University of Tennessee Health Science Center
UTHSC Digital Commons

Theses and Dissertations (ETD)

College of Graduate Health Sciences

5-2010

Transcriptional Regulation of Azole Antifungal Resistance and Tolerance in *Candida Glabrata*

Kelly E. Caudle

University of Tennessee Health Science Center

Follow this and additional works at: <https://dc.uthsc.edu/dissertations>

Part of the [Bacterial Infections and Mycoses Commons](#), [Fungi Commons](#), and the [Pharmacy and Pharmaceutical Sciences Commons](#)

Recommended Citation

Caudle, Kelly E. , "Transcriptional Regulation of Azole Antifungal Resistance and Tolerance in *Candida Glabrata*" (2010). *Theses and Dissertations (ETD)*. Paper 39. <http://dx.doi.org/10.21007/etd.cghs.2010.0045>.

This Dissertation is brought to you for free and open access by the College of Graduate Health Sciences at UTHSC Digital Commons. It has been accepted for inclusion in Theses and Dissertations (ETD) by an authorized administrator of UTHSC Digital Commons. For more information, please contact jwelch30@uthsc.edu.

Transcriptional Regulation of Azole Antifungal Resistance and Tolerance in *Candida Glabrata*

Abstract

Azole antifungal resistance has emerged as a significant problem in the management of infections caused by fungi including *Candida* species. In recent years, *Candida glabrata* has become the second most common cause of mucosal and invasive fungal infections in humans second to *Candida albicans*. Not only are systemic *C. glabrata* infections characterized by high mortality rates, treatment failures to the azole class of antifungals, the most widely used antifungal for treatment of *Candida* infections, have been reported. Contributing to this problem, *C. glabrata* exhibits intrinsic reduced susceptibility to the azole antifungals, and the development of high-level azole resistance during therapy has been reported in oral as well as bloodstream *C. glabrata* isolates in immunocompromised patients. The azole antifungals are fungistatic against *Candida* species, thus *C. glabrata* also exhibits tolerance to the azoles which may contribute to both therapeutic failures and ultimately the development of high-level azole resistance.

In *C. glabrata* clinical isolates, the predominant mechanism behind azole resistance is upregulated expression of multidrug transporter genes *CgCDR1* and *CgPDH1*. It was previously reported that azole-resistant mutants (MIC ≥ 64 $\mu\text{g/ml}$) of strain 66032 (MIC = 16 $\mu\text{g/ml}$) similarly show coordinate *CDR1-PDH1* upregulation, and in one of these (F15) a putative gain-of-function mutation was identified in the single molecule homologue of *Saccharomyces cerevisiae* transcription factors Pdr1p–Pdr3p. Here we show that disruption of *C. glabrata PDR1* conferred equivalent fluconazole hypersensitivity (MIC = 2 $\mu\text{g/ml}$) to both F15 and 66032 and eliminated both constitutive and fluconazole-induced *CDR1-PDH1* expression. Reintroduction of wild-type or F15 *PDR1* alleles fully reversed these effects; together these results demonstrate a role for this gene in both acquired and intrinsic azole resistance. *CDR1* disruption had a partial effect, reducing fluconazole trailing in both strains while restoring wild-type susceptibility (MIC = 16 $\mu\text{g/ml}$) to F15. In an azole-resistant clinical isolate, *PDR1* disruption reduced azole MICs eight- to 64-fold with no effect on sensitivity to other antifungals. To extend this analysis, *C. glabrata* gene expression microarrays were generated and used to analyze genome-wide expression in F15 relative to its parent. Homologues of 10 *S. cerevisiae* genes previously shown to be Pdr1p–Pdr3p targets were upregulated (*YOR1*, *RTA1*, *RSB1*, *RPN4*, *YLR346c* and *YMR102c* along with *CDR1*, *PDH1* and *PDR1* itself) or downregulated (*PDR12*); roles for these genes include small molecule transport and transcriptional regulation. However, expression of 99 additional genes was specifically altered in *C. glabrata* F15; their roles include transport (e.g. *QDR2*, *YBT1*), lipid metabolism (*ATF2*, *ARE1*), cell stress (*HSP12*, *CTA1*), DNA repair (*YIM1*, *MEC3*) and cell wall function (*MKC7*, *MNT3*). These azole resistance-associated changes could affect *C. glabrata* tissue-specific virulence; in support of this, we detected differences in F15 oxidant, alcohol and weak acid sensitivities. *C. glabrata* provides a promising model for studying the genetic basis of multidrug resistance and its impact on virulence.

We next examined the genome-wide gene expression profiles in four matched azole-susceptible and –resistant clinical isolate sets of *C. glabrata* in which *CgCDR1* gene expression was upregulated in the resistant isolate. Of all the genes identified in the gene expression profiles for these four matched pairs, there were nine genes that were commonly upregulated with *CgCDR1* in all four isolate sets (*YOR1*, *LCB5*, *RTA1*, *YIM1*, *YIL077c*, *POG1*, *HFD1*, *GLK1*, and *FMS1*). We then sequenced *CgPDR1* from each susceptible and resistant isolate and found two alleles with novel gain-of-function mutations. A third isolate, and its susceptible parent, harbored a *CgPDR1* allele with a frameshift mutation which presumably results in a truncated CgPdr1p. The final resistant isolate had no *PDR1* mutation. *CgPDR1* alleles with putative gain-of-function mutations were expressed in a common background strain in which *CgPDR1* had been disrupted, and genome-wide gene expression profiles were examined to determine if different mutations in *CgPDR1* result in different target gene activation and fluconazole MICs. Microarray analysis comparing

these re-engineered strains to their respective parent strains identified a core set of commonly differentially-expressed genes as well as genes uniquely regulated by specific mutations.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Pharmaceutical Sciences

Research Advisor

P. David Rogers, Pharm.D., Ph.D.

Keywords

azole resistance; azole tolerance; *Candida glabrata*; fluconazole; pdr1

Subject Categories

Bacterial Infections and Mycoses | Fungi | Medicine and Health Sciences | Pharmacy and Pharmaceutical Sciences

**TRANSCRIPTIONAL REGULATION OF AZOLE ANTIFUNGAL RESISTANCE
AND TOLERANCE IN *CANDIDA GLABRATA***

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Kelly E. Caudle, Pharm.D.
May 2010

Chapter 2 © 2006 by Molecular Microbiology
All other material © 2010 by Kelly E. Caudle

ACKNOWLEDGEMENTS

I would first like to thank my husband, Wes, for his love, support and patience during the past eight years it has taken me to finally graduate. I would also like to thank my family and friends for their unending love and support. Thank you to my parents for believing in me and inspiring me to set high goals and giving me the tools to accomplish these goals.

Next I would like to express my sincerest gratitude to my advisor, Dave Rogers, who has not only inspired me as a scientist but also as an academician. Above all and the most needed, he provided me unflinching encouragement and challenged me in many ways throughout both portions of the Pharm.D./Ph.D program. Without his guidance and persistent help this dissertation would not have been possible. I would also like to thank the members of my graduate committee, Ramin Homayouni, Keith English, Todd Reynolds, and Bernd Meibohm. I appreciate not only the guidance and critical review of my dissertation projects but also for the encouragement along the way. A special thanks to Ramin Homayouni and his laboratory for designing the microarrays and aiding in the annotation of the data. I would also like to thank Nathan Wiederhold for performing the MIC testing throughout my dissertation.

Last but not least, I would like to express my gratitude to the members and past members of the Rogers lab, Kathy Barker, Teresa Liu, Chris Hoehamer, Erin Vasicek, Stephanie Flowers, Sarah Whaley, and Andy Nishimoto, for their friendship and support. A special thanks to Kathy Barker for everything she has taught me and for her patience and encouragement throughout the last eight years.

ABSTRACT

Azole antifungal resistance has emerged as a significant problem in the management of infections caused by fungi including *Candida* species. In recent years, *Candida glabrata* has become the second most common cause of mucosal and invasive fungal infections in humans second to *Candida albicans*. Not only are systemic *C. glabrata* infections characterized by high mortality rates, treatment failures to the azole class of antifungals, the most widely used antifungal for treatment of *Candida* infections, have been reported. Contributing to this problem, *C. glabrata* exhibits intrinsic reduced susceptibility to the azole antifungals, and the development of high-level azole resistance during therapy has been reported in oral as well as bloodstream *C. glabrata* isolates in immunocompromised patients. The azole antifungals are fungistatic against *Candida* species, thus *C. glabrata* also exhibits tolerance to the azoles which may contribute to both therapeutic failures and ultimately the development of high-level azole resistance.

In *C. glabrata* clinical isolates, the predominant mechanism behind azole resistance is upregulated expression of multidrug transporter genes *CgCDR1* and *CgPDH1*. It was previously reported that azole-resistant mutants ($\text{MIC} \geq 64 \mu\text{g/ml}$) of strain 66032 ($\text{MIC} = 16 \mu\text{g/ml}$) similarly show coordinate *CDR1-PDH1* upregulation, and in one of these (F15) a putative gain-of-function mutation was identified in the single molecule homologue of *Saccharomyces cerevisiae* transcription factors Pdr1p–Pdr3p. Here we show that disruption of *C. glabrata PDR1* conferred equivalent fluconazole hypersensitivity ($\text{MIC} = 2 \mu\text{g/ml}$) to both F15 and 66032 and eliminated both constitutive and fluconazole-induced *CDR1-PDH1* expression. Reintroduction of wild-type or F15 *PDR1* alleles fully reversed these effects; together these results demonstrate a role for this gene in both acquired and intrinsic azole resistance. *CDR1* disruption had a partial effect, reducing fluconazole trailing in both strains while restoring wild-type susceptibility ($\text{MIC} = 16 \mu\text{g/ml}$) to F15. In an azole-resistant clinical isolate, *PDR1* disruption reduced azole MICs eight- to 64-fold with no effect on sensitivity to other antifungals. To extend this analysis, *C. glabrata* gene expression microarrays were generated and used to analyze genome-wide expression in F15 relative to its parent. Homologues of 10 *S. cerevisiae* genes previously shown to be Pdr1p–Pdr3p targets were upregulated (*YOR1*, *RTA1*, *RSB1*, *RPN4*, *YLR346c* and *YMR102c* along with *CDR1*, *PDH1* and *PDR1* itself) or downregulated (*PDR12*); roles for these genes include small molecule transport and transcriptional regulation. However, expression of 99 additional genes was specifically altered in *C. glabrata* F15; their roles include transport (e.g. *QDR2*, *YBT1*), lipid metabolism (*ATF2*, *ARE1*), cell stress (*HSP12*, *CTA1*), DNA repair (*YIM1*, *MEC3*) and cell wall function (*MKC7*, *MNT3*). These azole resistance-associated changes could affect *C. glabrata* tissue-specific virulence; in support of this, we detected differences in F15 oxidant, alcohol and weak acid sensitivities. *C. glabrata* provides a promising model for studying the genetic basis of multidrug resistance and its impact on virulence.

We next examined the genome-wide gene expression profiles in four matched azole-susceptible and –resistant clinical isolate sets of *C. glabrata* in which *CgCDR1* gene expression was upregulated in the resistant isolate. Of all the genes identified in the

gene expression profiles for these four matched pairs, there were nine genes that were commonly upregulated with *CgCDR1* in all four isolate sets (*YORI*, *LCB5*, *RTA1*, *YIM1*, *YIL077c*, *POG1*, *HFD1*, *GLK1*, and *FMS1*). We then sequenced *CgPDR1* from each susceptible and resistant isolate and found two alleles with novel gain-of-function mutations. A third isolate, and its susceptible parent, harbored a *CgPDR1* allele with a frameshift mutation which presumably results in a truncated CgPdr1p. The final resistant isolate had no *PDR1* mutation. *CgPDR1* alleles with putative gain-of-function mutations were expressed in a common background strain in which *CgPDR1* had been disrupted, and genome-wide gene expression profiles were examined to determine if different mutations in *CgPDR1* result in different target gene activation and fluconazole MICs. Microarray analysis comparing these re-engineered strains to their respective parent strains identified a core set of commonly differentially-expressed genes as well as genes uniquely regulated by specific mutations.

Because understanding the initial stress response of *C. glabrata* to fluconazole may facilitate a better understanding of how this organism is able to survive in the presence of fluconazole, we used microarray analysis to determine how fluconazole exposure affects the gene expression profile in *C. glabrata*. We identified four transcriptional regulators, *CgPDR1*, *UPC2*, *RLM1*, and *CRZ1*, and several of their respective putative target genes that are upregulated in response to this stress. There were also many genes differentially expressed in response to fluconazole that encode proteins involved in small molecule transport (*CgCDR1*, *CgPDH1*, and *YORI*), lipid, fatty acid, and sterol metabolism (*ERG1*, *ERG2*, and *ERG24*), cell wall maintenance (*CWP1*, *CRH1*, and *CHS1*), and cell stress (*DDR48*). As *CgPDR1* has been implicated as a key regulator of azole resistance by activating transcription of the genes encoding ABC transporters CgCDR1p and CgPDH1p and has been shown to be activated in response to azole exposure, we also identified genes that respond to fluconazole exposure in a *CgPDR1*-dependent fashion. Surprisingly, only five genes were Pdr1p-dependent in response to fluconazole, including *CgCDR1*. Our results provide a more comprehensive picture of the gene expression changes of *C. glabrata* in response to fluconazole.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION	1
<i>CANDIDA GLABRATA</i>	1
Biology and Classification	1
Genetics and Genetic Manipulation	1
Pathogenicity and Virulence	2
Infections Caused by <i>Candida</i> Species	5
Treatment of <i>Candida</i> Infections	7
AZOLE RESISTANCE AND TOLERANCE	9
Clinical Impact of Azole Resistance	9
Clinical Impact of Azole Tolerance	10
Molecular Mechanisms of Azole Tolerance	11
Molecular Mechanisms of Azole Resistance in <i>C. glabrata</i>	11
TRANSCRIPTIONAL REGULATORS OF AZOLE RESISTANCE	13
Gene Expression	13
Zinc-Cluster Transcription Factors	14
Zinc-Cluster Transcription Factors and Azole Resistance	15
CENTRAL HYPOTHESIS AND RESEARCH OBJECTIVES	17
CHAPTER 2. <i>PDR1</i> REGULATES MULTIDRUG RESISTANCE IN <i>CANDIDA GLABRATA</i> : GENE DISRUPTION AND GENOME-WIDE EXPRESSION STUDIES	19
INTRODUCTION	19
MATERIALS AND METHODS	20
Media, Inhibitors, and Strains	20
Isolation of <i>ura3</i> Strains	20
Gene Disruption and Replacement	21
Broth Microdilution Assay	21
RNA Hybridization	26
Construction of <i>C. glabrata</i> Microarrays	26
RNA Preparation for Microarrays	26
cRNA Synthesis and Labeling	27
Oligonucleotide Array Hybridization and Analysis	27
Microarray Data Analysis	28
Quantitative Real-Time RT-PCR	28
RESULTS AND DISCUSSION	28
<i>PDR1</i> Disruption in F15 and Parent	28
<i>CgCDR1</i> Disruption	29

