MDR1* gene from a clinical isolate containing an Mrr1 gain-of-function mutation reversed resistance for some of Mdr1 substrates such as brefeldin-A, it also resulted in a higher fluconazole MIC than the deletion of Mrr1 in the same background (167). This implies additional fluconazole resistance elements beyond Mdr1 are regulated by Mrr1b(167).

**Zinc-cluster transcription factors**

Zinc cluster transcription factors are in a fungal-specific group of zinc-finger proteins that bind DNA utilizing a conserved $CysX_2CysX_6CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$

motif (169). These transcription factors are involved in regulating a large variety of biological processes and multiple ZCFs, namely Upc2, Tac1, and Mrr1, have been shown to regulate genes mediating antifungal resistance. Each ZCF consists of three main functional domains, a regulatory middle homology domain, an acidic activation domain and the DNA binding domain (169). The DNA binding domain can be subdivided into three regions, the binuclear zinc finger, a linker region, and the dimerization domain which allows for DNA interaction in monomer, homodimer or heterodimer formations (169).

DNA binding studies and transcriptional analysis from *S. cerevisiae* and *C. albicans* have shown the role ZCFs play in the transcription of other ZCFs (170), with some ZCFs demonstrating an autoregulatory function which, upon activation, form positive-feedback loops (169, 171). As such, some activating mutations in the transcription factors Mrr1 and Upc2 have been shown to not only increase expression of direct fluconazole resistance effectors, but to also increase their own expression (167, 172). While activating mutations have not been identified in clinical isolates, the artificial activation of additional ZCF genes such as *ZNC1*, *STB5*, *CTA4*, *ARO80*, *ZCF35*, *MRR2*, and *ZCF25* demonstrate increased fluconazole resistance in-vitro (171).

### Genomic rearrangement

Chromosomal aneuploidy due to environmental stress can lead to antifungal resistance and antifungal tolerance (173, 174). Antifungal exposure has been shown to drive rapid and reversible amplification of the genes located near distinct long inverted repeats (175). These recurrent variations in copy number appear to most frequently involve the amplification of *CDR1*, *CDR2*, and *MRR1* in *C. albicans* (175). The development of sustainable triazole resistance in *Candida albicans* has also been linked to the appearance of a heterozygous mutation in *MRR1* or *TAC1*, followed by a loss of heterozygosity event (142, 176).

## Emerging Trend of Azole Resistance in *C. parapsilosis*

The development of triazole resistance in *Candida* spp. is an ongoing and sizable concern (94), and the World Health Organization (WHO) identifies *Candida parapsilosis* as a high priority fungal pathogen (177). Fluconazole is the recommended alternative to the first line antifungals, echinocandins, and in some medical situations, the first line antifungal therapy against invasive candidiasis (58). Fluconazole is also the primary antifungal treatment for invasive candidiasis in many developing countries due to its comparable safety and efficacy profiles and the prohibitive cost of echinocandin therapy (3, 40, 75, 178). Worryingly, when compared to fluconazole-susceptible *C. parapsilosis*, fluconazole-resistant *C. parapsilosis* candidemia is associated with worse medical outcomes, increased treatment failure, and increased mortality (76, 179, 180). *C. parapsilosis* is capable of persisting within a hospital environment for years without detection or source identification (181, 182) and recently, persistent fluconazole resistant *C. parapsilosis* strains have also been observed (76, 179, 183). Similar to the estimations

for large-scale incidence of *C. parapsilosis* candidemia and associated mortality, it can be difficult to make large-scale estimates on the prevalence of fluconazole resistant *C. parapsilosis*. Localized outbreaks of *C. parapsilosis* candidemia within single healthcare facilities have reported fluconazole resistance rates of > 80% (184) within countries where fluconazole resistance is normally found in just 17% of *C. parapsilosis* isolates (185). Additionally, localized outbreaks of clonal fluconazole resistant *C. parapsilosis* candidemia have been reported in many countries worldwide, including Mexico(186), Brazil (180), Spain (187, 188), Italy (189), France (179), Turkey (76), Kuwait (182), South Africa (38), India (184), China (28, 190) and Korea (191).

**Africa**

A recent literature review by Okoye et al. found *C. parapsilosis* accounted for 30.4% of reported invasive candidiasis cases in Africa, however reports containing cases of invasive candidiasis were only available for 21 of the 54 African countries (75). With just 5 countries reporting for all of western and central Africa, a true epidemiological picture of candidiasis and antifungal resistance for the entire continent remains largely unknown. Of the 21 reporting countries, South Africa reported the highest number of cases, in part due to increased surveillance of fungal diseases nationwide. Laboratory surveillance of candidemia from 2009-2010 by Govender et al. identified a stratification of *C. parapsilosis* candidemia between private and public sector hospitals within South Africa. *C. parapsilosis* was responsible for 35% of cases from public hospitals but 55% of candidemia cases in private sector hospitals (192). Additionally, 60% of the isolates from private hospitals were fluconazole resistant compared to just 44% of the isolates from public hospitals (192). Thirty-six percent of isolates collected from cases of neonatal candidemia in South Africa between 2009-2010 were identified as *C. parapsilosis*, of which, 54% were fluconazole resistant. Further genotypic analysis of these isolates indicated NICU outbreaks of *C. parapsilosis* candidemia and intra-hospital transmission of closely related genotypes (38). Nationwide laboratory-based surveillance of pediatric and neonatal candidemia in South Africa from 2012-2017 found *C. parapsilosis* accounted for 42% of isolates, with 55% resistant to fluconazole (193). These findings are consistent with the most recent retrospective analysis from a single hospital in Johannesburg, South Africa by Chibabhai et al. in which *C. parapsilosis* was isolated from 31% of all candidemia cases and was overrepresented in candidemia reported from neonatal and pediatric departments, representing 51% and 36% of the clinical isolates, respectively (194).

**Asia**

A 2015 surveillance of candidemia isolates from 6 mainly eastern Asian countries found only 12.1% were *C. parapsilosis*, (195) however, the incidence of *C. parapsilosis* fungemia varies widely across the Asian continent as does the occurrence of fluconazole resistant *C. parapsilosis*. Reports from countries in eastern Asia show varying incidence of *C. parapsilosis* candidemia and tend to report fewer fluconazole-resistant strains

among clinical isolates. In contrast, areas of Western Asia including Turkey and Kuwait, report a preponderance of *C. parapsilosis* candidemia compared to other NAC spp., and report higher frequencies of fluconazole resistance among *C. parapsilosis* clinical isolates as well.

Analysis of 57001 candidemia isolates from Japan identified *C. albicans* (43.6%) as the predominant the species followed by *C. glabrata* (19.5%), *C. parapsilosis* (18.8%), and *C. tropicalis* (1.4%) and found low rate of fluconazole resistance among *C. parapsilosis* isolates overall (196). However, when evaluating bloodstream isolates specifically from bone-marrow transplant hospitals, both the proportion of isolates identified as *C. parapsilosis* and the rate of fluconazole resistance among those isolates increased compared to average across all hospitals (21.6% vs 18.8%; 5.41% vs 1.72%) (196). National observations from Korea identified *C. tropicalis* (20.2%) and *C. parapsilosis* (18.2%) in nearly equal proportion as the predominant NAC spp. causing healthcare-associated candidemia while *C. glabrata* (14.0%) was not far behind (197). The most recent surveillance for Korea reported a smaller proportion of candidemia was caused by *C. parapsilosis* for 2021 compared to 2020 however *C. parapsilosis* isolated in 2021 had slightly decreased fluconazole susceptibility (95.7% to 92.6% of isolates) (198).

A systematic analysis of 65 article published since 2011 revealed *C. tropicalis* (21.89%) as the most predominant NAC spp. causing candidiasis in mainland China, followed by *C. parapsilosis* (13.92%) and *C. glabrata* (11.37%) (199). However, studies from the China Hospital Invasive Fungal Surveillance Net (CHIF-NET) found *C. parapsilosis* to be most common NAC spp. causing invasive candidiasis (17%) and was consistently recovered from approximately 25% of candidemia cases in 2009 to 2017 (200, 201). A disparity of species distribution across China is not wholly unexpected seeing as the country encompasses both a great number of people and a large area of land. On average, 4% to 7% of *C. parapsilosis* isolates are reported as resistant to fluconazole, with slight increases, up to 9.8%, for those isolated from the northern region of China (199, 202, 203). These finding are consistent with species distribution and *C. parapsilosis* fluconazole resistance rates have identified by multiple retrospective studies from individual hospitals throughout China (204-207).

Multiple reports out of India have shown the inter-hospital variation for both the proportion of *C. parapsilosis* causative candidemia and the rates of fluconazole resistance. A hospital-based retrospective study from Rajasthan reported *C. parapsilosis* as the causative agent in 20% of all candidemia episodes from their ICUs from 2014-2018 (208). A similar study out of Chandigarh for the same time period reported *C. parapsilosis* as the causative agent in just 6.6% of candidemia cases from their ICUs (209). While only 7.8% the *C. parapsilosis* isolates from a Chandigarh medical center demonstrated fluconazole resistance (209), fluconazole resistance was observed in an alarming 63% of the *C. parapsilosis* isolates from Rajasthan (208). Furthermore, a multicenter analysis of 199 *C. parapsilosis* isolates identified an overall resistance rate of 27.6%, however the rates of fluconazole resistance varied among different medical centers, ranging from 12% to 76% of tested isolates (184). A systematic review of 106 studies of candidemia and candidiasis from India, found *C. parapsilosis* only represented

8.36% of the total 11,429 isolates studied (185) but importantly, fluconazole resistant strains accounted for 17.63% of the total 693 *C. parapsilosis* isolates tested for antifungal susceptibility (185).

An observational study from a multi-center cohort in Turkey found *C. albicans* (39.7%) and *C. parapsilosis* (33.6%) to be the predominant species isolated from adults with candidemia (210). *C. parapsilosis* is a predominant NAC spp. causing invasive candidiasis in pediatric populations worldwide, however recent laboratory surveillance in Turkey has found *C. parapsilosis* to be more prevalent than *C. albicans* within some medical centers regardless of patient age (211). Immense variation in rate of fluconazole resistance among *C. parapsilosis* has been reported by medical centers in Turkey with reports of 33.8% (211), 0% (212), and 49% (213) all published in the past few years. Additionally, one of Turkey's largest medical centers has reported increased rates of fluconazole resistance among *C. parapsilosis* candidemia isolates, and has identified the candidemia cases caused by clonal strains of fluconazole resistant *C. parapsilosis* all of which harbored a Y132F substitution in CpErg11 (76).

**Australia and New Zealand**

*C. parapsilosis* is the second most common NAC spp. causing candidemia in Australia (16.5%) following *C. glabrata* (26.7%) (214). Fluconazole resistant *C. parapsilosis* is rare in Australia and New Zealand with nearly 98.8% and 96% of *C. parapsilosis* isolates reported as fluconazole susceptible, respectively (214, 215).

**Europe**

Incidence of *C. parapsilosis* candidemia seemingly varies with geographic latitude across Europe however the rates of fluconazole resistance are not as clearly defined. A recent systematic review by Galia et al. combined information from national surveillance networks for the years 2015-2020 with epidemiological studies from 2005-2020 and found just 5 European countries, United Kingdom, Austria, Italy, Spain and Norway, had published *Candida* species stratification for both the infection type and antifungal susceptibility testing (216).

*C. parapsilosis* is the most common NAC spp. causing candidemia in neonates and children throughout Europe (217). *C. parapsilosis* represents the second or third most commonly isolated NAC spp., for much of Northern Europe according to recent surveillance studies from Austria (218), France (219), Sweden (220), Switzerland (221), Finland (222), Belgium (223), Germany (224), and England (225). Overall, rates of fluconazole resistance among *C. parapsilosis* isolates from northern Europe remain low and SENTRY data for participating countries show fluconazole resistance in < 5% of clinical isolates (226).

A different *C. parapsilosis* picture has recently emerged in southern Europe. A retrospective observational study from a tertiary hospital in Croatia found *C. parapsilosis* represented 31.76% of the isolates causing invasive *Candida* infections between 2018 and 2020 (227). Antifungal susceptibility testing of these isolates revealed a staggering 83.33% of the *C. parapsilosis* isolates were fluconazole resistant (227). Nationwide studies of bloodstream *Candida* infections in Greece reported almost a doubling of *C. parapsilosis* isolation rates between 2009 to 2018 (28% to 49%), surpassing the isolation rate of *C. albicans* to become the most common *Candida* species (228). The increase in incidence coincided with a concomitant rise in rates of fluconazole resistance from just 1% of *C. parapsilosis* isolates collected between 2009-2011 to 27% of isolates between 2015 and 2018 (228). A tertiary hospital in Genoa, Italy revealed *C. parapsilosis* as the causative species in 28% of candidemia cases between 2012-2016 with a fluconazole resistance rate of 33.0% (189). Similar results were obtained from a tertiary hospital in Rome, with *C. parapsilosis* accounting for 28.5% of the *Candida* bloodstream infections and a fluconazole resistance rate of 22.0% (229). Furthermore, as observed among isolates from Turkey, multi-locus sequencing of Italian isolates identified clonal distribution of a fluconazole resistant *C. parapsilosis* strain harboring the CpErg11 amino acid substitution Y132F (229, 230).

The incidence of fluconazole resistant *C. parapsilosis* has also risen in many hospitals across Spain. A single hospital in the Balearic Islands reported fluconazole resistance in 77.7% the *C. parapsilosis* strains from patients (188). Interestingly, a combination of genotypic analysis and patient data showed less than 20% of patients with infections caused by the resistant *C. parapsilosis* had previous fluconazole exposure, suggesting the presence of endemic fluconazole resistant *C. parapsilosis* strains (188). In mainland Spain, a prospective study of *C. parapsilosis* causing invasive *Candida* infections across 16 Madrid hospitals found the rate of fluconazole resistance among *C. parapsilosis* isolates ranged from 0.0% to 37.7% per hospital but overall, there was a significant rise in the rate of fluconazole resistance between 2019 and 2021 (3.8% in 2019 to 29.1% in 2021) (187). When susceptibility profiles for 1315 *C. parapsilosis* isolates collected between 2000 and 2021 were examined, there was significant increases in fluconazole resistance in 2019-2021 compared to previous years (approximately 60% vs 3% - 7%) (231).

**South America**

*C. parapsilosis* incidence across South America is comparable to the observations from southern Europe. A multi-center analysis of candidemia in Lima, Peru found *C. parapsilosis* to be the most frequently isolated NAC spp. (25.3%) with low rates of fluconazole resistance among all *Candida* species (2.6%) (232). Comparable reports have been published for Argentina, Chile, Costa Rica, Ecuador, and Paraguay (233-235) and *C. parapsilosis* has been reported as the most frequently isolated *Candida* species causing candidemia in Venezuela and Colombia (236, 237). Fortunately, despite the relative abundance of *C. parapsilosis* within these countries, overall rates of fluconazole resistance remain low (226, 238).

As with other large and highly populated countries such as China, India and the United States, Brazil presents a more complicated picture of incidence of invasive candidiasis caused by *C. parapsilosis* and for rates of fluconazole resistance among clinical isolates. For many areas of Brazil, *C. parapsilosis* is the most common NAC spp. causing candidemia and overall rates of resistance to fluconazole and other triazoles remain low, analogous to many other South American countries (239, 240). One representative retrospective study of eight hospitals around the southern state of Paraná identified fluconazole susceptible *C. parapsilosis* as the etiological agent in 23% of candidemia cases reported between 2016-2017 (241). Comparable results were also found in a 5-year study, between 2011-2016 for Northeast Brazil with *C. parapsilosis* representing 21.6% of candidemia cases, and fluconazole resistance only reported in one of the nine isolates tested for antifungal susceptibility (242).

Antifungal susceptibility testing of candidemia isolates obtained from 22 hospitals around São Paulo collected between 2016 and 2017 established a fluconazole resistance rate of 6.4% among *C. parapsilosis* isolates (243). However, an investigation of a 2018-2019 candidemia outbreak in a São Paulo cancer institute reported *C. parapsilosis* detection from positive blood cultures had increased 254% between 2017 and 2018 (180). Subsequently, 67.9% all *C. parapsilosis* strains isolated from environmental and healthcare worker screens (10/39, 25.6%), catheter swabs (3/3 100%), and bloodstream cultures (63/70, 90%) demonstrated fluconazole resistance (180). Concerningly, candidemia caused by these clonal fluconazole resistant *C. parapsilosis* strains showed a significantly higher 30-day crude mortality rate compared to candidemia by the fluconazole susceptible *C. parapsilosis* strains (63.8% vs 20% p=0.008) (180). Additionally, the identification of environmental reservoirs and inter-hospital horizontal transmission of the fluconazole resistant *C. parapsilosis* clones among cardiology centers (244), COVID-19 ICUs (244), and neonatal ICUs (29) is a cause for concern in one of Brazil's most populous cities.

**North America**

*C. parapsilosis* is a common NAC spp. accounting for 14.8% of candidemia and invasive candidiasis isolates in North America (226) and routinely represents more than 20% of *Candida* isolated from neonatal and infant populations (245-247). *Candida parapsilosis* represented 17.4% of the nosocomial bloodstream *Candida* infections in 52 hospitals across the United States between 1998 and 2006 (248). Antifungal susceptibility testing for those same isolates found a fluconazole resistance rate of only 2.9% (248). Notably, while *C. parapsilosis* continuously accounted for approximately 15% of candidemia in the United States between 2012 and 2016, resistance to fluconazole increased from 4.4% to 14% for those same years (2). As with other countries with sizable landmass and population differences in *C. parapsilosis* incidence has been reported across the United States. Analysis of bloodstream *Candida* isolates collected between 2008 and 2011 demonstrated the fluconazole resistance rate among *C. parapsilosis* isolates from Atlanta to be double that of isolates from Baltimore (5.6% vs

2.3%) (249). The majority of the resistant isolates were concentrated among three hospitals in Atlanta and microsatellite analysis indicated the persistence of a local fluconazole resistant strain (250). Similarly, between 2011 and 2016 *C. parapsilosis* was the causative species for 12.0% of the bloodstream *Candida* infections reporting to tertiary hospitals across Canada, with 4.9% demonstrating resistance to fluconazole (251).

Prevalence of *C. parapsilosis* etiologic invasive candidiasis in Mexico varies considerably from hospital to hospital and patient population. A 2017 review identified *C. parapsilosis* as the fourth most common *Candida* strain causing candidemia behind both *C. tropicalis* and *C. glabrata* (252). Antifungal susceptibility testing of *C. parapsilosis* clinical isolates collected from a Monterrey medical center detected a fluconazole resistance rate of 4.5% (253). Whereas nationwide surveillance of *C. parapsilosis* isolated from the same time in pediatric populations reported no fluconazole resistant strains (254). Outbreaks of invasive candidiasis by *C. parapsilosis* have been reported in both neonatal and adult hospitals (186, 255). Additionally, an outbreak in one general hospital was caused by a fluconazole resistant strain of *C. parapsilosis* containing the Y132F substitution in CpErg11 (186).

### Global Estimates

While the temporal nature of localized outbreaks can affect the reported frequencies with which antifungal resistant *C. parapsilosis* isolates are identified for a given year, it appears overall rates of fluconazole resistance among *C. parapsilosis* isolates are on the rise. The SENTRY antifungal surveillance program found 3.9% of *C. parapsilosis* isolates, collected from 39 countries, were fluconazole resistant between 2006 and 2016, with gradual increases in each two-year period starting in 2009 (2.5%, 3.2%, 5.5%) (226). Using isolates collected from the SENTRY program between 2016-2017, Castanheira et al. found 8.8% of *C. parapsilosis* isolates to be fluconazole resistant, the majority of which were from European countries (89.4%) (104). This trend continued further as fluconazole resistance among *C. parapsilosis* isolates across healthcare centers in North America, Europe, and Asia-Pacific from the first year of the COVID-19 pandemic increased from 9.8% in 2018 and 2019 to 13.9% in 2020 (256). These trends of increasing fluconazole resistance are in line with a 2022 meta-analysis of antifungal resistance in cases of *C. parapsilosis* candidemia by Yamin et al., which estimated the fluconazole resistance rate among *C. parapsilosis* candidemia isolates to be 15.2% (257).

## Objectives

Rates of fluconazole resistance are on the rise; understanding the development of triazole resistance represents a key first step to preserving the utility of one the most widely prescribed antifungals worldwide. As with other prominent NAC species, previous studies performed in both *S. cerevisiae* and *C. albicans* can provide a model for the identification of putative resistance mechanisms in *Candida parapsilosis.* However,

mechanisms of resistance vary between different species of *Candida* and we cannot simply presume that each resistance mechanism functions the same for all NAC species (258). In the following chapters, we describe two key mediators of fluconazole resistance in *C. parapsilosis*, identify the direct contribution individual resistance effectors have on fluconazole resistance, and provide in-depth analysis of the genotypes and phenotypes associated with clinical resistance for a collection of *C. parapsilosis* isolates.

# CHAPTER 2. *CANDIDA PARAPSILOSIS MDR1B* AND *CDR1B* ARE DRIVERS OF *MRR1*-MEDIATED CLINICAL FLUCONAZOLE RESISTANCE[1]

**NOTE:** This chapter refers frequently to content in **Appendix A.** When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See **Preface** for further details.

## Introduction

As introduced in Chapter 1, identified resistance mechanisms for *C. parapsilosis* are largely presumed from those identified and studied in *Candida albicans*. The purpose of this project is to understand triazole, specifically fluconazole, resistance in *C. parapsilosis* as its own unique species. In *C. albicans*, increased expression of the target gene *MDR1* by the zinc cluster transcription factor Mrr1 through either gain-of-function mutation or artificial activation via C-terminal fusion with a Gal4 activation domain, results in decreased fluconazole susceptibility (167, 171).

Early surveillance for antifungal resistance mechanisms among *C. parapsilosis* clinical isolates found increased frequency for non-synonymous mutations in the *C. albicans MRR1* orthologous gene, CPAR2_807270 (*CpMRR1*) among fluconazole-resistant isolates compared to those that were fluconazole-susceptible (250). Multiple amino acid substitutions in CpMrr1 have been previously observed among fluconazole-resistant clinical isolates (**Figure A-4**) (135, 191, 250, 259-262). Additionally, our lab previously identified that three isolates (Cp29, Cp30, Cp36) within our collection of 39 clinical isolates displayed elevated expression of the *MDR1* ortholog, CPAR2_301760 (*CpMDR1*) (261). Among these isolates we identified three homozygous *CpMRR1* mutations, including two novel mutations resulting in the I283R and A854V amino acid substitutions, and a previously identified mutation resulting in the R479K substitution (250). These isolates were also among those with the highest measured fluconazole resistance with MICs of ≥ 64 µg/mL (250, 261). To determine the direct impact of *CpMDR1* expression on CpMrr1 mediated fluconazole resistance, *CpMDR1* was deleted from the corresponding *C. parapsilosis* isolates. While in *C. albicans* the deletion of *MDR1* from backgrounds known to have activating mutations in *MRR1* is associated with a 2- to 4-fold decrease to fluconazole MIC (167), there was little to no change in fluconazole MIC upon *CpMDR1* deletion in *C. parapsilosis* (261).

The primary goal of Chapter 2 is to characterize clinical fluconazole resistance mediated by CpMrr1 gain-of-function mutation. In the referenced publication (**Appendix A**), we were able to demonstrate that activating mutations in CpMrr1 are a

[1] Final PDF used with permission. **Doorley, Laura A.**; Rybak, Jeffrey M.; Berkow, Elizabeth L.; Zhang, Qing; Morschhäuser, Joachim; Rogers, P. David. *Candida parapsilosis* Mdr1B and Cdr1B Are Drivers of Mrr1-Mediated Clinical Fluconazole Resistance. Antimicrob Agents Chemother. 2022;66(7):e0028922. DOI: https://doi.org/10.1128/aac.00289-22 [277] (**Appendix A**).

common contributor to fluconazole resistance in *C. parapsilosis* and elicit their effect not through overexpression of the gene encoding CpMdr1, but rather through overexpression of the gene encoding the MFS transporter here named *CpMDR1B* and, unexpectedly, in conjunction with overexpression of the gene encoding the ABC transporter we have designated *CpCDR1B*.

## Summary

A study of 62 fluconazole resistant C. *albicans* isolates identified 20.9% with clinically relevant *MDR1* overexpression and subsequent *MRR1* mutations (139). Previous RTqPCR analysis for *CpMDR1* expression within our collection of 35 fluconazole resistant *C. parapsilosis* isolates revealed overexpression only occurred within Cp29, Cp30, and Cp36, or 8.5% of the fluconazole resistant isolates (261). Nevertheless, previous surveillance of *C. parapsilosis* clinical isolates had revealed increased frequency of non-synonymous mutation in *CpMRR1* occurred both with and without accompanying *CpMDR1* overexpression. Our first task was to identify the frequency with which mutations in *CpMRR1* occurred within our collection of *C. parapsilosis* clinical isolates. We found mutations leading to amino acid substitutions in CpMrr1 to be present in 31% (12/35) of the resistant clinical isolates (**Table A-1**). These results show that while overexpression of *CpMDR1* may not be common among fluconazole-resistant *C. parapsilosis* isolates, mutations in *CpMRR1* were relatively common.

In *C. albicans*, *MRR1* mutations contribute to fluconazole resistance but are not in-and-of-themselves sufficient to impart high-level resistance; however, Cp29, Cp30 and Cp36 all displayed high fluconazole MICs by broth microdilution (**Table A-1**). To determine the direct contribution of these mutations, we introduced the three homozygous *CpMRR1* mutations into a susceptible background. Introduction of the mutations leading to the I283R, R479K, and V854A substitutions in both alleles of the susceptible isolate Cp13 resulted in dramatic 128- to 256-fold increases in fluconazole resistance (**Figure A-1**) Conversely, correction of the mutations in both alleles to the wild-type *CpMRR1* sequence in clinical isolates Cp36, Cp30, and Cp29 resulted in similar 32- to 128-fold fluconazole MIC decreases. All three corrected strains demonstrated fluconazole MICs at or below the clinical breakpoint for fluconazole susceptibility. These results indicated *CpMRR1* mutations were in-and-of-themselves sufficient to impart high level fluconazole resistance in clinical isolates of *C. parapsilosis* and highlight the pronounced fluconazole resistance mediated by CpMrr1 substitution in *C. parapsilosis* compared to that of *C. albicans* Mrr1.

To characterize how gain-of-function mutations in *CpMRR1* were able to mediate high level fluconazole resistance, we performed transcriptional profiling on isolates Cp36, Cp30, and Cp29 and their respective derivatives with the *CpMRR1* alleles corrected to wild-type. A total of 41 genes were commonly up-regulated, and 23 were down-regulated in the presence of the gain-of-function mutation compared to the respective wild-type *CpMRR1* derived strains (**Figure A-2**). Among the up-regulated

genes associated with *CpMRR1* gain-of-function were those homologous to genes up-regulated by *MRR1* gain-of-function in *C. albicans* strains, including homologs of *GRP2*, *LPG20*, orf19.7306, orf19.7166, orf19.6586, *OYE32*, *MDR1* and *MRR1* (**Table A-2**) (157, 168). Interestingly, we identified two *MDR1* homologs, *CpMDR1* and *CpMDR1B*, were upregulated with *CpMRR1* gain-of-function along with a gene encoding a homolog of the *C. albicans* ABC transporter gene *CDR1*, designated *CpCDR1B*. As mentioned in Chapter 1, ABC transporters Cdr1 and Cdr2 play an important role in *C. albicans* fluconazole resistance and increased expression of homologous transporter genes are known to be major determinants of fluconazole resistance in other *Candida* species as well (149, 263). However, in *C. albicans* neither *CDR1* nor *CDR2* expression appears to be regulated by Mrr1 (168).

While homologs of *C. albicans CDR1*, and *MDR1* have been observed to be overexpressed in resistant *C. parapsilosis* clinical isolates, their contribution to fluconazole resistance has been unclear. To determine whether the upregulation of these newly identified *MDR1* and *CDR1* homologs were directly involved in driving the fluconazole resistance observed with CpMrr1 gain-of-function, we derived constitutive *CpMDR1B* and *CpCDR1B* overexpression strains in a fluconazole susceptible clinical isolate background. Antifungal susceptibility testing for these strains revealed a direct association with clinically relevant *CpMDR1B* and *CpCDR1B* expression (**Figure A-3A**) and decreased fluconazole resistance (**Figure A-3B**). To determine the direct contributions of *CpMDR1*, *CpMDR1B*, and *CpCDR1B* to CpMrr1-mediated fluconazole resistance, we disrupted these genes individually and in combination in a resistant clinical isolate. Disruption of *CpMDR1* resulted in a single dilution decrease on fluconazole MIC similar to the previous observations with *CpMDR1* deletion (261). Disruption of either *CpMDR1B* or *CpCDR1B* resulted in a modest reduction in both fluconazole MIC and voriconazole MIC as measured by broth microdilution (**Table A-3**). Combined disruption of both *CpMDR1B* and *CpCDR1B* was sufficient to impart fluconazole susceptibility with no effect observed with the addition of *CpMDR1* disruption (**Table A-3**). These data show how activating mutations in *CpMRR1* increase fluconazole resistance primarily through the increased expression of *CpMDR1B* and *CpCDR1B* in *C. parapsilosis* and not through overexpression of the presumed effector *CpMDR1*.

## Conclusions

The findings presented in Chapter 2 and the referenced publication (**Appendix A**) delineate fluconazole resistance mediated by the presence of relatively common *CpMRR1* activating mutations. Fluconazole resistance characterization performed with antifungal surveillance commonly involves expression analysis for *CpMDR1*, *CpCDR1* and *CpERG11*. However, as this data demonstrates the measurement of *CpMDR1* should not be used for characterization of fluconazole resistance in *C. parapsilosis*. Not only is overexpression of *CpMDR1* insufficient to impart any effects on fluconazole susceptibility, but a lack of *CpMDR1* overexpression appears to have no association in whether a given isolate possesses CpMrr1 gain-of-function mutation. Differences in the resistance determinants regulated, and the strength of fluconazole resistance imparted by

CpMrr1 GOF, highlight the need investigate other mechanisms of resistance assumed to be in *C. parapsilosis* based on observations from *C. albicans*. In chapter 3, we focus on two other resistance mechanisms that has been assumed in *C. parapsilosis* based on the acknowledged fluconazole resistance mediated in *C. albicans*, the presence of the Y132F substitution in CpErg11, and the elevation of *CpCDR1* expression by the transcriptional regulator CpTac1.

# CHAPTER 3. MUTATIONS IN *TAC1* AND *ERG11* ARE MAJOR DRIVERS OF TRIAZOLE ANTIFUNGAL RESISTANCE IN CLINICAL ISOLATES OF *CANDIDA PARAPSILOSIS*[2]

**NOTE:** This chapter refers frequently to content in **Appendix B.** When using Adobe Acrobat, after going there, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See **Preface** for further details.

## Introduction

Chapter 1 presented some of the mutations leading to amino acid substitutions in the triazole drug target Erg11 and emphasized their role as a prominent resistance mechanism among C. *albicans* clinical isolates. Recent surveillance studies from the United States, South America, South Africa, and parts of Western Asia over the past decade have highlighted notable increases in the rate of fluconazole resistance in *C. parapsilosis* isolates associated both with and without a mutation leading the substitution Y132F in CpErg11 (2, 75, 228, 243, 264). Varied fluconazole MICs have been reported among *C. parapsilosis* clinical isolates carrying the Y132F mutation A395T ranging between 2 μg/mL to 256 μg/mL. Unlike the vast array of mutations leading to amino acid substitution in Erg11 identified among clinical *C. albicans* isolates, very few CpErg11 substitutions have been identified among *C. parapsilosis* clinical isolates (129).

Increased *CDR1* and *CDR2* expression by *TAC1* activation also play a vital role in the development of fluconazole resistance in *C. albicans* isolates (151). While expression of the *C. albicans CDR1* ortholog, CPAR2_405290 (*CpCDR1*), is routinely measured in the determination of fluconazole resistance mechanisms among *C. parapsilosis* clinical isolates, sequencing for the *C. albicans TAC1* ortholog CPAR2_303510 (*CpTAC1*) has been reported less frequently. However, some *CpTAC1* mutations have been reported among fluconazole-resistant *C. parapsilosis* clinical isolates suggesting its role in resistance (135, 191, 261).

Previous characterization of fluconazole resistance mechanisms among our collection of 39 clinical *C. parapsilosis* isolates revealed the CpErg11 substitution in eleven isolates and the overexpression of *CpCDR1* in three of these isolates, Cp35, Cp38, and Cp40 (261). Sequence analysis revealed non-synonymous mutations present in *CpTAC1* for each of the three *CpCDR1* overexpressing isolates (261). Among these *CpTAC1* mutations was the homozygous mutation leading to the amino acid substitution G650E, present in Cp35 and Cp38, and the heterozygous mutation leading to L978W in

[2] Article is reused from the prepared manuscript with the authors' permission. **Doorley LA**, Barker KS, Zhang Q, Rybak JM, Rogers PD. "Mutations in *TAC1* And *ERG11* are Major Drivers of Triazole Antifungal Resistance in Clinical Isolates of *Candida parapsilosis*" (**Appendix B**).

Cp40. Isolates Cp35, Cp38, and Cp40 were all highly resistant with fluconazole MICs of ≥ 32 μg/mL. In *C. albicans*, deletion of *CDR1* results in a highly susceptible phenotype (153), however deletion of *CpCDR1* from these three resistant isolates had little to no effect on fluconazole MIC (261).

The primary goals for Chapter 3 research were two-fold. We sought to determine the contribution of the A395T mutation in *CpERG11* to fluconazole resistance and to characterize clinical fluconazole resistance mediated by the presence these putative CpTac1 gain-of-function mutations. In the referenced article submitted for publication (**Appendix B**), we demonstrated that the CpErg11 substitution Y132F is not sufficient in-and-of itself to impart high level fluconazole resistance. We were also able to show that the G650E substitution in CpTac1 contributes to triazole resistance through the elevated expression of *CpCDR1*, *CpCDR1B*, and *CpCDR1C*.

## Summary

To determine the contribution of the Y132F substitution in CpErg11 to triazole resistance in *C. parapsilosis* clinical isolates, we placed the *CpERG11* mutation A395T into three susceptible clinical isolates, Cp3, Cp13, and Cp23. Resulting fluconazole MICs increased four- to eight- fold, however, no derived strain surpassed the clinical breakpoint for fluconazole resistance in *C. parapsilosis* (**Figure B-1**). Increases in the MICs of other triazoles were minimal. Voriconazole MICs increased one dilution for Cp3 and Cp23 with CpErg11$^{Y132F}$, but no effects were observed in itraconazole, isavuconazole or posaconazole MICs (**Figure B-S1**). The modest increases for fluconazole MICs are similar to the 8-fold increase in fluconazole resistance observed with the presence of the Erg11 Y132H substitution in *C. albicans (129)*. These results imply that while the Y132F substitution in CpErg11 does contribute to fluconazole resistance in *C. parapsilosis*, its presence does not affect the susceptibility to other triazoles and that CpErg11$^{Y132F}$ alone is insufficient for the high-level fluconazole resistance observed in Cp35, Cp38, and Cp40.

Previous sequence analysis of *C. albicans* clinical isolates suggests *TAC1* mutations to be present in 55-75% of those with fluconazole resistance (265, 266). One of our first tasks was to determine the prevalence of *CpTAC1* mutation in our collection of 35 fluconazole resistant isolates. We identified non-synonymous mutations in 29% or 9 of 35 resistant clinical isolates (**Table B-1**). Far fewer isolates than would be expected in a comparable collection of fluconazole resistant *C. albicans* isolates. We selected the G650E CpTac1 substitution identified in Cp35 and Cp38 for further gain-of-function characterization due to its presence as a homozygous mutation and its previously association with elevated *CpCDR1* expression (261).

Depending on the mutation, *TAC1* gain-of-function is associated with 4- to 16-fold increases in fluconazole resistance in *C. albicans* (267). To determine the direct contribution of these mutations, we introduced the homozygous *CpTAC1* mutation into the susceptible background Cp13 (**Figure B-2A**). The conversion to CpTac1$^{G650E}$ resulted

in an 8-fold increase in fluconazole MIC. Conversely, correction of the mutation in *CpTAC1* leading to G650E substitution to that of the wildtype *CpTAC1* sequence in Cp35 and Cp38 led to 32-fold decreases in fluconazole MICs (**Figure B-2A**). These results demonstrate the contribution of CpTac1 gain-of-function to fluconazole resistance in *C. parapsilosis* clinical isolates.

To delineate the resistance effectors regulated by activation of CpTac1, we performed transcriptional analysis of Cp35 and Cp38 compared to their derived $CpTac1^{WT}$ strains. Our analysis revealed the shared elevated expression for four genes, of which three were ABC transporter genes (**Figure B-2B**). The *C. albicans CDR1* homologs *CpCDR1* and *CpCDR1B* were identified along with a previously classified pseudogene, CPAR2_300010 (**Figure B-2C**). CPAR2_300010 was deemed a pseudogene due to the presence of multiple early stop codons in the *C. parapsilosis* reference genome based on the clinical isolate CDC317. Due to its consistent elevated expression among our CpTac1 gain-of-function strains, we performed sequencing of the CPAR2_300010 gene within our clinical isolate collection. The results revealed that when compared to the sequence present in CDC317, our clinical isolates contained an insertion at position 3424 (GAA – GAAA). This insertion results in a shift at R1144 in the codon reading frame and allows for the transcription of a full ABC transporter sequence. As such we designated the ABC transporter associated with the CPAR2_300010 as *CpCDR1C*, in accordance with the nomenclature based on discovery of function and homology to *C. albicans CDR1*.

To determine the direct contribution of each ABC transporter toward fluconazole resistance we constitutively overexpressed *CpCDR1*, *CpCDR1B*, and *CpCDR1C* in susceptible isolate Cp13 (**Figure B-S2A**). Individual overexpression of *CpCDR1* and *CpCDR1B* increased fluconazole resistance 16-fold while the overexpression of *CpCDR1C* increased fluconazole resistance 4-fold (**Figure B-S2B**). Additionally, the combined disruption of these three transporters in the clinical isolate Cp35 decreased fluconazole resistance, as measured by broth microdilution (**Table B-2**). Interestingly, the disruption of *CpCDR1*, *CpCDR1B* and *CpCDR1C* displayed a more pronounced effect on fluconazole susceptibility when MICs were measured by a fluconazole general diffusion test strip (**Figure B-S3**). This antifungal susceptibility test showed simultaneous disruption of the three ABC transporters in Cp35 to be more consistent with the MICs that were observed with the *CpTAC1* correction to wild-type sequence.

## Conclusions

The findings detailed in the referenced publication (**Appendix B**) delineate the contribution of the most common *CpERG11* mutation toward fluconazole resistance and characterize the resistance mediated by activating mutations in *CpTAC1*. While often overlooked, *CpTAC1* mutations appear to be prevalent among highly resistant *C. parapsilosis* clinical isolates. Identification of the *CpTAC1* mutation represents an important determinant of triazole resistance in *C. parapsilosis* clinical isolates. High-level fluconazole resistance in *C. parapsilosis* is not driven by the presence of $CpErg11^{Y132F}$

alone. Rather it appears to work in concert with activating *CpTAC1* mutations to drive high-level fluconazole resistance. The identification of three ABC transporters associated with *CpTAC1* gain-of-function, and the predominance for their overexpression either singularly or in combination among resistant isolates (**Figure B-3**), once again highlights unique features of fluconazole resistance mechanisms at play in *C. parapsilosis*.

In Chapter 4, the focus shifts to genotypic and phenotypic characterization of our clinical isolate collection. Chapters 2 and Chapter 3 focused on characterization of the mutations among the three genes most often associated with fluconazole resistance in the literature, however mutations in *CpMRR1*, *CpTAC1*, and *CpERG11* do not account for the fluconazole resistance for all the clinical isolates within our collection.

# CHAPTER 4. GENOTYPIC AND PHENOTYPIC ANALYSIS OF FLUCONAZOLE-RESISTANT *CANDIDA PARAPSILOSIS* CLINICAL ISOLATES

**NOTE:** When using Adobe Acrobat, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See **Preface** for further details.

## Introduction

In neonatal populations, *C. albicans* and C. *parapsilosis* account for 80% to 90% of all invasive *Candida* infections (7). With only three antifungal drug classes available for invasive candidiasis treatment, development of resistance to any one of them presents a major scientific and therapeutic concern. The triazole fluconazole is an alternative first line antifungal drug used for prophylaxis and treatment of invasive *Candida* infections (58). Fluconazole is a fungistatic drug which reduces the production of ergosterol through inhibition of the enzyme lanosterol 14 α-demethylase encoded by *ERG11*. Resistance to fluconazole in C. *albicans* and other *Candida* species is most often due to the combinatorial effects of mutations in *ERG11*, increased drug efflux, and altered sterol biosynthesis (92).

Concern surrounding increased fluconazole resistance among *C. parapsilosis* isolates are amplified by the inherent vulnerability of the populations associated with *C. parapsilosis* and reports of breakthrough infection following antifungal therapy. Consequently, robust biofilm formation on medically implanted devices and horizontal transmission from healthcare workers to patients by hand carriage readily allow for persistence and dissemination of drug resistant strains (179, 182). Previous studies have found *C. parapsilosis* varies from *C. albicans* in virulence (268, 269), immune evasion (270-272), and biofilm regulation (273), however many of the fluconazole resistance mechanisms are presumed similar. In the two previous chapters, we investigated three triazole resistance mechanisms for *C. parapsilosis*, in-depth. The regulation of both ABC and MFS transporters by the transcription factor CpMrr1 and the relatively narrow transcriptional regulation by CpTac1 highlight some of the differences between *C. albicans* and *C. parapsilosis* and warrant a more complete understanding of the species-specific antifungal resistance mechanisms. Understanding the development of triazole resistance in the different species of *Candida* is key to preserving the utility of one the most widely prescribed antifungals worldwide.

The most common fluconazole resistance mechanism cited among clinical *C. parapsilosis* isolates in the literature is the presence of the Y132F substitution in CpErg11. In our collection of clinical isolates, the most common CpErg11 substitution identified among clinical isolates appears to be R389I, which is found in both susceptible and resistant isolates, followed by the Y132F which is only found among resistant isolates. The Y132F substitution in CpErg11 has been identified in clinical isolates

worldwide (104, 188, 197) and has been specifically associated with localized candidemia outbreaks caused by clonal *C. parapsilosis* isolates (274). As such, antifungal surveillance among *C. parapsilosis* clinical isolates has primarily focused on *CpERG11* sequencing (104). As we previously demonstrated, the placement of CpErg11$^{Y132F}$ into susceptible isolates results in moderate decreases in fluconazole susceptibility, however as explained Chapter 3, this substitution alone is not sufficient to cause the high-level resistance observed among *C. parapsilosis* clinical isolates.

Other CpErg11 amino acid substitutions have also recently been identified among resistant *C. parapsilosis* clinical isolates including K143R and the combination of Y132F + K143R (135). Additional substitutions in CpErg11 have been identified in resistant isolates, however many have been identified in isolates that also contain substitutions in CpMrr1 and CpTac1. Amino acid substitutions that have only been associated with non-susceptible *C. parapsilosis* strains to date are summarized in **Table 4-1**. Compared to *CpMRR1* and *CpERG11*, relatively few studies have scrutinized the sequences of genes encoding other fluconazole resistance determinants in *C. parapsilosis*. We emphasized the role *CpTAC1* activating mutations play in high-level clinical resistance, and additional mutations have been identified in the literature (**Table 4-1**).

To date, a single CpErg3 substitution, G111R, has been classified as causing triazole resistance in a *Candida parapsilosis* clinical isolate (146). The CpErg3 substitutions R135I and D14Y have also been found in triazole resistant *C. parapsilosis* strains following prolonged posaconazole exposure in the laboratory (145, 262). Moreover, deletion of the *CpUPC2* from a posaconazole evolved strain of *C. parapsilosis* demonstrated fluconazole hyper-susceptibility and down regulated expression ergosterol biosynthesis genes (143) although, antifungal surveillance studies have yet to identify any individual gain-of-function mutations in *CpUPC2* among clinical isolates.

Our collection of 39 *C. parapsilosis sensu stricto* clinical isolates was curated based on resistance or susceptibility to fluconazole by the University of Iowa and provided by Daniel J Diekema. Previous studies on this collection led by Berkow et al. identified sterol composition for all 39 clinical isolates along with potential gain-of-function mutations in CpTac1 and CpMrr1 based on the expressions of *CpCDR1* and *CpMDR1*, respectively. We have also reported fluconazole susceptibility testing and *CpERG11*, *CpTAC1*, and *CpMRR1* sequencing for these isolates in previous chapters and are summarized in **Table 4-2**. Our investigations into fluconazole resistance mechanisms thus far have identified eight isolates with fluconazole MICs ≥ 16 μg/mL for which CpErg11$^{Y132F}$ is the only explanation for resistance. A further eight isolates with fluconazole MICs ≥ 16 μg/mL have no known mechanism driving resistance.

The work presented in this chapter seeks to further describe these clinically relevant fluconazole resistant genotypes and phenotypes using next-generation sequencing and help guide future investigations into *Candida parapsilosis* resistance mechanisms and related species.

**Table 4-1. Amino acid substitutions in fluconazole resistant clinical isolates.**

| *C. parapsilosis* protein | Amino acid substitution | FLU MIC[#] (μg/mL) | Reference |
|---|---|---|---|
| **CpMrr1** | P250S | 8 | (191) |
| | I283R | 64 | (261) |
| | P295R | 32 | (191) |
| | P295L+Q1074* | 16 | (135) |
| | R479K | 128 | (261) |
| | G583R | > 64 | (275) |
| | L779F | 32 | (250) |
| | A854V | 64 | (261) |
| | A859T | 8 | (250) |
| | W872C | 32 | (191) |
| | K873N | 64 | (275) |
| | L926* | 32 | (135) |
| | G927D | 16 | (276) |
| | L986P | 32 | (259) |
| | S1081P | 8 | (191) |
| | G472V | 32 | (135) |
| | G427V | 8 | (135) |
| | L419F | 8 | (135) |
| | Q1027R | 8 | (135) |
| | [‡]K129*fs*, [‡]G982R | 32 | (277) |
| | [‡]P255L, [‡]A854V | 16 | (277) |
| | [‡]G294E | 32 | (277) |
| | A808T | 128 | (145) |
| | N394Y | > 256 | (145) |
| | G927C | ≥ 16 | (278) |
| | Y552H | 32 | (128) |
| **CpTac1** | A21V | ≥ 8 | (135) |
| | G490R+S760R+A761G | 8 | (135) |
| | D603V + P803L | 8 | (135) |
| | G650E | 256 | (261) |
| | N900D | 8 | (191) |
| | Q965K+M966V | > 32 | (135) |
| | L978W | 128 | (261) |
| | P150H | 8 | (135) |
| | [‡]HI221T | 128 | (L.A. Doorley, unpublished manuscript) |
| **CpErg11** | Y132F | ≥ 2 | (250) |
| | K143R | ≥ 4 | (184) |
| | Y132F+K143R | 32 | (135) |
| | G458S | ≥ 16 | (135) |
| | G307A+Y132F | ≥ 16 | (135) |
| | Q250K+G458S | 16 | (135) |
| | G458S+T519A | 16 | (135) |
| | K128N | 32 | (191) |
| **CpErg3** | R135I | 64 | (262) |
| | G111R | 64 | (146) |
| | D14Y | 256 | (145) |

**Table 4-1. Continued.**

| *C. parapsilosis* protein | Amino acid substitution | FLU MIC[#] (μg/mL) | Reference |
|---|---|---|---|
| **CpUpc2** | P45H | ≥ 8 | (135) |
| | Q371H | 16 | (135) |
| | G342S | 16 | (135) |
| | E7* | 8 | (135) |

Note: [#]Fluconazole MICs reported by reference, ≥ or ≤ symbols denote a range of MICs when reported, [‡]Denotes heterozygous mutation leading to stated amino acid substitution, *early stop codon, *fs* frameshift. Fluconazole MICs are the lowest reported by the first reference identifying the substitution in clinical isolates.

**Table 4-2. Current characterizations for clinical *C. parapsilosis* isolate collection.**

| Isolate ID | Origin country | FLU MIC[#] (µg/mL) | CpMrr1 | CpTac1 | CpErg11 |
|---|---|---|---|---|---|
| Cp 1 | South Africa | 16 | ‡A854V | | |
| Cp 2 | South Africa | 16 | ‡A854V | | |
| Cp 3 | Malaysia | 0.25 | | | R398I |
| Cp 4 | South Africa | 16 | | | R398I, ‡Y132F |
| Cp 5 | United States | 0.5 | K177N, ‡Q1053* | | |
| Cp 6 | Italy | 16 | | | |
| Cp 7 | South Africa | 16 | | | Y132F, R398I |
| Cp 8 | South Africa | 16 | | | |
| Cp 9 | South Africa | 16 | ‡A854V | | |
| Cp 10 | South Africa | 16 | ‡A854V | | |
| Cp 11 | South Africa | 16 | | | |
| Cp 12 | South Africa | 16 | ‡P255L, ‡A854V | | |
| Cp13 | United States | 0.25 | | | R398I, S216L |
| Cp 14 | Finland | 16 | | | |
| Cp 15 | Ecuador | 16 | | N900D | F145L |
| Cp 16 | South Africa | 8 | | | |
| Cp 17 | South Africa | 16 | | | |
| Cp 18 | South Africa | 16 | | | |
| Cp 19 | South Africa | 8 | | | |
| Cp 20 | South Africa | 16 | A854V‡ | | |
| Cp 21 | United States | 8 | | L978W‡ | Y132F, R398I |
| Cp 22 | South Africa | 32 | | | Y132F, R398I |
| Cp 23 | South Africa | 0.12 | | | |
| Cp 24 | South Africa | 16 | | | Y132F, R398I |
| Cp 25 | South Africa | 16 | | | Y132F, R398I |
| Cp 26 | South Africa | 32 | | | Y132F, R398I |
| Cp 27 | United States | 32 | ‡K129*fs*, ‡G982R | R208G | |
| Cp 28 | South Africa | 64 | ‡A854V | | |
| Cp 29 | South Africa | 64 | A854V | | |
| Cp 30 | Slovakia | 128 | R479K | | R398I |
| Cp 31 | South Africa | 32 | | | Y132F, R398I |
| Cp 32 | Venezuela | 128 | | I221T(h) | R398I |
| Cp 34 | South Africa | 32 | | | Y132F, R398I |
| Cp 35 | South Africa | 32 | | G650E | Y132F, R398I |
| Cp 36 | United States | 64 | I283R | | |
| Cp 37 | South Africa | 256 | | ‡L978W | Y132F, R398I |
| Cp 38 | South Africa | 32 | | G650E | Y132F, R398I |
| Cp 39 | Slovakia | 32 | ‡G294E | R208G | Y132F |
| Cp 40 | South Africa | 128 | | ‡L978W | ‡R398I, ‡Y132F |

Note: [#]Fluconazole MICs performed in accordance with CLSI guidelines, read visually at 24 hours. ‡Denotes heterozygous allele, *stop codon, *fs* frameshift. Data Sources: Berkow EL, Manigaba K, Parker JE, Barker KS, Kelly SL, Rogers PD. 2015. Multidrug Transporters and Alterations in Sterol Biosynthesis Contribute to Azole Antifungal Resistance in *Candida parapsilosis*. Antimicrobial agents and chemotherapy 59:5942-5950. https://doi.org/10.1128/AAC.01358-15. (261); Doorley LA, Rybak JM, Berkow EL, Zhang Q, Morschhäuser J, Rogers PD. 2022. *Candida parapsilosis* Mdr1B and Cdr1B Are Drivers of Mrr1-Mediated Clinical Fluconazole Resistance. Antimicrobial agents and chemotherapy https://doi.org/10.1128/aac.00289-22 (277).

## Materials and Methods

### Strains and Media

All *C. parapsilosis* isolates used in this study have been previously described (261). Isolates and derived strains were kept at -80°C in 40% glycerol stock. All strains and isolates were maintained on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plates at 30°C or in YPD liquid media at 30°C in a 220 rpm shaking incubator. RPMI with MOPS and 2% glucose pH 7.0 was used for both drug susceptibility testing growth prior to RNA isolation techniques. Chemically competent DH5α cells were utilized for plasmid construction and grown in Luria-Bertani media supplemented with 100 μg/mL ampicillin.

### Potential Resistance-Associated Genes in *C. parapsilosis* Genome

Searches for ABC, MFS, and ZCF DNA binding domains were conducted using the list of genes with identifiable protein domains in CDC317 by Butler et al. downloaded from the *Candida* Genome Database website (*www.candida*genome.org) (24, 279, 280). Lists of *Candida parapsilosis* genes orthologous or classified as a 'best hit' to genes of interest in *S. cerevisiae* and *C. albicans* based on blastP mappings by the *Candida* genome database were also downloaded for comparison (www.*Candida*genome.org/download/homology) (280, 281). Finally, the sequences of putative genes of interest were analyzed using Uniprot (282), eggnog (283), interpro (284), and TMHMM (285) databases to confirm presence of putative protein domains.

### Whole Genome Sequencing

Genomic DNA was isolated utilizing a Triton SDS and phenol-chloroform method previously described (286). DNA concentrations were quantified using both the Qubit Fluorometer and Nanodrop spectrophotometer using the manufacturers' protocols. Whole genome libraries were prepared and sequenced on the NovaSeq600 platform (150bp, paired-end reads) by the University of Maryland School of Medicine Institute for Genomic Sciences. performed with initial analysis for protein variants determined by code4dna.com. (www.code4DNA.com). Forward and reverse paired-reads FASTQ files imported into CLC genomics workbench 22.0 (QIAGEN Aarhus A/S) for additional variant, copy number, and phylogenetic analysis. Raw sequencing reads were trimmed using a quality limit of 0.05, with automatic read-through adaptor removal. Reads were mapped to the *C. parapsilosis* reference genome (GCA_000182765.2_ASM18276v2) with considerations made for global alignment and default CLC-workbench recommendations for match scores, mismatch, insertion, and deletion cost parameters (GCA_000182765.2) (24, 287).

Genetic variants called based on a fixed ploidy of 2x targeting entire chromosome scaffolds. Variants in consensus coding sequence determined, 90% required variant probability, min coverage =10, min count=4, min frequency 25%, and base quality filters set to the defaults for CLC-genomics workbench, thresholds applied. Adjacent MNVs and SNVs were joined, and variants tracks for resistant isolates were formed by filtering for MNVs and SNVs encoded by fluconazole susceptible isolates Cp13, Cp5, and Cp3 (min. read count 10). Resulting tracks within CDS regions were analyzed for amino acid substitution utilizing alternative genetic code table 12 and GCA_000182765.2 compiled with GCA_000182765.2 mRNA for exon determination. Predicted amino acid substitutions with QUAL scores < 20 discarded (99% call accuracy). Copy number variations determined utilizing CNV detection2.1. Read mappings were compared to isolate Cp13 with alignment to GCA_000182765.2_ASM18276v2 gene scaffold annotations, minimum fold change for amplification and deletion set to 1.5, significance < 0.05, coverage cutoff = 30.

In CLC genomics workbench 22.0 (QIAGEN), mapped reads with 2x fixed ploidy variant track parameters for each isolate were aligned for all SNPs, coverage > 10. Hierarchical likelihood ratio model testing performed on SNP alignments. Maximum likelihood phylogeny1.3 used for final tree construction: UPGMA construction, Kimura80 substitution modelling (Ts/Tv ratio =2, with estimated topology) bootstrapped for 1000 replicates.

**RNA Sequencing**

*C. parapsilosis* cultures were grown as described for MIC preparation with minimal modification. *C. parapsilosis* strains were grown in biological triplicate at 30°C overnight in YPD liquid media and subsequently plated onto Sabouraud-Dextrose (BD companies) minimal medium agar for 24h growth at 30°C. Sterile loops were used to transfer cells into 20mL RPMI for OD600=0.1 inoculums. Cultures were incubated at 35°C with110rpm shaking for 8h, after which the cells were centrifuged at 4000rpm for 5 min. Supernatants were removed and the pellets were stored at -80°C for a minimum of 24hrs. RNA isolation was performed using the RiboPureTM Yeast (Invitrogen) system per manufacturer's instructions. RNA Sequencing performed using Illumina NextSeq for stranded mRNA.

Libraries were prepared with paired-end adapters using Illumina chemistries per manufacturer's instructions, with read lengths of approximately 150bp with at least 50 million raw reads per sample. RNA-sequencing was analyzed using CLC Genomics Workbench version 20.0 (QIAGEN), and reads were trimmed using default settings for failed reads and adaptor sequences and then subsequently mapped to the *C. parapsilosis* genome (GenBank accession: GCA_000182765.2) with paired reads counted as one and expression values set to RPKM. Principal-component analysis was utilized for the assessment the clustering of biological replicates. Whole transcriptome differential gene expression analysis was performed with the prescribed algorithm of CLC Genomics Workbench version 20.0. Mismatch, insertion, and deletion costs were set to default

parameters and a Wald test was used for all group pairs against the pool of susceptible isolates. Genes were considered differentially regulated when a fold change of $\geq 2$ or $\leq -2$ was observed accompanied by an FDR p value $\leq 0.05$.

## Results

### Fluconazole Susceptibility Testing

Within the collection of 39 clinical *C. parapsilosis* isolates there are 35 fluconazole-resistant (MIC ≥ 8μg/mL) and 4 fluconazole-susceptible (MIC ≤ 2μg/mL) clinical isolates. As shown in **Table 4-2,** this collection consists of both highly susceptible strains fluconazole MIC of less than 0.125 μg/mL at 24 hours and highly resistant strains measuring at 256 μg/mL at 24 hours. Isolates were collected between 2001 and 2009 in Europe, Asia, North America, and South America however a majority, 27/39, were isolated in South Africa.

### Phylogenetic Analysis Reveals Closely Related Isolates within Collection

Single nucleotide polymorphisms detected within each isolate were aligned through a neighbor-end joining construction method and alignments were then used in the construction of a maximum likelihood phylogenetic tree (**Figure 4-1**). While many of the isolates with a mutation in *CpTAC1* also possessed CpErg11$^{Y132F}$ these isolates were distinct from those isolates with *CpMRR1* mutations. Only Cp39 contained substitutions in both CpMrr1 and CpTac1, and CpErg11$^{Y132F}$. Isolates Cp14, Cp6, Cp15, and Cp13 diverged as outgroups from larger clusters of isolates while the susceptible US isolate Cp5 formed an outgroup off the primary node. The Cp21 and Cp27, both isolated in the United States, formed monophyletic pairs with South African isolate Cp37 and Slovenian isolate Cp39, respectively. The relative diversity of the isolates from the United States mirrors the geographic variation inherent in large countries.