<i>PDR1</i> Replacement.....	32
Characterization of CgPdr1p-Independent Azole Resistance.....	32
<i>PDR1</i> Disruption in Azole-Resistant Clinical Isolates.....	32
Microarray Analysis: Upregulated Genes.....	35
Microarray Analysis: Downregulated Genes.....	39
Confirmation of Microarray Results.....	41
Promoter Sequence Analysis.....	41
<i>Candida glabrata</i> F15 Exhibits Additional Phenotypes Predicted by Microarray Analysis Which May Alter Virulence.....	43
CONCLUSIONS.....	45
CHAPTER 3. GENOME-WIDE EXPRESSION PROFILE ANALYSIS OF THE <i>CANDIDA GLABRATA</i> PDR1P REGULON.....	47
INTRODUCTION.....	47
MATERIALS AND METHODS.....	48
Antifungal Agents.....	48
Strains and Growth Media.....	48
Plasmid Constructions.....	48
<i>PDR1</i> Gene Disruption and Replacement by the SAT1-Flipper Method.....	51
Isolation of Genomic DNA and Southern Hybridization.....	51
Drug Susceptibility Tests.....	51
Culture Growth for Microarray Experiments.....	52
RNA Preparation for Microarrays.....	52
Microarray cRNA Synthesis and Hybridization.....	52
Microarray Data Analysis.....	53
Quantitative Real-Time RT-PCR.....	53
Sequence Analysis of <i>C. glabrata</i> Cg <i>PDR1</i>	54
RESULTS.....	54
Genes Coregulated with Cg <i>CDR1</i> among Matched Sets of Clinical Isolates.....	54
Identification of <i>PDR1</i> Mutations in Azole Resistant Clinical Isolates.....	56
T2837C and G822T Are Gain-of-Function Mutations in Cg <i>PDR1</i> and Each Confer Increased Resistance to Fluconazole.....	61
Identification of Putative Cg <i>PDR1</i> Target Genes.....	64
DISCUSSION.....	64
CHAPTER 4. GENOME-WIDE GENE EXPRESSION PROFILING OF THE RESPONSE TO FLUCONAZOLE IN <i>CANDIDA GLABRATA</i>	73
INTRODUCTION.....	73
MATERIALS AND METHODS.....	74
Antifungal Agent.....	74

Strains and Growth Media	74
Cell Culture and Fluconazole Exposure for Microarray Experiments.....	74
Microarray cRNA Synthesis and Hybridization	74
Microarray Data Analysis	75
Quantitative Real-Time RT-PCR.....	75
RESULTS	76
Global Changes in Gene Expression in Response to Fluconazole	76
Effects of <i>CgPDR1</i> on Global Gene Expression Responses to Fluconazole.....	78
DISCUSSION	78
CHAPTER 5. SUMMARY	94
LIST OF REFERENCES	97
VITA.....	115

LIST OF TABLES

Table 2-1. Primers used in this study (grouped by application)	23
Table 2-2. <i>C. glabrata</i> genes upregulated $\geq$ twofold in fluconazole-resistant mutant F15	36
Table 2-3. <i>C. glabrata</i> genes downregulated $\geq$ twofold in fluconazole-resistant mutant F15 compared with parent 66032	40
Table 3-1. <i>C. glabrata</i> isolates/strains used in this study.	49
Table 3-2. Primers used in this study	50
Table 3-3. Genes upregulated in all four of the clinical matched isolate sets (A) and in at least three of the clinical matched sets (B, C, D, and E)	58
Table 3-4. Genes down-regulated in all four of the clinical matched isolate sets (A) and in at least three of the clinical matched sets (C, D, and E)	60
Table 3-5. Fluconazole MICs for each of the revertant strains as compared to the parent strains	63
Table 3-6. Genes upregulated in SM1 Δ <i>pdr1</i> / <i>PDR1</i> ^{L946S} compared to SM1 Δ <i>pdr1</i> / <i>PDR1</i> ^{SM1} as well as SM3 compared to SM1	65
Table 3-7. Genes upregulated in SM1 Δ <i>pdr1</i> / <i>PDR1</i> ^{L946S} compared to SM1 Δ <i>pdr1</i> / <i>PDR1</i> ⁶⁸⁵⁶ as well as 6955 compared to 6856	67
Table 4-1. Primers used for quantitative real-time PCR expression analysis	77
Table 4-2. <i>C. glabrata</i> putative target genes of Pdr1p upregulated by $\geq$ 1.5 fold in response to fluconazole	80
Table 4-3. <i>C. glabrata</i> putative target genes of Upc2p, Crz1p, Rlm1p, and Cin5p upregulated by $\geq$ 1.5 fold in response to fluconazole	81
Table 4-4. <i>C. glabrata</i> putative target genes of CgAp1p upregulated by $\geq$ 1.5 fold in response to fluconazole	83
Table 4-5. <i>C. glabrata</i> genes found to be Pdr1p-dependent in response to fluconazole	88

LIST OF FIGURES

Figure 2-1. Disruption of <i>PDR1</i> and effects on azole sensitivity	22
Figure 2-2. Expression of multidrug transporter genes <i>CDR1</i> and <i>PDH1</i> and transcriptional activator gene <i>PDR1</i> in parent 66032, mutant F15 and their respective $\Delta pdr1$ disruptants.....	30
Figure 2-3. Disruption of <i>CgCDR1</i> and effects on azole sensitivity	31
Figure 2-4. <i>PDR1</i> replacement confirms role in azole resistance.....	33
Figure 2-5. Antifungal sensitivities and <i>CgCDR1-ERG11</i> expression in a $\Delta pdr1$ derivative of azole-resistant clinical isolate BG14.....	34
Figure 2-6. Confirmation of F15/66032 microarray results for selected genes by RNA hybridization and real-time RT-PCR.....	42
Figure 2-7. Spot assays examining sensitivity of 66032 and its azole-resistant mutant F15 to hydrogen peroxide, ethanol and sorbic acid	44
Figure 3-1. Quantitative real-time RT-PCR analysis of <i>CgPDR1</i> , <i>CgCDR1</i> , <i>CgPDH1</i> , and <i>CgSNQ2</i> in SM3 versus SM1 (Set 1), 62-17 versus 62-16 (Set 2), 7755 versus 7754 (Set 3), and 6955 versus 6856 (Set 4)	55
Figure 3-2. Venn Diagram showing the number of genes that are upregulated (A) or down-regulated (B) in the fluconazole-resistant isolates SM3, 62-17, 7755, and 6955 as compared to their matched fluconazole-susceptible isolates SM1, 62-16, 7754, and 6856, respectively	57
Figure 3-3. Location of gain-of-function mutations found in previous studies and in the present study.....	62
Figure 3-4. Quantitative real-time RT-PCR analysis of <i>CgPDR1</i> , <i>CgCDR1</i> , <i>CgPDH1</i> , and <i>CgSNQ2</i> in SM1 $\Delta pdr1$ / <i>PDR1</i> ^{T2837C} compared to SM1 $\Delta pdr1$ / <i>PDR1</i> ^{SM1} (Set 1), SM1 $\Delta pdr1$ / <i>PDR1</i> ⁶²⁻¹⁷ compared to SM1 $\Delta pdr1$ / <i>PDR1</i> ⁶²⁻¹⁶ (Set 2), SM1 $\Delta pdr1$ / <i>PDR1</i> ^{G822T} compared to SM1 $\Delta pdr1$ / <i>PDR1</i> ⁶⁸⁵⁶ (Set 4).....	68
Figure 4-1. Distribution of genes upregulated by ≥ 1.5 fold in response to fluconazole	79
Figure 4-2. Distrubution of genes downregulated by ≥ 1.5 fold in response to fluconazole	86
Figure 4-3. Quantitative real-time RT-PCR analysis of relative gene expression in 200989 and 200989 $\Delta pdr1$ treated with fluconazole versus 200989 treated with DMSO (control)	87
Figure 4-4. Proposed model for the calcium-signaling pathway in <i>C. glabrata</i>	91
Figure 4-5. Cell wall integrity pathway in <i>S. cerevisiae</i>	93
Figure 5-1. Mechansim of <i>CDR1</i> induction by Pdr1p	95

CHAPTER 1. INTRODUCTION

CANDIDA GLABRATA

Biology and Classification

Candida glabrata is a commensal organism found among normal flora of the skin, mouth, vagina, and gastrointestinal tract. It is a budding yeast and belongs to the largest taxonomic group of the Fungal Kingdom, ascomycetes, and the class Saccharomycotina. *C. glabrata* is considered a non-dimorphic yeast that exist as small blastoconidia (1 to 4 μm) both as a commensal and as a pathogen; however, pseudohyphae have been observed in *C. glabrata* when grown on synthetic low-ammonium dextrose agar (45). In fact, *C. glabrata* was once classified in the genus *Torulopsis* secondary to its lack of pseudohyphae production. However, it was later determined that the ability to produce pseudohyphae was not a reliable distinguishing factor for members of the genus *Candida* and *T. glabrata* was reclassified in the genus *Candida* (152).

The evolutionary relationship within the genus *Candida* was compared by Barnes *et al.* on the basis of homology of 18S rRNA sequence, and it was concluded that despite its name, *C. glabrata* is evolutionarily more related to *Saccharomyces cerevisiae* than to other pathogenic fungi such as *Candida albicans* (16). However, in contrast to the sexually active *S. cerevisiae* that can shift in its life cycle between diploid and haploid forms of cells (226), *C. glabrata* is exclusively a haploid and asexual organism despite the fact that genetic analyses revealed the presence of genes potentially responsible for conjugation and mating-type switching (112, 202). Like with *S. cerevisiae*, *C. glabrata* is a “petite-positive” yeast in that a loss of mitochondrial genome is not lethal. Interestingly, these “petite mutants” were found to be more resistant to antifungals than the wild-type strains (50).

Genetics and Genetic Manipulation

The genome sequence of *C. glabrata* strain CBS138 was published in 2004 (50). It has 13 chromosomes encompassing a total of 12.3 Mbp and consists of approximately 207 tRNA-encoding genes and 5283 protein-encoding genes. Its G-C content is 38.8% which is similar to *S. cerevisiae* (38.3%)(60). The genomes of *C. glabrata* and *S. cerevisiae* display a high degree of synteny with a majority of the *S. cerevisiae* genes having *C. glabrata* orthologs (60). However, *C. glabrata* has lost many metabolism genes, such as genes involved in galactose and sucrose assimilation, phosphate, nitrogen and sulfur metabolism, and thiamine, pyridoxine, and nicotinic acid biosynthesis. This is possibly a result of its close relationship with the mammalian host.

The lack of a sexual cycle in *C. glabrata* is a drawback for classical genetics in this species. Methods used in *C. glabrata* for gene disruption rely on integrative

transformation by homologous recombination. Furthermore, two methods have been developed for transformation in *C. glabrata*, lithium acetate transformation and electroporation transformation (adapted from *Cryptococcus neoformans*) (75, 214). There are several gene disruption protocols that have been used in *C. glabrata* (82, 100, 132, 225, 230); however, all of these methods depend on the use of nutritional markers for the selection of prototrophic transformants from auxotrophic host strains. One of these systems, modified from a *S. cerevisiae* method for use in *C. glabrata*, has been termed the PRoMoter-dependent DIruption of Genes (PRODIGE) method (19, 63, 119). This method uses polymerase-chain reaction (PCR) rather than cloning to generate disruption cassettes. These cassettes are generated with a promoterless marker gene (*URA3*) such that the expression of the selection marker gene is dependent, following recombination, on the promoter of the targeted gene. Selection then takes place on minimal medium plates lacking uracil. One obvious drawback of this method is that it requires at least a minimal level of promoter activity of the targeted gene for *URA3* expression. Another drawback is that using the PRODIGE method, genes can only be disrupted in *C. glabrata* strains with a *URA3*-deficient genetic background.

It is becoming increasingly evident that strategies like the one mentioned above that make use of auxotrophic markers may cause difficulties in the interpretation of mutant phenotypes (204). Recently, a transformation method that makes use of a dominant selection marker has been developed (176). This method, originally developed for use in *C. albicans*, relies on the use of a cassette that contains a dominant nourseothricin resistance marker (*SAT1*) for the selection of integrative transformants and the *FLP* recombinase gene under the control of an inducible promoter that allows the subsequent excision of the cassette, which is flanked by *FLP* target sequences, from the genome. When used in the diploid *C. albicans*, two rounds of integration/excision generate homozygous mutants that differ from the wild-type parent strain only by the absence of the target gene, and reintegration of an intact gene copy for complementation of mutant phenotypes is performed in the same way. Transformants are obtained after only one day of growth on a selective medium, and integration into the target locus occurs with high specificity after adding homologous flanking sequences on both sides of the cassette. *FLP*-mediated excision of the *SAT1*-flipper cassette is achieved by simply growing the transformants for a few hours in medium without selective pressure, and nourseothricin-sensitive derivatives can easily be identified by their slower growth on indicator plates containing a low concentration of nourseothricin. Although optimized for the alternative codon usage in *C. albicans*, this method has now been successfully used in the haploid *C. glabrata*, and requires only one round of integration/excision (68). This approach has allowed for the rapid and efficient generation of disruption mutants in *C. glabrata*.

Pathogenicity and Virulence

Candida species are found as part of normal microflora of human mucosal surfaces, although sometimes they can cause infection. They enter the host through the urogenital tract, lungs and other mucosal surfaces and induce formation of purulent

infiltrates (83). *Candida* species are known to cause vaginitis, oropharyngeal infections, thrush, urogenital tract infections and have been isolated from the feces. Furthermore, the increasing number of immunocompromised individuals has added to the problem of increasing fungal infections, in particular, oral and systemic candidiasis (15, 24, 191, 211). Over the past three decades, there has been an increase in non-*albicans* species causing infection, with *C. glabrata* being the second most common cause of mucosal and invasive fungal infections in humans. Although *C. glabrata* is phylogenetically closer to *S. cerevisiae* than other *Candida* species like *C. albicans*, the one distinct difference is that *C. glabrata* can cause infection in humans. It is likely that the differences in the *S. cerevisiae* and *C. glabrata* genome and the similarities between *C. glabrata* and *C. albicans* characterize the pathogenicity of *C. glabrata*; however, the virulence factors of *C. glabrata* are poorly understood. Therefore, the following discussion describes several known virulence factors in *C. albicans* and whether there are orthologs in *C. glabrata*.

Secreted hydrolases play an important role in virulence of *C. albicans*. It is thought that secreted hydrolases can contribute to host tissue invasion by digesting host cell membranes or cells and molecules of the host immune system, or by degrading host surface molecules to enhance adhesion. The most significant hydrolases produced by *C. albicans* are secreted aspartic proteinases (SAPs) and phospholipases. Although the *C. glabrata* genome encodes multiple orthologs of the *S. cerevisiae* extracellular glycosylphosphatidylinositol (GPI)-linked aspartyl proteases, *C. glabrata* does not produce significant levels of extracellular proteinase activity *in vivo* (94, 129). Furthermore, *C. albicans* secretes phospholipase B which is implicated in the spread of the organism from the gastrointestinal tract and in hematogenous dissemination (57, 74, 142). The *C. glabrata* genome encodes three phospholipase B genes and it has been shown that they do generate phospholipase activity (94). To date, no published studies have analyzed the role of *C. glabrata* phospholipases in virulence; however, one unpublished study did find an association between phospholipase B activity and persistent *C. glabrata* candidemia (74).

Another virulence factor that has been identified in *C. albicans* is its ability to grow in different morphological forms (i.e. yeast, pseudohyphae, and hyphae). Although both yeast and hyphal forms of *C. albicans* are pathogenic, the invasive properties of hyphae are suggested by their ability to penetrate several cell layers deeper than yeast forms in superficial candidiasis. For example, histological examination of lingual tissue from a patient with candidiasis shows yeast and hyphae forms within vacuolated regions of tissue whereas only hyphae are present in deeper layers (106, 205). This observation suggests that hyphae are more invasive than the yeast form and are thought to increase the level of damage to host tissue. Although *C. glabrata* has been shown to form pseudohyphae in response to nitrogen starvation, this form has not been found in clinical specimens. However, components of the mitogen-activated protein (MAP) kinase signaling cascade that regulates pseudohyphal formation of *S. cerevisiae* have been studied in *C. glabrata*. Ste12p is a transcriptional regulator in *C. glabrata* and is required for nitrogen starvation pseudohyphal formation. Interestingly, *ste12* *C. glabrata* mutants demonstrate attenuated virulence in a mouse model (33). The significance of *C. glabrata*

pseudohyphal growth on virulence is not known, and it is possible that *C. glabrata* has developed different strategies than *C. albicans* to invade its host.

One of the ways *C. albicans* regulates its virulence-associated traits such as secretion of proteinases and hyphal formation is by phenotypic switching, a process by which the cell can switch between epigenetic metastable states (7, 88, 140, 223). In general, phenotypic switching allows for rapid adaptation of the organism to different stressful environments. In *C. albicans*, “white cells” are ovoid, budding yeast cells that form white, domed colonies, and “opaque cells” are large, elongated cells that form gray flat colonies; these cells have different infectious properties and different transcriptional profiles (200). For example, phenotypic switching from the white to the opaque form significantly increases mating (133) and allows the organism to survive in different host tissues (108, 109). White cells are more suited for bloodstream infection while opaque cells are better at colonizing skin surfaces in mice (111). Similar to *C. albicans*, *C. glabrata* can undergo phenotypic switching exhibiting different colony colors on plates that contain copper sulphate: white (Wh), light brown (LB), dark brown (DB) and very dark brown (vDB) (28, 110, 112). A second switch phenotype, 'irregular wrinkle' (IW_r), has been identified, which exhibits a highly wrinkled colony morphology (110). In patients infected with a single strain of *C. glabrata*, differences were seen in the proportions of switch phenotypes at oral and vaginal locations. This suggests selection of particular switch phenotypes due to differences in microenvironments (28).

Adherence of *Candida* species to host cells is an important initial step in the establishment of infection. The fungal proteins involved in mediating these adhesive interactions are termed “adhesins”. Unlike *S. cerevisiae*, *C. glabrata* encodes adhesins, specifically a family of GPI-anchored cell wall proteins, the *EPA* (Epithelial adhesin) family. The *C. glabrata* genome contains at least 23 *EPA*-related genes most of which are encoded in subtelomeric clusters; however, only three, *EPA1*, *EPA6* and *EPA7* have been shown to mediate adhesion to human epithelial cells *in vitro* (35, 40). Due to their subtelomeric localization, *EPA* genes are subject to chromatin-based transcriptional silencing. In *S. cerevisiae*, silencing of subtelomeric genes is dependent on a complex of proteins encoded by *SIR2*, *SIR3*, and *SIR4* (79, 172). *C. glabrata* orthologs of these silencing factors have been identified and cells with silencing mutations in these genes are hyperadherent to a variety of cultured epithelial cells due to derepression of three *EPA* genes (*EPA1*, *EPA6* and *EPA7*) (35, 58). Furthermore, hyperadherent Δ *SIR3* mutants have been shown to colonize the kidney more efficiently than wild-type strains in a murine model of disseminated candidiasis (35). *EPA* gene expression may play an important role in the virulence of *C. glabrata*. Although *C. glabrata* and *C. albicans* are different in respect to their interactions with the host (i.e. hyphal formation and secreted hydrolases), the aforementioned similarities are a few potential reasons to why *C. glabrata* is virulent and *S. cerevisiae* is not.

Infections Caused by *Candida* Species

Since the early 1980s, the incidence and spectrum of fungal infections have increased significantly partly due to the improved supportive care of critically-ill patients (165). One study showed the annual incidence of fungal sepsis in the United States increased by 207% between 1979 and 2000 (131). The expanding number of immunocompromised individuals has added to the problem of increasing fungal infections due to mucosal or cutaneous barrier disruption, defects in the number and function of neutrophils or in cell-mediated immunity, metabolic dysfunction, and extremes of age. Moreover, increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies, and transplantation increases the risk for both common and uncommon opportunistic fungi (123, 137, 190). Several surveillance studies have reported *Candida* species as the most important cause of mycoses worldwide followed by *Aspergillus* and *Cryptococcus* species. Although *C. albicans* is still the most common cause of candidiasis, over the last two decades there has been an increase in non-*albicans* species causing infection with *C. glabrata* being the second most common cause of mucosal and invasive fungal infections in humans in the United States. The remainder of this section will discuss the clinical spectrum of superficial and systemic infections caused by *C. glabrata* as it compares to *C. albicans*.

Candida infections other than those on the skin and mucous membranes are referred to as invasive candidiasis, which include candidemia, acute or chronic hematogenously disseminated candidiasis, and infection of a single deep organ, either by hematogenous seeding or direct inoculation. A majority of these infections are a result of bloodstream invasion and hematogenous spread. The frequency of candidemia among hospitalized patients has doubled over the last three decades (165). There are 17 *Candida* species known to cause bloodstream infections; however, 90% can be attributed to five species: *C. albicans*, *C. glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (165). Furthermore, the 2004 Surveillance and Control of Pathogens of Epidemiological Importance study identified *Candida* species as the fourth most common cause of nosocomial bloodstream infection and the third most common cause of bloodstream infections in the intensive care unit (195). Not only has the incidence of *Candida* infections increased, mortality rates from disseminated candidiasis have been reported as high as 30-40% (162). Risk factors for invasive candidiasis include immune suppression (neutropenia, immunosuppressive drugs including glucocorticoids, cancer and cancer chemotherapy) and changes in the physical barriers that facilitate the organism's access to the bloodstream (prior gastrointestinal surgery or perforation, hemodialysis, parenteral nutrition, indwelling catheters, treatment with broad-spectrum antibiotics, and colonization with *Candida* species). *C. albicans* account for 45-60% of incidences of invasive candidiasis or candidemia in the United States and worldwide (35, 159, 165). In North America, *C. glabrata* accounts for 20-22% of cases invasive candidiasis (35, 159, 165). *C. glabrata* infections are difficult to treat due to intrinsic resistance to the most widely used antifungal, fluconazole. In fact, *C. glabrata* candidemia is associated with a 43% mortality rate in adults and 13% in children (159).

Approximately 75% of all adult women have suffered from vulvovaginal candidiasis (VVC) at least once in their lives, with 40-50% of women experiencing recurrence (89). *Candida* species can be found as a commensal in the vagina in 10-25% of all women (151). Predisposing factors for VVC include: African American, pregnancy, contraceptives, diabetes mellitus, and use of antibiotics. Pregnant HIV-infected women are at higher risk for VVC with a 40-60% prevalence of vaginitis and colonization (20-40% in non-HIV pregnant women). *C. albicans* accounts for 85-95% of yeast strains isolated from the vagina (113, 196, 197). Non-albicans *Candida* species are isolated in approximately 10-20% cases of VVC, with *C. glabrata* being the most common (32, 41, 153). In small clinical studies, a variety of risk factors have emerged for *C. glabrata* vaginitis which include uncontrolled diabetes mellitus and douching (73).

Urinary tract infections (UTI) due to *Candida* species has increased over the past three decades (13). In fact, one study found that *Candida* species accounts for 22% of all UTIs in the critical care setting (6). Risk factors for development of *Candida* UTI are elderly, female (vaginal colonization), pregnancy, diabetes mellitus, urinary tract disease producing obstruction (i.e. congenital abnormalities, neurogenic bladder), urinary tract catheterization, and antibiotic exposure (9, 97, 148, 155). Unlike OPC and vaginal candidiasis, *C. albicans* only accounts for about 50% of *Candida* UTIs with 50% caused by non-albicans *Candida* species. Interestingly, *C. glabrata* causes approximately 25% of *Candida* UTIs (199); however, no *C. glabrata* specific risk factors have been reported.

Symptomatic mucosal candidiasis arises in humans who are colonized with *Candida* and who are immunocompromised (i.e. Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS), neonates, elderly) or have local reduction in host resistance, thereby promoting the overgrowth of their own endogenous yeast flora. The most common mucosal infections include oropharyngeal, esophageal, and vaginal candidiasis. Oropharyngeal candidiasis, or thrush, is the general term given to oral infection caused by *Candida* species. *Candida* species are part of the normal mouth flora in 25-50% of healthy individuals (151). OPC is often the first manifestation of HIV infection with approximately 80-90% of HIV/AIDS patients ultimately developing OPC during their disease progression (54). Although there has been a decrease in OPC in HIV-infected individuals since the availability of antiretroviral therapy (ART), OPC still remains a significant infection in this population (208).

The most important predisposing factor for OPC other than HIV is the wearing of removable dentures (192). The dentures provide a surface for *Candida* to adhere; therefore, sufficient hygiene care is very important (31). Patients with hematological malignancies and solid tumors are also affected by OPC due to the breakdown of natural barriers to colonization and invasion after anticancer therapy. *C. albicans* is the main organism isolated from OPC patients; however, other species, such as *C. glabrata*, have often been implicated in patients not only with HIV/AIDS but also patients undergoing bone marrow transplantation and patients receiving therapy for head and neck cancer (31, 174, 175, 177). It is thought that the intrinsic resistance of *C. glabrata* to fluconazole, the most widely used antifungal for treatment and prophylaxis of OPC, allows for the

selection of *C. glabrata* in OPC (177). The emergence of fluconazole resistant organisms remains a significant clinical problem.

The second most common site of gastrointestinal candidiasis is the esophagus. Esophageal candidiasis occurs in patients with chronic diseases, usually after the use of antibiotics, steroids, or omeprazole; however, the disease most frequently is seen in individuals affected with advanced HIV infection (38). In fact 10-15% of HIV/AIDS patients will suffer from *Candida* esophagitis during their disease progression (62, 66, 138). Recently, there have been case reports linking *Candida* esophageal candidiasis to the use of inhaled corticosteroids (143). Like with OPC, *C. albicans* is the main causative organism of esophageal candidiasis (11). *C. glabrata* has also been implicated in esophagitis but the occurrence is rare (101).

Treatment of *Candida* Infections

Although the number of antifungal agents has increased since 2000, treatment of fungal infections is still limited to only 15 agents currently approved for clinical use. There are many similarities between fungal cells and mammalian cells that make the development of antifungals difficult. They are both eukaryotes with similar organelles, DNA replication, and protein synthesis. Antifungal agents must exploit the differences between host and fungal cells; however, there are a few biochemical differences between the host and the fungal pathogen. At the cellular level, the greatest difference is that fungal cells have cell walls and mammalian cells do not. Inhibitors of bacterial cell wall biosynthesis, such as penicillins, have been used for years with little associated toxicities. Only recently have a few potent inhibitors of fungal cell wall biosynthesis become available for clinical use. Other antifungal targets include inhibitors of DNA biosynthesis, disruption of mitotic spindles, and interference with intermediary metabolism. The most widely exploited difference between mammalian and fungal cells is that the cell membranes contain different sterols. Mammalian cells contain cholesterol whereas the fungal cells contain ergosterol.

The polyenes are natural antifungal compounds discovered in the early 1950s. The two commercially-available polyenes are Amphotericin B (AmB) and nystatin. The polyenes bind to ergosterol in the membrane bilayer and disrupt cell wall permeability by forming oligodendromes functioning as pores, with subsequent efflux of potassium and intracellular molecules causing fungal death (20). Due to the toxicities of nystatin, it is only used as a topical agent. AmB is not orally or intramuscularly absorbed; therefore, it is given intravenously or sometimes through the inhalational route. AmB has a broad antifungal spectrum including *Candida* species except *Candida lusitanae*. Although AmB is an effective antifungal drug, toxicities associated with its use have limited its utility. The most common and most serious side effect of AmB is nephrotoxicity. Some degree of reduction of renal function occurs in more than 80% of patients receiving the drug, and though this generally recovers after treatment has stopped, some impairment of glomerular filtration may remain (27). To allow for higher doses of AmB and to reduce its toxic effects to mammalian cells, AmB has been formulated in liposomes, lipid

complexes, and colloidal suspensions. Before the development of alternative agents such as the azole and echinocandin antifungals, AmB was the drug of choice for invasive *Candidal* infections. However, the most recent candidiasis guidelines recommend the newer and less toxic classes of antifungals for first-line use of many conditions caused by *Candida* species with the exception of neonatal candidiasis (158).

Flucytosine (5FC) is a prodrug that is deaminated by 5-fluorouracil by fungal cytosine deaminases. 5-fluorouracil is further converted to 5-fluorodeoxyuridylic acid, which interferes with DNA synthesis in the fungus. Mammalian cells lack cytosine deaminase allowing for selective inhibition of fungal organisms. This drug is only available orally and has a short half-life so it must be administered frequently. 5FC has activity against most *Candida* species, except *C. krusei*. Because of the rapid development of resistance, 5FC is rarely used in monotherapy but used in combination with AmB for the treatment of *Candida* endocarditis or meningitis (158).

The echinocandins, caspofungin, micafungin, and anidulafungin, are the only class of fungal cell wall inhibitors. They exert their mechanism of action by non-competitively inhibiting 1, 3- β -D glucan synthase, an enzyme that is necessary for the synthesis of 1, 3- β -D glucan, an essential component of the fungal cell wall. The inhibition of this enzyme destabilizes the integrity of the fungal cell wall, leaving it less rigid and unable to resist osmotic pressure resulting in cell lysis (18); therefore, this class of antifungals is fungicidal against most *Candida* species. All of the echinocandins are available in parenteral preparations only and have very few side effects. Echinocandins have activity against most *Candida* species but have less activity against some isolates of *Candida parapsilosis* and *Candida guilliermondii* (163). Each of these drugs has been studied in esophageal candidiasis (48, 105, 218, 219) and invasive candidiasis (107, 139, 160, 173) and each has been shown to be effective in these clinical situations. The new updated Candidiasis guidelines recommend use of this class for refractory esophageal disease and as an alternative for OPC or when azole-resistant pathogens are suspected or when azole antifungals are not tolerated (158). Echinocandins are also recommended as an alternative to fluconazole for primary therapy for most non-neutropenic adults with candidemia or suspected invasive candidiasis. Echinocandins are favored therapy for patients with moderate-to-severe illness, recent azole antifungal exposure, candidemia caused by *C. glabrata* or *C. krusei*. However, fluconazole is preferred over an echinocandin for *C. parapsilosis* infections. For the treatment of candidemia in a neutropenic patient, echinocandins are the drug of choice (158).