Conversely, the isolates from South Africa were nearly indistinguishable from one another, except for Cp23 and Cp40 (**Figure 4-1**). A maximum likelihood alignment and phylogenetic tree containing just the South African isolates from Bloemfontein and Johannesburg shows two distinct clusters with very few unique SNPs identified between members of the same subgroup (**Figure 4-2**). The formation of a single node containing both Cp23 and Cp40 indicated high genetic similarity. A look at the SNP alignment for these two isolates revealed no unique SNPs between them despite the fluconazole susceptibility of Cp23 and fluconazole resistance of Cp40. There is a possibility that the Cp23 DNA sample became contaminated, therefore we removed this isolate from the pool of susceptible controls for subsequent whole genome sequencing analysis. This second phylogenetic analysis also highlighted the close relation between the Bloemfontein isolates Cp1, Cp2, Cp10, Cp12, Cp20, Cp28, Cp 29, all of which possess a

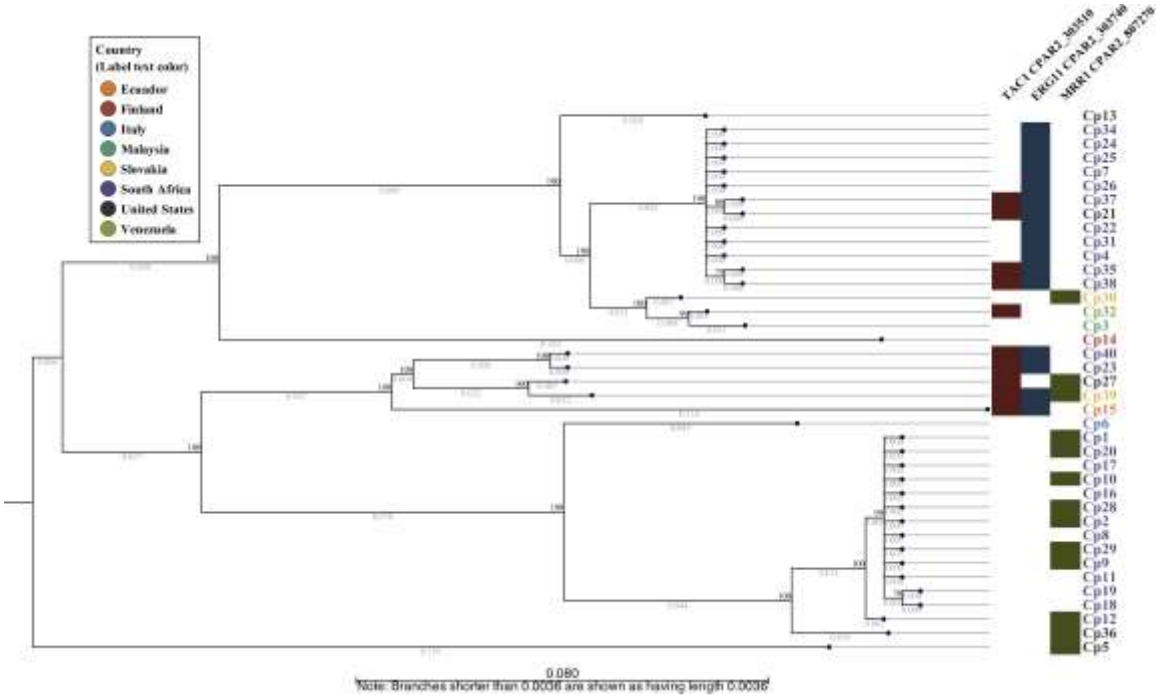


**Figure 4-1. Maximum likelihood phylogeny of 39 clinical *C. parapsilosis* isolates.**

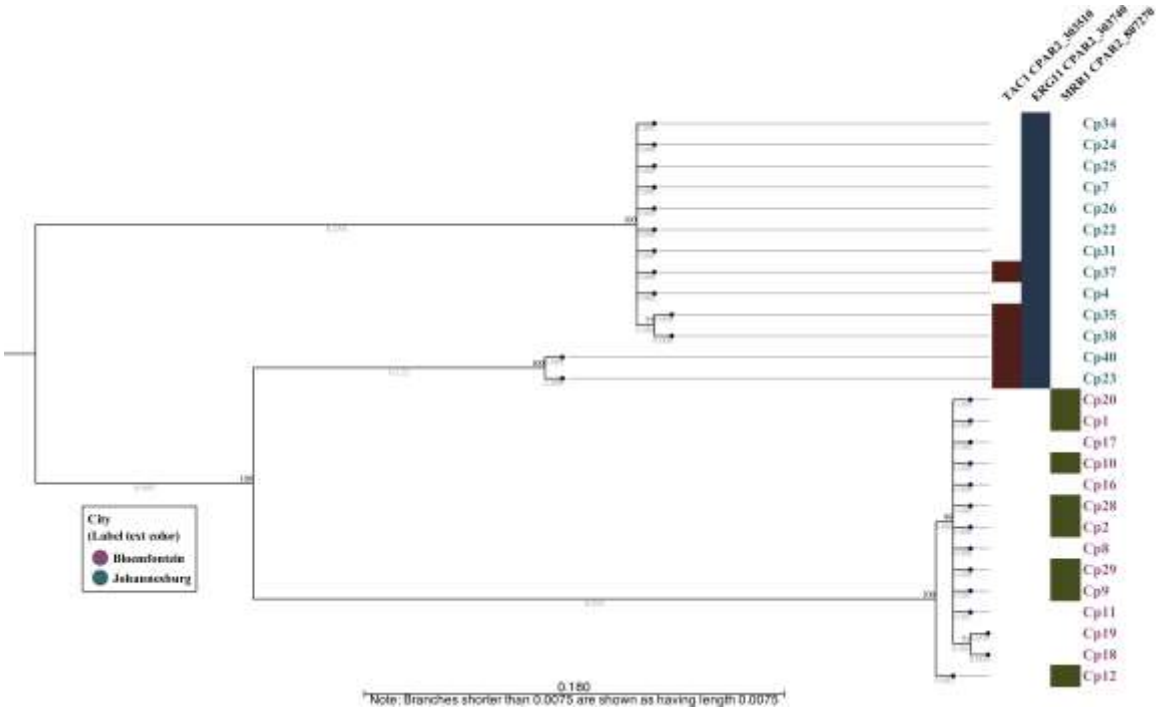


**Figure 4-2. Phylogeny of *C. parapsilosis* isolates from South African hospitals.**

mutation in at least one *CpMRR1* allele, with the isolates Cp8, Cp11, Cp16, Cp17, Cp18, and Cp19, all of which contain the wildtype sequence for *CpMRR1*.

## Copy Number Variation Contributes to Overall Genomic Variability

The total number of nucleotide variants within chromosomes was determined for each genome as mapped to the reference strain CDC317 based on a fixed diploid model. Compared to other *Candida* species *C. parapsilosis* is highly homozygous (24). Additionally, while other *Candida* species have approximately 1 SNP every 200 – 500 bases, *C. parapsilosis* strains exhibit only 1 SNP per 4000 – 15000 bases (24, 34). As such, the total number of detected variants for these clinical isolates was relatively low, ranging from 9104 nucleotide variants for Cp15 to 1963 for Cp14, equating to 1 SNP per 1431 – 6621 bases. To identify potential genetic determinants of resistance within the isolate collection, we considered variants with an associated amino acid substitution, and filtered out those that were present in the susceptible isolates (**Table 4-3**). Overall, the average SNP density for the coding regions of the resistant *C. parapsilosis* isolates was 0.0688 SNPs/kb or 1 SNP every 14536 bp. Resistant isolates Cp30, Cp32, and Cp14 all had the lowest number of mutations leading to amino acid substitution following filtering by those present in the coding regions of susceptible isolates Cp3, Cp5, and Cp13, with 233, 194, and 162 variants respectively. Of these 194 mutations in Cp32, only 35 were found to be homozygous.

Copy number variations were estimated for regions containing *C. parapsilosis* protein coding sequences using an analysis of expected coverage with a minimum length of 50 bases and a conservative p-value of 0.0001 to eliminate identification of false positive coverage regions. The number of gene sequences affected ranged from six genes in isolate Cp7 to an exceptionally high 1750 genes in Cp19. A plurality of genes affected by copy number variations in Cp19 occurred along chromosome 4 (669 protein coding genes), 82.2% (550/669) of which were characterized as deletion events. However, among all resistant isolates the chromosomes with the highest occurrence of duplications and deletions for protein coding genes seemed to be isolate dependent rather than geographically clustered (**Table 4-4**). Together these findings indicate that while *C. parapsilosis* isolates, as a whole, tend to have lower rates of polymorphisms for a diploid species, this does not portend to a lack of genomic diversity for resistant isolates.

## No Putative Gain-Of-Function Mutations in Resistance Associated Genes *CpERG3* and *CpUPC2*

Our characterization of the collection began with a search of the WGS for mutations leading to amino acid substitutions among genes previously shown to influence antifungal susceptibility in *Candida parapsilosis* CpErg3, and CpUpc2. There were no mutations leading to amino acid substitutions in CpErg3 within this collection. A heterozygous mutation in CpUpc2 was identified in isolate Cp36 (N455D) with very low read coverage. However isolate Cp36 also possesses the mutation leading to

**Table 4-3. Number of variants leading to amino acid substitutions in each resistant *C. parapsilosis* isolate.**

| | Variants | | |
|---|---|---|---|
| **Isolate ID** | **Homozygous** | **Heterozygous** | **Total** |
| Cp 1 | 466 | 296 | 762 |
| Cp 2 | 464 | 314 | 778 |
| Cp 4 | 124 | 257 | 381 |
| Cp 6 | 419 | 311 | 730 |
| Cp 7 | 118 | 299 | 417 |
| Cp 8 | 467 | 334 | 801 |
| Cp 9 | 468 | 300 | 768 |
| Cp 10 | 449 | 341 | 790 |
| Cp 11 | 469 | 332 | 801 |
| Cp 12 | 470 | 374 | 844 |
| Cp 14 | 59 | 103 | 162 |
| Cp 15 | 667 | 615 | 1282 |
| Cp 16 | 483 | 271 | 754 |
| Cp 17 | 471 | 292 | 763 |
| Cp 18 | 494 | 273 | 767 |
| Cp 19 | 468 | 284 | 752 |
| Cp 20 | 481 | 335 | 816 |
| Cp 21 | 129 | 268 | 397 |
| Cp 22 | 126 | 232 | 358 |
| Cp 24 | 119 | 282 | 401 |
| Cp 25 | 130 | 249 | 379 |
| Cp 26 | 125 | 313 | 438 |
| Cp 27 | 328 | 450 | 778 |
| Cp 28 | 460 | 351 | 811 |
| Cp 29 | 467 | 315 | 782 |
| Cp 30 | 49 | 184 | 233 |
| Cp 31 | 126 | 301 | 427 |
| Cp 32 | 35 | 159 | 194 |
| Cp 34 | 124 | 279 | 403 |
| Cp 35 | 125 | 253 | 378 |
| Cp 36 | 450 | 328 | 778 |
| Cp 37 | 118 | 301 | 419 |
| Cp 38 | 125 | 276 | 401 |
| Cp 39 | 355 | 464 | 819 |
| Cp 40 | 225 | 308 | 533 |

Note: Number of variants present in coding regions, following fixed ploidy (2x) alignment with reference genome, that were not identified in susceptible isolates Cp3, Cp5, or Cp13 that lead to amino acid substitutions based on the alternative yeast nuclear codon usage for *C. parapsilosis.*

**Table 4-4. Number of genes affected by copy number variation for each isolate.**

| Isolate ID | Chromosome number | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | |
| Cp 1 | 0 | 4 | 0 | 6 | 0 | 2 | 4 | 7 | 23 |
| Cp 2 | 1 | 3 | 1 | 1 | 4 | 2 | 4 | 11 | 27 |
| Cp 4 | 33 | 1 | 3 | 4 | 0 | 0 | 2 | 4 | 47 |
| Cp 6 | 3 | 6 | 7 | 3 | 11 | 8 | 12 | 7 | 57 |
| Cp 7 | 0 | 0 | 2 | 1 | 1 | 0 | 2 | 0 | 6 |
| Cp 8 | 0 | 3 | 2 | 4 | 5 | 2 | 7 | 6 | 29 |
| Cp 9 | 0 | 4 | 5 | 4 | 1 | 0 | 7 | 5 | 26 |
| Cp 10 | 0 | 2 | 1 | 0 | 1 | 0 | 8 | 6 | 18 |
| Cp 11 | 26 | 11 | 1 | 115 | 26 | 7 | 8 | 3 | 197 |
| Cp 12 | 0 | 4 | 1 | 4 | 5 | 13 | 15 | 20 | 62 |
| Cp 14 | 1 | 0 | 2 | 2 | 2 | 17 | 0 | 0 | 24 |
| Cp 15 | 3 | 3 | 9 | 3 | 7 | 3 | 5 | 6 | 39 |
| Cp 16 | 0 | 70 | 0 | 6 | 0 | 2 | 6 | 13 | 97 |
| Cp 17 | 0 | 192 | 1 | 5 | 1 | 2 | 11 | 7 | 219 |
| Cp 18 | 3 | 5 | 0 | 5 | 1 | 2 | 3 | 13 | 32 |
| Cp 19 | 8 | 215 | 103 | 669 | 286 | 83 | 188 | 198 | 1750 |
| Cp 20 | 0 | 5 | 1 | 6 | 4 | 2 | 7 | 6 | 31 |
| Cp 21 | 1 | 0 | 2 | 1 | 1 | 1 | 2 | 0 | 8 |
| Cp 22 | 1 | 1 | 3 | 1 | 1 | 25 | 35 | 0 | 67 |
| Cp 24 | 1 | 0 | 3 | 3 | 4 | 4 | 2 | 0 | 17 |
| Cp 25 | 0 | 0 | 0 | 1 | 6 | 10 | 2 | 0 | 19 |
| Cp 26 | 4 | 0 | 2 | 1 | 6 | 8 | 2 | 0 | 23 |
| Cp 27 | 4 | 0 | 3 | 8 | 32 | 1 | 3 | 4 | 55 |
| Cp 28 | 0 | 3 | 0 | 2 | 1 | 2 | 15 | 7 | 30 |
| Cp 29 | 1 | 2 | 0 | 5 | 0 | 2 | 4 | 7 | 21 |
| Cp 30 | 3 | 2 | 0 | 0 | 3 | 0 | 1 | 0 | 9 |
| Cp 31 | 125 | 0 | 0 | 4 | 6 | 5 | 3 | 0 | 143 |
| Cp 32 | 1 | 3 | 0 | 4 | 0 | 23 | 2 | 1 | 34 |
| Cp 34 | 108 | 236 | 97 | 287 | 215 | 19 | 137 | 54 | 1153 |
| Cp 35 | 109 | 69 | 131 | 101 | 132 | 14 | 61 | 51 | 668 |
| Cp 36 | 24 | 243 | 2 | 25 | 39 | 9 | 231 | 297 | 870 |
| Cp 37 | 47 | 1 | 107 | 24 | 38 | 2 | 51 | 318 | 588 |
| Cp 38 | 132 | 76 | 129 | 185 | 104 | 4 | 69 | 54 | 753 |
| Cp 39 | 40 | 38 | 74 | 68 | 90 | 3 | 39 | 27 | 379 |
| Cp 40 | 15 | 0 | 10 | 1 | 4 | 0 | 6 | 1 | 37 |

Note: Number of genes with copy number variation determined by a fixed ploidy of 2.0. Only genes with fold change $\geq 1.5$ or $\leq -1.5$ and p-value $\leq 0.05$. Susceptible isolate Cp13 used for comparison control.

CpMrr1$^{I283R}$ which, when corrected to wild-type, decreased fluconazole MICs from 64μg/mL to 2μg/mL (277). Therefore, the presence of this heterozygous mutation in *CpUPC2* likely has little impact on the clinically relevant fluconazole resistance within this isolate.

## Identification of Resistance Associated Genes Encoded in the *C. parapsilosis* Genome

The lack of mutation within previously characterized genes within Cp6, Cp8, Cp11, Cp14, Cp16, Cp17, Cp18, and Cp19, each of which demonstrates fluconazole MICs above the clinical breakpoint made them prime targets in our search for novel resistance mechanisms in *C. parapsilosis*. Due to number of non-synonymous mutations within the fluconazole resistant clinical isolates, we sought to use transcriptomics to identify potential associations between observed genotypes known resistance effectors. To do so we curated a list of putative ABC and MFS transporters along with the ZCFs in *C. parapsilosis.*

To identify the number of putative ABC transporters encoded in the *C. parapsilosis* genome, a search was performed from the predicted *C. parapsilosis* protein domains downloaded from the *Candida* genome database. Genes were filtered for "ABC" and "AAA" terms within Interpro descriptions, then for listing the Pfam domain pf00005, and, finally, genes without the identification of transmembrane domains listed by TMHMM were eliminated, revealing a list of putative 27 ABC protein genes. This list was then compared to the identification of ABCG/PDR genes (KOG0065) and ABCC/MRP genes (KOG0054) by the EggNOG 6.0.0 website. A total of 11 putative ABCG transporters, containing 1 classified pseudogene, and 10 ABCC transporters containing 1 classified pseudogene were identified within the *Candida parapsilosis* reference genome CDC317 (**Table 4-5**).

Syntenic and phylogenetic analysis by Dias et al. identified 12, 18, 18, and 10 full length DHA1 transporters that have been identified within the reference genomes for *S. cerevisiae*, *C. albicans*, *C. tropicalis*, and *C glabrata* strains respectively (161). Based on sequence similarity, they were also able to identify 31 potential DHA1 transporters in *C. parapsilosis*, however further topology analysis revealed that 28 genes contained sequences capable of the forming the necessary 12 transmembrane helices for DHA1 classification (**Table 4-6**) (161). Potential ZCF genes were identified through a blastp of a typical DNA binding motif along with a search for associated interpro domains curated by the *Candida* genome database.

The number of ZCFs encoded in the genome varies between species. The *C. albicans* genome encodes a large number with 82 ZCFs, while *S. cerevisiae* and closely related *C. glabrata* only encode 55 and 41 ZCFs, respectively (169, 171, 288). Our analysis of orthologous ZCF genes along with interpro classification and blastp searches for the cysteine rich consensus sequence confirms a total of 69 ZCF proteins are encoded in the *C. parapsilosis* reference genome (**Table 4-7**).

**Table 4-5. Putative ATP-binding cassette transporters encoded in *C. parapsilosis* genome.**

| Gene ID | *C. parapsilosis* gene | *S. cerevisiae* homolog | *C. albicans* homolog | Protein ID |
|---|---|---|---|---|
| **ABCG-PDR Transporters** | | | | |
| CPAR2_108270 | | *YOL075C* | *ROA1* | G8B8K4 |
| CPAR2_205160 | | *ADP1* | *ADP1* | G8BG89 |
| CPAR2_300010 | | *PDR5* | *CDR1* | pseudogene |
| CPAR2_304370 | *CDR1B* | *PDR5* | *CDR1* | G8B9Z6 |
| CPAR2_403600 | | *PDR5* | *CDR3* | G8BIU2 |
| CPAR2_405280 | | *PDR5* | *CDR1* | G8BJA9 |
| CPAR2_405290 | *CDR1* | *PDR5* | *CDR1* | G8BJB0 |
| CPAR2_600730 | | *PDR15* | *CDR4* | G8B4W5 |
| CPAR2_600750 | | *SNQ2* | *SNQ2* | G8B4W7 |
| CPAR2_603800 | | *PDR5* | *CDR1* | G8B5S0 |
| CPAR2_700030 | | *PDR5* | *CDR1* | G8BL10 |
| **ABCC-MRP Transporters** | | | | |
| CPAR2_103160 | | *YBT1* | *YCF1* | G8B746 |
| CPAR2_205440 | | *YOR1* | *YOR1* | G8BGB7 |
| CPAR2_205610 | | *BPT1* | CR_08200C_A | G8BGD4 |
| CPAR2_207420 | *MLT1* | *YCF1* | *MLT1* | G8BCV4 |
| CPAR2_207430 | | *YCF1* | *MLT1* | G8BCV5 |
| CPAR2_407200 | | *YOR1* | *YOR1* | G8BJV2 |
| CPAR2_407510 | | *YOR1* | *YOR1* | pseudogene |
| CPAR2_503260 | | *BPT1* | *MLT1* | G8BGV3 |
| CPAR2_702790 | | *YCF1* | *YCF1* | G8BKK0 |
| CPAR2_703390 | | *YBT1* | *YCF1* | G8BKR0 |

Note: Homologs listed for *S. cerevisiae* and *C. albicans* represent orthologs or 'best hits' to the identified *C. parapsilosis* gene. Data Sources: Skrzypek MS BJ, Binkley G, Miyasato SR, Simison M, and Sherlock G. 2022. *Candida* Genome Database. www.candidagenome.org. (280); Binkley J, Arnaud MB, Inglis DO, Skrzypek MS, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G. 2013. The *Candida* Genome Database: The new homology information page highlights protein similarity and phylogeny. Nucleic Acids Research 42:D711-D716. https://doi.org/10.1093/nar/gkt1046. (281); Uniprot-Consortium. 2022. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research 51:D523-D531. https://doi.org/10.1093/nar/gkac1052. (282).

**Table 4-6. Major facilitator superfamily type 1 drug-hydrogen antiporters encoded in the *C. parapsilosis* genome.**

| Gene ID | *C. parapsilosis* gene | *S. cerevisiae* homolog | *C. albicans* homolog | Protein ID |
|---|---|---|---|---|
| **MFS-DHA1+ Transporters** | | | | |
| CPAR2_100470 | *HBT3* | *QDR1* | orf19.341 | G8B6C7 |
| CPAR2_300670 | | *TPO1* | *FLU1* | G8B8X9 |
| CPAR2_300760 | | *TPO1* | *FLU1* | G8B8Y8 |
| CPAR2_300740 | | *TPO1* | *FLU1* | G8B8Y6 |
| CPAR2_203870 | | *DTR1* | CR_04620C_A | G8BFW0 |
| CPAR2_300750 | | *TPO1* | *FLU1* | G8B8Y7 |
| CPAR2_300680 | | *TPO1* | *FLU1* | G8B8Y0 |
| CPAR2_804630 | | *HOL1* | C1_10200C_A | G8BA41 |
| CPAR2_202130 | | *QDR1* | *QDR1* | G8BFD6 |
| CPAR2_102760 | | *TPO3* | C3_03440C_A | G8B706 |
| CPAR2_300730 | | *TPO1* | *FLU1* | G8B8Y5 |
| CPAR2_300590 | | *TPO1* | *FLU1* | G8B8X1 |
| CPAR2_300770 | | *TPO1* | *TPO2* | G8B8Y9 |
| CPAR2_204840 | *HBT2* | *TPO2* | orf19.341 | G8BG57 |
| CPAR2_700120 | | *TPO3* | *NAG4* | G8BL19 |
| CPAR2_504220 | | *TPO1* | *FLU1* | G8BH50 |
| CPAR2_700110 | *NAG4* | *TPO3* | *NAG3* | G8BL18 |
| CPAR2_804310 | *TPO3* | *TPO2* | *TPO3* | G8BA09 |
| CPAR2_808400 | | *HOL1* | orf19.2517 | G8BB67 |
| CPAR2_603010 | *MDR1B* | *FLR1* | *MDR1* | G8B5J0 |
| CPAR2_301760 | *MDR1* | *FLR1* | *MDR1* | G8B987 |
| CPAR2_704330 | *HBT1* | *TPO2* | orf19.341 | G8BL03 |
| CPAR2_108860 | | *HOL1* | *HOL4* | G8B5T8 |
| CPAR2_207540 | | *FLR1* | *MDR1* | G8BCW6 |
| CPAR2_602760 | | *QDR3* | *QDR3* | G8B5G5 |
| CPAR2_100460 | *HBT4* | *QDR1* | orf19.341 | G8B6C6 |
| CPAR2_202420 | | *TPO4* | *TPO4* | G8BFG5 |
| CPAR2_802890 | | *HOL1* | CR_01340W_A | G8BC74 |

Note: Homologs for *S. cerevisiae* and *C. albicans* represent orthologs or 'best hits' for *C. parapsilosis* gene. Data Sources: Dias PJ, Sá-Correia I. 2014. Phylogenetic and syntenic analyses of the 12-spanner drug:H(+) antiporter family 1 (DHA1) in pathogenic *Candida* species: evolution of *MDR1* and *FLU1* genes. Genomics 104:45-57. https://doi.org/10.1016/j.ygeno.2014.05.005 (161); Skrzypek MS BJ, Binkley G, Miyasato SR, Simison M, and Sherlock G. 2022. *Candida* Genome Database. (280); Uniprot-Consortium. 2022. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research 51:D523-D531. https://doi.org/10.1093/nar/gkac1052. (282).

**Table 4-7. Zinc-cluster transcription factors encoded in the *C. parapsilosis* genome.**

| Gene ID | *C. parapsilosis* gene | *S. cerevisiae* homolog | *C. albicans* homolog | Protein ID |
|---|---|---|---|---|
| **$CysX_2CysX_6CysX_{5-12}CysX_2CysX_{6-8}Cys$ DNA binding motif** | | | | |
| CPAR2_704130 | | *OAF1* | *ZCF29* | G8BKY3 |
| CPAR2_405400 | | *UME6* | *WOR2* | G8BJC1 |
| CPAR2_700930 | | *LYS14* | *LYS143* | G8BLA0 |
| CPAR2_400210 | | *UME6* | *UME7* | G8BHV6 |
| CPAR2_805700 | | *UPC2* | *RHA1* | G8BAE8 |
| CPAR2_807270 | *MRR1* | *PIP2* | *MRR1* | G8BAV4 |
| CPAR2_401970 | | *THI2* | *AHR1* | G8BID1 |
| CPAR2_400190 | | *RGT1* | *RGT1* | G8BHV4 |
| CPAR2_101730 | | *LEU3* | *LEU3* | G8B6Q3 |
| CPAR2_400920 | | *YKL222C* | *ZCF27* | G8BI25 |
| CPAR2_108570 | | *ARO80* | *ARO80* | G8B8N4 |
| CPAR2_700900 | | *LYS14* | *LYS142* | G8BL97 |
| CPAR2_503620 | | *MAL13* | C3_01590W_A | G8BGZ0 |
| CPAR2_700130 | | *MAL13* | *SUC1* | G8BL20 |
| CPAR2_501580 | | *HAP1* | *ZCF13* | G8BHK8 |
| CPAR2_202000 | | *UPC2* | *ECM22* | G8BFC4 |
| CPAR2_103120 | | | *ZCF8* | G8B742 |
| CPAR2_303520 | | *HAL9* | *HAL9* | G8B9R2 |
| CPAR2_303510 | *TAC1* | *HAL9* | *TAC1* | G8B9R1 |
| CPAR2_700880 | | *ARG81* | *ARG81* | G8BL95 |
| CPAR2_405770 | | *TEA1* | *TEA1* | G8BJF9 |
| CPAR2_302900 | | *THI2* | *ZCF4* | G8B9K0 |
| CPAR2_401480 | | *ASG1* | *ZCF15* | G8BI81 |
| CPAR2_406460 | | *CAT8* | *ZCF10* | G8BJM7 |
| CPAR2_109760 | | *STB5* | *STB5* | G8B610 |
| CPAR2_208030 | | *GSM1* | *ZCF23* | G8BD15 |
| CPAR2_204560 | | *RDS2* | *CWT1* | G8BG29 |
| CPAR2_203610 | | | *ZCF3* | G8BFT4 |
| CPAR2_602060 | | *LYS14* | *ZCF18* | G8B595 |
| CPAR2_704370 | | *ASG1* | *ZCF25* | G8BL07 |
| CPAR2_110360 | | *WAR1* | *WAR1* | G8B669 |
| CPAR2_502960 | | *LYS14* | *LYS144* | G8BGS4 |
| CPAR2_501640 | | *HAP1* | *ZCF20* | G8BHL4 |
| CPAR2_501570 | | *OAF1* | *ZCF14* | G8BHK7 |
| CPAR2_101530 | | *PPR1* | *PPR1* | G8B6N3 |
| CPAR2_207280 | *UPC2* | *UPC2* | *UPC2* | G8BCU0 |
| CPAR2_802610 | | *ERT1* | *ZCF11* | G8BC46 |
| CPAR2_803820 | | *UME6* | *UME6* | G8BCG7 |
| CPAR2_103550 | | *SIP4* | *ZCF16* | G8B784 |
| CPAR2_501290 | | *UME6* | *CZF1* | G8BHH9 |
| CPAR2_802630 | | *ASG1* | *ASG1* | G8BC48 |
| CPAR2_200790 | *UGA3* | *UGA3* | *UGA3* | G8BEX8 |
| CPAR2_210200 | | *PDR1* | *CTF1* | G8BDM8 |

**Table 4 7. Continued.**

| Gene ID | *C. parapsilosis* gene | *S. cerevisiae* homolog | *C. albicans* homolog | Protein ID |
|---|---|---|---|---|
| CPAR2_404560 | | | *ZCF31* | G8BJ37 |
| CPAR2_303270 | | *SEF1* | *SEF2* | G8B9N7 |
| CPAR2_808620 | | *CAT8* | *FCR1* | G8BB89 |
| CPAR2_207490 | | | *ZCF30* | G8BCW1 |
| CPAR2_807260 | | *PIP2* | *ZCF35* | G8BAV3 |
| CPAR2_405260 | | *OAF1* | *CTA4* | G8BJA7 |
| CPAR2_213280 | | *ARG81* | *ZCF9* | G8BEP7 |
| CPAR2_400180 | | *ARG81* | *ARG83* | G8BHV3 |
| CPAR2_800040 | | *TEA1* | *ZCF38* | G8BBD7 |
| CPAR2_801430 | | *SEF1* | *SEF1* | G8BBS6 |
| CPAR2_207400 | *CAT8* | *CAT8* | *CAT8* | G8BCV2 |
| CPAR2_401180 | | *ECM22* | *ZCF21* | G8BI51 |
| CPAR2_806830 | | *LYS14* | *AHR1* | G8BAR0 |
| CPAR2_600300 | | *LYS14* | *LYS14* | G8B4S5 |
| CPAR2_302880 | | *RDR1* | *FGR27* | G8B9J8 |
| CPAR2_800890 | *DAL81* | *DAL81* | *DAL81* | G8BBM2 |
| CPAR2_204950 | | | *ZCF19* | G8BG68 |
| CPAR2_303500 | | *STB4* | *ZNC1* | G8B9R0 |
| CPAR2_107840 | | *STB5* | *STB5* | G8B8G4 |
| CPAR2_302555 | | *SUT1* | *SUT1* | G8B9G7 |
| CPAR2_101320 | | *STB4* | *CTA7* | G8B6L2 |
| CPAR2_405270 | | *OAF3* | *CTA4* | G8BJA8 |
| CPAR2_407920 | | *GAL4* | *GAL4* | G8BK22 |
| CPAR2_208790 | *PUT3* | *PUT3* | *PUT3* | G8BD88 |
| CPAR2_109790 | | *UPC2* | *ZCF17* | G8B613 |
| CPAR2_102000 | | | *ZCF1* | G8B6T0 |

Note: Homologs for *S. cerevisiae* and *C. albicans* represent orthologs or 'best hits' for *C. parapsilosis* gene. Data Sources: Skrzypek MS BJ, Binkley G, Miyasato SR, Simison M, and Sherlock G. 2022. *Candida* Genome Database. www.candidagenome.org. (280); Uniprot-Consortium. 2022. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research 51:D523-D531. https://doi.org/10.1093/nar/gkac1052 (282).