The second class of antifungals that exert their efforts on the fungal cell membrane are the azole antifungals. Azole antifungals work by inhibiting lanosterol demethylase in the ergosterol biosynthesis pathway which prevents the conversion of lanosterol to ergosterol. This mechanism results in the accumulation of toxic methylsterols and results in inhibition of cell growth and replication. The azole antifungals include two classes, imidazoles and triazoles. Imidazoles have a two-nitrogen azole ring; they are predominantly used topically and have been replaced for systemic administration by triazoles, which have three nitrogens in the azole ring. Triazoles have a

more favorable pharmacokinetic profile than imidazoles and do not significantly inhibit human sterol synthesis.

The triazole antifungals include fluconazole, itraconazole, voriconazole and posaconazole. Fluconazole is water soluble and has good bioavailability regardless of food or gastric pH (26); therefore, it is available orally and intravenously. In contrast, itraconazole and voriconazole are poorly water soluble. Itraconazole capsule is best absorbed with food and the oral solution is better absorbed in the fasting state (17) while voriconazole as a tablet or solution is better absorbed without food. Both itraconazole and voriconazole are available intravenously; however, both are formulated with cyclodextrin derivatives and the intravenous form of both of them is contraindicated in patients with severe renal dysfunction. Posaconazole, available only as a suspension for oral use, has variable bioavailability and must be taken with high fat foods (42). Triazoles as a class have numerous clinically significant drug interactions. With exception of posaconazole, most of the interactions are due to inhibition of the CYP450 system enzymes specifically, CYP3A4, CYP2C9, and CYP2C19. Interestingly, itraconazole and posaconazole inhibit gastric P-glycoprotein (222). As a class, the triazoles are generally well-tolerated. Of note, itraconazole can often cause significant nausea and vomiting and voriconazole treatment can cause visual changes.

All the triazoles demonstrate similar activity against most *Candida* species, with less activity against *C. glabrata* and *C. krusei* (64, 166). Fluconazole is considered the standard therapy for OPC, esophageal candidiasis and VVC (76, 198). Fluconazole has the greatest penetration into the cerebrospinal fluid and vitreous body; therefore, it is used in the treatment of central nervous system and intraocular *Candida* infections (10). Fluconazole concentrates in the urine achieving 10-20 times higher levels than in serum making it an optimal drug for treatment of *Candida* UTIs (158). As few data has examined the use of itraconazole in the treatment of invasive candidiasis, it is generally reserved for patients with mucosal candidiasis who have failed fluconazole treatment (5). Voriconazole is effective for both mucosal and invasive candidiasis. For treatment of *Candida* infections, its major role has been used for step-down oral therapy for patients with susceptible *C. krusei* and *C. glabrata* infections (158). The use of voriconazole versus fluconazole for treatment of esophageal candidiasis has been evaluated (5). Voriconazole was found to be as effective as fluconazole but was associated with more adverse event-related discontinuation of treatment. Posaconazole does not have an indication for primary candidiasis therapy (158).

AZOLE RESISTANCE AND TOLERANCE

Clinical Impact of Azole Resistance

Resistance to antifungals relies on two components: clinical resistance and microbial resistance. Clinical resistance is the failure of treatment or persistence of fungal infection despite appropriate antifungal drug therapy. Successful treatment not only

depends on the susceptibility of the organism to the antifungal, but also on the host immune system, drug penetration and distribution, and the absence of a protected or persistent focus infection. Microbial resistance is a quantifiable variable and is defined by the minimum inhibitory concentration (MIC), the lowest concentration of antimicrobial agent which inhibits the growth of the microorganism. Ideally, microbial resistance will be predictive of clinical resistance. In other words, above a given MIC for a fungal pathogen or a “breakpoint”, the infection will not be cured with that antifungal. There are three terms used to describe the susceptibility of any organism to a particular drug; “susceptible”, “intermediate” (“susceptible-dose dependent” or SDD) or “resistant”. The Clinical and Laboratory Standards Institute (CLSI) Subcommittee for Antifungal Testing has developed and standardized broth microdilution, broth macrodilution and disk diffusion methods for *in vitro* susceptibility testing of *Candida* species against fluconazole (145, 146). Breakpoints for *Candida* species were determined in 1997 by the CLSI Subcommittee for Antifungal Testing which are as follows: susceptible, MIC $\leq$ 8 $\mu\text{g/ml}$; SDD, MIC = 16 to 32 $\mu\text{g/ml}$; resistant, MIC $\geq$ 64 $\mu\text{g/ml}$. However, there were concerns over the clinical relevance of these breakpoints in more invasive and microbial resistant organisms as they were derived mostly (80%) from studies of mucosal candidiasis and there was little clinical data for which the MICs were elevated (178). Recently, further evaluation of these breakpoints for fluconazole and *Candida* have been conducted including expanded MIC data and clinical efficacy studies (166). These data more strongly validate the CLSI MIC interpretive breakpoints for fluconazole and *Candida* species.

When discussing resistance in *Candida* species, one must also differentiate between primary or “intrinsic resistance” and secondary or “development of resistance”. Intrinsic resistance is the natural or innate resistance of a species to antimicrobial therapy even though the organism has had no previous exposure to the agent. In contrast, development of resistance develops in previously susceptible organisms after exposure to an antimicrobial agent. For example, this occurs in patients infected with HIV who receive multiple courses of fluconazole for recurrent OPC. It is thought that exposure to fluconazole inhibits the growth of susceptible populations and the less susceptible populations are allowed to grow (117). Moreover, this situation also selects for less susceptible species, such as *C. glabrata*, to grow. In fact, over the past two decades there has been an increase in fluconazole-resistant non-*albicans* species causing infection, with *C. glabrata* being the second most common cause of mucosal and invasive fungal infections in humans (39). The molecular basis for the intrinsic resistance of *C. glabrata* to azole antifungals remains undefined; however, the molecular basis for the development of resistance has been studied (21, 185, 215).

Clinical Impact of Azole Tolerance

Candida species inhabit a range of environments in humans and these environments can change as the organism colonizes and spreads within a host. *Candida* species have evolved responses to protect it from environmental changes that cause physiological stress to the cell. The administration of antifungal drugs also represents a

stress to which the cell must respond (43). The nature of these responses to antifungal drugs depends on the organism, the dose, its duration, and the mechanism of action of the drug. For fungicidal drugs at appropriate doses above the MIC, stress responses fail to prevent cell death. However, for fungistatic drugs, such as the azoles, even above the MIC, growth is inhibited but the cells are not killed. This phenomenon is referred to as drug tolerance (186). As azole resistance mechanisms are usually due to genetic mutations leading to constitutive gene over-expression, tolerance is mediated by immediate reversible stress responses. These stress responses are important because they give cells time to develop long-term genetically stable resistant mechanisms. Thus, the development of a drug that can be used in co-therapy with fluconazole and abolish tolerance could prevent the development of azole resistance.

Molecular Mechanisms of Azole Tolerance

Fluconazole is fungistatic against *Candida* species, thus *C. glabrata* exhibits tolerance to azoles. It has recently been shown that the calcineurin inhibitors cyclosporine A and FK506 exhibit potent fungicidal synergism with azoles against *C. albicans*, *C. glabrata*, and *C. krusei* (44, 121, 126, 127, 154). Calcineurin is a serine/threonine-specific protein phosphatase that functions as a heterodimer composed of a catalytic subunit and a regulatory subunit B. In the presence of calcium, the phosphatase activity of calcineurin is activated when it binds calmodulin and it effects gene expression via transcriptional regulators such as Crz1p. It is possible that exposure to azoles causes an intracellular calcium spike that induces this pathway (186). In *C. glabrata*, disruption of *CgCRZI* did not affect the antifungal activity of fluconazole; however, disruption of the gene encoding the calcineurin regulatory subunit B resulted in fungicidal activity of fluconazole (136). Thus, the precise mechanism calcineurin-dependent azole tolerance in *C. glabrata* remains to be elucidated.

Molecular Mechanisms of Azole Resistance in *C. glabrata*

Development of high-level resistance to the azoles in *C. glabrata* has been reported in oral and bloodstream isolates from head and neck radiation patients, stem cell transplant patients, and HIV patients (21, 74, 185). Also, the development of azole resistance has been implicated in the fluconazole treatment failure and death of a patient suffering from *C. glabrata* candidemia (122). In *Candida* species, four primary mechanisms of azole resistance have been identified: (i) drug efflux mechanisms, (ii) changes in the gene expression of the target of fluconazole, lanosterol demethylase, (iii) amino acid changes in lanosterol demethylase that alter the binding of fluconazole, (iv) alterations in the sterol biosynthesis pathway that may bypass the accumulation of toxic sterols. In *C. glabrata*, the expression of drug efflux pumps and upregulation of the gene that encodes lanosterol demethylase, are the only two known mechanism of fluconazole resistance.

Azole resistance in fungi has been linked to the upregulation of membrane-associated protein pumps and, consequently, efflux of a wide range of molecules, including azoles. These transporter proteins consist of members of the ATP-binding cassette (ABC) or major facilitator superfamily (MFS) of transporters. ABC transporters are found in all cells of all organisms usually in the plasma membrane but also in the membranes of organelles. They are primary transporters that use the hydrolysis of ATP. The basic structure of the ABC transporters consist of two cytoplasmic nucleotide binding domains (NBDs), involved in ATP binding and hydrolysis and two transmembrane domains (TMDs) that span the membrane via putative α -helices. The arrangement of these domains depends on the type of ABC transporter (71, 86). In *S. cerevisiae*, these ABC transporters have been divided into subfamilies: pleiotropic drug resistance (PDR), multidrug resistance (MDR), and multidrug resistance-associated protein (MRP). The PDR ABC transporters usually contain the domain arrangement, from the N- terminus, (TMD-NBD)₂, and the MDR and MRP ABC transporters usually have the arrangement, (NBD-TMD)₂ (49). The subfamily of ABC transporters most often associated with antifungal drug resistance is the PDR subfamily. MFS transporters also comprise large superfamilies of proteins with high similarity to proteins found in plants, animals, bacteria, and fungi. These pumps are secondary transporters that utilize the proton-motive force across the plasma membrane. There are two subfamilies of MFS transporters involved in drug efflux. These subfamilies are defined by the number of transmembrane spans (TMS) within the TMD: DHA1 (12 TMS) and DHA2 (14 TMS).

C. glabrata contains 18 putative ABC transporters (six in the PDR subfamily) and 15 putative MFS transporters (10 in the DHA1 subfamily and five in the DHA2 subfamily) as predicted by comparison of the *C. glabrata* and *S. cerevisiae* genomes (72). Only four of these transporters have been implicated in fluconazole resistance in *C. glabrata*; the ABC transporters CgCdr1p, CgPdh1p, CgSnq2p, and CgAus1p. RNA analyses of fluconazole-resistant *C. glabrata* clinical isolates suggest that the predominant basis for azole resistance is the upregulation of CgCDR1 and to a lesser extent CgPDH1 (21, 135, 184, 185, 187, 216, 221). Deletion of CgCDR1 or CgCDR1-CgPDH1 renders fluconazole-resistant *C. glabrata* isolates susceptible to fluconazole (90, 185). Interestingly, one study found a fluconazole-resistant isolate that did not over-express CgCDR1 or CgPDH1, but upregulated another ABC transporter, CgSNQ2 (210). Disruption of CgSNQ2 decreased azole resistance. The expression of these transporters is controlled by CgPDR1 which will be discussed in detail in a later section.

Although not an efflux pump, CgAUS1 is a homolog of a *S. cerevisiae* gene that encodes an ABC transporter involved in uptake of sterols and anaerobic growth. Nakayama *et al.* investigated the effects of CgAUS1 under the control of a tetracycline-regulatable promoter. In the presence of bovine serum, they found that cells were more susceptible to fluconazole in the CgAUS1 “knockdown” strain suggesting that sterol transport can counteract ergosterol deficiency and azole toxicity.

CgERG11 is the gene encoding the target of the azole antifungal agents, lanosterol demethylase. Two studies have shown an increase in CgERG11 expression in azole-resistant *C. glabrata* isolates (130, 174). In one of these studies this upregulation

was due to duplication of the entire chromosome containing the *ERG11* gene; however, loss of the duplicated chromosome was seen when the isolate was grown in fluconazole-free medium (130). Increased expression of *CgERG11* may also be due to point mutations in the *CgERG11* gene itself or in upregulation of a transcriptional regulator that controls *CgERG11*. While several point mutations in *ERG11* have been linked to azole resistance in *C. albicans*, such mutations in *C. glabrata* have not been identified. A recent study examined a collection of azole resistant *C. glabrata* isolates and found no point mutations in *CgERG11* that were associated with the azole-resistant phenotype (187). Recently, our laboratory found a single-nucleotide substitution in one *UPC2* allele in *C. albicans* that resulted in an amino acid exchange in the encoded protein. Complementation of the mutated allele into a fluconazole susceptible strain resulted in upregulation of *ERG11* (61). The molecular basis for constitutive upregulation of *CgERG11* has not been elucidated in *C. glabrata*.

TRANSCRIPTIONAL REGULATORS OF AZOLE RESISTANCE

Gene Expression

The over-expression of aforementioned ABC transporters, *CgCDR1*, *CgPDH1*, and *CgSNQ2* could be due to several mechanisms: gene amplification, increased half-life of mRNA, or increased transcription. Gene amplification occurs when there is chromosomal duplication. Recently, Polakova *et al.* found that while gene sequences were well-conserved among different strains of *C. glabrata*, their chromosome structures differed drastically (171). Aneuploidy by gain of small chromosomes and segmental aneuploidy were the most prevalent events on the left arm of chromosome F. Chromosome F encodes the ABC transporters, *CgAUS1* as well as *CgPDH1*. The isolate carrying the minichromosome could tolerate 129.6 mg/L of fluconazole; however, the isolate which had lost the minichromosome could only tolerate 14.4 mg/L of fluconazole suggesting a role for aneuploidy in tolerance to azoles. However, when this isolate was grown in the absence of azoles, the loss of minichromosome was observed in 70% of the progeny. Although this study raises the possibility of aneuploidy contributing to the over-expression of these ABC transporters in the presence of azoles, it does not explain the constitutive over-expression of these genes in fluconazole-resistant isolates.

Another possibility of increased expression of these transporters could be the possibility of posttranscriptional control. Large amounts of the ABC transporter proteins could correlate with increased stability of its mRNA and protein. One study has demonstrated that the over-expression of *CgCDR1* in fluconazole-resistant *C. albicans* isolates is in part due to decreased mRNA turnover (125). However, transcriptional regulation is considered to be the key step accounting for basal and induced patterns of ABC transporter expression. There are two prominent families of transcriptional regulators in yeast that regulate PDR: zinc-cluster proteins and basic region-leucine zipper proteins. Since only the zinc-cluster proteins have been found to regulate

fluconazole PDR in *C. glabrata*, the remainder of this review will focus on this class of transcriptional regulators.

Zinc-Cluster Transcription Factors

Zinc-binding proteins represent one of the largest families of transcriptional regulators in eukaryotes. They all contain a zinc finger motif in which they are categorized into various families according to these motifs. One family, the zinc-cluster or binuclear cluster protein family, are found only in fungus and possess a well-conserved motif, CysX₂CysX₆CysX₅₋₁₂CysX₂CysX₆₋₈Cys (120). These cysteine residues bind to two zinc atoms and coordinate folding of the domain involved in DNA binding. At least 12 of these zinc-cluster proteins have been implicated multidrug resistance in *S. cerevisiae* (120).