**Distinct Expression Patterns of Identified ABC and MFS Transporters**

Compared to the expression in the susceptible control strains, there was elevated *CpCDR1B* for all isolates with homozygous mutations in *CpMRR1,* as well as in most of the isolates with heterozygous *CpMRR1* mutations (**Figure 4-3**). Similarly, we observed slight upregulation of *CpCDR1* (CPAR2_405290), *CpCDR1B* (CPAR2_304370), and *CpCDR1C* (CPAR2_300010) among the CpTac1$^{G650E}$ isolates (**Figure 4-3**). The expression of ABCG transporter *CpCDR1B* was elevated >2 fold in 18 of the 35 resistant isolates, 7 of which, Cp4, Cp7, Cp14, Cp22, Cp25, Cp31, and Cp34, are wildtype at both the *CpMRR1* and *CpTAC1* loci. For five of these isolates, increased *CpCDR1B* expression coincided with increased incidence of duplication events at the *CpCDR1B* loci (Cp4: 2, Cp7: 2, Cp14: 4, Cp22: 3, Cp34: 7). Increased expression of CPAR2_207420 (a putative transporter gene orthologous to *MLT1* in *C. albicans* and *YBT1* in *S. cerevisiae*) and CPAR2_407200 (a *C. albicans YOR1* ortholog) was observed among the isolates from Bloemfontein, South Africa including Cp8, Cp11, Cp16, Cp17, Cp18, and Cp19. In *S. cerevisiae*, the transporter Ybt1 has been shown to actively pump azoles into the vacuole, thus preventing the ability of the drug to bind Erg11 within the membrane of the endoplasmic reticulum (150). *C. albicans* transporter Yor1 has been associated with altered susceptibility to beauvericin and Hsp90 inhibiting drugs, however no effect on fluconazole susceptibility has been observed for this organism (289, 290).

Increased expression of the MFS transporter gene *CpMDR1B* (CPAR2_603010) has been shown to decrease fluconazole susceptibility in *C. parapsilosis* (277). Expression of *CpMDR1B* was upregulated > 2-fold (FDR p-value ≤ 0.05) in twenty of the resistant isolates (**Figure 4-4**), including six out of the eight isolates for which no other known resistance mechanism is present (Cp8, Cp11, Cp16, Cp17, Cp18, and Cp19). Other MFS transporter genes also displayed low level differential expression; however it is unknown at this time how those transporters influence fluconazole resistance.

**Transcriptional Analysis of Ergosterol Biosynthesis Genes and ZCF Genes Revealed Little About Underlying Fluconazole Resistance Mechanisms**

Some zinc cluster transcription factors involved in stress response and xenobiotic resistance have been reported as having some degree of autoregulatory function, therefore we examined each resistant clinical isolate for ZCF differential expression. Out of the 69 identified zinc-cluster transcription factors in the *C. parapsilosis* genome, 40 were shown to be increased and 45 were decreased within at least one resistant isolate compared to fluconazole susceptible isolates (**Figure 4-5**). The ZCF gene increased in the largest number of resistant isolates was CPAR2_803820, a homolog of the C. *albicans UME6*. While there were two mutations identified within CPAR2_803820 in a number of isolates, neither was unique to fluconazole-resistant isolates. Of the genes with the highest incidence for mutation among the resistant isolates only CPAR2_704130 presented any appreciable change in expression. Similarly, many resistant isolates reported non-synonymous mutations in at least one allele of CPAR2_107840 (28/35),

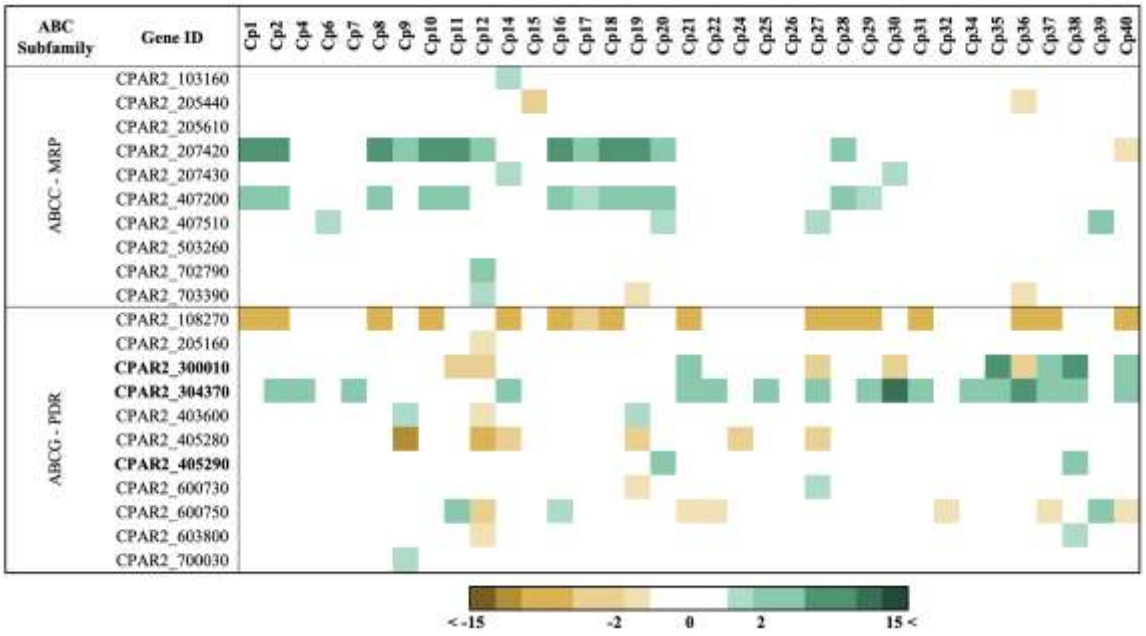


**Figure 4-3. Differential expression of ABCG and ABCC transporter genes in resistant isolates.**

Note: Bolded Gene IDs represent those of *CpCDR1* (CPAR2_405290), *CpCDR1B* (CPAR2_304370), and *CpCDR1C* (CPAR2_300010). Differential expression values from pairwise comparisons for each fluconazole resistant *C. parapsilosis* isolate against four fluconazole susceptible isolates. Only fold changes ≥ 2 or ≤ -2 with an FDR p-value of ≤ 0.05 are color coded.

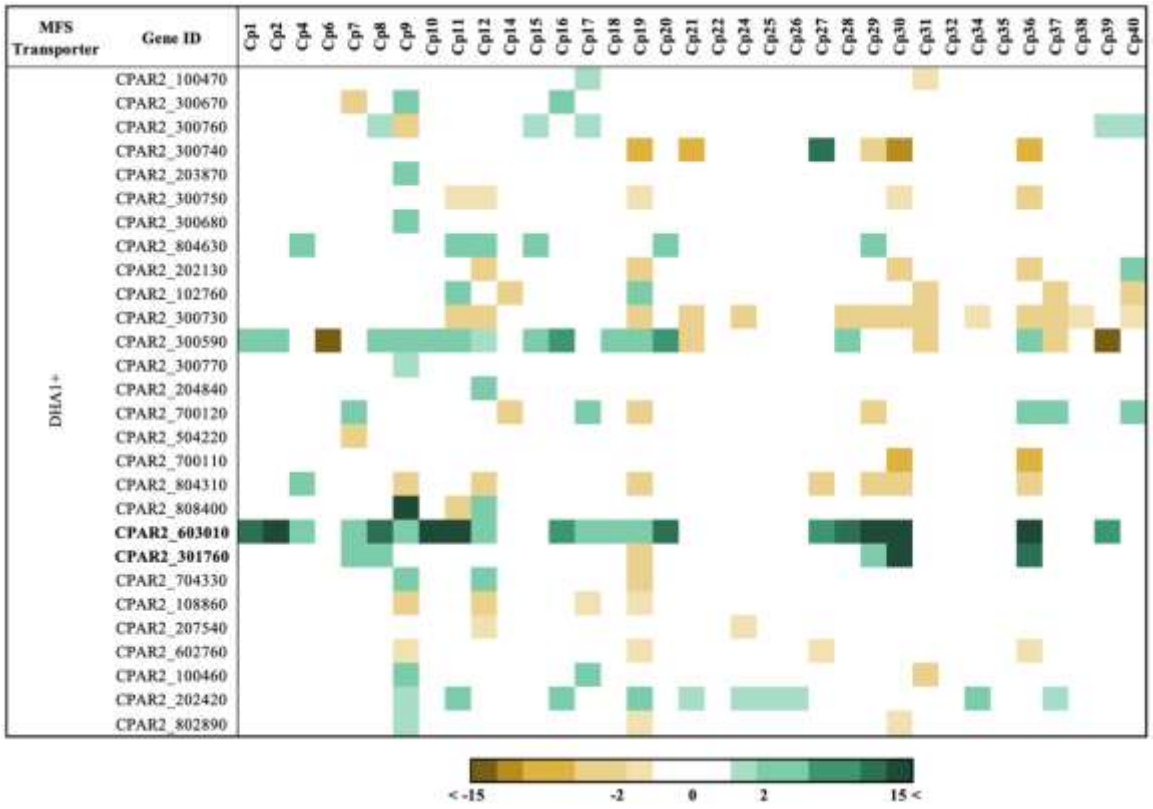


**Figure 4-4. Differential expression of MFS-DHA1 transporter genes in resistant isolates.**

Note: Bolded Gene IDs represent those of *CpMDR1* (CPAR2_301760) and *CpMDR1B* (CPAR2_603010). Differential expression values from pairwise comparisons for each fluconazole resistant *C. parapsilosis* isolate against four fluconazole susceptible isolates. Only fold changes ≥ 2 or ≤ -2 with an FDR p-value of ≤ 0.05 are color coded.


**Figure 4-5. Differential expression of zinc cluster transcription factor genes in resistant isolates.**

Note: Differential expression values from pairwise comparisons for each fluconazole resistant *C. parapsilosis* isolate against four fluconazole susceptible isolates. Only fold changes ≥ 2 or ≤ -2 with an FDR p-value of ≤ 0.05 are color coded.

CPAR2_704130 (20/35), CPAR2_805700 (17/35), CPAR2_101730 (17/35), and CPAR2_502960 (17/35). However, due to the low number of susceptible isolates used for comparison it is unfair to claim any association to fluconazole resistance at this time.

Of the six isolates, Cp8, Cp11, Cp16, Cp17, Cp18, and Cp19 with upregulated *CpMDR1B* but no mutations in *CpMRR1*, four isolates, Cp8 Cp11, Cp16 and Cp18 also had increased expression of the *ZCF29* homolog CPAR2_704130 (*CpZCF29*). Whole genome sequencing revealed sixteen isolates, including Cp8, Cp11, Cp16, and Cp18 contained a mutation in *CpZCF29* leading to the substitution N861K. The combined sequencing data implied the potential for a novel regulator of *CpMDR1B* which, in turn, would imply a direct impact on clinical fluconazole susceptibility. Utilizing the plasmid-based CRISPR Cas9 system for single-base editing we had previously employed for manipulations in *CpMRR1* and *CpTAC1*, we placed the C2583G mutation into both alleles of *CpZCF29* in the susceptible isolates Cp13 and Cp5. Transformants were confirmed via sequencing, however antifungal susceptibility testing by broth microdilution revealed no change in fluconazole MIC.

Only a few of the ergosterol biosynthesis genes were regulated differentially within the *C. parapsilosis* resistant isolates (**Figure 4-6**). Apart from the expression of *CpUPC2* and *CpERG11*, any differential expression as measured by fold change (FC) was relatively discreet (decreased FC between -3 and -2; increased FC between 2.0 and 2.5). Whole genome sequencing analysis confirmed 20 of the 39 clinical isolates contained mutation in at least one allele *CpERG11*. Only 2 resistant isolates were found to overexpress *CpERG11* when compared to the susceptible isolates, Cp15 (FC: 3.53) and Cp27 (FC: 3.63). Neither isolate contained a mutation within *CpUPC2* and the expression of *CpUPC2* was found to be slightly decreased for both isolates (Cp27 FC: -2.28, Cp15 FC: -1.54).

## Discussion

Our investigations into fluconazole resistance mechanisms within our collection of 35 resistant *C. parapsilosis* isolates show how much is still unknown about this pathogen. In contrast to previous phylogenetic analysis of *C. parapsilosis* that demonstrated clustering of isolates based on isolation context rather than geographical source, our phylogenetic trees based on SNP alignments demonstrated distinctive geographical clustering for these isolates (34). The amount of single nucleotide variants within this collection is comparable to previously published genomes and isolate Cp14 showed incredible similarity to the reference genome. Despite genetic similarity, CDC317 and Cp14 are both distinct both in their isolation context and their geographic origin. CDC317 was isolated from the hands of a healthcare worker during an outbreak of candidemia in a community hospital in Mississippi, USA in 2001 (291) while Cp14 was isolated from an infant with nosocomial candidemia in Helsinki, Finland in 2002. Interestingly, both isolates lack mutations in CpTac1 and CpMrr1 and while CDC317 is heterozygous for the mutation leading to CpErg11$^{Y132F}$, isolate Cp14 (CpErg11$^{WT}$) demonstrates a 4-fold higher fluconazole MIC.

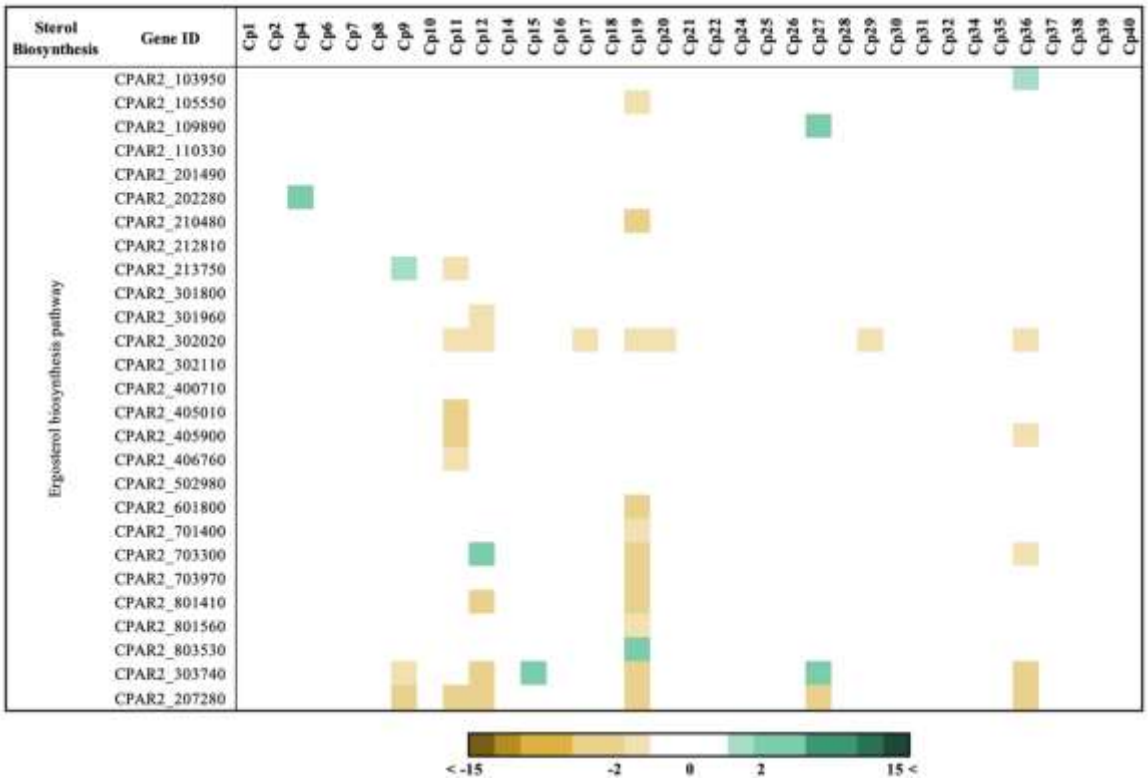


**Figure 4-6. Differential expression of ergosterol biosynthesis pathway genes in resistant isolates.**

Note: Differential expression values from pairwise comparisons for each fluconazole resistant *C. parapsilosis* isolate against four fluconazole susceptible isolates. Only fold changes ≥ 2 or ≤ -2 with an FDR p-value of ≤ 0.05 are color coded.

The Y132F substitution in CpErg11 has been identified in *C. parapsilosis* isolates all over the world (104). Additionally, CpErg11$^{Y132F}$ has been associated with clonal *C. parapsilosis* strains that are capable forming endemic, and in some cases persistent, candidemia outbreaks within healthcare facilities (76, 183). Our phylogenetic analysis lends support to both of these previous findings with CpErg11$^{Y132F}$ identified among isolates from multiple countries and among the closely-related cluster isolated from a single South African health center in Johannesburg between 2001 and 2009. While CpErg11$^{Y132F}$ has not been linked to any cell fitness advantage, the ability this mutation to persist even in the absence of triazole selective pressure is concerning due to the increased negative patient outcomes associated with candidemia by fluconazole-resistant *C. parapsilosis* (179). Interestingly, a similar clustering was observed among those isolates collected between 2001 and 2005 from Bloemfontein, South Africa, none of which contained CpErg11$^{Y132F}$. The lack of CpErg11$^{Y132F}$ implies other *C. parapsilosis* fluconazole resistance mechanisms are also capable of persisting in healthcare facilities however further *C. parapsilosis* surveillance and genotyping is necessary.

Interesting variation in gene copy numbers were observed for genes involved in *C. parapsilosis* fluconazole resistance. The varied number of duplications surrounding *CpCDR1B* near the telomeric end of chromosome 3 show promising associations to fluconazole resistance (292). Unfortunately, sequence similarity between *CpCDR1B* and other ABCG transporter genes make further analysis of this region difficult due to the length of reads used in our whole genome sequencing. Longer read sequencing techniques would be needed to understand exactly how many multiple intact copies of the *CpCDR1B* may be present in each isolate and their orientation along the chromosome.

Our differential expression analysis for potential ABCC drug transporters revealed promising candidates for future studies, particularly, CPAR2_ 407200 and CPAR2_207420 which have not been studied in *C. parapsilosis* as of this writing. Additionally, the decreased expression of CPAR2_108270, a putative ABCG transporter orthologous to *C. albicans CDR6,* and formerly referred to as *ROA1,* was observed among 15 resistant isolates. In *Candida albicans,* loss of *CDR6* results in altered TOR (Target-Of-Rapamycin) signaling contributes to fluconazole resistance through increased membrane rigidity and subsequent decreased azole accumulation inside the cell (293). No studies have investigated a role for CPAR2_108270 in *C. parapsilosis*. Our analysis of putative ZCFs in *C. parapsilosis* revealed very few associations between differential expression and the presence of a potential gain-of-function mutation. Of the six *CpMRR1$^{WT}$* isolates with upregulated *CpMDR1B* (Cp8, Cp11, Cp16, Cp17, Cp18, and Cp19), four exhibited increased expression of *CpZCF29* along with the N861K substitution (Cp8 Cp11, Cp16 and Cp18). However, when we placed CpZcf29$^{N861K}$ into two susceptible backgrounds, there was no change in fluconazole MICs.

Transcriptional analysis of ergosterol biosynthesis genes revealed increased *CpERG11* expression in only two clinical isolates. This is vastly different from observations made in C. *albicans,* where analysis of 63 clinical isolates found 74.6% of them overexpressed *ERG11,* and 46% displayed associated mutations in *UPC2* (139). Isolate Cp36 was the only isolate to contain a heterozygous non-synonymous mutation in

*CpUPC2*. Conversely, Cp36 exhibited decreased expression for both *CpUPC2* and *CpERG11* (FC: -2.28 and FC: -2.41, respectively) indicating that the presence of the heterozygous mutation does not provide a gain-of-function in terms of fluconazole resistance. A cursory look at the copy number variable regions around the *CpERG11* loci for the two overexpressing isolates revealed a sharp increase in coverage right before the start codon in Cp15 and an apparent duplication across the entire open reading frame in Cp27. Increased *ERG11* and *TAC1* copy number has been shown to result in the rapid development of fluconazole resistance in C. *albicans* (175) and could be an area for future examination in *C. parapsilosis*.

# CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

**NOTE:** When using Adobe Acrobat, return to the last viewed page using quick keys Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For the next page, use Alt/Ctrl or Command + Right Arrow. See **Preface** for further details.

*Candida parapsilosis* is a significant pathogen in neonatal, pediatric, and critically ill populations (3, 4, 11). The ability to form robust biofilms, proliferate on a variety of biosynthetic materials, and the well-documented history of healthcare worker to patient transmission highlight some of the species-specific challenges associated with *C. parapsilosis* candidiasis (14, 19, 30, 36). Additionally, the persistence of *C. parapsilosis* within healthcare facilities has led to outbreaks of candidemia by both resistant and non-resistant clonal strains (179). Polyenes, echinocandins, and azoles are currently the only antifungal drugs classes approved for the treatment of invasive *Candida* infections and candidemia (58){Demir 2022}. While echinocandins are the recommended first line antifungal therapy in the United States and in much of Europe, there can be hesitation in its use for *C. parapsilosis* etiological infection due to increased MICs in vitro and increased incidence of *C. parapsilosis* associated with increased echinocandin usage (58, 122). Additionally, there are parts of South America, Western Asia, and Africa, where *C. parapsilosis* is the predominant NAC spp., where the widespread use of echinocandins is cost prohibitive. For these areas, rising rates of resistance to the alternative first-line antifungal drug used to treat invasive candidiasis represents a significant therapeutic concern. Historically, presumed mechanisms of fluconazole resistance in *C. parapsilosis* were based on studies performed in other *Candida* species, namely *C. albicans*. However, as this body of work demonstrates, fluconazole resistance among *C. parapsilosis* clinical isolates differ in small but substantiative ways from those in other *Candida* species (**Figure 5-1**). Understanding clinically relevant fluconazole resistance mechanisms is key to the treatment of *C. parapsilosis* infection in the future.

Accurate calculation for rates of triazole resistance among *C. parapsilosis* clinical isolates is confounded by the geographical distribution of isolation, proper species identification, economic burden of antifungal susceptibility testing, and a general lack of criteria-specific surveillance systems for many of the countries where *C. parapsilosis* is most predominant. Many of the recent *C. parapsilosis* antifungal surveillance reports have focused on isolates from localized outbreaks and fluconazole resistance is often solely attributed to the CpErg11$^{Y132F}$ substitution. However, as multilocus sequencing typing of these isolates reveals, many of these isolates are clonal in nature and this could lead to a sampling bias in favor of this substitution being represented as the canonical pathway causing resistance in *C. parapsilosis* (294). While the identification of mutations in *CpERG11* can rapidly identify a common effector of fluconazole susceptibility, more attention needs to be paid to ABC and MFS drug efflux pumps, their transcriptional regulators, and other heretofore undiscovered resistance mechanisms that appear to be operative in clinical isolates.

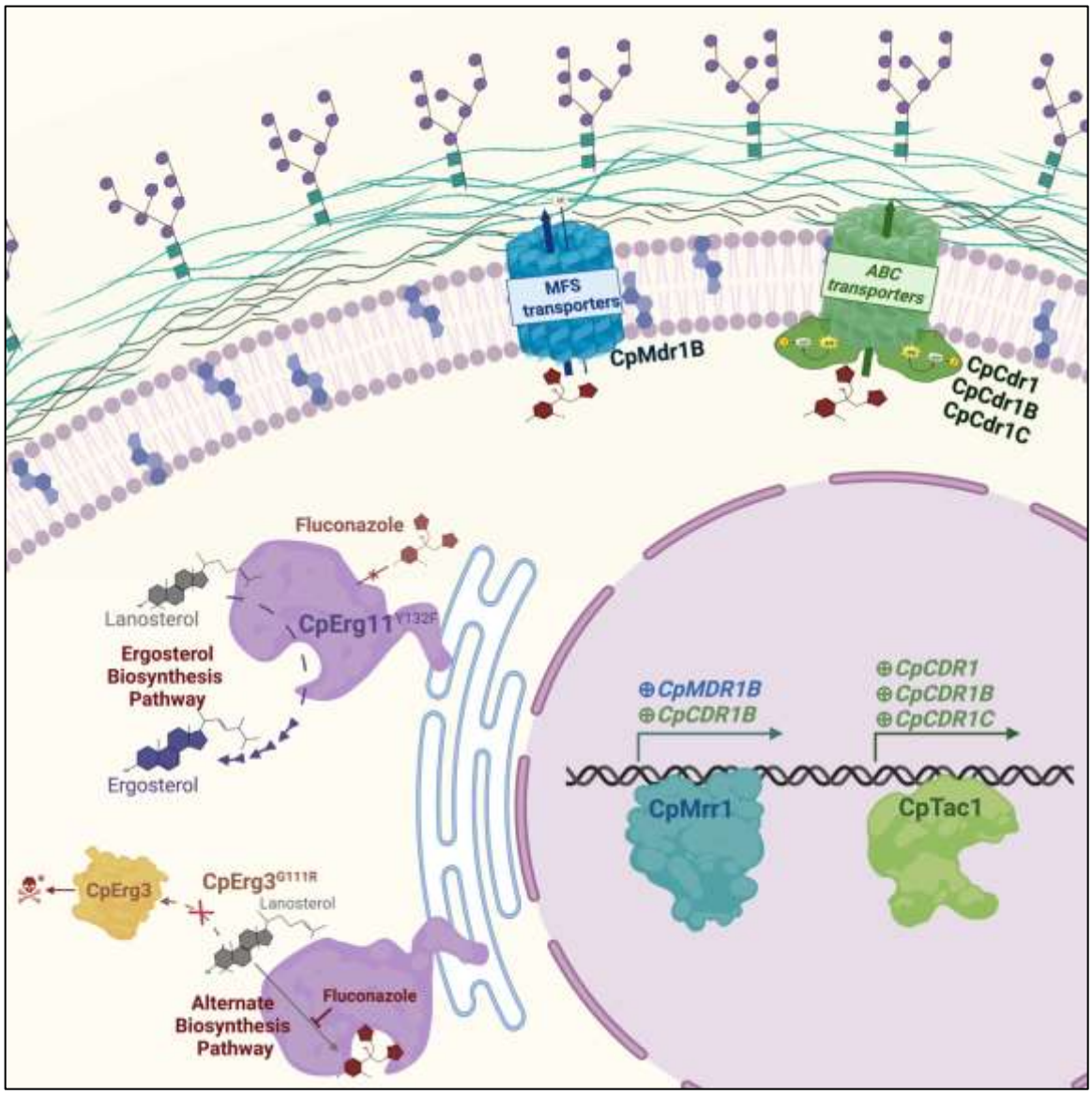


**Figure 5-1. Summary of clinical triazole resistance mechanisms identified in *C. parapsilosis.***
Note: Figure created with Biorender.com.

The placement of the CpErg11 substitution Y132F into susceptible isolates had a direct impact on fluconazole susceptibility (**Figure 5-1**), however this substitution demonstrated little-to-no impact on voriconazole, itraconazole, isavucoanzole or posaconazole susceptibility. This substitution disrupts the formation of a water mediated hydrogen bond between fluconazole and the heme molecule within the active site of the Erg11 enzyme (295). Molecular modeling has shown that the loss of this hydrogen bond disrupts fluconazole's ability to bind and inhibit the CYP51 active site in *S. cerevisiae* (295, 296). A similar mechanism is believed to also drive reduced susceptibility to other small triazole structures such as voriconazole, although perhaps to a smaller degree due to varied binding potentials among other key residues in the binding pocket (296). The substitution K143R, which also affects the hydrogen binding of the heme molecule in *C. albicans* Erg11, has recently been identified in fluconazole resistant *C. parapsilosis* isolates however this substitution was not associated with voriconazole resistance (297). This is most likely due to the retainment of a positively charged side chain with arginine substitution causing less disruption of the active-site (295). Decreased occurrence for *CpERG11* mutations compared to observations in *C. albicans,* along with the low-level effects for other triazoles could be reflective of adaptation driven by fluconazole exposure due to its predominance both in the prophylaxis and treatment of invasive infection by *C. parapsilosis* compared to other triazoles (58). However, these findings fail to explain the increasing reports of high-level fluconazole (MICs > 32μg/mL) resistance with concurrent voriconazole resistance and the reports of increased itraconazole resistance among clinical *C. parapsilosis* isolates with, and without, the Y132F substitution in CpErg11 (231, 256, 298). Indicating that while CpErg11$^{Y132F}$ is frequent among fluconazole non-susceptible clinical isolates, additional mechanisms are likely increasingly common among *C. parapsilosis* isolates but are currently under-appreciated in the literature.

Our investigations into CpMrr1 and CpTac1 gain-of-function mutations revealed increased expression of multiple transporters capable of directly effecting fluconazole, voriconazole, and itraconazole susceptibility. Increased expression of MFS transporter *CpMDR1B* displayed increased fluconazole MICs and minimal but measurable effects on voriconazole MIC. Increasing *CpCDR1*, *CpCDR1B*, and *CpCDR1C* expression not only impacted fluconazole and voriconazole MICs but also itraconazole MICs. Overall, isavuconazole and posaconazole MICs remained largely unaffected by the increased expression of ABC and MFS transporters. These triazoles warrant additional therapeutic consideration especially in countries with increased rates of voriconazole or itraconazole resistance among *C. parapsilosis* isolates such as Brazil, Mexico South Africa, and Iran (257, 299). However, it is important to note that there is currently little evidence linking isavuconazole and posaconazole MICs with treatment failure of *C. parapsilosis* infections in the clinic. This information void is also represented in the CLSI guidelines which provides clinical breakpoints for fluconazole and voriconazole but does not list any interpretations for isavuconazole MICs and only provides ECVs for itraconazole and posaconazole in *C. parapsilosis* (88).

Multiple reviews have underscored drug efflux pumps as the prime target for novel chemosensitizing drugs in *C. albicans* by either directly inhibiting the mechanism

or structures involved in efflux or by down-regulating the efflux pump expression (300, 301). High throughput screening methods have led to the discovery of drugs such as estrogen receptor inhibitor ospemifene and the efflux inhibitor azoffluxin that can directly inhibit the active transport of triazoles by *CDR1* in *C. albicans* and *C. auris*, respectively (302, 303). Additionally molecules such as the flavonoid, kaempferol, certain quinone derivatives, and cis-2-dodecenoic acid are capable of reducing expression of *CDR1*, *CDR2*, and *MDR1* in *C. albicans* (304-306). The identification of *CpCDR1B*, *CpCDR1C*, and *CpMDR1B*, and the dual regulation of *CpCDR1B* by both CpTac1 and CpMrr1 also highlights the significance of studying MFS and ABC transporters as potential targets to overcome clinical fluconazole resistance in *C. parapsilosis*. Previously, the calcium-channel blocker tetrandrine has been shown to exhibit synergism with fluconazole and voriconazole in resistant *C. parapsilosis* isolates via decreased *CpCDR1* expression however the exact mechanism behind this regulation remains unknown (307). The classification of *CpCDR1C* along with the potential for *CpCDR1B* paralogs, suggests there is still much to learn about the ABCG transporters encoded in the *C. parapsilosis* genome. As such, it is important to note for future investigations that there is high sequence similarity between many of these transporters. *CpCDR1*, *CpCDR1B*, and *CpCDR1C* all share high levels of sequence similarity between 81% and 87%, and proper considerations must be made for future studies involving these genes individually. However, this increased sequence similarity also lends to their potential as a target for fluconazole-potentiating drug discovery. A chemosensitizing drug capable of binding one of the shared functional domains or residues among the closely-related ABCG transporters could hypothetically work to overcome the fluconazole and voriconazole resistance associated with CpMrr1, CpTac1, and increased *CpCDR1B* copy variations.

Genetic modification systems available for *C. parapsilosis* have expanded greatly in recent years. Our ability to investigate transcriptional regulators, efflux pump expression, and individual point mutations in *CpERG11* were greatly improved by the development of the plasmid-based pCP-tRNA system (308) These systems allowed for single base editing and the insertion of short disruptive barcode sequences without the need for entire full allelic replacement or the insertion of recombinase recognition site scars into the genome. Importantly, these systems lessened the risk for off target effects and the accidental manipulation of upstream or downstream sequences. For overexpression, pJMR5 was created with *C. parapsilosis* specific components for constitutive gene overexpression. This system allowed for insertion of the *CpTEF1*promoter immediately upstream of the transporter start codon and resulted in expression that met or exceeded the maximum levels observed among the highly resistant clinical isolates. It important to note that these systems are not perfect and do not always result in perfect sequence editing. Each gene manipulation displayed differing transformation efficiencies. This was partly due to availability of nearby CRISPR cut sites but also its propensity to use the provided repair template rather than its own second allele for homologous recombination. The increasing accuracy in identification of *C. parapsilosis*, along with the quality of genetic manipulation tools available, allows for rapid testing of proposed genetic determinants within this species.

Improving surveillance programs, increasing antifungal susceptibility testing, and determination of resistance mechanisms among clinical isolates will allow for better understanding and treatment of fluconazole-resistant *C. parapsilosis* infection in the future. Collectively, the findings of this dissertation further our understanding of clinically relevant fluconazole resistance effectors and their regulation in *C. parapsilosis*, providing a foundation for future investigations into ways to detect and overcome triazole resistance in this important fungal pathogen.

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# APPENDIX A. CHAPTER 2 ARTICLE

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For "Next view," use Alt/Ctrl+Right Arrow on PC or Command+Right Arrow on Mac. See **Preface** for further details. If needed, use this link to return to **Chapter 2** after navigating within this appendix.

## Introduction

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## Article

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# *Candida parapsilosis* Mdr1B and Cdr1B Are Drivers of Mrr1Mediated Clinical Fluconazole Resistance

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## ABSTRACT

*Candida parapsilosis* is a common cause of invasive candidiasis worldwide and is the most commonly isolated *Candida* species among pediatric and neonatal populations. Previous work has demonstrated that nonsynonymous mutations in the gene encoding the putative transcription factor CpMrr1 can influence fluconazole susceptibility. However, the direct contribution of these mutations and how they influence fluconazole resistance in clinical isolates are poorly understood. We identified 7 nonsynonymous *CpMRR1* mutations in 12 isolates from within a collection of 35 fluconazole-resistant clinical isolates. The mutations leading to the A854V, R479K, and I283R substitutions were further examined and found to be activating mutations leading to increased fluconazole resistance. In addition to *CpMDR1*, we identified two other genes, one encoding a major facilitator superfamily (MFS) transporter (*CpMDR1B*, CPAR2_603010) and one encoding an ATP-binding cassette (ABC) transporter (*CpCDR1B*, CPAR2_304370), as being upregulated in isolates carrying *CpMRR1*-activating mutations. Overexpression of *CpMDR1* in a susceptible strain and disruption in resistant clinical isolates that overexpress *CpMDR1* had little to no effect on fluconazole susceptibility. Conversely, overexpression of either *CpMDR1B* or *CpCDR1B* increased resistance, and disruption in clinical isolates overexpressing these genes decreased fluconazole resistance. Our findings suggest that activating mutations in *CpMRR1* represent important genetic determinants of fluconazole resistance in clinical isolates of *C. parapsilosis*, and unlike what is observed in *Candida albicans*, this is primarily driven by upregulation of both MFS (CpMdr1B) and ABC (CpCdr1B) transporters.

**KEYWORDS** *Candida parapsilosis*, fluconazole, resistance, *MRR1*

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## INTRODUCTION

The triazole antifungal fluconazole remains an important antifungal agent in the treatment of candidemia and accounts for approximately 80% of all antifungal prescribing in the United States (1). Triazole antifungals are fungistatic against susceptible *Candida* species and act by binding to and competitively inhibiting sterol demethylase, a key enzyme in the fungal sterol biosynthesis pathway. This leads to reduced production of ergosterol and an accumulation of 14-amethyl-sterols that are thought to be deleterious to the fungal cell membrane.

Invasive candidiasis is among the most common nosocomial fungal infections and is associated with significant morbidity and mortality (2). *Candida parapsilosis* is the most common causative agent in non-albicans invasive *Candida* disease in pediatric and neonatal populations and is the most common *Candida* species isolated from intensive care units (ICUs) (3). In a CDC surveillance of candidemia between 2012 and 2016, all cause in-hospital mortality rose to 30%, and antifungal resistance was found in 7% of all *Candida* isolates, while *C. parapsilosis* was the only species with a notable increase in fluconazole resistance from 4.4% in 2012 to 14% in 2016 (4). The rates of fluconazole resistance in *C. parapsilosis* can vary depending on geographic region; however, on average, the rate remains around 3.4% worldwide (5).

Resistance to fluconazole has been well-studied in *Candida albicans*, where it has been shown to often be the result of multiple mechanisms working in concert. Two of these involve the gene (*ERG11*) encoding the triazole target, sterol-demethylase. Mutations in *ERG11* can lead to amino acid substitutions in sterol-demethylase that impair the ability of fluconazole to inhibit its activity. Overexpression of *ERG11*, often due to activating mutations in the gene encoding the transcriptional regulator of sterol biosynthesis genes, Upc2, leads to increased production of this enzyme, requiring more triazole to inhibit its activity (6). Another mechanism of resistance involves the gene (*ERG3*) encoding sterol-desaturase. Mutations leading to loss of function of this enzyme preclude the accumulation of deleterious 14-a methyl sterols and allow the fungus to survive in the presence of fluconazole (7). While activating mutations in *UPC2* have not been described in fluconazole-resistant *C. parapsilosis* clinical isolates, mutations in *ERG3* and *ERG11* have been reported, with the mutation in *ERG11* leading to the Y132F amino acid substitution being quite common among resistant isolates. (8–15).

Another well-characterized mechanism of fluconazole resistance in *C. albicans* involves drug efflux through the overexpression of the genes encoding the ATP-binding cassette (ABC) transporters Cdr1 and Cdr2 and the major facilitator superfamily (MFS) transporter Mdr1. Overexpression of *CDR1* and *CDR2* is due to activating mutations in the gene encoding the transcription factor Tac1 (transcriptional activator of *CDR* genes), whereas overexpression of *MDR1* is due to activating mutations in the gene encoding the transcription factor Mrr1 (multidrug resistance regulator) (16, 17). While fluconazole-resistant clinical isolates of *C. parapsilosis* have been observed to overexpress homologs of *C. albicans CDR1* and *MDR1*, these genes have not been shown to have any direct impact on fluconazole susceptibility (9, 18– 20). Moreover, while mutations in the homologs of *TAC1* and *MRR1* have been observed in fluconazole-resistant *C. parapsilosis* clinical isolates (11, 15, 21, 22) and activating mutations in *CpMRR1* have been evolved experimentally, such mutations have not yet been well characterized in clinical isolates (23).

Previously, we examined known mechanisms of fluconazole resistance for *Candida* spp. in a collection of 35 fluconazole-resistant *C. parapsilosis* clinical isolates (22). We found 11 resistant isolates with a mutation leading to the Y132F amino acid substitution in the fluconazole drug target, CpErg11, and three resistant isolates each overexpressing the gene encoding the putative drug transporter CpMdr1 (22). Among these three isolates, we identified three homozygous *CpMRR1* mutations, including two novel mutations resulting in the I283R and A854V amino acid substitutions, and a previously identified mutation resulting in the R479K substitution. These isolates were also among those with the highest measured fluconazole resistance, with MICs of $64 mg/mL (22). To determine the direct impact of *CpMDR1* expression on *CpMRR1* mediated fluconazole resistance, *CpMDR1* was deleted from the corresponding *C. parapsilosis* isolates. While in *C. albicans* the deletion of *MDR1* from backgrounds known to have activating mutations in MRR1 is associated with a 2- to 4-fold decrease in fluconazole MIC, there was little to no change in fluconazole MIC upon *CpMDR1* deletion in *C. parapsilosis* (22). In the present study, we show that activating mutations in CpMrr1 are a common contributor to fluconazole resistance in *C. parapsilosis* and elicit their effect not through overexpression of the gene encoding CpMdr1 but rather through overexpression of the gene encoding the MFS transporter, here named CpMdr1B, and unexpectedly in conjunction with overexpression of the gene encoding the ABC transporter, here named CpCdr1B.

## RESULTS

***CpMRR1* polymorphisms are common among fluconazole-resistant clinical isolates of *C. parapsilosis*.** In *C. albicans*, *MRR1*-activating mutations leading to increased expression of *MDR1* and increased fluconazole resistance were found in around 20% of clinical isolates in one large collection of resistant isolates (6). To determine the frequency with which mutations in *CpMRR1* occur within our collection, we first sequenced the *CpMRR1* alleles for all 35 fluconazole-resistant *C. parapsilosis* clinical isolates (**Table 1**). We found mutations leading to amino acid substitutions in CpMrr1 to

**TABLE 1 Amino acid substitutions in CpMrr1 identified in a collection of *C. parapsilosis* clinical isolates.**

| Clinical isolate identifier[a] | Fluconazole MIC (mg/mL)[b] | CpMrr1 amino acid substitution(s)[c] |
|---|---|---|
| Cp 1 | 16 | A854V[d] |
| Cp 2 | 16 | A854V[d] |
| Cp 9 | 16 | A854V[d] |
| Cp 10 | 16 | A854V[d] |
| Cp 12 | 16 | P255L[d], A854V[d] |
| Cp 20 | 16 | A854V[d] |
| Cp 27 | 32 | K129frameshift[d], G982R[d] |
| Cp 28 | 64 | A854V[d] |
| Cp 29 | 64 | A854V |
| Cp 30 | 64 | R479K |
| Cp 36 | 64 | I283R |
| Cp 39 | 32 | G294E[d] |

Note: [a]Clinical isolate collection previously described by Berkow et al. (22). [b]Fluconazole MIC obtained by broth microdilution assay as described in the CLSI document M27, 4th edition (32). [c]Mutations encoding amino acid substitutions identified through Sanger sequencing. [d]Heterozygous mutation.

be present in 12 isolates (31%). These included the three isolates (Cp36, Cp30, and Cp29) previously found to be homozygous for mutations encoding either the I283R, R479K, or A854V substitutions (22). Seven isolates were heterozygous for the A854V substitution, with one of these also being heterozygous for a P255L substitution. One isolate was heterozygous for the G294E substitution, and one isolate presented heterozygous mutations for both a frameshift at K129 and a G982R substitution. These results suggest that mutations in *CpMRR1* are relatively common among resistant clinical isolates of *C. parapsilosis*, especially those without *CpERG11* mutation.

**Fluconazole resistance in clinical isolates of *C. parapsilosis* is mediated in part by activating mutations in *CpMRR1*.** While *MRR1* mutations contribute to fluconazole resistance in *C. albicans*, they are not sufficient to impart high-level fluconazole resistance. To determine the direct contribution of mutations in *CpMRR1* to fluconazole resistance in *C. parapsilosis*, we introduced the three mutations found to be homozygous within isolates in our collection into fluconazole-susceptible isolate Cp13 (fluconazole MIC, 0.25 mg/mL) using the plasmid-based CRISPR-Cas9 gene editing system pCP-tRNA (24). Introduction of the mutations leading to the I283R, R479K, and V854A substitutions in both alleles resulted in a 128- to 256-fold increase in fluconazole MIC

and was sufficient to impart fluconazole resistance (MIC, 32 to 64 mg/mL) (**Fig. 1**). Conversely, correction of the mutations in both alleles to the wild-type *CpMRR1* sequence in clinical isolates Cp36, Cp30, and Cp29 resulted in a 32- to 128-fold decrease in fluconazole MIC and was sufficient to impart fluconazole susceptibility (MIC, 1 to 2 mg/mL). These results indicate that, unlike *C. albicans*, *CpMRR1* mutations are sufficient to impart high level fluconazole resistance in clinical isolates of *C. parapsilosis*.

**Increased expression of specific genes in clinical isolates of *C. parapsilosis*, including *CpMDR1*, *CpMDR1B*, and *CpCDR1B* is driven by activating mutations in *CpMRR1***. In *C. albicans*, activation of Mrr1 leads to upregulation of a distinct repertoire of genes, including the gene encoding the transporter Mdr1 (17, 25). To determine which genes are differentially expressed in the presence of mutations in *CpMRR1*, we subjected isolates Cp36, Cp30, and Cp29 and their respective derivatives with the wildtype corrected *CpMRR1* allele to transcriptional profiling by transcriptome sequencing (RNA-seq). We found 41 genes to be commonly upregulated and 23 to be downregulated among the three clinical isolates when mutations in *CpMRR1* were present compared to the respective wild-type *CpMRR1* derivatives (**Fig. 2**). Among upregulated genes were those homologous to genes upregulated in *C. albicans* strains carrying activating mutations in *MRR1*, including homologs of *GRP2*, *LPG20*, orf19.7306, orf19.7166, orf19.6586, *OYE32*, *MDR1*, and *MRR1* (**Table 2**) (17).

Interestingly, two homologs of *C. albicans MDR1* (orf19.5604) were among those genes upregulated in the presence of a *CpMRR1* mutation, *CpMDR1* (CPAR2_301760), which shares 66% predicted peptide sequence identity with *C. albicans* Mdr1, as well as a second homolog sharing 54.0% predicted peptide sequence identity that we have designated *CpMDR1B* (CPAR2_603010). Also upregulated was a gene encoding a homolog of the *C. albicans* ABC transporter gene *CDR1*, which we have designated *CpCDR1B* (CPAR2_304370). This gene shares 77% predicted peptide sequence identity with *C. albicans* Cdr1 and is distinct from the previously named *C. parapsilosis CpCDR1* (CPAR2_405290), which shares 79% predicted peptide sequence identity. For relative comparison of the expression levels of these genes, in clinical isolate Cp29, which carries a mutation leading to the A854V amino acid substitution, average reads per kilobase per million (RPKM) values for *CpCDR1*, *CpCDR1B*, *CpMDR1*, and *CpMDR1B* were 43.4, 341.9, 468.4, and 966.3, respectively.

In *C. albicans*, Cdr1 (*Candida* drug resistance) is an ATP-binding cassette transporter, and homologs are known to be a major determinant of fluconazole resistance in other *Candida* species (26, 27). However, in *C. albicans*, expression of *CDR1* is not known to be regulated by Mrr1 but rather by the zinc-cluster transcription factor Tac1 (16). Additionally, some of the genes associated with upregulation in C. albicans Mrr1 activation were downregulated in the isolates that contained *CpMRR1* mutations, including the homologs of *HGT2*, *HGT1*, *GLX3*, and *ADH4*. These results highlight similarities and differences in the genes regulated by *CpMRR1* in *C. parapsilosis* and *MRR1* in *C. albicans* and raise the possibility that both MFS and ABC transporters may participate in *CpMRR1*-mediated fluconazole resistance in *C. parapsilosis*.

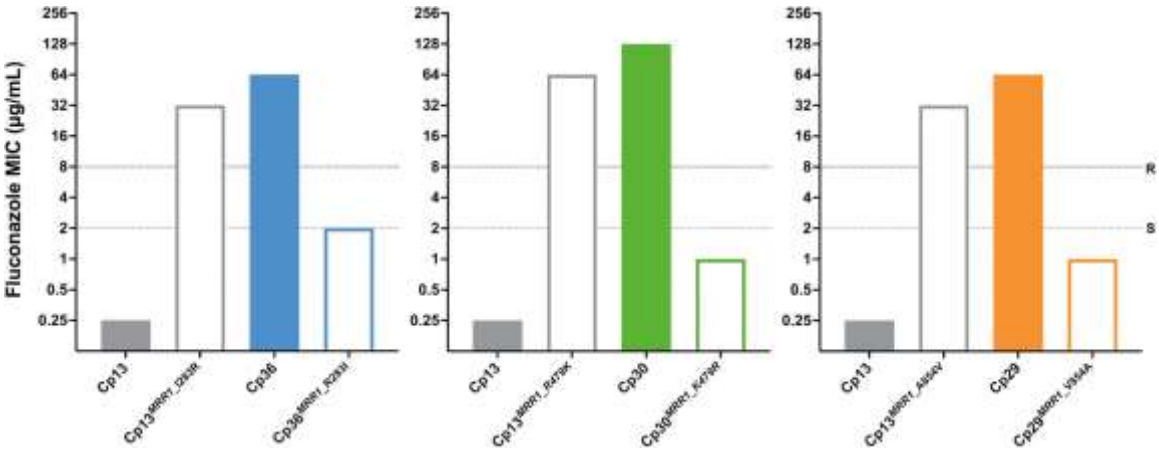


**FIG. 1 Impact of *CpMRR1* mutations on fluconazole susceptibility.** Susceptibility testing was performed according to CLSI guidelines. Data shown are representative of three independent MIC measurements. MICs represented in micrograms per milliliter were measured at 24 h. Graphs are divided by specific isolates with complemented transformant strains, each with specific homozygous CpMrr1 amino acid substitution. "R" represents the clinical breakpoint for fluconazole resistance in *C. parapsilosis*; "S" represents the clinical breakpoint for fluconazole susceptibility for *C. parapsilosis* (34).

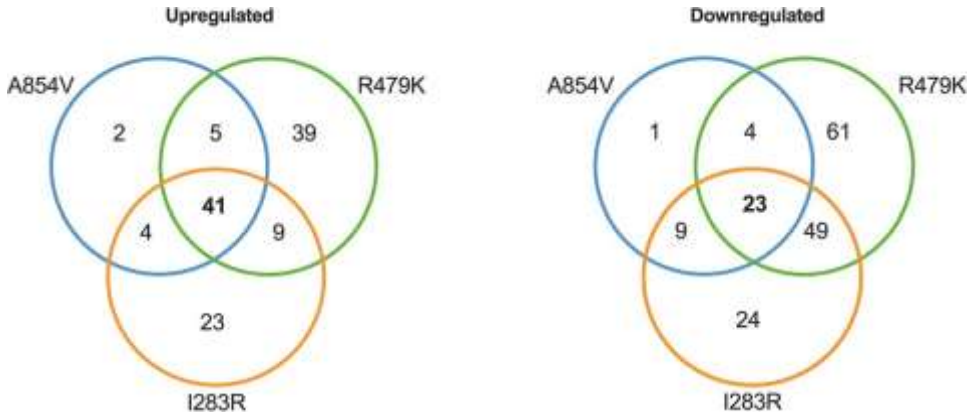


**FIG. 2 Differentially expressed genes in clinical isolates with homozygous mutations leading to amino acid substitutions in CpMrr1 compared to the respective corrected strain as determined by RNA sequencing.** Upregulated genes include genes with a ≥ 2-fold change for the clinical isolate with CpMrr1 mutation versus each clinical isolate with CpMrr1 corrected to wild type. Downregulated genes include genes with a ≤ -2 fold change for the clinical isolate with CpMrr1 mutation versus each clinical isolate with CpMrr1 corrected to wild type. FDR p values ≤ 0.05.

**TABLE 2 Genes with differential expression in clinical isolates containing CpMrr1 mutation compared to wild type.**

| *C. parapsilosis* gene [a] | *C. albicans* homolog [b] | Fold change [c] | | | Description [d] |
|---|---|---|---|---|---|
| | | A854V vs WT | R479K vs WT | I283R vs WT | |
| CPAR2_100480 | *GRP2* | 518.37 | 4861.44 | 201.20 | Similar to *S. cerevisiae* Gre2p (methylglyoxal reductase); expression increased in fluconazole-and voriconazole-resistant strains |
| *GRP2* | *GRP2* | 136.77 | 756.03 | 47.92 | Similar to *S. cerevisiae* Gre2p (methylglyoxal reductase); expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_105750 | | 107.70 | 8.08 | 24.03 | Has domain(s) with predicted DNA binding activity |
| CPAR2_103330 | orf19.2812 | 12.32 | 88.35 | 11.11 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_103600 | orf19.320 | 11.77 | 69.14 | 24.01 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_603010 | *MDR1* | 10.51 | 218.69 | 7.00 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains; ortholog(s) localize to the endoplasmic reticulum |
| *MDR1* | *MDR1* | 9.41 | 43.78 | 14.33 | Member of the MDR family of major facilitator transporter superfamily; putative drug transporter; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_206630 | orf19.3544 | 8.66 | 75.34 | 15.97 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_103320 | | 7.81 | 8.00 | 7.09 | Uncharacterized |
| CPAR2_601840 | orf19.5517 | 7.61 | 57.44 | 8.14 | Putative alcohol dehydrogenase; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_401490 | *GST2* | 6.53 | 30.58 | 5.36 | *GST2/URE2* family protein; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_404080 | orf19.5860 | 4.90 | 9.56 | 6.12 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |

| ***C. parapsilosis* gene** [a] | ***C. albicans* homolog** [b] | **Fold change** [c] | | | **Description** [d] |
|---|---|---|---|---|---|
| | | **A854V vs WT** | **R479K vs WT** | **I283R vs WT** | |
| *SADH* | *ADH5* | 4.83 | 10.44 | 3.35 | Butyraldehyde dehydrogenase, carbonyl reductase involved in amino acid degradation pathways |
| CPAR2_701130 | *PLB3* | 4.81 | 6.13 | 5.73 | Has domain(s) with predicted phospholipase activity and role in metabolic process, phospholipid catabolic process |
| CPAR2_404090 | orf19.345 | 4.64 | 5.13 | 6.38 | Putative succinate-semialdehyde dehydrogenase [NAD(P)$^+$]; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_703250 | | 4.60 | 24.67 | 5.76 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| *LPG20* | *LPG20* | 4.50 | 43.42 | 9.14 | Aldo-keto reductase family protein; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_805920 | orf19.1075.1 | 4.50 | 15.54 | 3.65 | Uncharacterized |
| CPAR2_304370 | *CDR1* | 4.35 | 15.28 | 5.82 | Has domain(s) with predicted ATP binding, ATPase activity, ATPase-coupled transmembrane transporter activity, nucleoside-triphosphatase activity, nucleotide binding activity |
| *OYE32* | *OYE32* | 4.13 | 14.15 | 3.17 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains; ortholog(s) have role in cell redox homeostasis |
| CPAR2_702640 | orf19.6586 | 4.08 | 15.93 | 11.22 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_701460 | orf19.7306 | 4.00 | 9.32 | 7.67 | Aldo-keto reductase family protein; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_300590 | *FLU1* | 3.33 | 3.91 | 2.96 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane localization |
| CPAR2_503210 | orf19.6943 | 3.23 | 5.29 | 2.91 | Uncharacterized |

| ***C. parapsilosis* gene [a]** | ***C. albicans* homolog [b]** | **Fold change [c]** | | | **Description [d]** |
|---|---|---|---|---|---|
| | | **A854V vs WT** | **R479K vs WT** | **I283R vs WT** | |
| CPAR2_402120 | orf19.1430 | 3.22 | 3.24 | 2.88 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_807720 | *POX1*-3 | 3.21 | 2.82 | 3.27 | Ortholog(s) have role in fatty acid beta-oxidation, long-chain fatty acid catabolic process and peroxisome localization |
| *PDR16* | *PDR16* | 3.00 | 3.61 | 2.82 | Phosphatidylinositol transfer protein; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_301750 | orf19.4779 | 2.90 | 3.23 | 2.03 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane localization |
| CPAR2_103670 | orf19.2446 | 2.88 | 8.42 | 5.36 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_109500 | orf19.6066 | 2.77 | 3.75 | 2.78 | Ortholog(s) have 4-hydroxybenzaldehyde dehydrogenase activity, carboxylate reductase activity |
| *STP4* | *STP4* | 2.72 | 2.91 | 2.80 | Putative transcription factor with zinc finger DNA-binding motif; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_200870 | orf19.6600 | 2.67 | 2.90 | 2.05 | Ortholog(s) have phosphatidic acid transfer activity and role in cardiolipin metabolic process, phospholipid translocation, phospholipid transport, positive regulation of phosphatidylcholine biosynthetic process |
| CPAR2_702300 | orf19.7166 | 2.65 | 5.79 | 2.86 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains; ortholog(s) localize to the Golgi apparatus and endoplasmic reticulum |
| CPAR2_700540 | orf19.7235 | 2.57 | 3.36 | 3.72 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |

| ***C. parapsilosis* gene** [a] | ***C. albicans* homolog** [b] | **Fold change** [c] | | | **Description** [d] |
|---|---|---|---|---|---|
| | | **A854V vs WT** | **R479K vs WT** | **I283R vs WT** | |
| *MRR1* | *MRR1* | 2.47 | 9.22 | 3.36 | Regulator of *MDR1* transcription; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_400630 | orf19.4609 | 2.45 | 3.32 | 2.88 | Protein of unknown function; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_701900 | orf19.1985 | 2.27 | 3.61 | 2.83 | Has domain(s) with predicted protein serine/threonine kinase activity, transferase activity, transferring phosphorus-containing groups activity and role in protein phosphorylation |
| CPAR2_602390 | orf19.3442 | 2.25 | 2.68 | 2.63 | Putative oxidoreductase; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_211640 | orf19.5785 | 2.17 | 3.08 | 4.66 | Uncharacterized |
| CPAR2_201400 | orf19.6348 | 2.10 | 4.02 | 2.84 | Putative ubiquitin thiolesterase; predicted role in ubiquitin-dependent protein catabolism; expression increased in fluconazole- and voriconazole-resistant strains |
| *LAP4* | *LAP4* | 2.04 | 4.43 | 3.12 | Similar to aminopeptidase I; expression increased in fluconazole- and voriconazole-resistant strains |
| CPAR2_203940 | | −2.02 | −2.92 | −4.19 | Uncharacterized |
| CPAR2_800070 | *PGA28* | −2.13 | −4.8 | −4.36 | Ortholog of *C. albicans* SC5314: C7_03110W_A/*PGA28* |
| CPAR2_106690 | *DIP5* | −2.13 | −4.9 | −2.23 | Ortholog(s) have l-aspartate transmembrane transporter activity, l-glutamate transmembrane transporter activity, dicarboxylic acid transmembrane transporter activity |
| CPAR2_500170 | *ZRT1* | −2.2 | −2.34 | −2.81 | Has domain(s) with predicted metal ion transmembrane transporter activity, role in metal ion transport, transmembrane transport and membrane localization |
| CPAR2_407280 | orf19.1370 | −2.34 | −5.5 | −3.43 | Ortholog of *C. albicans* SC5314: C2_09800C_A |
| CPAR2_102140 | *ADH4* | −2.47 | −4.56 | −3.33 | Has domain(s) with predicted oxidoreductase activity and role in metabolic process |

| ***C. parapsilosis* gene** [a] | ***C. albicans* homolog** [b] | **Fold change** [c] | | | **Description** [d] |
|---|---|---|---|---|---|
| | | **A854V vs WT** | **R479K vs WT** | **I283R vs WT** | |
| CPAR2_702930 | orf19.6475 | −2.57 | −4.77 | −2.99 | Ortholog of *Candida metapsilosis*: CMET_1690 |
| CPAR2_403560 | orf19.1318 | −2.57 | −2.89 | −3.05 | Ortholog of *C. albicans* SC5314: C4_03580W_A |
| CPAR2_601420 | orf19.3475 | −2.59 | −3.21 | −3.51 | Ortholog of *C. albicans* SC5314: C6_02330W_A |
| CPAR2_106680 | *PUT4* | −2.65 | −3.87 | −2.91 | Has domain(s) with predicted amino acid transmembrane transporter activity, role in amino acid transmembrane transport, transmembrane transport and membrane localization |
| CPAR2_701480 | orf19.7300 | −2.71 | −11.84 | −7.32 | Ortholog of *C. albicans* SC5314: CR_09040W_A |
| CPAR2_403880 | | −2.74 | −3.95 | −3.7 | Uncharacterized |
| CPAR2_502450 | *MRV4* | −3.29 | −5.43 | −6.65 | Ortholog of *C. albicans* SC5314: C5_04210C_A/*MRV4* |
| CPAR2_103080 | *GLX3* | −4.2 | −5.87 | −5.28 | Ortholog(s) have glyoxalase III activity, protein folding chaperone activity |
| *HGT10* | *HGT10* | −4.99 | −9.23 | −4.09 | Ortholog(s) have solute/proton symporter activity, role in glycerol transport, transmembrane transport and plasma membrane localization |
| *TNA1* | *TNA1* | −5.8 | −3.37 | −2.99 | Ortholog(s) have carboxylic acid transmembrane transporter activity and role in carboxylic acid transport, quinolinic acid transmembrane transport |
| CPAR2_502460 | *MRV2* | −6.41 | −49.42 | −19.66 | Ortholog of *S. cerevisiae*: YDL218W, *C. albicans* SC5314: C5_04190W_A/*MRV2* |
| CPAR2_701510 | *SLP3* | −6.68 | −9.98 | −11.84 | Has domain(s) with predicted membrane localization |
| *NAG4* | *NAG3* | −8.41 | −4.32 | −6.87 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane localization |
| CPAR2_802720 | orf19.3232 | −11.99 | −5.19 | −3.3 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane localization |

| ***C. parapsilosis* gene** [a] | ***C. albicans* homolog** [b] | **Fold change** [c] | | | **Description** [d] |
|---|---|---|---|---|---|
| | | **A854V vs WT** | **R479K vs WT** | **I283R vs WT** | |
| CPAR2_108370 | *HGT1* | −12.9 | −5.89 | −13.54 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane, membrane localization |
| CPAR2_102120 | orf19.2633.1 | −20.74 | −5.08 | −11.07 | Uncharacterized |
| CPAR2_108340 | *HGT2* | −33.69 | −8.52 | −7.04 | Has domain(s) with predicted transmembrane transporter activity, role in transmembrane transport and integral component of membrane, membrane localization |

Note: [a]CPAR2 identifier from genome annotation of *C. parapsilosis*, CDC317, *Candida* Genome Database. Gene name provided where available. [b]*Candida albicans* homologs are identified where possible from *Candida* Genome Database. Gene names provided may be true orthologs and best hits. [c]Fold change ($\geq 2$ or $\leq -2$) for clinical isolates with *CpMRR1* mutation versus each clinical isolate with *CpMRR1* corrected to wild type. Performed in 3 biological replicates with FDR *p* values or $\leq 0.05$.[d]Gene descriptions taken from *Candida* Genome Database.