Zinc-cluster proteins contain a DNA-binding domain (DBD), a regulatory domain, and an acidic domain. The cysteine-rich DBD is separated into three regions: the zinc finger, linker, and dimerization regions and is usually located at the N- terminus. There are six cysteine residues that are important for DNA binding and protein function (14, 51). The linker region, located C-terminally to the zinc-cluster motif, has no similarities to linker regions of other zinc-cluster proteins, and changes in this region alter the DNA-binding specificity (123). Therefore, it is proposed that this region provides a rigid scaffold that mediates DNA binding to a preferred sequence (123). The dimerization region is typically positioned C-terminally to the linker. Most zinc proteins contain this region and it is made up of heptad repeats that form a highly conserved coiled-coiled structure that is most likely for dimerization and protein-protein interaction (189).

The regulatory domain displays lesser homology among most members of the zinc-cluster protein class which is why it also termed the middle homology region (MHR). This domain is thought to play an inhibitory role since removal of this domain renders the zinc-cluster proteins constitutively active (169). Finally, the acidic domain acts as an activation domain (189). This domain is not well-defined as its function and structure within this superfamily is varied. Interestingly, gain-of-function mutations for Pdr1p and Pdr3p, transcriptional regulators involved in PDR, have been identified which make these transcriptional regulators constitutively activated.

Zinc-cluster proteins recognize elements containing trinucleotide sequences usually CGG triplets that can be everted, inverted, or direct repeats (120). The orientation of the trinucleotide sequences and the spacing between the triplets are very important determinants of DNA-binding specificity. There are several DNA-binding motifs (also termed “response elements”) that have been identified in *S. cerevisiae* zinc-cluster transcription factors (120).

Zinc-Cluster Transcription Factors and Azole Resistance

***S. cerevisiae*.** Due to the phylogenetically close relationship, *S. cerevisiae* represents a well-characterized model for studying *C. glabrata*. *S. cerevisiae* is also one of the most intensively studied eukaryotic model organisms in molecular and cell biology. The availability of the *S. cerevisiae* genome sequence and the complete set of deletion mutants have further enhanced the power of *S. cerevisiae* as a model for understanding the regulation of eukaryotic cells. Fluconazole resistance in *S. cerevisiae* is usually due to gain-of-function mutations within some of the zinc-cluster transcriptional regulators. Two of these, Pdr1p and Pdr3p, have been named the master regulators of drug resistance in *S. cerevisiae*. Together these proteins are responsible for the regulation of multiple genes in the PDR network, including the ABC transporters, *PDR5*, *SNQ2*, and *YOR1*. In fact, each of these genes contains at least one PDRE, pleiotropic drug response element, in its promoter region. Pdr1p and Pdr3p act by binding to PDREs in the promoters of target genes (95). The PDRE contains the consensus sequence TCCGCGGA, which displays CGG triplets in an everted repeat orientation. The *PDR3* gene promoter also contains two PDREs allowing for autoregulation as well as regulation by Pdr1p (95). Several studies have shown that single point mutations in the regulatory domain and in the activation domain of both ScPdr1p and ScPdr3p make these transcription factors hyperactive (34, 46, 65).

Although our understanding of the molecular basis of regulation of ScPdr1p and ScPdr3p is incomplete, there have been several interesting observations regarding their regulation. Cells that have lost their mitochondrial genome (known as petite mutants and designated “p⁰”) strongly induce levels of *ScPDR5* in a ScPdr3p-dependent manner (81). Also, zinc-cluster transcription factors, including Pdr3p, have been found to be negatively regulated by the Hsp70 protein Ssa1p (193). Most recently, downstream effectors of these transcription factors have been identified. Thakur *et al.* showed that regulation of ScPdr1p is similar to regulation of multidrug resistance (MDR) in vertebrates by the PXR nuclear receptor where ScPdr1p directly binds to fluconazole resulting in stimulation of drug efflux pumps. They also found a specific role of the Gal11p/MED15 subunit of the mediator co-activator and its activator-targeted KIX domain in antifungal-dependent regulation of MDR (207).

Yrr1p is a zinc-cluster transcriptional regulator found to also induce the expression of PDR genes in *S. cerevisiae* (228). Many Pdr1p, Pdr3p, and Yrr1p targets overlap suggesting cross-talk between regulators. *YRR1*, like *PDR3*, is also self regulated and contains a putative Yrr1p response element (YRRE) as well as a PDRE in its promoter (228). In addition, Yrr1p is regulated by zinc-cluster protein Yrm1p (228). Furthermore, Yrr1p has been shown to have point mutations in the regulatory domain which cause it to act as a hyperactive transcriptional regulator (228).

Other zinc-cluster proteins known to regulate drug resistance in *S. cerevisiae* include Pdr8p, Stb5p, Rds1p, Rds2p, and Rds3p and Rdr1p (120). Pdr8p is known to bind the promoters of PDR genes such as the ABC transporters *YOR1*, *PDR15*, and *AZRI* (MFS transporter) and disruption of this gene in *S. cerevisiae* increases ketoconazole

susceptibility (87). Disruption of *STB5* leads to reduced expression of the ABC transporters *SNQ5*, *PDR16*, and *PDR5* and increased susceptibility to cycloheximide but not ketoconazole. Furthermore, this same study showed evidence that Stb5p binds to the *SNQ2* promoter *in vivo* (114). Disruption of *RDS1* and *RDS3* (Regulators of Drug Sensitivity) are involved in conferring resistance to cycloheximide while disruption of *RDS2* confers resistance to ketoconazole. Lastly, a negative regulator of PDR genes has been identified named *RDR1* for repressor of drug resistance (85). Disruption of *RDR1* significantly increased the expression of five genes which encode membrane or membrane-associated proteins and increased resistance to cycloheximide. Rdr1p appears to bind to the same PDREs as Pdr1p and Pdr3p and the authors of this study postulate the formation of heterodimers between Rdr1p and Pdr1p or Pdr3p that would impair the activation of target genes. However, whether Rdr1p represses the gene expression of its target genes by directly binding the PDRE has not been determined (85).

S. cerevisiae can also overcome the effects of fluconazole by up-regulating genes in the ergosterol biosynthesis pathway. Several zinc- cluster transcriptional regulators are involved in this process. The *ERG* genes of the ergosterol biosynthesis pathway are regulated by two highly homologous zinc-cluster proteins, Upc2p and Ecm22p. They regulate transcription by binding to sterol response elements (SREs) in the promoters of their targets (217). Eleven of the *ERG* genes encoding enzymes in the ergosterol biosynthesis pathway contain putative sterol response elements in their promoters. Upc2p also plays a role in the uptake of exogenous sterols through the activation of the ABC transporters, *AUS1* and *PDR11*. Furthermore, it also has role in the upregulation of *DAN/TIR* genes. A gain-of-function mutation has been identified in Upc2p that was responsible for the upregulation of *AUS1* and *PDR11*, *DAN/TIR* genes, as well as Upc2p itself in *S. cerevisiae*. Hap1p is another zinc-cluster transcriptional regulator known to regulate *ERG11* which encodes the target of the azoles (213). *HAP1* is also overexpressed in the presence of the Upc2p gain-of-function mutation, and it is postulated that a regulatory interaction may exist between Hap1p and Upc2p (224). Interestingly, Stb5p has also been identified as a regulator of ergosterol biosynthesis as it is a direct activator of *ERG* genes in the presence of oxidative stress (114).

***C. glabrata*.** Multiple studies have linked the development of high-level azole resistance to the over-expression of ABC transporter genes in *C. glabrata*, such as *CgCDR1*, *CgPDH1*, and *CgSNQ2* (21, 68, 210, 212, 215). These are homologs to the *ScPDR5*, *ScPDR15*, and *ScSNQ2*, respectively. These mechanisms are closely related to the resistance mechanisms described above for *S. cerevisiae*. A significant difference between *S. cerevisiae* and *C. glabrata* is that CgPdr1p represents a single best homolog of the ScPdr1p and ScPdr3p transcription factor heterodimer found in *S. cerevisiae* (216). Like with *S. cerevisiae*, single point mutations in the regulatory domain of *CgPDR1* make this transcription factor hyperactive resulting in high-level transcription of *CgCDR1* and *CgPDH1* as well as increased resistance to azoles (68, 210, 212, 215). The majority of this dissertation (Chapters 2 and 3) discusses the role of these gain-of-function mutations in azole resistance in *C. glabrata*.

As with *S. cerevisiae*, the over-expression of ABC transporters in *C. glabrata* petite mutants has been documented (184). *CgPDR1* has been shown to be required for azole resistance due to mitochondrial deficiency (212). Moreover, it has also been shown that the regulation of *CgPDR1* is analogous to the regulation of *ScPDR1* where *CgPDR1* also directly binds to fluconazole resulting in stimulation of drug efflux pumps. The study also found a specific role of the Gal11p/MED15 subunit of the mediator co-activator and its activator-targeted KIX domain in antifungal-dependent regulation of MDR in *C. glabrata* (207).

CENTRAL HYPOTHESIS AND RESEARCH OBJECTIVES

C. glabrata has emerged as the second major cause of mucosal and invasive fungal infection in the United States. The azole class of antifungal agents has proven effective management of *Candida* infections. Recent reports have described the acquisition of azole resistance in *C. glabrata* clinical isolates from hematopoietic stem cell transplant patients, AIDS patients, and patients receiving radiation for head and neck cancer. Not only does *C. glabrata* have the ability to develop high-level resistance, this fungal pathogen also displays an intrinsic reduced susceptibility to azole antifungal agents which makes this a significant problem in the management of candidiasis. Furthermore, fluconazole is fungistatic against *Candida* species. The fungistatic nature of the azoles allows the organism to survive in the presence of drug, a phenomenon known as drug tolerance. Yeasts have developed ways to adapt to its environment. Such adaptations are often regulated at the transcriptional level. For example, in *S. cerevisiae*, multidrug resistance is mostly due to gain-of-function mutations of the transcriptional regulators, Pdr1p and Pdr3p. It is likely that multiple transcriptional activation networks are involved in both azole resistance and azole tolerance in *C. glabrata*. Therefore, my central hypotheses are as follows: 1) Gain-of-function mutations in activators of transcriptional activation networks represent the primary mechanism for the development of high-level resistance in *C. glabrata* and 2) Key transcriptional activation networks are activated in *C. glabrata* in the presence of azole antifungal agents which allow this organism to survive at higher azole concentrations as compared to other *Candida* species. It is hoped that identification of transcriptional activation networks found to influence azole susceptibility will serve as targets for future work towards the development of compounds that will both abrogate resistance and enhance the therapeutic utility of this class of antifungal agent.

It has been previously reported that azole-resistant *C. glabrata* mutants (MIC $\geq$ 64 μ g/ml) of strain 66032 (MIC = 16 μ g/ml) similarly show coordinate *CgCDR1-CgPDH1* upregulation, and in one of these (F15) a putative gain-of-function mutation was identified in the single homolog of *S. cerevisiae* transcriptional regulators, Pdr1p-Pdr3p (216). In the first chapter, we hypothesized that CgPdr1p plays a central role in development of azole resistance as well as intrinsic resistance to fluconazole. Our research aims were several-fold. First, we characterized the effect of *PDR1* disruption as well as gene replacement on fluconazole susceptibility and the expression of the ABC transporters *CgCDR1* and *CgPDH1* in the fluconazole susceptible isolate 66032 as well

as the resistant isolate F15. To more directly assess the role of acquired or intrinsic resistance of the ABC transporter *CgCDR1*, we also characterized the effect of *CgCDR1* disruption in these same isolates. Next, we used genome-wide expression studies in *C. glabrata* to identify other putative Pdr1p targets that are co-regulated with *CgCDR1*. This work was the first to identify CgPdr1p as a key regulator of the ABC transporter genes *CgCDR1* and *CgPDH1* and azole resistance.

Since the publication of the above study, many novel gain-of-function mutations in *C. glabrata* clinical isolates have been reported (68, 210, 212). These mutations cause upregulation of the ABC transporters, *CgCDR1*, *CgPDH1*, and *CgSNQ2*, but to varying degrees. For example, one mutation resulted in upregulation of *CgSNQ2* but not *CgCDR1* and *CgPDH1*. In Chapter 3, I hypothesized that the not only do these gain-of-function mutations in *CgPDH1* result in different levels of azole resistance, but also different target gene expression. To determine genes co-regulated with *CgCDR1*, I examined the genome-wide gene expression profiles in four matched azole-susceptible and –resistant clinical isolate sets of *C. glabrata* in which *CgCDR1* gene expression was upregulated in the resistant isolate. Next, we identified two gain-of-function mutations in two of the fluconazole resistant isolates and identified putative target genes specific to each mutation. We found that the different mutations do result in different levels of azole resistance and different target gene expression.

Understanding the initial stress response of *C. glabrata* to fluconazole may facilitate a better understanding of how this organism copes with such stresses to its environment. In Chapter 4, I hypothesized that *C. glabrata* responds to fluconazole treatment by upregulating transcriptional regulators that allow for this organism to survive in the presence of fluconazole. I examined the stress response of *C. glabrata* to exposure to fluconazole. Among the upregulated genes were the transcriptional regulators *CgPDR1*, *RLM1*, *CRZ1*, *UPC2*, *CgAPI1*, and *CIN5* and putative target genes for each of these regulators. Because the transcriptional regulator CgPdr1p is upregulated in response to fluconazole and has previously been shown to be involved in fluconazole intrinsic resistance, we studied the role of *CgPDR1* in the initial response to fluconazole. Using a *C. glabrata* strain disrupted for *CgPDR1*, we identified putative CgPdr1p targets that respond to fluconazole in a CgPdr1p-dependent and –independent manner. Together this work suggests other transcriptional pathways associated with azole intrinsic resistance and azole tolerance.

CHAPTER 2. *PDR1* REGULATES MULTIDRUG RESISTANCE IN *CANDIDA GLABRATA*: GENE DISRUPTION AND GENOME-WIDE EXPRESSION STUDIES*

INTRODUCTION

Increasing numbers of individuals are immunocompromised in association with AIDS, organ and tissue transplantation, aggressive treatments for cancer and immune-related diseases, diabetes, premature birth and advanced age. These individuals are at high risk for opportunistic fungal infection, in particular mucosal or systemic candidiasis. In the previous decade, *Candida glabrata* emerged as a common cause of these infections (10–30% of yeast isolates), trailing only *Candida albicans* (156, 168, 179, 183). In some populations such as diabetics and the elderly, *C. glabrata* may be the dominant pathogen (55, 77, 78, 104). In *C. glabrata* candidemia, mortality rates of 38–53% have been reported (102, 182, 220). Nevertheless, the basis for *C. glabrata* pathogenicity is not yet clear, because it is deficient in the virulence factors implicated in *C. albicans* infection: dimorphism, strong adhesion, secreted hydrolases and biofilm formation (59, 99, 150, 188). On the other hand, *C. glabrata* demonstrates relative resistance to azoles, the most widely used antifungal group which includes topical imidazoles such as miconazole and oral/parenteral triazoles such as fluconazole. Specifically, the fluconazole MIC inhibiting 50% of clinical isolates is 8 µg/ml, compared with 0.25 µg/ml for *C. albicans* (156, 167). Azoles inhibit lanosterol demethylase, product of the *ERG11* gene (CYP51 in moulds), which results in depletion of the membrane component ergosterol and accumulation of toxic sterol products (3). The emergence of *C. glabrata* (from ≤ 5% of yeast isolates in the 1980s) parallels the introduction in the early 1990s of fluconazole and over-the-counter imidazoles, along with widespread application of agricultural azole fungicides. Indeed, its intrinsic low-level azole resistance, the molecular basis for which remains undefined, may represent a *C. glabrata* 'virulence factor'.