**Overexpression of *CpMDR1B* and *CpCDR1B* results in increased resistance to fluconazole in *C. parapsilosis*.** To determine if increased expression of the genes encoding the CpMdr1, CpMdr1B, and CpCdr1B transporters are capable of influencing fluconazole resistance, these genes were overexpressed by placing them under the control of the strong *CpTEF1* promoter, resulting in a 58-, 15-, and 20-fold increase in relative expression of *CpMDR1*, *CpMDR1B*, and *CpCDR1B*, respectively (**Fig. 3A**). These levels of overexpression not only replicated the expression observed in the highest expressing resistant clinical isolate, Cp30, but also resulted in a 16-fold increase in fluconazole resistance for the *CpMDR1B* and *CpCDR1B* overexpression strains. Conversely, no change in fluconazole MIC was observed for the strain overexpressing *CpMDR1* (**Fig. 3B**). These results indicate that overexpression of *CpMDR1B* and *CpCDR1B* to levels comparable to those observed in resistant clinical isolates is capable of influencing fluconazole susceptibility.

**Disruption of *CpMDR1B* and *CpCDR1B*, but not *CpMDR1*, increases susceptibility of fluconazole in resistant clinical isolates carrying activating mutations in *CpMRR1*.** In order to determine the direct contributions of *CpMDR1*, *CpMDR1B*, and *CpCDR1B* to CpMrr1-mediated fluconazole resistance, we disrupted these genes individually and in combination in a resistant clinical isolate. We used the isolate Cp29 as the background strain, as it overexpresses all three of these genes and is homozygous for the most commonly observed CpMrr1 substitution, A854V. To accomplish this, the pCP-tRNA method was used to insert a STOP-codon containing a segment of DNA at the 59 end for each desired open reading frame. Disruption of *CpMDR1* resulted in a single dilution decrease in fluconazole MIC, whereas disruption of either *CpMDR1B* or *CpCDR1B* resulted in a modest reduction in fluconazole MIC from 64 mg/mL to 16 mg/mL. Combined disruption of both *CpMDR1B* and *CpCDR1B* was sufficient to impart fluconazole susceptibility resulting in a reduction in MIC from 64 mg/mL to 4 mg/mL (**Table 3**). No additional effect was observed for deletion of *CpMDR1* either in combination with *CpMDR1B* or *CpCDR1B* or when all three genes were disrupted. Notably, similar effects were also observed for voriconazole MICs. These data indicate that activating mutations in *CpMRR1* increases fluconazole resistance, not through overexpression of *CpMDR1* but primarily through the increased expression of *CpMDR1B* and *CpCDR1B*.

## DISCUSSION

Polymorphisms leading to amino acid substitutions in *C. parapsilosis* CpMrr1 have been previously observed among fluconazole-resistant (MIC, ≥ 8 mg/mL) and susceptible dose dependent (MIC, 4 mg/mL) clinical isolates (**Fig. 4**) (11, 13, 15, 19, 21–23). Additionally, the presence of certain amino acid substitutions, specifically, I283R, R479K, A854V, G583R, and K873N, have been associated with increased expression of *CpMDR1* and increased resistance to fluconazole and voriconazole (22, 23, 28). *CpMRR1* mutations have previously been evolved in the laboratory and experimentally shown to confer resistance to fluconazole (23, 28). Among fluconazole-resistant clinical isolates, *CpMRR1* mutations were first reported in nine isolates collected from a population-based

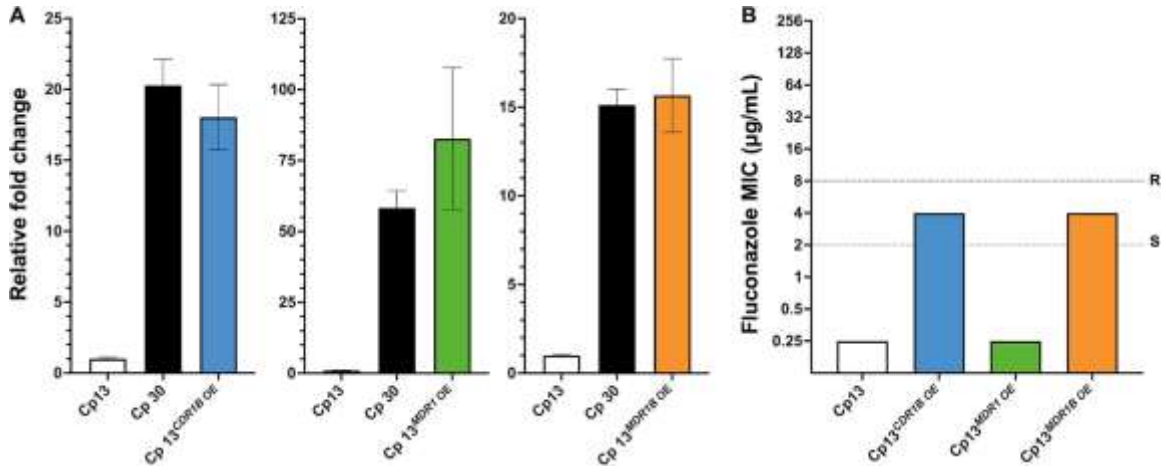


**FIG. 3 Constitutive overexpression (OE) of *CpCDR1B, CpMDR1*, and *CpMDR1B* in a susceptible clinical isolate.** (A) Relative fold change of efflux pump expression as measured by reverse transcriptase quantitative PCR (RT-qPCR). Expression is measured compared to the average for the susceptible isolate Cp13. Error bars represent standard deviation for three independent experiments. (B) Fluconazole MICs for overexpressing strains. Susceptibility testing was performed according to CLSI guidelines. Data shown are representative of three independent MIC measurements. Values are represented in micrograms per milliliter measured at 24 h. "R" represents the clinical breakpoint for fluconazole resistance in *C. parapsilosis*; "S" represents the clinical breakpoint for fluconazole susceptibility for *C. parapsilosis*.

**TABLE 3 Triazole MICs[a] for efflux pump disruption strains made in CpMrr1 activating mutation containing background.**

| | MIC (mg/mL) | | | | |
|---|---|---|---|---|---|
| **Strain[b]** | **Fluconazole** | **Voriconazole** | **Isavuconazole** | **Itraconazole** | **Posaconazole** |
| Cp29 | 64 | 0.5 | 0.125 | 0.125 | 0.125 |
| Cp29$^{mdr1}$ | 32 | 0.5 | 0.125 | 0.125 | 0.06 |
| Cp29$^{mdr1b}$ | 16 | 0.25 | 0.125 | 0.125 | 0.06 |
| Cp29$^{cdr1b}$ | 16 | 0.25 | 0.06 | 0.125 | 0.06 |
| Cp29$^{mdr1,mdr1b}$ | 16 | 0.25 | 0.125 | 0.125 | 0.06 |
| Cp29$^{mdr1,cdr1b}$ | 16 | 0.25 | 0.06 | 0.125 | 0.06 |
| Cp29$^{mdr1b,cdr1b}$ | 4 | 0.06 | 0.06 | 0.125 | 0.06 |
| Cp29$^{mdr1,mdr1b,cdr1b}$ | 4 | 0.06 | 0.06 | 0.125 | 0.06 |

Note: [a]MICs obtained by broth microdilution assay as described in the CLSI document M27, 4th edition (32). [b]Clinical isolate collection previously described by Berkow et al. (22).

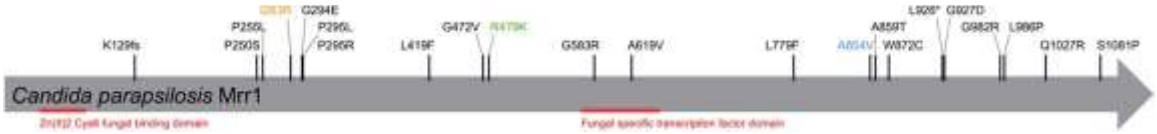


**FIG. 4 Amino acid substitutions identified in *Candida parapsilosis* CpMrr1.** Substitutions identified in *C. parapsilosis* clinical isolates with fluconazole MICs of ≥ 8mg/mL (11, 13, 15, 19, 21, 22). Red labeled domains were identified through the *Candida* Genome Database and a search of the InterPro database. I283R, R479K, and A854V were identified as homozygous mutations within our collection.

surveillance study of candidemia by the CDC (11). Only six of these exhibited ≥ 10-fold increases in *CpMDR1* expression relative to the average expression of susceptible controls (11). Our initial examination of the 35 clinical isolates with fluconazole MIC values of ≥ 8mg/mL in our collection revealed that none carry a mutation in *CpERG3*, whereas 11 carry a mutation in *CpERG11* leading to the Y132F substitution. Three isolates were observed to have *CpMDR1* expression ≥ 10-fold above the average of susceptible controls, and all three were homozygous for mutations in *CpMRR1* (22). However, deletion of *CpMDR1* in these isolates had no effect on fluconazole resistance in one isolate and reduced resistance by only one dilution in the remaining two isolates (128 to 64mg/mL and 64 to 32mg/mL).

Our findings here delineate the direct contribution of *CpMRR1* activating mutations to fluconazole resistance in clinical isolates and suggest that such mutations represent a relatively common mechanism of resistance in *C. parapsilosis*. Indeed, we observed *CpMRR1* mutations in 12 of our 35 resistant isolates, with heterozygous mutations being generally associated with lower fluconazole MICs (16 to 32 mg/mL), whereas isolates with homozygous mutations exhibited MICs of 64 mg/mL. Only one isolate (isolate Cp39) carried a mutation in both *CpMRR1* (leading to the G294E substitution) and *CpERG11* (leading to the Y132F substitution). The data presented in this study clearly demonstrate that the *CpMRR1* mutations leading to the I283R, R479K, and A854V substitutions confer resistance to fluconazole. Furthermore, these findings support the notion that the levels of resistance observed in 11 isolates in this collection can be fully explained by the mutations present in *CpMRR1*.

While homologs of *C. albicans CDR1* and *MDR1* have been observed to be overexpressed in resistant *C. parapsilosis* clinical isolates, their contribution to fluconazole resistance has been unclear. Transcriptional profiling of the clinical isolates with *CpMRR1* mutations leading to I283R, R479K, or A854V substitutions compared to that of their respective strains with *CpMRR1* edited to the wild-type sequence revealed changes in gene expression with similarities to those observed when Mrr1 is activated in *C. albicans*, as well as similarities to the transcriptional profiles of isolates previously experimentally evolved to have resistance to voriconazole. These include overexpression of *CpMDR1*, *CpMDR1B* (CPAR2_603010), and *CpCDR1B* (CPAR2_304370).

Importantly, a *CDR1* homolog has been shown to be regulated by an Mrr1 homolog in the related species *Clavispora lusitaniae* (also referred to as *Candida lusitaniae*) and has been shown to contribute to Mrr1-mediated fluconazole resistance. Moreover, there are similarities between the genes observed to be upregulated in that species when Mrr1 is activated and those that we observe here with the activation of CpMrr1 (29, 30). In contrast, in *C. albicans*, Mrr1 regulates fluconazole resistance primarily through overexpression of *MDR1* with no additional MFS or ABC transporter having been identified as a target of CaMrr1, influencing fluconazole susceptibility (17).

Our observation that overexpression of *CpMDR1* had no impact on fluconazole susceptibility and that deletion of *CpMDR1* in clinical isolate Cp29 resulted in only a single dilution reduction in fluconazole MIC was consistent with our previous findings that deletion of *CpMDR1* had little to no effect on fluconazole susceptibility in isolates in which it was highly overexpressed (22). Indeed, *CpMDR1B* appears to be more functionally similar to *C. albicans MDR1*, as overexpression at levels observed in resistant isolates resulted in an increase in fluconazole MIC, and disruption in clinical isolate Cp29 reduced the MIC from 32 to 8 mg/mL. We were surprised to observe a role for *CpCDR1B*, as Mrr1-mediated resistance in other *Candida* species appears to be driven primarily by overexpression of *MDR1*. CpMrr1-mediated fluconazole resistance in *C. parapsilosis* instead appears to be driven by overexpression of both *CpMDR1B* and *CpCDR1B*, with both making an equal contribution. Disruption of these transporter genes had similar effects on susceptibility to voriconazole, while isavuconazole and posaconazole resistance appeared only minimally affected by the disruption of *CpCDR1B* with a decrease in observed MIC of a single dilution. No change in itraconazole susceptibility was observed, suggesting that these latter three agents may represent useful alternatives to fluconazole for the treatment of *C. parapsilosis* exhibiting CpMrr1-mediated resistance.

We have shown that, in addition to mutations in *CpERG11*, *CpMRR1* mutations represent an important mechanisms of fluconazole resistance in *C. parapsilosis* clinical isolates. However, it is important to note that 8 out of our 35 clinical isolates have no sufficient explanation for the fluconazole resistance observed. It is therefore apparent that additional mechanisms of fluconazole resistance are operative in these isolates, and as such, the discovery and characterization of these remaining mechanisms of fluconazole resistance in *C. parapsilosis* are needed.

## MATERIALS AND METHODS

**Strains and media.** All *C. parapsilosis* isolates used in this study have been previously described (22). Isolates and derived strains were kept at 280°C in 40% glycerol stock. All strains and isolates were maintained on YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plates at 30°C or in YPD liquid medium at 30°C in a 220-rpm shaking incubator. RPMI with morpholinepropanesulfonic acid (MOPS) and 2% glucose, pH 7.0, was used for both drug susceptibility testing growth prior to RNA isolation techniques. Chemically competent DH5a cells were utilized for plasmid

construction and grown in LuriaBertani (LB) medium supplemented with 100 mg/mL ampicillin.

***CpMRR1* sequence analysis.** Genomic DNA was isolated as previously described (31), and the coding sequence of *C. parapsilosis CpMRR1* (CPAR2_807270) was amplified via PCR with CpMrr1-AmpF and CpMrr1-AmpR primers. PCR products were purified by QIAquick PCR purification kit (Qiagen). Single nucleotide polymorphisms were determined using evenly spaced primers for multiple Sanger sequencing reactions (Applied Biosystems; Veriti). Sequencing primers and all other primers used in this study can be found in Table S1 in the supplemental material.

**Drug susceptibility determination.** Antifungal susceptibilities for triazoles were determined by broth microdilution for all clinical isolates and strains according to the Clinical and Laboratory Standards Institute document M27, 4th edition (32). Triazoles were obtained from Sigma-Aldrich, and concentrated stocks were prepared in dimethyl sulfoxide. Drug stocks were diluted 1:50 for serial inoculation of RPMI medium on 96-well plates. MICs were recorded after 24 h of incubation at 35°C. All drug susceptibility testing was performed in biological triplicates. Graphical representation was made using GraphPad Prism version 9.2.0.

***C. parapsil*osis transformation**. Transformation of *C. parapsilosis* isolates was performed using electroporation methods previously described with alteration (22). Cells were grown for 6 h at 30°C in 2 mL YPD liquid medium. After incubation, 25 to 400 mL of cell suspension was used to inoculate 25 mL fresh YPD liquid medium, depending on *C. parapsilosis* isolate growth, for overnight growth at 30°C. Optical density was read at 600 nm ($OD_{600}$) for a minimum of 2.0, and competent cells were prepared as previously described (22). Electroporation was performed on competent cells using the *C. albicans* protocol on a Gene Pulsar Xcell (Bio-Rad). After recovery for 6 h at 30°C in a 50-50 YPD and 1 M D-Sorbitol medium, transformed cells were plated on YPD plates containing 200 mg/mL of nourseothricin for selection.

**Modification of putative drug transporter genes**. For overexpression of the genes of interest, the 1,000-bp upstream region of *CpTEF1* was amplified utilizing CpTEF1-1 and CpTEF1-2 primers, and a nourseothricin resistance marker, originally derived from pV1200 (33), was amplified using pJMR_forward_p1 and pJMR_reverse_p3. The two amplified templates were then combined via fusion PCR (annealing temperature, 58°C) with the primers pJMR5_p5_NOT1 and pJMR5_p2_ApaI (see **Table S1**). This fusion product was then digested with ApaI and NotI-HF restriction enzymes, and products were ligated into a plasmid backbone containing an f1 bacteriophage origin of replication, an ampicillin resistance marker, and an origin of replication sequence to make pJMR5. Repair templates for overexpression were amplified from pJMR5 using 59 and 39 primers, which introduced 50 to 70 bases of homology to the sequence immediately upstream of the gene of interest (GOI) and were purified using the Gene Clean II kit (MP Biomedicals). Using the Cas9-RNP method previously described (27) with some modification, approximately 1 mg of the purified repair template was mixed with 4 mM Cas9-RNP complexes targeting the immediate

upstream sequence for *CpMDR1* (CPAR2_301760), *CpMDR1B* (CPAR2_603010), and *CpCDR1B* (CPAR2_304370) and added to the electrocompetent *C. parapsilosis* cells. Promoter insertion was confirmed via PCR screening.

**pCP-tRNA-guided transformation.** For the precise manipulation of *CpMRR1* and the disruption of putative efflux pump genes, the pCP-tRNA plasmid was used as previously described (24) with minor modification. Briefly, two 23-bp oligonucleotides with SapI restriction sites (Integrated DNA Technologies) were annealed to generate site-specific guide sequences (see Table S1). SapI-digested pCP-tRNA plasmid was purified with the QIAquick PCR purification kit (Qiagen) and subsequently cloned with the designated guideRNA. Repair templates were generated by primer extension using 50- to 70-base microhomology from the gene of interest and contained either the desired point mutations for *CpMRR1* investigation or the addition of a 22-base STOP-codon sequence for GOI disruption (Integrated DNA Technologies). Representative mutation strains were identified by Sanger sequencing (Applied Biosystems; Veriti). Plasmid ejection was induced by overnight growth in liquid YPD followed by isolation on YPD-agar plates and subsequent replicative patching on YPD agar plates containing 200mg/mL nourseothricin.

**RNA sequencing.** *C. parapsilosis* cultures were grown similar to those described for MIC preparation with some modification. *C. parapsilosis* strains were grown in biological triplicate at 30°C overnight in YPD liquid medium and subsequently plated onto Sabouraud dextrose (BD) minimal medium agar for 24 h growth at 30°C. Sterile loops were used to transfer cells into 20 mL RPMI for inoculums with an $OD_{600}$ of 0.1. Cultures were incubated at 35°C with shaking at 110 rpm for 8 h, after which the cells were centrifuged at 4,000 rpm for 5 min. Supernatants were removed, and the pellets were stored at 280°C for a minimum of 24 h. RNA isolation was performed using the RiboPure yeast (Invitrogen) system per manufacturer's instructions. RNA sequencing was performed using Illumina NextSeq for stranded mRNA. Libraries were prepared with paired-end adapters using Illumina chemistries per manufacturer's instructions, with read lengths of approximately 150 bp with at least 50 million raw reads per sample. RNA-seq data were analyzed using CLC Genomics Workbench version 20.0 (Qiagen), and reads were trimmed using default settings for failed reads and adaptor sequences and then subsequently mapped to the *C. parapsilosis* genome (GenBank accession number GCA_000182765.2) with paired reads counted as one and expression values set to RPKM. Principal-component analysis was utilized for assessment of the clustering of biological replicates. Whole-transcriptome differential gene expression analysis was performed with the prescribed algorithm of CLC Genomics Workbench version 20.0. Mismatch, insertion, and deletion costs were set to default parameters, and the Wald test was used for all group pairs against the matched parent control strain. Genes were considered differentially regulated when a fold change of ($\geq 2$ or $\leq -2$) was observed accompanied by a false-discovery rate (FDR) P value of $\leq 0.05$.

**Reverse transcriptase quantitative PCR**. *C. parapsilosis* overexpression strains were grown in YPD medium, and RNA was isolated using methods as previously described (22). cDNA was synthesized from isolated RNA using SuperScript first-strand

synthesis system according to the manufacturer's protocol. *CpACT1* (CPAR2_201570), *CpMDR1*, *CpMDR1B*, and *CpCDR1B* were amplified from synthesized cDNA by PCR utilizing SYBR green master mixes in accordance with the manufacturer's instructions. Gene-specific primers were used in PCR at 95°C for 10 min for AmpliTaq Gold activation and then 40 cycles of denaturation at 95°C for 15 s followed by annealing/extension at 60°C. The dissociation curve was determined using the 7500 detection real-time PCR system (Applied Biosystems). Changes in expression among isolates and transformants were calculated using the $2^{-\Delta\Delta CT}$ method and performed in triplicate. Data representation was made using GraphPad Prism version 9.2.0.

**DATA AVAILABILITY.** Study data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE196409.

**SUPPLEMENTAL MATERIAL.** Supplemental material is available online only. SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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# APPENDIX B. CHAPTER 3 ARTICLE

**NOTE:** Navigation with Adobe Acrobat Reader or Adobe Acrobat Professional: To return to the last viewed page, use key commands Alt/Ctrl+Left Arrow on PC or Command+Left Arrow on Mac. For "Next view," use Alt/Ctrl+Right Arrow on PC or Command+Right Arrow on Mac. See **Preface** for further details. If needed, use this link to return to **Chapter 3** after navigating within this appendix.

## Introduction

Article is reused from the prepared manuscript with the authors' permission. **Doorley LA**, Barker KS, Zhang Q, Rybak JM, Rogers PD. "Mutations in *TAC1* And *ERG11* are Major Drivers of Triazole Antifungal Resistance in Clinical Isolates of *Candida parapsilosis*," 2023.

## Article

**Mutations in *TAC1* and *ERG11* are major drivers of triazole antifungal resistance in clinical isolates of *Candida parapsilosis***

**Keywords:** *Candida parapsilosis,* triazole, fluconazole, resistance, *ERG11*, *TAC1*

**Running Title:** Mutations in *Candida parapsilosis TAC1* and *ERG11* work in concert to drive high-level fluconazole resistance.

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## ABSTRACT

### Objectives

*Candida parapsilosis* is the most common cause of non-*albicans Candida* infections in pediatric and neonatal populations and the rates of fluconazole resistance are increasing. The aim of this study was to determine how mutations in *CpERG11* and *CpTAC1* contribute to fluconazole resistance in a collection of clinical isolates.

### Methods

*CpERG11* and *CpTAC1* were sequenced in thirty-nine *C. parapsilosis* clinical isolates. Gene manipulations were achieved using a plasmid-based CRISPR-Cas9 system. Differential expression of *CpTAC1* mutation-associated genes was determined by RNA sequencing. Antifungal susceptibility testing was performed by broth microdilution and E-test. Relative expression of specific genes of interest was determined by RT-qPCR.

### Results

Six isolates carried a mutation in *CpTAC1* in combination with the *CpERG11* mutation leading to the Y132F substitution. When introduced into susceptible isolates, the *CpERG11* mutation led to a 4- to 8-fold increase in fluconazole MIC. When introduced into a susceptible isolate, the *CpTAC1* mutation leading to the G650E substitution resulted in an 8-fold increase in fluconazole MIC whereas correction of this mutation in resistant isolates led to a 16-fold reduction. RNA-seq analysis revealed four genes to be commonly up-regulated in the presence of the *CpTAC1* mutation including the transporter genes *CpCDR1*, *CpCDR1B, and CpCDR1C*. These genes were also found to be up-regulated among resistant clinical isolates including those with *CpTAC1* mutations. Overexpression of these transporter genes in a susceptible isolate led to reduced fluconazole susceptibility and their disruption in a resistant isolate led to increased susceptibility to fluconazole and other triazoles.

### Conclusions

These results define the specific contribution made by the Y132F substitution in *CpERG11* to fluconazole resistance, demonstrate a role for activating mutations in *CpTAC1* in triazole resistance, and underscore that multiple mechanisms are often required to act in concert to achieve clinically relevant levels of resistance.

## INTRODUCTION

Candidemia is associated with significant morbidity and mortality and is one of the most common healthcare-associated bloodstream infections in the United States, with approximately 25,000 cases diagnosed each year. (1) *Candida parapsilosis* is the most common causative agent of non-*albicans Candida* (NAC) infections in pediatric and neonatal populations and is responsible for a growing proportion of invasive *Candidiasis* in many parts of the world. (2, 3) Triazole antifungals such as fluconazole are important antifungal agents in the treatment of *Candida* infections. (4) Triazoles are fungistatic drugs that reduce production of the membrane sterol ergosterol in susceptible *Candida* species by competitively inhibiting the activity of lanosterol 14-a-demethylase. Disruption of the ergosterol biosynthesis pathway leads to a reduction in ergosterol and an accumulation of sterol precursors and 14-a-methyl-sterols, the latter of which are thought to be detrimental to fungal cell membrane integrity. (5)

Resistance to fluconazole has been well studied in the related species *Candida albicans* and has often been shown to be the result of multiple mechanisms working in combination. These mechanisms include mutations in the lanosterol 14-a-demethylase (*ERG11*) or sterol desaturase *(ERG3)* genes, overexpression of *ERG11* due to activating mutations in the gene encoding the transcription factor Upc2, and overexpression of the drug transporters encoded by *CDR1*, *CDR2 and MDR1* due to activating mutations in their respective transcription factors Tac1 and Mrr1. (5, 9-12)

Recent surveillance studies have highlighted notable increases in the rate of fluconazole resistance in *C. parapsilosis* isolates from the United States, South America, South Africa, and parts of western Asia over the past decade. (1, 3, 6-8) Similarities are apparent between the molecular and genetic basis of fluconazole resistance in *C. parapsilosis* and *C. albicans*, however, important differences exist. Unlike the wide repertoire of *ERG11* mutations found in *C. albicans*, (13) only a few have been identified in worldwide surveillance studies of *C. parapsilosis* clinical isolates, most predominantly the A395T mutation in *CpERG11* (CPAR2_303740). (14) Mutations in *CpERG3* (CPAR2_105550) leading to loss of sterol desaturase activity have been found to contribute to resistance, but as in *C. albicans*, these appear to be rare. (15, 16) Finally, as in *C. albicans*, mutations in the genes orthologous to *MRR1* and *TAC1* have been identified in resistant *C. parapsilosis* isolates, and increased expression of genes homologous to those encoding the Mdr1 and Cdr1 transporters have also been observed. (17-20) Indeed, experimentally evolved *CpMRR1* (CPAR2_807270) mutations have been shown to drive overexpression of *CpMDR1* (CPAR2_301760). (18) We have recently shown that such *CpMRR1* mutations occur relatively frequently among resistant *C. parapsilosis* clinical isolates and appear to drive resistance primarily through overexpression of the *CpMDR1B* (CPAR2_603010) and *CpCDR1B* (CPAR2_304370) transporters. (20)

Our previous examination of 35 fluconazole resistant clinical *C. parapsilosis* isolates identified 11 that carried the A395T mutation leading to the Y132F substitution in CpErg11. Of these, three isolates also exhibited elevated expression of the ATP-

Binding Cassette (ABC) transporter gene *CpCDR1* (CPAR2_405290) and when examined further were found to carry non-synonymous mutations in the putative *CpTAC1* gene, CPAR2_303510. (19) In two of the isolates, we found a homozygous mutation leading to the amino acid substitution G650E and a heterozygous mutation leading to the L978W substitution in the third isolate. Additional *CpTAC1* mutations have since been observed by others in fluconazole-resistant *C. parapsilosis* clinical isolates further suggesting a role for CpTac1 in resistance. (21, 22) Here we show that the mutation in *CpERG11* leading to the Y132F substitution and the mutation in *CpTAC1* leading to the G650E substitution both contribute in part to resistance and together lead to clinically relevant, high-level fluconazole resistance. We further demonstrate that in addition to fluconazole, the mutation leading to the G650E substitution in *CpTAC1* also confers increased resistance to other triazoles through the increased expression of the ATP-Binding Cassette (ABC) transporter genes *CpCDR1*, *CpCDR1B* and a third *CDR1* homolog that we have designated *CpCDR1C* which was previously classified as a pseudogene in the *C. parapsilosis* reference strain CDC317.

## MATERIALS AND METHODS

### Strains and media

*C. parapsilosis* isolates used in this study have been previously described. (19, 20) Isolates and derived strains (**Table 1**) were stored at -80°C in 40% glycerol stocks. Cultures were propagated with YPD media at 30°C and RPMI supplemented with MOPS, 2% glucose, pH 7.0 was used for drug susceptibility testing. Plasmids were constructed in DH5α cells and cultured in LB media+100 μg/mL ampicillin.

### Sequence confirmation

Primers and oligonucleotides are listed in **Table S1**. Genomic DNA was isolated as previously described. (23) PCR products purified with QIAquick PCR purification kit (Qiagen) and used for Sanger sequencing (Hartwell Center, St. Jude Children's Research Hospital). Sequences aligned to GCA_000182765.2_ASM18276v2 (SnapGene V6.2).

### Drug susceptibility

Minimum Inhibitory concentrations (MIC) for triazoles were determined by broth microdilution according to CLSI M27-A4 guidelines. 100x fluconazole (Sigma Aldrich), voriconazole, itraconazole, isavuconazole, and posaconazole stocks in DMSO were used for RPMI serial dilutions. Triplicate MIC runs determined visually for 50% growth inhibition at 24hrs.

### *C. parapsilosis* transformation and genetic manipulation

Cultures were grown for transformation as previously described. (19, 20) Electroporation performed using *C. albicans* presets on GenePulser Xcell (BioRad). Previously described plasmid-based CP-tRNA system was used for efflux pump disruption and insertion of *CpTAC1*/*CpERG11* point mutations. (20, 25) Repair templates were generated using microhomology to genes of interest (GOI) containing either the desired point mutations or the addition of a 22-nucelotide STOP-codon sequence for GOI disruption (Integrated DNA Technologies) (**Table S1**). Positive transformants identified by Sanger sequencing.

### RNA Sequencing

Isolates were cultured based on cell culture described in the CLSI guideline for MIC with slight modification. (20) RNA was isolated with the RiboPureTM Yeast (Invitrogen) system per manufacturer's instructions. Stranded mRNA sequencing was performed using Illumina NovaSeq (Hartwell Center, St. Jude Children's Research Hospital). Libraries were prepared with paired-end adapters by Illumina chemistries per the manufacturer, ~150bp read lengths, targeting >50 million paired-reads per sample. Analysis was performed with CLC Genomics Workbench version 20.0 (Qiagen) (**Table S2**).

### RTqPCR

Biological triplicates grown as previously described. (19) RNA isolation by RIBOpure yeast kit (Invitrogen). Complementary DNA synthesized from 2μg total RNA with SuperScript first-strand synthesis kit (Invitrogen) and SYBR green master mixes per manufacturer's instructions. Primers (**Table S1**) combined with cDNA (1:50) and GOI were amplified with SSOAdvanced Universal SYBR green SuperMix (BioRad) for 40 cycles in CFX96-RealTimeSystem (BioRad) in technical triplicate. Relative expression then calculated as $2^{-(\Delta\Delta CT)}$.

## RESULTS

### *CpERG11* mutation resulting in the Y132F amino acid substitution contributes directly to fluconazole resistance

To understand the specific contribution of the mutation leading to the Y132F substitution in *CpERG11*, we first attempted to correct this mutation to the wild-type sequence in both alleles of resistant isolate Cp35 and were unsuccessful. We then successfully introduced this mutation into both alleles of *CpERG11* in susceptible isolates Cp13, Cp23, and Cp3 resulting in a four- to eight-fold increase in MIC depending on the

clinical isolate background, however no MIC surpassed the clinical breakpoint for fluconazole resistance (**Figure 1**) and only minimal if any effects were observed for other triazoles (**Figure S1**). These results demonstrate the direct influence of this mutation on fluconazole MIC and indicate that the high level of fluconazole resistance observed for isolates carrying this mutation is not explained by this mutation alone.

**Fluconazole resistance is mediated in part by activating mutations in *CpTAC1* which drive overexpression of *CpCDR1, CpCDR1B* and *CpCDR1C***

We previously observed that three fluconazole resistant isolates which overexpressed *CpCDR1* in our collection of 35 fluconazole-resistant clinical isolates, also had *CpTAC1* mutations. (19) In the current study, sequencing of *CpTAC1* for the entire collection confirmed the presence of the *CpTAC1* mutations reported previously by Berkow et al. for isolates Cp35, Cp38, and Cp40 and further revealed mutations leading to amino acid substitutions in a total of 9 of the 35 resistant isolates (29%) (**Table 1**). (19)

To determine if the *CpTAC1* mutations influence fluconazole susceptibility, we focused on a putative gain-of-function (GOF) mutation leading to the substitution G650E in Cp35 and Cp38 and introduced this mutation into both alleles of the fluconazole susceptible isolate Cp13. This resulted in an 8-fold increase in fluconazole MIC. We then corrected the G650E mutation in both *CpTAC1* alleles for both Cp35 and Cp38 to the wild-type sequence, resulting in a 16-fold reduction in fluconazole MIC for both isolates (**Figure 2A**). Similar changes in voriconazole, itraconazole, isavuconazole, and posaconazole susceptibility were also observed (**Table 2**). Importantly, both Cp35 and Cp38 carry *CpERG11* mutations which likely contributed to the residual elevated MICs within these strains.

Our previous observations of Cp35 and Cp38 revealed overexpression of *C. albicans CDR1* ortholog, *CpCDR1*. However, *CpCDR1* deletion in these isolates only resulted in a single dilution reduction in fluconazole MIC, indicating involvement of other resistance determinants. (19) We therefore compared the transcriptional profiles of Cp35 and Cp38 to their respective derivatives in which the *CpTAC1* mutation leading to the G650E substitutions were corrected to the wild-type sequence. RNA sequencing revealed differential expression of eight genes in Cp35 as compared to its *CpTAC1* corrected derivative, all of which were up-regulated in the presence of $CpTAC1^{G650E}$ (**Table S2**). Compared to its *CpTAC1* corrected derivative, Cp38 differentially expressed 45 genes, 24 of which were up-regulated in the presence of $CpTAC1^{G650E}$ (**Table S2**). Only four genes were commonly up-regulated in both isolates (**Figure 2B**). These were *CpCDR1* (CPAR2_405290), a second *CDR1* homolog, previously designated as *CpCDR1B* (CPAR2_304370) (20), a *Candida* Genome Database-classified pseudogene with homology to *CDR1*, CPAR2_300010, and a *CIP1* homolog, CPAR2_301450 (**Figure 2C**). The elevation of the pseudogene in each data set led us to sequence the CPAR2_300010 open reading frame in Cp13, Cp35 and Cp38. When compared to CPAR2_300010 in CDC317, each isolate contained the insertion 3424GAA – 3424GAAA causing a frameshift at R1144. This single nucleotide insertion creates a

continuous open reading frame (ORF), eliminating the early stop codons observed in the reference sequence. Additionally, the insertion was observed in this location of CPAR2_300010 for all 39 *C. parapsilosis* clinical isolates within our collection. We therefore designated the CPAR2_300010 ORF as *CpCDR1C.* These results demonstrate that activating mutations in *CpTAC1* contribute directly to triazole resistance and suggest that the mutation leading to CpTac1$^{G650E}$ influences susceptibility through multiple ABC transporters, *CpCDR1, CpCDR1B,* and *CpCDR1C*.

**Expression of *CpCDR1, CpCDR1B, and CpCDR1C* directly affect fluconazole resistance**

Due to genetic sequence similarity between the three *CDR1* homologs, (*CpCDR1*: 80.9% identity to *CpCDR1B* and *CpCDR1C*; *CpCDR1B:* 87.8% identity to *CpCDR1C*) we determined the relative expression for each across all 39 clinical isolates by RTqPCR (**Figure 3**). We observed the expression of *CpCDR1*, *CpCDR1B*, and *CpCDR1C* to be upregulated by ≥2-fold in 15, 21, and 11 resistant clinical isolates, respectively (**Figure 3**). While *CpCDR1* and *CpCDR1C* were upregulated in 9 isolates, *CpCDR1* and *CpCDR1B* were upregulated in 7 isolates. Only 6 isolates had upregulation of all three homologs. Two of these Cp20 and Cp28 are heterozygous for the mutation encoding the A854V substitution in CpMrr1. (20) The remaining four isolates Cp21, Cp35, Cp37, and Cp38 each contain a mutation in at least one *CpTAC1* allele.

To determine if *CpCDR1, CpCDR1B,* and *CpCDR1C* are capable of influencing fluconazole susceptibility, we overexpressed these genes individually in isolate Cp13 by placing them under the control of a strong constitutive *CpTEF1* promoter. (20) We achieved a 10-fold increase in expression of *CpCDR1*, a 19-fold increase in *CpCDR1B* expression and a 75-fold increase in *CpCDR1C* in relation to their baseline constitutive expression (**Figure S2A**). Overexpression increased fluconazole MIC 16-fold for *CpCDR1 and CpCDR1B and* 4-fold for *CpCDR1C* (**Figure S2B**). Overexpression of *CpCDR1* and *CpCDR1B* increased voriconazole and itraconazole MICs to a varied extent, however *CpCDR1C* overexpression had no effect on voriconazole MIC and minimal effects on itraconazole MIC (**Figure S2B**). These results indicate that all three transporters are capable of contributing to triazole resistance.

To determine the individual and combined contribution for all three ABC transporters to the resistance conferred by *CpTAC1 GOF*, we disrupted each gene individually and simultaneously in isolate Cp35. Disruption of *CpCDR1* resulted in a two-fold reductions in fluconazole MIC, whereas individual disruption of *CpCDR1B* and *CpCDR1C* did not result in measurable reductions in fluconazole MIC as measured by broth microdilution (**Table 2**). Disruption of all three genes simultaneously resulted in a four-fold reduction in fluconazole MIC. Generally, similar incremental decreases in MIC were observed for the other triazoles tested by microbroth dilution (**Table 2**). Importantly, while the disruption of *CpCDR1*, *CpCDR1B* and *CpCDR1C* displayed appreciable differences in MIC when measured by microbroth dilution assays, a more pronounced effect on fluconazole susceptibility was observed when measured by a

general diffusion test strip (**Figure S3**). Here, simultaneous disruption of all three genes resulted in a change in susceptibility more consistent with that observed when *CpTAC1* was corrected to the wild-type sequence (**Figure S3**). However, for all the triazole MICs tested by broth microdilution assay, none of the disruptions completely returned the MICs to the levels observed when *CpTAC1* was corrected to the wild-type sequence (**Table 2**). These results suggest *CpCDR1*, *CpCDR1B*, and *CpCDR1C* are determinants of *CpTAC1*-mediated fluconazole resistance, but that additional CpTac1 targets may contribute to the residual differences in susceptibility.

## DISCUSSION

The mutation leading to the Y132F substitution in CpErg11 is by far the most common mutation associated with fluconazole resistance in *C. parapsilosis.* Indeed, this mutation is present in at least one allele in 14 of the 35 fluconazole-resistant clinical isolates in our collection, six of which also carry a mutation in *CpTAC1*. Based on the contribution of similar mutations in the *ERG11* active-site to fluconazole resistance in *C. albicans*, it has been presumed to have a similar impact on resistance in *C. parapsilosis*. We show here that while this mutation contributes to fluconazole resistance in *C. parapsilosis*, it is not sufficient in-and-of itself to achieve clinical resistance to fluconazole as defined by current CLSI clinical breakpoints and often occurs in tandem with mutations in *CpTAC1* to achieve high-level fluconazole resistance.

Activation of Tac1, and the subsequent overexpression of the genes encoding ABC transporters Cdr1 and Cdr2, is an important driver of fluconazole resistance in *C. albicans*. Overexpression of *CDR1* can be detected in nearly 70% of fluconazole-resistant *C. albicans* isolates (26, 27), and sequence analysis suggests *TAC1* mutations are present in 55-75% of fluconazole-resistant *C. albicans* isolates. (28, 29) Here, we found that 85% (30/35) of our fluconazole-resistant *C. parapsilosis* isolates overexpressed *CpCDR1*, *CpCDR1B*, or *CpCDR1C* $\geq$ 2-fold, and increased *CpCDR1B* alone accounted for 43% (13/30) of these isolates. However, only 9 (26%) isolates contained nonsynonymous mutations in at least one *CpTAC1* allele. Therefore, while ABC transporter overexpression appears prevalent among fluconazole-resistant *C. parapsilosis* isolates, potentially activating *CpTAC1* mutations appear less frequently compared to fluconazole-resistant *C. albicans* clinical isolates. Additionally, the homozygous mutations leading to the N900D and I221T substitutions in CpTac1 did not coincide with increased expression for *CpCDR1*, *CpCDR1B*, or *CpCDR1C*. Isolates with the CpTac1 R208G substitution did have increased *CpCDR1B* expression. However, this is most likely due to the simultaneous presence of *CpMRR1* mutations in these isolates given that CpTac1$^{R208G}$ has also been previously observed among fluconazole-susceptible isolates (22). Importantly, we observed increased expression for all three *CDR1* homologs in all isolates containing a G650E or L978W mutation, suggesting that both of these mutations activate CpTac1.

In *C. albicans*, activation of Tac1 leads to the up-regulation of a repertoire of genes associated with the response to biotic stimulus and oxidative stress. We found the

upregulated genes associated *CpTAC1* mutation to be very different from those regulated by activation of *TAC1* in *C. albicans*, except for the *CDR1* homologs *CpCDR1*, *CpCDR1B*, and *CpCDR1C*. (30) While, mutations *CpMRR1* have been shown to increase *CpCDR1B* expression (20), here, we show mutations in *CpTAC1* increase expression for *CpCDR1*, *CpCDR1B*, and *CpCDR1C*. The shared ability to regulate *CpCDR1B* by both CpMrr1 and CpTac1 is in striking contrast to the *C. albicans* Tac1 and Mrr1 regulation of specific transporter types. (11, 12) Taken together, our work expands our understanding of fluconazole resistance in *C. parapsilosis* by showing that high-level resistance can be achieved by CpErg11$^{Y132F}$ working in concert with activating *CpTAC1* mutations. Understanding the genetic basis of fluconazole resistance reveals opportunities for improved surveillance, diagnostics, and therapeutics to combat such resistance in the clinical setting.

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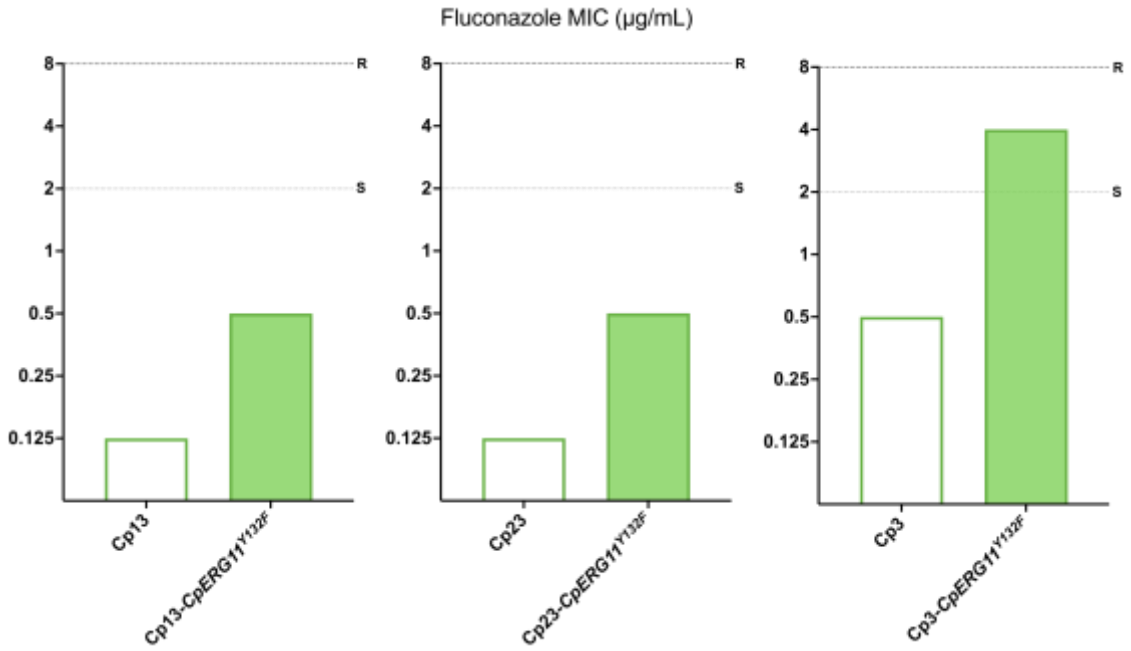
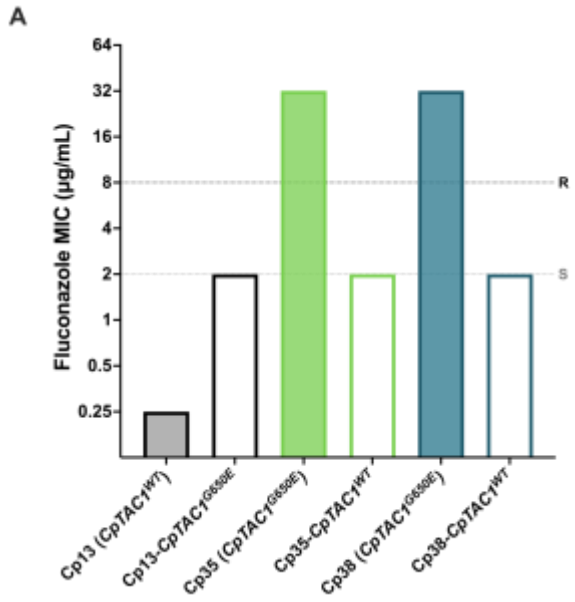



**Figure 1. Effect of Erg11 Y132F substitution on fluconazole susceptibility**. Susceptibility testing representative of three independent MIC measurements as determined by broth microdilution in accordance with CLSI guidelines. MICs represented in micrograms per milliliter were measured at 24hrs. R represents the clinical breakpoint for fluconazole resistance in *C. parapsilosis;* S represents the clinical breakpoint for fluconazole susceptibility for *C. parapsilosis*.





C

| Systematic ID | Standard Name | Fold Change CpTac1$^{G650E}$ vs CpTac1$^{WT}$ | |
|---|---|---|---|
| | | Cp35 | Cp38 |
| CPAR2_300010 | *CpCDR1C** | 7.96 | 9.64 |
| CPAR2_405290 | *CpCDR1* | 3.91 | 4.42 |
| CPAR2_304370 | *CpCDR1B* | 2.54 | 2.8 |
| CPAR2_301450 | | 2.51 | 2.54 |

**Figure 2. Effects of CpTac1 G650E substitution.**

Note: A) Susceptibility testing representative of three independent MIC measurements as determined by broth microdilution in accordance with CLSI guidelines. MICs represented in micrograms per milliliter were measured at 24hrs. R represents the clinical breakpoint for fluconazole resistance in *C. parapsilosis*; S represents the clinical breakpoint for fluconazole susceptibility for *C. parapsilosis*. B) Differentially expressed genes in clinical isolates with homozygous mutation leading to amino acid substitution in CpTac1 compared to the corrected strain as determined by RNA sequencing. Upregulated genes include genes with ≥2 fold change for clinical isolate with CpTac1 mutation vs each clinical isolate with CpTac1 corrected to wild type. No downregulated gene were determined to fit criteria and be common between strains. FDR p-values ≤ 0.05. C) Genes upregulated in isolates with CpTac1$^{G650E.}$

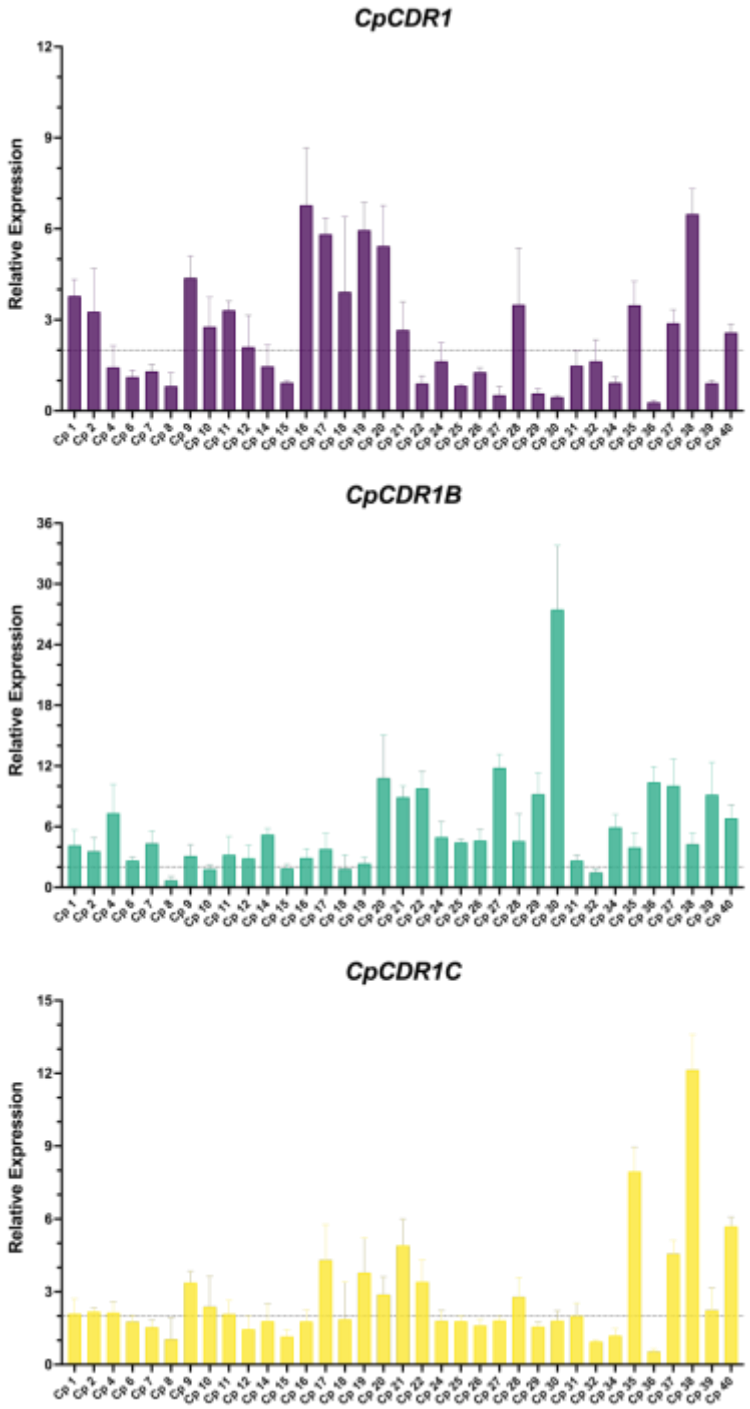



**Figure 3. Increased *CpCDR1*, *CpCDR1B*, and *CpCDR1C* expression identified within multiple *C. parapsilosis* clinical isolates.**

Note: Differential expression for *CpCDR1*, *CpCDR1B*, and *CpCDR1C* among fluconazole resistant clinical isolates. Relative quantitation for efflux pump expression as measured by RTqPCR. The average for each biological replicate is compared to a single biological replicate of the susceptible isolate Cp13. Error bars represent standard error and the 2-fold increase threshold is indicated by the dotted line.

**Table 1. CpTac1 amino acid substitutions, with corresponding CpErg11 substitutions, identified from a collection of 35 fluconazole-resistant *C. parapsilosis* clinical isolates.**

| Clinical Isolate | FLU MIC (μg/mL)[b] | Amino acid substitution [c] | |
|---|---|---|---|
| | | CpTac1 | CpErg11 |
| Cp 15 | 16 | N900D | F145L |
| Cp 21 | 8 | L978W‡ | Y132F, R398I |
| Cp 27 | 32 | R208G | |
| Cp 32 | 128 | I221T‡ | R398I |
| Cp 35 | 32 | G650E | Y132F, R398I |
| Cp 37 | 256 | L978W‡ | Y132F, R398I |
| Cp 38 | 32 | G650E | Y132F, R398I |
| Cp 39 | 32 | R208G | Y132F |
| Cp 40 | 128 | L978W‡ | Y132F‡, R398I‡ |

Note: [a] Clinical isolate collection previously described by Berkow EL, et al. 2015, Doorley LA, et al. 2022. [b] Fluconazole MIC obtained by broth microdilution assay as described in the CLSI document M27, 4th ed. [c] Mutations encoding amino acid substitutions identified via Sanger sequencing. ‡ Heterozygous mutation

**Table 2. Summary of triazole[a] MICs (μg/mL) for efflux pump disruption strains.**

| Strain ID[b] | FLU | VOR | ISAV | ITR | POSA |
|---|---|---|---|---|---|
| Cp35 | 32 | 1 | 0.0625 | 0.25 | 0.125 |
| Cp35-*CpTAC1*$^{WT}$ | 2 | 0.125 | 0.0039 | 0.03125 | 0.03125 |
| Cp35-1A | 16 | 1 | 0.03125 | 0.0625 | 0.03125 |
| Cp35-1B | 32 | 1 | 0.0625 | 0.0625 | 0.03125 |
| Cp35-1C | 32 | 1 | 0.03125 | 0.0625 | 0.03125 |
| Cp35-1A1B | 16 | 0.5 | 0.03125 | 0.0625 | 0.03125 |
| Cp35-1A1C | 16 | 0.5 | 0.03125 | 0.0625 | 0.03125 |
| Cp35-1B1C | 32 | 0.5 | 0.03125 | 0.0625 | 0.03125 |
| Cp35-1A1B1C | 8 | 0.5 | 0.03125 | 0.0625 | 0.03125 |

Note: [a] FLU: Fluconazole, VOR: Voriconazole, ISAV: Isavuconazole, ITR: Itraconazole, POSA: Posaconazole. MICs read at 24hr and in accordance with CLSI standards for microbroth dilution. Values represent μg/mL.[d] Disrupted transporter abbreviations: *CpCDR1*, 1A; *CpCDR1B*, 1B; *CpCDR1C*, 1C.

## SUPPLEMENTAL MATERIALS

**Mutations in *TAC1* and *ERG11* are major drivers of triazole antifungal resistance in clinical isolates of *Candida parapsilosis***

### Expanded Methods

#### Figure S2. Putative drug transporter overexpression.

Overexpression of drug transporters *CpCDR1* (CPAR2_405290), *CpCDR1B* (CPAR2_304370), and *CpCDR1C* (CPAR2_300010) via *CpTEF1* (CPAR2_407690) promoter insertion was performed as previously described. (20) The Cas9-RNP method was used as previously described with modification for *C. parapsilosis*. (20, 24) Repair templates were purified using the Gene Clean II kit (MP Biomedicals). Transformants were confirmed via multiple PCR screenings and subsequent electroporation on agarose gel (**Table S1**).

#### Figure S3. Fluconazole MICs measured by diffused test strip.

Strains were cultured according to CLSI M27-A4 guidelines. Fluconazole Etests using diffusion test strips (bioMérieux USA, Chicago, IL) and were performed according to manufacturer's instructions.