C. glabrata also demonstrates a high capacity for acquired high-level azole resistance, with 8–27% of isolates demonstrating a fluconazole MIC ≥ 64 µg/ml (156, 164, 182). RNA analysis of these clinical isolates suggests that the predominant basis for acquired azole resistance is the constitutively upregulated expression of multidrug transporter genes *CgCDR1* and, to a lesser extent, *CgPDH1* (135, 175, 184, 185, 187, 216). In support of this, *CgCDR1* or *CgCDR1-CgPDH1* disruption was shown to confer azole hypersensitivity (90, 184). In this respect, *C. glabrata* resembles *C. albicans* and other fungi in which azole resistance has been attributed to upregulated expression of multidrug transporters (3). Initial laboratory studies of *C. glabrata* acquired azole resistance using standard glucose-supplemented medium yielded a virulent respiratory-deficient mitochondrial mutants (30, 184). Using glycerol-supplemented medium, we isolated respiratory-competent mutants with coordinately upregulated *CgCDR1-CgPDH1*

* Vermitsky, J. P., K. D. Earhart, W. L. Smith, R. Homayouni, T. D. Edlind, and P. D. Rogers. 2006. *Pdr1* regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Mol Microbiol* 61:704-722. Reprinted by permission of publisher. (NOTE: Kelly D. Earhart was my name prior to current married name.)

analogous to that observed in azole-resistant clinical isolates (216). Coordinate upregulation of these genes was also observed following brief exposure of susceptible cells to azoles, representing a potential basis for intrinsic low-level resistance.

Coordinate *CgCDR1-CgPDH1* upregulation implies a common transcription factor. Although very distinct in terms of niche and human pathogenicity, *C. glabrata* is an evolutionary close relative of *Saccharomyces cerevisiae* (16, 60). In the latter, the coordinate upregulation of multidrug transporter genes *PDR5* and *SNQ2* is mediated by the paralogous Pdr1p and Pdr3p zinc-cluster transcription factors (103). Many gain-of-function mutations within Pdr1p–Pdr3p have been identified that result in constitutive upregulation of *PDR5-SNQ2* along with a diverse group of additional genes (34, 52, 53). Our analysis of the recently released *C. glabrata* genome sequence (60) revealed a single *PDR1–PDR3* homolog, and a putative gain-of-function mutation in this gene was identified in azole-resistant laboratory mutant F15 (216). Here we demonstrate the central role of *C. glabrata PDR1* in acquired azole resistance, and identify a likely role in intrinsic resistance, by characterizing $\Delta pdr1$ derivatives of laboratory strains and clinical isolates. Furthermore, we report the first application of microarrays to this organism, which identified multiple genes co-regulated with *CgCDR1-CgPDH1* that are likely to impact *C. glabrata* virulence. This work was done in collaboration with Dr. Tom Edlind at Drexel University who created the disruption and reintegrated *CgPDR1* and *CgCDR1* mutants and performed the broth microdilution assays.

MATERIALS AND METHODS

Media, Inhibitors, and Strains

For most experiments, the medium employed was YPD (1% yeast extract, 2% peptone, 2% dextrose). Gene disruptants and *ura3* mutants were selected on DOB (synthetic defined medium with dextrose) with complete supplement mixture (CSM) or CSM lacking uracil/uridine (-URA) (Qbiogene BIO 101). Drugs were obtained from the following sources: fluconazole (Pfizer), itraconazole (Janssen), terbinafine (Novartis); caspofungin (Merck), amphotericin B, miconazole and cyloheximide (Sigma-Aldrich). They were dissolved in dimethyl sulphoxide (DMSO); the final DMSO concentration was $\leq 0.5\%$ in all experiments which had no detectable effect on growth. Sorbic acid, lactic acid, acetic acid and hydrogen peroxide (Sigma) were diluted as necessary in water. Strains were previously described (203) or constructed as described below.

Isolation of *ura3* Strains

Wild-type *URA3* yeast strains are sensitive to 5-fluoroorotic acid (5FOA). To isolate 5FOA-resistant mutants, a single colony from a fresh YPD plate was streaked on DOB + CSM agar containing 0.1% 5FOA (Research Products International) and incubated at 35°C for 3 days. Colonies were streaked for isolation on YPD and DOB-

URA; those that failed to grow on the latter were then tested for *URA3* complementation by transformation with pRS416 (shuttle vector with *S. cerevisiae URA3*) and selection on DOB-URA plates. Yeast transformations employed the Frozen-EZ Yeast Transformation II Kit (Zymo Research) as previously described (63).

Gene Disruption and Replacement

The PRODIGE method for PCR product-mediated gene disruption was employed (Figure 2-1) (63). Briefly, primers (80 mers; Table 2-1) were designed to precisely replace, after homologous recombination, a *C. glabrata* coding sequence (CDS) with the selection marker CDS. These primers consisted of approximately 60 nucleotides at the 5' end complementary to *C. glabrata* sequences directly upstream and downstream of the targeted CDS and approximately 20 nucleotides at the 3' end complementary to the *S. cerevisiae URA3* CDS contained in plasmid template pRS416. PCR products generated with these primers were used to transform *C. glabrata ura3* strains. Following selection on DOB-URA medium, transformants were screened by PCR with specific primer pairs (Table 2-1; Figure 2-1) to confirm replacement of the targeted CDS with *URA3* CDS. DNA was generally prepared by phenol extraction of glass bead-disrupted cells (63); some screens employed colony PCR in which a small volume of cells was added directly to the PCR mix.

For *PDR1* replacement, a PCR product representing the *PDR1* CDS plus 430–680 bp upstream and downstream sequence was amplified with primers PDR1uF-PDR1dR (Table 2-1) from 66032 or F15 genomic DNA. These products were used to transform 66032 Δ *pdr1* strain with selection on 1 μ g/ml cycloheximide-containing YPD plates. Colonies were screened as above with primer pair PDR1uF2-PDR1iR (Table 2-1).

Broth Microdilution Assay

Fresh overnight cultures from a single colony were diluted 1:100 in YPD, incubated for 3 h with aeration, and then counted in a haemocytometer and diluted again to 1×10^4 cells/ml. Aliquots of 100 μ l were distributed to wells of a 96-well flat-bottomed plate, except for row A which received 200 μ l. Inhibitor was added to row A to the desired concentration and then serially twofold diluted to rows B through G; row H served as inhibitor-free control. Plates were incubated at 35°C for the indicated times. Absorbance at 630 nm was read with a microplate reader; background due to medium was subtracted from all readings. The MIC (minimum inhibitory concentration) was defined as the lowest concentration inhibiting growth at least 80% relative to the drug-free control.

Figure 2-1. Disruption of *PDR1* and effects on azole sensitivity. A. Diagram illustrating PRODIGE primer design for disruption of *PDR1* with *URA3* CDS amplified from pRS416. Also shown are the upstream forward and two internal reverse primers used to screen transformants. B. PCR screen of representative $\Delta pdr1$ transformants selected on DOB-URA and their parent 66032 and F15. DNA was purified from isolated colonies, amplified with the indicated primer pairs, and analyzed by gel electrophoresis; loss of the Pdr1uF-PDR1iR product and formation of the PDR1uF-URA2iR product identified $\Delta pdr1$ clones. C. Broth microdilution assays examining fluconazole sensitivities of parent 66032, azole-resistant mutant F15, and their respective $\Delta pdr1$ disruptants. Absorbance at 630nm was recorded after 24 or 48 h incubation as indicated; growth was plotted as percentage of drug-free control.

Table 2-1. Primers used in this study (grouped by application).

Primer name	Primer sequence
PRODIGE-based gene disruption	
PDR1-URA3F	5'-GCCTTTTTTTTTAGAATATATTGGTAAAGTCATTCTTTAGC TACGTTATTGAGAGAATATGTCGAAAGCTACATATAAGG-3'
PDR1-URA3R	5'-TGATTTTTCAGATTAAATATAAAATTATACAGGCTATGCAC ACTGTCTAAATTAATAGCATTAGTTTTGCTGGCCGCATC-3'
CDR1-URA3F	5'-TACTTACAGGAAAAAGAATTTACAACCTCTTGATATATACAAA GTAAAGAAAAAGTAACAATGTCGAAAGCTACATATAAGG-3'
CDR1-URA3R	5'-TTTTCCGAATGCAATATGTATTAATACCAGAGCCAGATTAT GAGCGCAGGCTAAATAAAATTAGTTTTGCTGGCCGCATC-3'
PCR screening and <i>PDR1</i> replacement	
PDR1uF	5'-GGCGTATTCATAGAATCCGAA-3'
PDR1uF2	5'-GGTCCTTCTAATAGTCATCTTT-3'
PDR1iR	5'-CCATAGTATTCGTCGAGAGCA-3'
PDR1dR	5'-GACCTCTGTGAAAAGCTACTG-3'
URA3iR	5'-CAGCAACAGGACTAGGATGAG-3'
CDR1uF	5'-GCAGCTATGAGTTGAGGAAG-3'
CDR1iR	5'-ACGCCACATCGGCATCCTT-3'
DNA probes for RNA hybridization	
ACT1F	5'-TTGACAACGGTTCCGGTATG-3'
ACT1R	5'-CCGCATTCCGTAGTTCTAAG-3'
CDR1F	5'-ACAATGTCTCTTGCAAGTGAC-3'
CDR1R	5'-AAGTGTTTTCTGATGTGCTTT-3'
PDH1F	5'-GTGATGAACCCCGATGA-3'
PDH1R	5'-TTCTTGATCTCGTTGGGCGT-3'
PDR1F	5'-AGTGCCACCACTAAGTCACT-3'
PDR1R	5'-CCATAGTATTGCTGCAGAGCA
YLR346F	5'-GGAAGTGAACGCAGAACCA-3'

Table 2-1 (continued).

Primer name	Primer sequence
DNA probes for RNA hybridization, continued	
YLR346R	5'-ATCCTTCCATGTGTCGGCAT-3'
YOR1F	5'-GAACAAGCCACAGACGTATC-3'
YOR1R	5'-CAAATTGCCAAGATGGCTGG-3'
YNL134F	5'-CCACCATGAAAGCTGCTGTA-3'
YNL134R	5'-AACTTAGGATCAGCTGGCAG-3'
YML131F	5'-AATGAACCCACACCGGGTTA-3'
YML131R	5'-TTCACCAGTTGCATCAACCAT-3'
RTA1F	5'-CGTTCGCGGTGTTGTTTCTT-3'
RTA1R	5'-CATCTTCAATATCGGCTTCGA-3'
MEC3F	5'-TAGCGTCATTACGGAGCCTT-3'
MEC3R	5'-TATCGGGACCGCTTTCTTGT-3'
YJL163F	5'-TAGGTGCCTCGCATTCTGAT-3'
YJL163R	5'-ATCTTGCCAGCTAATCCAGG-3'
Real-time RT-PCR	
18SrtF	5'-TCGGCACCTTACGAGAAATCA-3'
18SrtR	5'-CGACCATACTCCCCCAGA-3'
CDR1rtF	5'-CATACAAGAAACACCAAAGTCGGT-3'
CDR1rtR	5'-GAGACACGCTTACG TTCACCAC-3'
PDH1rtF	5'-ACGAGGAGGAAGACGACTACGA-3'
PDH1rtR	5'-CTTTACTGGAGAACTCATCGCTGTT-3'
CSR1rtF	5'-TGGATTTTTTCTCCCATCTGGA-3'
CSR1rtR	5'-ACCACAGGGTCAAGCCATTTT-3'
PDR1rtF	5'-TTTGACTCTGTTATGAGCGATTAC-3'
PDR1rtR	5'-TTCGGATTTTTCTGTGACAATGG-3'
KAD2rtF	5'-AACCCGCAGTCATCGTGG-3'
KAD2rtR	5'-CCTGTCTCTCAGTTCTTGGAACC-3'
YOR1rtF	5'-CCATCGGTGCTTGTGTAATGTTA-3'

Table 2-1 (continued).

Primer name	Primer sequence
Real-time RT-PCR, continued	
YOR1rtR	5'-TTGAGAGGCGTGGAAAAAATG-3'
RTA1rtF	5'-TCCTGTTTGTTCATTAGGGTTAGGG-3'
RTA1rtR	5'-TGGCAATTTTGTTCCTTATTCCTCAG-3'
QDR2rtF	5'-GACGAATGAGGACGAGGCTG-3'
QDR2rtR	5'-GGTTGGACCTGGTTCTGTAAATAGG-3'
SUT1rtF	5'-ACGAGAGCCAGAAGTTGATGG-3'
SUT1rtR	5'-TGGAGGCGATAGGAATTGGT-3'
RPN4rtF	5'-AGCCAGTATGCTGACCCGAG-3'
RPN4rtR	5'-ACACGCCACATCGCCC-3'
SAC7rtF	5'-CGCTGGAGACGCCTGG-3'
SAC7rtR	5'-TCGTATCCGCTTGCTGTTCC-3'
YBT1rtF	5'-AAGTGCTTCTTCCGCCTCATT-3'
YBT1rtR	5'-AACAGGAGCTGGTGTAGTACCCA-3'
MET8rtF	5'-TCCACCGCTATGCGATTCT-3'
MET8rtR	5'-GGAGATGACCCATTGGATGAA-3'

RNA Hybridization

Log-phase cultures in YPD at 35°C were adjusted to 3×10^6 cells/ml and incubated for an additional 3 h. In some studies, cultures were divided into equal portions to which either fluconazole or a comparable volume of DMSO was added, followed by incubation for the indicated times. In all studies, culture volumes corresponding to 3×10^7 cells were removed and centrifuged to pellet cells. RNA preparation and hybridization analysis were as previously described (195). Briefly, cell pellets were suspended in sodium acetate-EDTA buffer and stored frozen. After thawing, RNA was extracted by vortexing in the presence of glass beads, SDS and buffer-saturated phenol alternating with incubation at 65°C for 10–15 min. Samples were cooled on ice and centrifuged, and RNA was ethanol precipitated from the aqueous phase. RNAs were dissolved in water and denatured in formaldehyde-SSPE with incubation for 15 min at 65°C. Either 40 µl (for *ACT1* probing) or 200 µl (for other probes) of denatured RNA (approximately 4 or 20 µg, respectively) was applied to nylon membrane by using a slot blot apparatus. Membranes were rinsed in SSPE, UV cross-linked, hybridized to purified PCR products (Table 2-1) labeled with ^{32}P by random priming (Takara), and exposed to film.