#### Table S2. RNA sequencing analysis used for comparison of *CpTAC1* gain-of-function.

Reads mapped to the *C. parapsilosis* reference genome (GenBank accession: GCA_000182765.2). Paired reads were counted as one and expression values set to RPKM. Principal-component analysis was performed for the assessment of biological replicates. Differential gene expression analysis was performed using the prescribed algorithm. Mismatch, insertion, and deletion costs were set to default parameters. Statistical analysis performed using the Wald test for all group pairs against matched parent control strains.

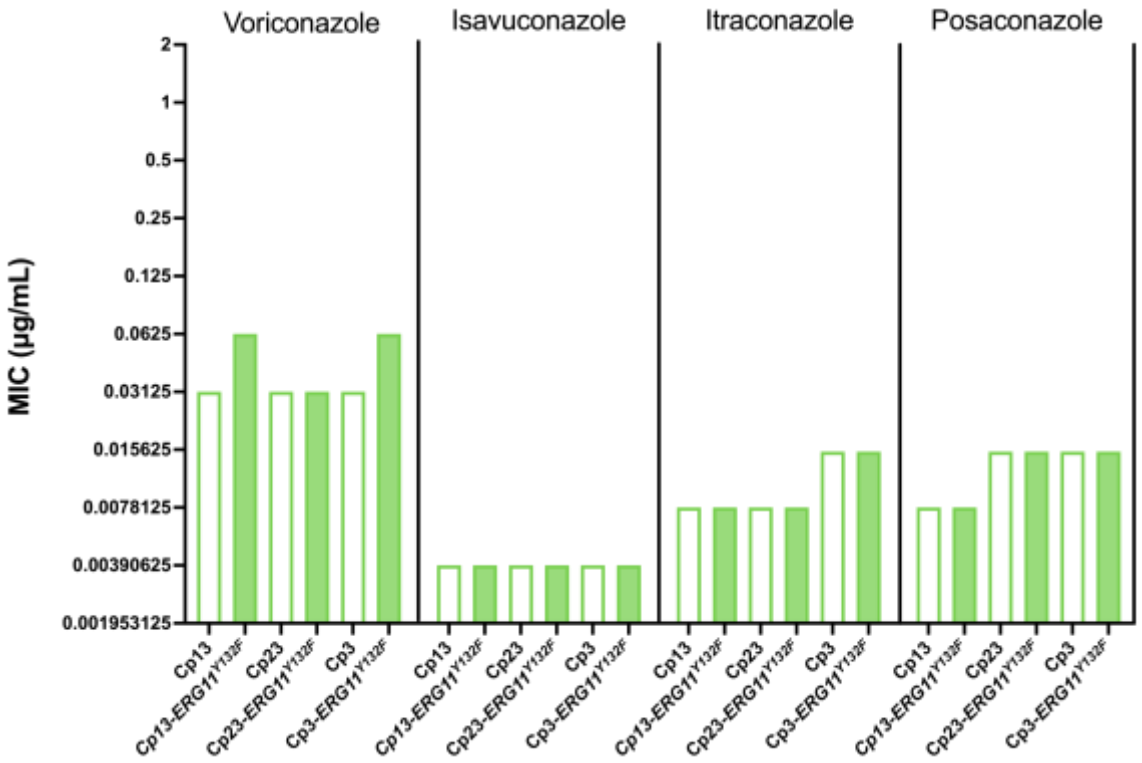



**Figure S1. Effect of Erg11 Y132F substitution on triazole susceptibility.**

Note: Susceptibility testing representative of three independent MIC measurements as determined by CLSI M27-A4 broth microdilution guidelines. MICs visually read at 24hrs.

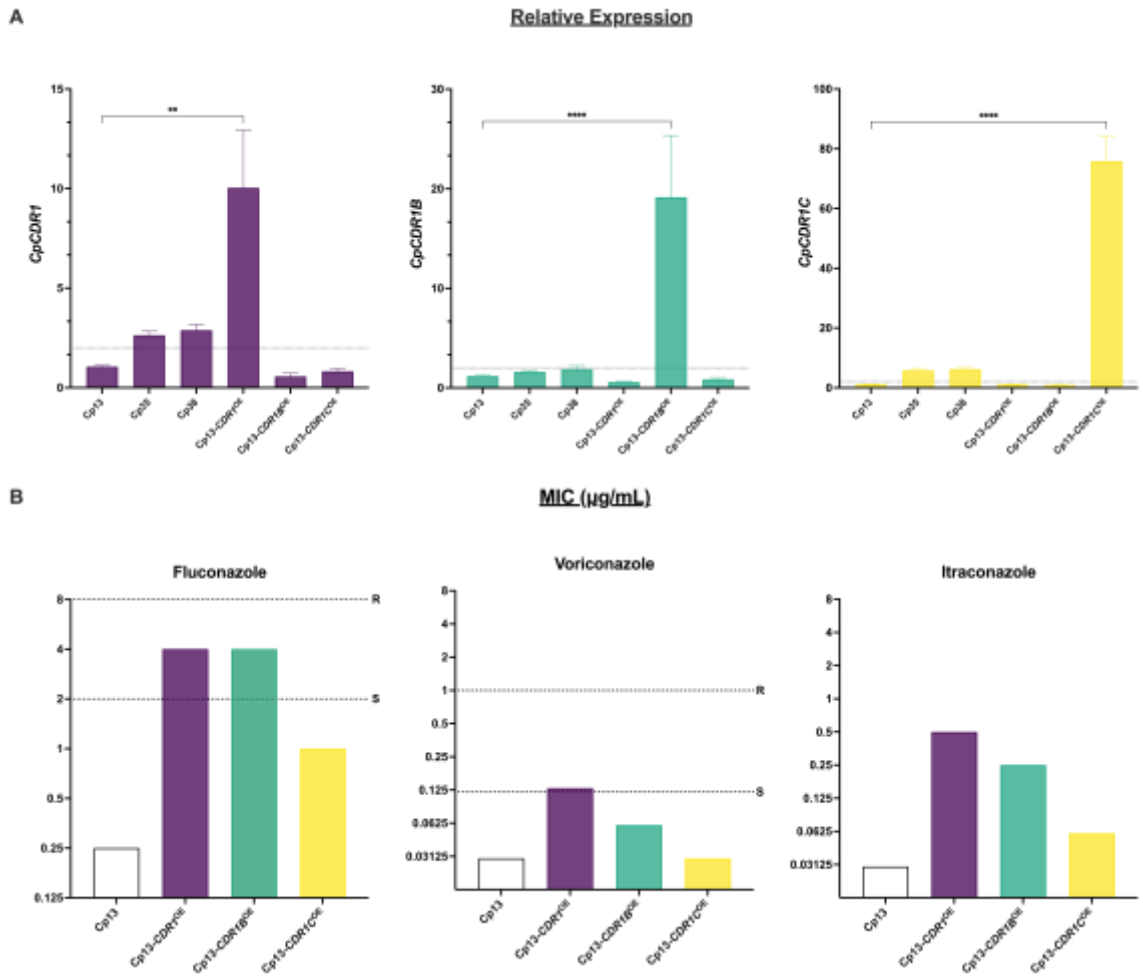



**Figure S2. Overexpression of *CpCDR1, CpCDR1B,* and *CpCDR1C.***

Note: A) Constitutive overexpression (OE) of *CpCDR1* and *CpCDR1C* Relative fold change of efflux pump expression as measured by RTqPCR. Each strain was grown in biological triplicate and RTqPCR performed in technical triplicate for each sample. The average for each biological replicate is compared to a single biological replicate of the susceptible isolate Cp13. Error bar standard deviation for 3 biological replicates. 2-fold increase threshold indicated by dotted line. One-way ANOVA statistical analysis compared to susceptible isolate Cp13 **p value <0.01, ***p value <0.0001. B) Impact of *CpCDR1*, *CpCDR1B,* and *CpCDR1C* OE on select triazole susceptibility in a susceptible clinical isolate. Susceptibility testing representative of three independent MIC measurements as determined by broth microdilution in accordance with CLSI guidelines. MICs represented in micrograms per milliliter were measured at 24hrs. R represents the CLSI clinical breakpoint for clinical resistance, S represents the clinical breakpoint for susceptibility where applicable for *C. parapsilosis*

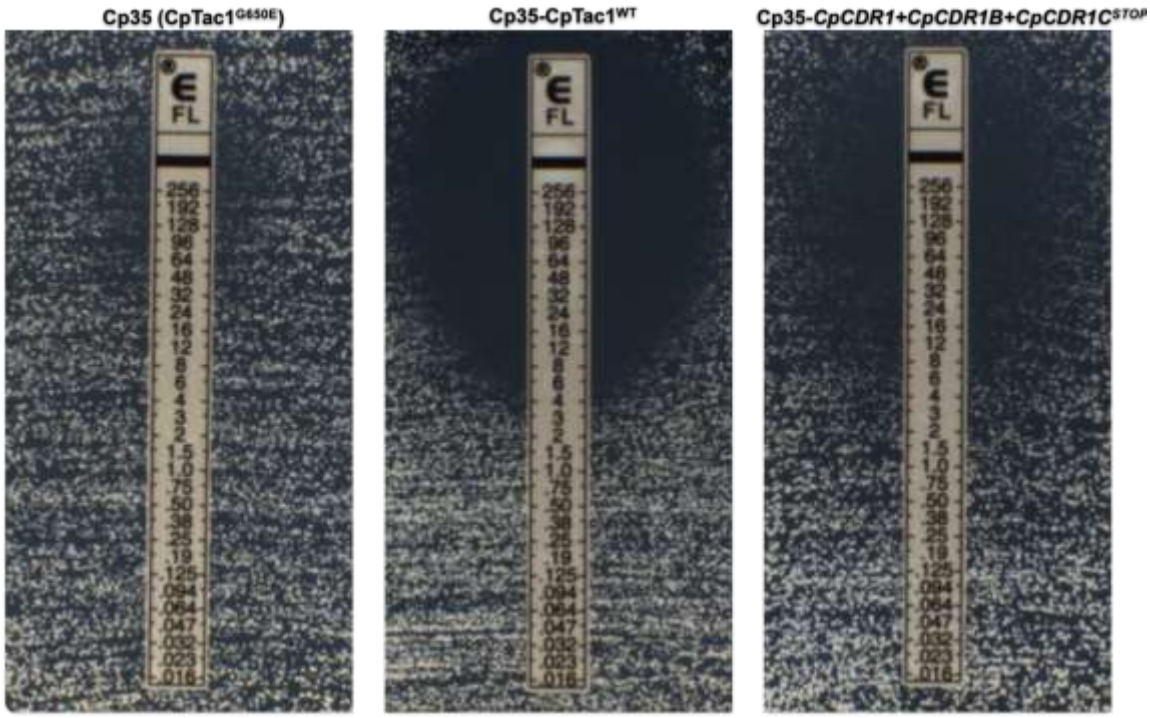



**Figure S3. Fluconazole Etests for comparison of CpTac1 correction and ABC transporter disruption with the parental clinical isolate Cp35.**

Note: Plating done in accordance with manufacturer's instructions and in accordance with CLSI recommendations. Plates imaged 24hrs and all images treated equally for zone of inhibition cropping.

**Table S1. Primer sequences used for CpTac1, CpErg11, and ABC transporter experimentation.**

| Primer Name* | 5'- Sequence-3' |
|---|---|
| | ***CpERG11* manipulation** |
| CpErg11_Y132F_RTF | GAATCGGTAGTCAATGCGGTCTTTGCGAACTTCTTTTGCTCCATAAGTCTTGCATT |
| CpErg11_Y132F_RTR | GGAAAAGGTGTTATTTTCGATTGTCCtAATGCAAGACTTATGGAGC |
| CpErg11_Y132F_guide1 | CCATTGTCCGAATGCAAGACTTA |
| CpErg11_Y132F_guide2 | AACTAAGTCTTGCATTCGGACAA |
| CpErg11_Y132F_AmpF | CCCTACCTTCGTTCATCCAGAC |
| CpErg11_Y132F_AmpR | CATAACTACACACCACTGACTCC |
| CpErg11_Y132F_Seq1 | GGGTAGAGTAATGACGGTGT |
| | ***CpTAC1* sequencing** |
| CpTAC1_SeqA | TGAACCATATCTGGGAGTTTAACAG |
| CpTAC1_SeqB | GGATATGCACTGTATATCGGTACC |
| CpTAC1_SeqC | GATGATGTCACAACCTGTACAGAG |
| CpTAC1_SeqD | CGATTTTGCCAAACCCGATAAG |
| CpTAC1_SeqE | CTAAACACCCCACTTGAGATGC |
| CpTAC1_SeqF | CTTATCGGGTTTGGCAAAATCG |
| CpTAC1_SeqG | CTCTGTACAGGTTGTGACATCATC |
| CpTAC1_SeqH | GGTACCGATATACAGTGCATATCC |
| | ***CpTAC1* manipulation** |
| CpTAC1_E650G _RTF | CGCTCATATGATGGTAATCAATCGATTTCCATTTGTTGTGCAAACGGACAAAGTTGATGCCGgGAGCCAGAT |
| CpTAC1_E650G _RTR | CACCAAAATAGTTCGAGCTGCATCCAACGATGTATTACGAAACTTTAAAATCTGGCTCcCGGCATC |
| CpTAC1_G650E _RTF | CGCTCATATGATGGTAATCAATCGATTTCCATTTGTTGTGCAAACGGACAAAGTTGATGCCGaGAGCCAG |
| CpTAC1_G650E _RTR | CACCAAAATAGTTCGAGCTGCATCCAACGATGTATTACGAAACTTTAAAATCTGGCTCtCGGCATCAAC |
| CpTAC1_E650G_guide1 | CCAAAACTTTAAAATCTGGCTCT |
| CpTAC1_E650G_guide2 | AACAGAGCCAGATTTTAAAGTTT |
| CpTAC1_G650E_guide1 | CCACAAACGGACAAAGTTGATGC |
| CpTAC1_G650E_guide2 | AACGCATCAACTTTGTCCGTTTG |
| | ***CpCDR1C* frameshift confirmation** |
| CpCDR1C_FS_amp1 | CTTTCCTAGCACTACAATTCAAGG |
| CpCDR1C_FS_amp2 | CTGCTTATGTCAACGATGAGGT |
| CpCDR1C_FS_Seq1 | CCATGCCAAACATGACTAT |
| CpCDR1C_FS_Seq2 | GATGGTTCTCCAAGTCAC |
| | **Efflux pump disruption** |
| CpCDR1_guide1 | CCAGAATTTGGGAACAGAGTCCA |
| CpCDR1_guide2 | AACTGGACTCTGTTCCCAAATTC |
| CpCDR1_Stop_RTF | CGGCACCGTAAATCGAAAAGAAGATTCAGCCATGTCGAATTTGGGAACAGAGCGGTAGATAGATAGCGGGGAAG |
| CpCDR1_Stop_RTR | GAGGATGAAGTTTGCTGCTTTTCCATTCTATTCAAAGAGTCGTCCTTGGACTTCCCCGCTATCTATCTACCG |
| CpCDR1_Amp_F | CCGCATATAGTCCTCGTAAAGTGG |
| CpCDR1_Amp_R | GCTGGAACATATTGTGCGCAC |
| CpCDR1_Seq1 | GGCGTCAAAGCTGTCCG |
| CpCDR1B_guide1 | AACACGACAGAGGAGACTAACGA |
| CpCDR1B_guide2 | CCATCGTTAGTCTCCTCTGTCGT |
| CpCDR1B_Stop_RTF | GAGTCGATTACATTTGCCAACCCACTTTTTTCAATTATGCGGTAGATAGATAGCGGGGAAG |

| | |
|---|---|
| CpCDR1B_Stop_RTR | GCTGCTTCTCCAAATTGTTGTAGGAGTCTTCGTTAGTCTCCTCTGT CGTTGGCTTCCCCGCTATCTATCTACCG |
| CpCDR1B_amp_F | GACTTGGCGCTTGTCGGG |
| CpCDR1B_amp_R | CCCAACATACTTGGCGTAGG |
| CpCDR1B_Seq1 | CTTCCATCCCTTGATGAGTTAGAC |
| CpCDR1C_guide1 | CCAAGAGGACTCGGTCAACAAGT |
| CpCDR1C_guide2 | AACACTTGTTGACCGAGTCCTCT |
| CpCDR1C_Stop_RTF | CTGTCCTTTCCTAGCACTACAATTCAAGGAGGAATGTCTGATTGC GAAAAACAATCCAAAGAGGACTCGGTCCGGTAGATAGATAGCGG GGAAGAAC |
| CpCDR1C_Stop_RTR | CATCAAACCCTGAATATTCATATATTGACGGTCCCGACGACGTTG AATTGGTTTGTTGTTTTTCCAACTTGTTCTTCCCCGCTATCTATCTA CC |
| CpCDR1C_Amp_F | GCTTTGCTGCCACCATCATAC |
| CpCDR1C_Amp_R | AGGATGCCCCACGGAAATAG |
| CpCDR1C_Seq1 | GTTGCTGAACTAGTATCCT |
| CpSTOPBARCODE_F | CGGTAGATAGATAGCGGGGAAG |
| **Efflux pump overexpression** | |
| CpCDR1_OE_RTF | GATTGCTGATTTTAATTTTTGATTTCATTAGCAAGGTTTTCATAGC TATTATTAGGACCCAACTTGATAACGTTTTCCCAGTCACGACGT |
| CpCDR1_OE_RTR | GAGGATGAAGTTTGCTGCTTTTCCATTCTATTCAAAGAGTCGTCCT TGGACTCTGTTCCCAAATTCGACATCGGCCGCTTTTGTTGTTGC |
| CpCDR1B_OE_RTF | GACCCAAAGTGCATATCTCCGAGTGCAATTGTCCATTACCAAAAT CAAAAATTGAACAATGAAAGCAACGGTTTTCCCAGTCACGACGT |
| CpCDR1B_OE_RTR | GATGAGGAAGAAGTTTGCTGCTTCTCCAAATTGTTGTAGGAGTCT TCGTTAGTCTCCTCTGTCGTTGGCATCGGCCGCTTTTGTTGTTGC |
| CpCDR1C_OE_RTF | GCTTTTCAATGCCTTTTACTAACAATTTATGAAATCCCTCATTGGA ATCGGGTCCTCTTATACTTTCGATGGGTTTTCCCAGTCACGACGTT G |
| CpCDR1C_OE_RTR | TTGAATTGGTTTGTTGTTTTTCCAACTTGTTGACCGAGTCCTCTTTG GATTGTTTTTCGCAATCAGACATCGGCCGCTTTTGTTGTTGC |
| CpCDR1_5'_crRNA | TGTCGAATTTGGGAACAGAG |
| CpCDR1B_5'_crRNA | ATGTCTAACTCATCAAGGGA |
| CpCDR1C_5'_crRNA | TTTGGAAAGCATGGTTGAGG |
| CpCDR1_ NaPr_Scn_R | CTTTTCGATTTACGGTGCCGTG |
| CpCDR1_5'Flank_F | CCGCATATAGTCCTCGTAAAGTGG |
| CpCDR1_ORF_R | GGCGTCAAAGCTGTCCG |
| CpCDR1B_ NaPr_Scn _F | CTTCCATCCCTTGATGAGTTAGAC |
| CpCDR1B_5'Flank_F | GACTTGGCGCTTGTCGGG |
| CpCDR1B_ORF _R | GGCGGTACCATAAGCTCTCAAG |
| CpCDR1C_NaPr_Scn _F | CTTTCCTAGCACTACAATTCAAGG |
| CpCDR1C_5'Flank_F | GCTTTGCTGCCACCATCATAC |
| CpCDR1C_ORF _R | CAACGTCACCGAGGGATCAC |
| **Efflux Pump RTqPCR** | |
| CpACT1_qF1 | GTCACTACAACTATGCAATGGC |
| CpACT1_qR1 | TCGAGGTAGCCACAAGATTG |
| CpCDR1_qF1 | GGGTTGGTCCAGGTGGATCAA |
| CpCDR1_qR1 | GCTGAGCATGCTCTATTCGCT |
| CpCDR1B_qF1 | GAACGAGTTCCATGGTCGAGAG |
| CpCDR1B_qR1 | CGTTACTCCCTGGTCTAGACCC |
| CpCDR1C_qF1 | GGTGATCCCTCGGTGACGTTG |
| CpCDR1C_qR1 | AGGATGCCCCACGGAAATAG |

**Table S1. Continued.**

Note: * Primer names with keywords to indicate experimental function: RNP based editing (‘crRNA’); plasmid-based Cas9 editing (‘guide’); repair template amplification (‘RTF’ and ‘RTR’); RTqPCR (‘qF’ and ‘qR’); Sanger sequencing reactions (‘Seq’); PCR amplification (‘Amp’, ‘Flank’, ‘Scn’, ‘Orf’).

**Table S2. Differentially expressed genes for clinical isolates Cp35 and Cp38 compared to same clinical isolate background with *CpTAC1* corrected to wildtype, as determined by RNA sequencing.**

| *C. parapsilosis* gene [1] | *C. albicans* homolog [2] | Fold change [3] | Description [4] |
|---|---|---|---|
| **Cp38 (CpTac1$^{G650E}$) vs Cp38 - CpTac1$^{WT}$** | | | |
| CPAR2_300010 | *CDR1* | 9.64 | Pseudogene |
| *CpCDR1* | *CDR1* | 4.42 | Ortholog(s) have ABC-type xenobiotic transporter activity, and plasma membrane localization |
| CPAR2_802720 | orf19.3232 | 3.88 | Has domain(s) with predicted transmembrane transporter activity |
| CPAR2_500640 | orf19.1267 | 3.30 | Ortholog(s) have cysteine desulfurase activity, role in iron-sulfur cluster assembly, extrinsic component of mitochondrial inner membrane |
| CPAR2_405470 | *TRY4* | 3.06 | Has domain(s) with predicted nucleic acid binding, zinc ion binding activity |
| CPAR2_808370 | orf19.6484 | 2.92 | Ortholog of *C. albicans* SC5314 : C7_02280W_A |
| *CpGAP2* | *GAP2* | 2.82 | Ortholog(s) have L-proline transmembrane transporter activity |
| CPAR2_301450 | *CIP1* | 2.8 | Ortholog of *C. albicans* SC5314 : C6_01070C_A/*CIP1* |
| CPAR2_407710 | orf19.5365 | 2.61 | Ortholog of C. albicans SC5314 C2_10830W_A |
| *CpCDR1B* | *CDR1* | 2.54 | Has domain(s) with predicted ATPase-coupled transmembrane transporter activity. Upregulated by activating mutation in *CpMRR1* |
| CPAR2_501350 | orf19.3130 | 2.35 | Ortholog(s) have role in mitotic sister chromatid cohesion, protein import into nucleus |
| CPAR2_504120 | orf19.5455 | 2.30 | Ortholog(s) have GTPase regulator activity, mRNA binding activity |
| CPAR2_300120 | *CSA1* | 2.27 | Ortholog of *C. albicans* SC5314: C7_00090C_A/*CSA1* |
| CPAR2_208530 | *PHO100* | 2.22 | Has domain(s) with predicted hydrolase activity |
| CPAR2_805340 | *SAP5* | 2.11 | Has domain(s) with predicted aspartic-type endopeptidase activity and role in proteolysis |
| CPAR2_502390 | orf19.5698 | 2.11 | Ortholog(s) have structural constituent of ribosome activity and mitochondrial large ribosomal subunit localization |
| CPAR2_400510 | *PHO89* | 2.09 | Ortholog(s) have sodium:inorganic phosphate symporter activity |
| CPAR2_500500 | orf19.1239 | 2.05 | Ortholog of *C. albicans* SC5314 : C4_05580C_A |
| CPAR2_603050 | | 2.04 | Uncharacterized |
| CPAR2_300130 | *FRP2* | 2.03 | Ortholog(s) have ferric-chelate reductase activity and role in cellular response to iron ion starvation |
| CPAR2_206490 | *AAT1* | 2.03 | Ortholog(s) have role in replicative cell aging and mitochondrion localization |
| CPAR2_406810 | *PST1* | -2.00 | Has domain(s) with predicted FMN binding, NAD(P)H dehydrogenase (quinone) activity, oxidoreductase activity and role in negative regulation of transcription, DNA-templated |

| *C. parapsilosis* gene [1] | *C. albicans* homolog [2] | Fold change [3] | Description [4] |
|---|---|---|---|
| *CpOYE32* | *OYE32* | -2.00 | Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains; ortholog(s) have role in cell redox homeostasis |
| CPAR2_406830 | *PST1* | -2.02 | Has domain(s) with predicted FMN binding, NAD(P)H dehydrogenase (quinone) activity, oxidoreductase activity and role in negative regulation of transcription, DNA-templated |
| CPAR2_702730 | *SAP9* | -2.02 | Has domain(s) with predicted aspartic-type endopeptidase activity and role in proteolysis |
| CPAR2_208580 | *OYE2* | -2.07 | Has domain(s) with predicted FMN binding, catalytic activity, oxidoreductase activity |
| *CpMDR1* | *MDR1* | -2.08 | Member of the MDR family of major facilitator transporter superfamily; putative drug transporter; expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_203720 | *CRP1* | -2.1 | Has domain(s) with predicted ATPase-coupled cation transmembrane transporter activity, metal ion binding, nucleotide binding activity |
| *CpNAG4* | *NAG3* | -2.22 | Has domain(s) with predicted transmembrane transporter activity |
| CPAR2_206630 | orf19.3544 | -2.25 | Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_404070 | *KRE9* | -2.31 | Has domain(s) with predicted role in (1->6)-beta-D-glucan biosynthetic process, cell wall biogenesis and extracellular region localization |
| CPAR2_209930 | orf19.5210 | -2.36 | Ortholog(s) have DNA-binding transcription factor activity, RNA polymerase II-specific activity |
| CPAR2_601840 | orf19.5517 | -2.54 | Putative alcohol dehydrogenase; expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_403210 | *AHP1* | -2.60 | Ortholog(s) have thioredoxin peroxidase activity, role in cell redox homeostasis, cellular response to oxidative stress |
| *CpMDR1B* | *MDR1* | -2.80 | Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains; upregulated by activating mutation in *CpMRR1* |
| CPAR2_108230 | *HSP30* | -2.93 | Has domain(s) with predicted ion channel activity, role in ion transport and membrane localization |
| CPAR2_103080 | *GLX3* | -3.04 | Ortholog(s) have glyoxalase III activity, protein folding chaperone activity |
| CPAR2_302720 | | -3.06 | Pseudogene |
| CPAR2_107580 | *PRN4* | -3.11 | Ortholog of *C. albicans* SC5314 : C1_05880W_A/*PRN4* |
| *CpGRP2* | *GRP2* | -4.67 | Similar to *S. cerevisiae* Gre2p (methylglyoxal reductase); expression increased in fluconazole and voriconazole resistant strains |

| *C. parapsilosis* gene [1] | *C. albicans* homolog [2] | Fold change [3] | Description [4] |
|---|---|---|---|
| CPAR2_100480 | *GRP2* | -6.68 | Similar to *S. cerevisiae* Gre2p (methylglyoxal reductase); expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_212360 | orf19.5826 | -33.20 | Has domain(s) with predicted amino acid transmembrane transporter activity |
| **Cp35 ($CpTac1^{G650E}$) vs Cp35 - $CpTac1^{WT}$** | | | |
| CPAR2_405740 | | 41.57 | Has domain(s) with predicted DNA binding activity |
| CPAR2_300010 | *CDR1* | 7.96 | Pseudogene |
| *CpCDR1* | *CDR1* | 3.91 | Ortholog(s) have ABC-type xenobiotic transporter activity, role in cellular cation homeostasis, response to drug |
| CPAR2_703250 | | 3.15 | Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_301450 | *CIP1* | 2.54 | Ortholog of *C. albicans* SC5314 : C6_01070C_A/*CIP1* |
| *CpCDR1B* | *CDR1* | 2.51 | Has domain(s) with predicted ATPase-coupled transmembrane transporter activity. Upregulated by activating mutation in *CpMRR1* |
| CPAR2_702640 | orf19.6586 | 2.27 | Protein of unknown function; expression increased in fluconazole and voriconazole resistant strains |
| CPAR2_601840 | orf19.5517 | 2.17 | Putative alcohol dehydrogenase; expression increased in fluconazole and voriconazole resistant strains |

Note: [1] 'CPAR2_'identifier pulled from genome annotation of *C. parapsilosis*, CDC317, in the *Candida* Genome Database. Gene name provided where available (www.candidagenome.org). [2] *Candida albicans* homologs identified where possible from *Candida* Genome Database. Gene names provided represent both true orthologs and/or homologous best hits. [3] Fold change (≥2 or ≤ -2) for clinical isolates with *CpTAC1* mutation versus each clinical isolate with *CpTAC1* corrected to wild type; performed in biological triplicate with FDR p values of ≤ 0.05. [4] Gene descriptions pulled from *Candida* Genome Database and reviewed for brevity.

# VITA

Laura Doorley was born in 1988 in Hazel Crest, Illinois to Ronald and Kathy Snook. After graduation from Cordova High School in 2006, she received the Provost, TN lottery, and Tiger Athletic Band scholarships to attend the University of Memphis in Memphis, Tennessee. Laura graduated magna cum laude with a Bachelor of Science Dual Degree in Biology and Chemistry and a concentration in Biochemistry in 2011. Laura married Stephen Doorley in November 2013, while working as a Science Educator for the Memphis Zoo. In 2015, she enrolled in the Microbiology, Immunology and Biochemistry track in Biomedical Sciences program in the University of Tennessee Health Science Center's College of Graduate Health Sciences where she received the Tarnowski family scholarship. Laura joined the Rogers' lab at UTHSC in the fall of 2017 and moved with the lab to St. Jude Children's Research Hospital in the summer of 2020. Laura and Stephen welcomed their child, Landon Doorley, in February 2022. She expects to complete her doctoral degree in April 2023.