Construction of *C. glabrata* Microarrays

The nucleotide sequences corresponding to 5272 *C. glabrata* ORFs were downloaded from the Génolevures Consortium (<http://cbi.labri.fr/Genolevures/about.php>, Build 2). Following the Affymetrix Design Guide, two separate probe sets for each ORF were designed, each consisting of 13 perfect match and 13 mismatch overlapping 25 base oligonucleotides targeted to the 3' 600 bp region. For ORFs < 600 bp the sequence was divided in two equal segments for subsequent design procedures. For quality control and normalization purposes, we designed two to three additional probe sets spanning the *C. glabrata* 18S rRNA, *TDH1* and *ACT1* genes in addition to standard Affymetrix controls (BioB, C, D, cre, DAP, PHE, LYS, THR). The probe selection was performed by the Chip Design group at Affymetrix, using their proprietary algorithm to calculate probe set scores, which includes a probe quality metric, cross-hybridization penalty, and gap penalty. The probe sets were then examined for cross-hybridization against all other sequences in the *C. glabrata* genome as well as a number of constitutively expressed genes and rRNA from other common organisms. Duplicate probe sets are made to distinct regions of the ORF, thereby allowing two independent measurements of the mRNA level for that particular gene. *C. glabrata* custom Affymetrix NimbleExpress Arrays were manufactured by NimbleGen Systems per our specification (4).

RNA Preparation for Microarrays

Total RNA was isolated using the hot SDS-phenol method (190). Frozen cells were suspended in 12 ml of 50 mM sodium acetate (pH 5.2), 10 mM EDTA at room temperature, after which 800 µl of 25% sodium dodecyl sulphate and 12 ml of acid phenol (Fisher Scientific) were added. This mixture was incubated 10 min at 65°C with

vortexing each minute, cooled on ice for 5 min, and centrifuged for 15 min at 12 000g. Supernatants were transferred to new tubes containing 15 ml of chloroform, mixed and centrifuged at $200 \times g$ for 10 min. The aqueous layer was removed to new tubes, RNA was precipitated with 1 volume isopropanol and 0.1 volume 2M sodium acetate (pH 5.0), and then collected by centrifugation at 17 000g for 35 min at 4°C. The RNA pellet was suspended in 10 ml of 70% ethanol, collected again by centrifugation, and suspended in diethyl pyrocarbonate-treated water.

cRNA Synthesis and Labeling

Immediately prior to cDNA synthesis, the purity and concentration of RNA samples were determined from A_{260}/A_{280} readings and RNA integrity was determined by capillary electrophoresis using the RNA 6000 Nano Laboratory-on-a-Chip kit and Bioanalyzer 2100 (Agilent Technologies) as per the manufacturer's instructions. First and second strand cDNA was synthesized from 15 µg total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and oligo-dT24-T7 primer (PrOligo) according to the manufacturer's instructions. cRNA was synthesized and labeled with biotinylated UTP and CTP by in vitro transcription using the T7 promoter-coupled double stranded cDNA as template and the Bioarray HighYield RNA Transcript Labeling Kit (ENZO Diagnostics). Double stranded cDNA synthesized from the previous steps was washed twice with 70% ethanol and suspended in 22 µl of RNase-free water. The cDNA was incubated as recommended with reaction buffer, biotin-labeled ribonucleotides, dithiothreitol, RNase inhibitor mix and T7 RNA polymerase for 5 h at 37°C. The labeled cRNA was separated from unincorporated ribonucleotides by passing through a CHROMA SPIN-100 column (Clontech) and ethanol precipitated at -20°C overnight.

Oligonucleotide Array Hybridization and Analysis

The cRNA pellet was suspended in 10 µl of RNase-free water and 10 µg was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to the *C. glabrata* NimbleExpress GeneChip arrays. Arrays were washed at 25°C with $6 \times$ SSPE, 0.01% Tween 20 followed by a stringent wash at 50°C with 100 mM MES, 0.1 M NaCl, 0.01% Tween 20. Hybridizations and washes employed the Affymetrix Fluidics Station 450 using their standard EukGE-WS2v5 protocol. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes) and the fluorescence intensities were determined using the GCS 3000 high-resolution confocal laser scanner (Affymetrix). The scanned images were analyzed using software resident in GeneChip Operating System v2.0 (GCOS; Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity (250). The signal intensity for each gene was calculated as the average intensity

difference, represented by $[\Sigma(\text{PM} - \text{MM})/(\text{number of probe pairs})]$, where PM and MM denote perfect-match and mismatch probes.

Microarray Data Analysis

The scaled gene expression values from GCOS software were imported into GeneSpring 7.2 software (Agilent Technologies) for preprocessing and data analysis. Probesets were deleted from subsequent analysis if they were called absent by the Affymetrix criterion and displayed an absolute value below 20 in all experiments. The expression value of each gene was normalized to the median expression of all genes in each chip as well as the median expression for that gene across all chips in the study. Pairwise comparison of gene expression was performed for each matched experiment (F15 vs. 66032). Genes were included in the final data set if their expression changed by at least twofold between strain F15 and strain 66032 in two independent experiments.

Quantitative Real-Time RT-PCR

First strand cDNAs were synthesized from 2 µg total RNA in a 21 µl reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer's instructions. Quantitative real-time PCR was performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems). Independent amplifications were performed using the same cDNA for both the gene of interest and 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and *18S rRNA* using Primer Express software (Applied Biosystems) and the Oligo Analysis and Plotting Tool (Qiagen). The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each cycle to verify that a single product was amplified using software provided with the 7000 Sequence Detection System. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the system software, and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of an arbitrary calibrator (e.g. untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$.

RESULTS AND DISCUSSION

PDR1 Disruption in F15 and Parent

The laboratory selection of spontaneous fluconazole-resistant mutants of *C. glabrata* ATCC strain 66032 was previously described (216). One of these mutants, F15,

exhibited strong upregulation of *CgCDR1* and *CgPDH1*, modest upregulation of *PDR1*, and a single base change predicted to alter the Pdr1p amino acid sequence. We reasoned that disruption of *CgPDR1* in F15 and parent 66032 would provide an initial test of the hypothesis that this single base change is responsible for the fluconazole resistance. To accomplish this, *ura3* derivatives of F15 and 66032 were isolated by selection on 5FOA and screening for complementation by a URA3-encoding plasmid. Homologous recombination is relatively non-specific in *C. glabrata*, especially with short homology regions, but can be enhanced by promoter-dependent disruption of genes (PRODIGE) as previously described (63). This method was used to disrupt *CgPDR1* (Figure 2-1A). Transformants were screened by polymerase chain reaction (PCR); loss of the uF-PDR1iR product and generation of the PDR1uF-URA3iR product confirmed *PDR1* disruption (Figure 2-1B).

Broth microdilution assays were used to examine fluconazole susceptibility of F15 Δ *pdr1*, 66032 Δ *pdr1* and their parents (Figure 2-1C). Similar to previous results with their parents (216), the 66032 and F15 *ura3* derivatives generated 24 h fluconazole MICs of 8–16 and > 64 μ g/ml, respectively. In contrast, their Δ *pdr1* derivatives were fluconazole hypersusceptible, with equivalent MICs of 2 μ g/ml. Although susceptible, 66032 exhibited trailing growth typical of many *Candida* species (178), and by 48 h was fully grown at all fluconazole concentrations tested (Figure 2-1C). Trailing growth was absent in the *PDR1* disruptants. These results support the role of CgPdr1p in F15 fluconazole resistance. Furthermore, the reduced MIC and trailing growth associated with *PDR1* disruption in 66032 suggests that CgPdr1p is an important contributor to the intrinsic low-level resistance that is characteristic of this species.

As hypothesized, RNA analysis showed that *PDR1* disruption reversed the constitutive upregulation of *CgCDR1* and *CgPDH1* in untreated mutant F15 (Figure 2-2A). Moreover, expression of these genes was reduced relative to their expression in untreated parent 66032. This can explain the greater susceptibility of the Δ *pdr1* derivatives relative to 66032. As previously described (216), fluconazole treatment induced the expression of *CgCDR1* and *CgPDH1*, most clearly in strains 66032 and F15 respectively (Figure 2-2A). *CgPDR1* disruption completely blocked this treatment-dependent upregulation. Finally, we note that *CgPDH1* itself, which is upregulated in F15 (216), is also induced by fluconazole treatment in 66032 and F15 (Figure 2-2A).

***CgCDR1* Disruption**

To more directly assess the role in acquired or intrinsic azole resistance of multidrug transporter gene *CgCDR1*, it was similarly disrupted in the *ura3* derivatives of 66032 and F15 (Figure 2-3A). This reversed the fluconazole resistance of F15 (Figure 2-3B), although the MIC (16 μ g/ml) remained eightfold above that observed with *PDR1* disruption (Figure 2-1C). With respect to 66032, *CgCDR1* disruption had minimal effect on fluconazole MIC at 24 h, but trailing growth most apparent at 48 h was eliminated as it was with *PDR1* disruption. These results are consistent with *CgCDR1* being a major but not exclusive contributor to F15 azole resistance.

Figure 2-2. Expression of multidrug transporter genes *CDR1* and *PDH1* and transcriptional activator gene *PDR1* in parent 66032, mutant F15 and their respective $\Delta pdr1$ disruptants. A. RNA was isolated from log phase cultures, slot-blotted to membranes, and hybridized to the indicated gene probes: *ACT1* served as loading control. U, untreated cultures; T, treated with 256 $\mu\text{g/ml}$ fluconazole for 2.5 h. B. Quantitative real-time RT-PCR analysis of relative *CgCDR1* and *PDH1* expression in F15 versus 66032, F15 $\Delta pdr1$ versus 66032, and 66032 $\Delta pdr1$ versus 66032. Data are shown as mean $\pm$ SD.

Figure 2-3. Disruption of *CgCDR1* and effects on azole sensitivity. A. PCR screen of representative Δ *cdr1* transformants selected on DOB-URA and their parents 66032 and F15. DNA was purified from isolated colonies, amplified with the indicated primer pairs, and analyzed by gel electrophoresis; loss of CDR1uF-CDR1iR product and formation of CDR1uF-URA3iR product identified Δ *cdr1* clones. B. Broth microdilution assays examining fluconazole sensitivities of parent 66032, azole-resistant mutant F15, and their respective Δ *cdr1* disruptants. Absorbance at 630nm was recorded after 24 or 48 h incubation as indicated; growth was plotted as percentage of drug-free control.

***PDR1* Replacement**

To rigorously test the role of F15 *PDR1* in azole resistance, we employed gene replacement. The 66032 $\Delta pdr1$ strain was transformed with PCR products representing wild-type and F15 *PDR1*, including 5' and 3' flanking sequences which should direct *PDR1* to its native locus (Figure 2-4A). We initially attempted to select homologous recombinants on fluconazole-containing medium. However, this was precluded by a background of spontaneous fluconazole-resistant mutants in control (no added DNA) transformations. As an alternative, the protein synthesis inhibitor cycloheximide is a known substrate for Cdr1p-like multidrug transporters, and indeed *C. glabrata* $\Delta pdr1$ strains are cycloheximide-hypersensitive (63). In contrast to fluconazole, cycloheximide-containing plates yielded no spontaneous mutants while five or six transformants were obtained with addition of wild-type or F15 *PDR1* respectively. PCR screening of these transformants confirmed homologous recombination into the native locus (Figure 2-4B). All F15 *PDR1* replacements demonstrated fluconazole resistance comparable to F15 itself, while all but one of the wild-type *PDR1* replacements demonstrated wild-type sensitivity (Figure 2-4B). Sequencing of a representative F15 *PDR1* replacement confirmed there were no mutations other than the previously described P927L (216).

Characterization of CgPdr1p-Independent Azole Resistance

As noted above, a background of resistant mutants arose on fluconazole-containing YP-glycerol medium in control transformations of strain 66032 $\Delta pdr1$, which involved plating approximately 2×10^7 cells. To more rigorously examine this CgPdr1p-independent resistance, equivalent numbers (3×10^5) of 66032 and 66032 $\Delta pdr1$ cells were plated on YP-glycerol medium with fluconazole ranging from 0 to 256 $\mu\text{g/ml}$ (184). After 4 days incubation, the MIC was 32 $\mu\text{g/ml}$ for 66032, and about 30 mutant colonies (frequency = 1×10^{-4}) were observed on each of the four plates at or above this concentration. With 66032 $\Delta pdr1$, the MIC was 4 $\mu\text{g/ml}$, one or two colonies were observed at 4 and 8 $\mu\text{g/ml}$, and no colonies at 16–256 $\mu\text{g/ml}$ (frequency $< 3 \times 10^{-6}$). Thus, CgPdr1p-independent azole resistance occurs at significantly reduced frequency.

***PDR1* Disruption in Azole-Resistant Clinical Isolates**

Strain BG14, a model for *C. glabrata* pathogenesis (48), is a *ura3* derivative of a clinical isolate from a patient who failed fluconazole therapy (32). Consistent with this, BG14 is fluconazole-resistant (MIC = 256 $\mu\text{g/ml}$), the molecular basis for which is unknown. *PDR1* disruption in BG14, conferring cycloheximide hypersensitivity, was previously reported (63). Here we show that this disruption also largely reversed BG14 azole resistance. The fluconazole MIC decreased 16-fold to 16 $\mu\text{g/ml}$ (Figure 2-5A); i.e. comparable to the typical clinical isolate but eightfold above that observed for 66032 $\Delta pdr1$. Ketoconazole, itraconazole and miconazole MICs were similarly reduced in BG14 $\Delta pdr1$, but susceptibilities to unrelated antifungals terbinafine, caspofungin and amphotericin B were unchanged. Expression of *CgCDR1* and *ERG11* was examined by

Figure 2-4. *PDR1* replacement confirms role in azole resistance. A. Diagram illustrating replacement and PCR screening strategies. B. PCR screen of representative *PDR1* replacements 66032R and F15R (wild-type and F15-derived *PDR1*, respectively) selected on cycloheximide-containing plates, and their parent 66032Δ*pdr1*; strains 66032 and F15 were included as positive controls. DNA was purified from isolated colonies, amplified with PDR1uF2-PdR1iR primer pair, and analyzed by gel electrophoresis; formation of product confirmed replacement of *PDR1* into its native locus in 66032Δ*pdr1*. C. Broth microdilution assay showing that replacement into 66032Δ*pdr1* of 66032-derived (66032R) or F15-derived (F15R) *PDR1* conferred the expected low or high-level fluconazole resistance associated with 66032 and F15. Absorbance at 630 nm was recorded after 24 h incubation; growth was plotted as percentage of drug-free control.

Figure 2-5. Antifungal sensitivities and *CgCDR1-ERG11* expression in a $\Delta pdr1$ derivative of azole-resistant clinical isolate BG14. A. MIC values (at 24 h) determined by broth microdilution for BG14 and BG14 $\Delta pdr1$. A log scale was used to facilitate comparison of MICs over a wide range. Numbers above the BG14 $\Delta pdr1$ bars indicate the fold-change relative to BG14. FLU, fluconazole; ITR, itraconazole; KET, ketoconazole; MIC, miconazole; TER, terbinafine; AMB, amphotericin B; and CAS, caspofungin. B. RNA was isolated from log-phase BG14 and BG14 $\Delta pdr1$ cultures exposed to 256 μ g/ml fluconazole for 0–2.5 h, slot-blotted to membranes, and hybridized to the indicated gene probes.

RNA hybridization (Figure 2-5B). In BG14, constitutive expression of *CgCDR1* appeared to be modestly upregulated, but remained responsive to fluconazole-dependent upregulation. Both of these were strongly reduced in the $\Delta pdr1$ derivative, while no effects on *ERG11* expression were observed.

Strain 8512 represents a second azole-resistant clinical isolate with high constitutive *CgCDR1-CgPDH1* expression (216). Following 5FOA-mediated conversion to *ura3*, *PDR1* was disrupted in strain 8512 (not shown). Broth microdilution assays demonstrated reduction of fluconazole MIC from > 256 to 32 μ g/ml. Taken together, these data suggest that *PDR1* is a major determinant of azole sensitivity in *C. glabrata*, although additional gene mutations may contribute to clinical resistance.

Microarray Analysis: Upregulated Genes

In light of the major role played by transcription activator gene *PDR1* in *C. glabrata* azole sensitivity, an examination of genome-wide changes in gene expression in mutant F15 was warranted. We first attempted this with *S. cerevisiae* microarrays, because these two yeasts are closely related. However, the only confirmable change was upregulation of the *PDR5* (18-fold) and *PDR15* (ninefold) homologs (data not shown); both of these genes share 73% nucleotide identity with *CgCDR1*.

Therefore, *C. glabrata* microarrays were developed for the Affymetrix platform (see Materials and Methods) and used to examine changes in F15 relative to 66032. In F15, 78 genes were upregulated $\geq$ twofold relative to 66032. These genes are listed in Table 2-2, grouped by probable function and ordered by expression level. Among the upregulated are homologs of nine genes previously identified in microarray studies of *S. cerevisiae* Pdr1p–Pdr3p gain-of-function mutants (52, 53). Five of these nine genes encode putative membrane proteins with roles in small molecule transport or lipid metabolism. These include, in addition to *CgCDR1* and *CgPDH1*, the upregulated genes *YOR1* involved in oligomycin efflux, *RSB1* involved in sphingoid base-resistance, and *RTA1* involved in 7-aminosterol resistance (see *Saccharomyces* Genome Database (SGD) website (<http://www.yeastgenome.org>) for further information on these genes and references).

The four remaining genes upregulated in both *S. cerevisiae* and *C. glabrata* gain-of-function mutants include *PDR1* itself (as previously reported; (216)), the stress-induced *RPN4* encoding a proteasome gene transcription factor, and the uncharacterized open reading frames (ORFs) *YLR346C* and *YMR102C*. The latter encodes a relatively large and evolutionarily conserved protein with a WD40 domain commonly found in signaling proteins, and its disruption has been associated with fluconazole resistance in *S. cerevisiae* (8). The *YLR346C* product, in contrast, is not conserved; indeed, the *C. glabrata* and *S. cerevisiae* genes are not detectably homologous in terms of sequence but rather in terms of chromosomal synteny, flanked in both yeast by unambiguous *YLR345W* and *YLR347C* homologs. Also, both *YLR346C* products are short (101 and 112 amino acids) and highly charged in their C-terminal regions. In *S. cerevisiae*, Ylr346cp is

Table 2-2. *C. glabrata* genes upregulated $\geq$ twofold in fluconazole-resistant mutant F15.

Group	Systematic name	<i>S. cerevisiae</i> homolog ^a	<i>C. glabrata</i> designation	Description	Expression ^b		PDRE ^c
					F15	F15/66032	
Small molecule transport	IPF6352	^a <i>PDR5 (CDR1)</i>	CAGL0M01760g	ABC transporter involved in azole/multidrug resistance	41	2.5	134, 298, 388, 516
	IPF9719	^a <i>PDR15(PDH1)</i>	CAGL0F02717g	ABC transporter involved in azole/multidrug efflux	29	9.6	521, 557
	IPF1620	^a <i>QDR2</i>	CAGL0G08624g	MFS transporter involved in quinidine/multidrug efflux	23	4.5	848
	IPF8922	^a <i>YOR1</i>	CAGL0G00242g	ABC transporter involved in multidrug efflux	16	11	648
	IPF982	^a <i>YBT1</i>	CAGL0C03289g	ABC transporter involved in bile acid transport	13	7.7	450
Lipid, fatty acid, and sterol metabolism	IPF3303	^a <i>OAC1</i>	CAGL0K11616g	Mitochondrial inner membrane transporter	4.7	2.5	839
	IPF5152	^a <i>RTA1</i>	CAGL0K00715g	Over-expression confers 7-aminosterol resistance	22	7.0	300, 379
	IPF2180	^a <i>HFD1</i>	CAGL0K03509g	Putative mitochondrial fatty aldehyde dehydrogenase	13	5.6	218
	IPF4136	^a <i>RSB1</i>	CAGL0L10142g	Sphingolipid flippase	12	2.8	641, 881
	IPF8678	^a <i>LCB5</i>	CAGL0K05995g	Minor sphingoid long-chain base kinase	12	2.4	804
	IPF8367	^a <i>LAC1</i>	CAGL0M10219g	Ceramide synthase component	5.7	2.5	531
	IPF1002	^a <i>ARE1</i>	CAGL0C02981g	acyl-CoA:sterol acyltransferase; sterol esterification	4.6	4.6	114
	IPF4884	^a <i>ATF2</i>	CAGL0D05918g	Alcohol acetyltransferase; steroid detoxification	3.1	9.6	30, 195, 560, 772
	IPF2739	^a <i>SPO14</i>	CAGL0L03135g	Phospholipase D	0.8	2.6	–
	IPF2620	^a <i>CSR1</i>	CAGL0D00946g	Phosphatidylinositol transfer protein	0.4	3.8	239
	IPF6847	^a <i>HSP12</i>	CAGL0J04202g	Stress-induced membrane protein	29	4.0	849
	IPF3173	^a <i>YNL134c</i>	CAGL0K09702g	Alcohol dehydrogenase motif; stress-induced	14	9.5	541
Cell stress	IPF4605	^a <i>YML131W</i>	CAGL0K12958g	Alcohol dehydrogenase motif; stress-induced	7.3	9.1	594
	IPF6629	^a <i>HSP31</i>	CAGL0C00275g	Possible chaperone and cysteine protease	3.5	2.0	–
	IPF4140	^a <i>YOR052C</i>	CAGL0L10186g	Uncharacterized; stress-induced	2.3	3.2	–
	IPF8736	^a <i>TPS3</i>	CAGL0H02387g	Trehalose-6-phosphate synthase/phosphatase subunit	1.5	4.4	–
	IPF5558	^a <i>HSP42</i>	CAGL0E00803g	Small cytosolic stress-induced chaperone	0.6	4.9	–
	IPF5076	^a <i>RPN4</i>	CAGL0K01727g	Transcription factor for proteasome genes	16	3.9	378, 394, 552
	IPF5932	^a <i>SUT1</i>	CAGL0I04246g	Transcription factor involved in sterol uptake	15	2.4	–
	IPF3325	^a <i>PDR1</i>	CAGL0A00451g	Transcription factor involved in multidrug resistance	9.6	2.3	557, 701
	IPF7202	^a <i>TAF9</i>	CAGL0M05005g	Subunit of TFIID and SAGA complexes	1.0	6.3	–
	IPF6366	^a <i>YPR013C</i>	CAGL0M01870g	Uncharacterized; potential zinc finger	0.8	2.9	–
Transcription	IPF2113	^a <i>SIP3</i>	CAGL0I01980g	Activates transcription through DNA-bound Snf1	0.4	2.2	–
	IPF118	^a <i>HOT1</i>	CAGL0H08866g	Transcription factor involved in osmotic stress response	0.1	4.9	228
	IPF9036	^a <i>YIM1</i>	CAGL0M09713g	Implicated in DNA damage response	40	12	127, 179
	IPF9035	^a <i>MEC3</i>	CAGL0M09735g	DNA damage checkpoint	1.6	15	110, 162
	IPF2521	^a <i>DBF4</i>	CAGL0E04576g	Regulatory subunit of Cdc7p-Dbf4p kinase complex	0.7	2.4	–
	IPF785	^a <i>DPB3</i>	CAGL0B03355g	DNA polymerase II subunit	0.2	2.7	–
	IPF3014	^a <i>OCH1</i>	CAGL0A01738g	Mannosyltransferase of <i>cis</i> -Golgi apparatus	5.2	2.5	–
Protein synthesis, modification, or degradation	IPF6742	^a <i>UFD1</i>	CAGL0J08096g	Recognition of polyubiquitinated proteins	4.4	2.2	–
	IPF3846	^a <i>NCE3</i>	CAGL0G01540g	Carbonic anhydrase-like; non-classical protein export	3.1	2.9	–
	IPF3072	^a <i>RPN8</i>	CAGL0K08866g	Non-ATPase regulatory subunit of 26S proteasome	2.9	2.9	–
	IPF8484	^a <i>PCI8</i>	CAGL0M12749g	Possible shared subunit of Cop9 signalosome and eIF3	0.2	2.4	–

Table 2-2 (continued).

Group	Systematic name	<i>S. cerevisiae</i> homolog ^a	<i>C. glabrata</i> designation	Description	Expression ^b		PDRE ^c
					F15	F15/66032	
Vesicular and protein transport	IPF7414	<i>GSF2</i>	CAGL0L01485g	ER membrane, hexose transporter secretion	10	2.4	208
	IPF8257	<i>YPT52</i>	CAGL0G07689g	GTPase required for vacuolar protein sorting	1.6	2.8	–
	IPF8439	<i>MEH1</i>	CAGL0L02211g	Component of the EGO complex; microautophagy	0.5	5.7	–
	IPF4445	<i>VPS28</i>	CAGL0H05181g	Component of ESCRT-I complex; protein trafficking	0.5	2.2	–
	IPF4173	<i>VTI1</i>	CAGL0L10604g	Involved in <i>cis</i> -Golgi membrane traffic	0.3	6.7	741
	IPF3260	<i>GYL1</i>	CAGL0K10934g	putativegAP for Ypt1 involved in polarized exocytosis	0.3	4.0	–
Signal transduction	IPF271	<i>VPS51</i>	CAGL0H06809g	Golgi-associated retrograde protein complex	0.2	6.1	–
	IPF1489	<i>BAG7</i>	CAGL0I07249g	GAP for Rho1; cell wall and cytoskeleton homeostasis	1.6	2.9	–
	IPF8227	<i>CDC25</i>	CAGL0D06512g	Membrane bound GEF for Ras1-Ras2	1.2	5.8	–
	IPF351	<i>GAC1</i>	CAGL0F04917g	Regulatory subunit forglc7 protein phosphatase	0.9	4.7	–
	IPF2382	<i>YNL234W</i>	CAGL0J07502g	Similar toglobins with haem-binding domain	0.5	3.1	–
	IPF512	<i>GPG1</i>	CAGL0F07117g	Subunit of heterotrimericg protein, interacts withgrp1	0.4	2.6	–
	IPF5914	<i>KIN3</i>	CAGL0I04422g	Protein kinase	0.2	5.6	–
	IPF2122	<i>FMP48</i>	CAGL0K04301g	Ser/Thr protein kinase; mitochondrial	11	2.8	–
Mitochondrial	IPF7121	<i>YGR046W</i>	CAGL0G03861g	Essential protein involved in mitochondria transport	0.4	4.4	–
Cell wall	IPF9549	<i>FLO1</i>	CAGL0E00209g	Flo1-like family of cell wall proteins	2.6	3.0	284, 419
Amino acid and carbohydrate metabolism	IPF496	<i>PYC1</i>	CAGL0F06941g	Pyruvate carboxylase isoform	4.6	2.3	–
	IPF4499	<i>STR3</i>	CAGL0L06094g	Cystathionine beta-lyase	0.7	5.0	–
	IPF5315	<i>MET8</i>	CAGL0K06677g	Bifunctional dehydrogenase and ferrochelatase	0.2	7.8	–
Chromatin/chromosome structure	IPF8319	<i>SMD3</i>	CAGL0M04631g	Core Sm spliceosome protein Sm D3	0.9	3.7	–
	IPF390	<i>SPC19</i>	CAGL0F05467g	Component of Dam1 spindle pole complex	0.5	13.1	–
	IPF8077	<i>SPC97</i>	CAGL0I02464g	Component of microtubule-nucleating Tub4 complex	0.5	2.8	–
	IPF2730	<i>SPC34</i>	CAGL0L03223g	Component of Dam1 spindle pole complex	0.4	2.5	–
Other metabolism	IPF6032		CAGL0M14091g	Putative quinone reductase/NADPH dehydrogenase	3.8	9.8	244, 532
	IPF6034	<i>ADH6</i>	CAGL0M14047g	NADPH-dependent cinnamyl alcohol dehydrogenase	2.3	2.7	–
	IPF1279	<i>YPR1</i>	CAGL0A02816g	2-methylbutyraldehyde reductase	0.6	5.6	–
	IPF4182	<i>INP1</i>	CAGL0L10736g	Peripheral membrane protein of peroxisomes	0.2	5.5	–
Uncharacterized	IPF6116	<i>YIL077C</i>	CAGL0M12947g	Uncharacterized	20	38	472, 502
	IPF8009	<i>YJL163C</i>	CAGL0M08426g	Uncharacterized; ARS in promoter	9.1	10	450
	IPF2520		CAGL0E04554g	Uncharacterized; no similarities	8.8	6.4	–
	IPF2196	^d <i>YMR102C</i>	CAGL0K03377g	Transcribed along with MDRgenes by Yrr1/Yrm1	8.1	4.4	898
	IPF3019		CAGL0A01650g	Uncharacterized; no similarities	7.2	4.4	564
	IPF3875	^d <i>YLR346C</i>	CAGL0G01122g	Uncharacterized; syntenic but minimal similarities	3.3	22	793, 803
	IPF3655	<i>YLR177W</i>	CAGL0B01078g	Uncharacterized	2.7	2.5	–
	IPF1546		CAGL0G09603g	Uncharacterized; very weak similarity to Yor186w	2.1	6.1	–
	IPF2382	<i>YNL234W</i>	CAGL0J07502g	Similar toglobins with haem-binding domain	0.5	3.1	–
	IPF4149	<i>YOR059C</i>	CAGL0L10318g	Uncharacterized	0.5	2.4	–
	IPF6420	<i>YGR126W</i>	CAGL0I10604g	Uncharacterized	0.1	19	–
	IPF2249	<i>YHL010C</i>	CAGL0K02563g	Uncharacterized; mammalian BRAP2 homolog	0.1	5.0	–

Table 2-2 (continued).

^aParentheses indicate a previously named *C. glabrata* gene.

^bExpression in *C. glabrata* 66032 is represented in arbitrary microarray units (for comparison, actin and β -tubulin gene homologs *ACT1* and *TUB2* had average expression levels of 66 and 8.6 respectively). F15/66032 represents the ratio of expression in the fluconazole-resistant mutant vs. its parent (for comparison, *ACT1* and *TUB2* had ratios of 0.6 and 0.8 respectively).

^cPromoter regions (900 bp) were searched for matches to the *S. cerevisiae* PDRE consensus TCCRYGSR. Numbers indicate the distance (in bp) upstream of the ATG start codon of the PDRE; hyphens (–) indicate the absence of a PDRE.

^dGenes similarly upregulated in *S. cerevisiae* Pdr1p–Pdr3p gain-of-function mutants (52, 53).

mitochondria-localized and forms a two-hybrid interaction with MAP kinase Slr2p, suggesting a possible role in mitochondria-nucleus retrograde signaling.

Among the 69 genes whose upregulation appears to be *C. glabrata* F15-specific (i.e. not similarly upregulated in *S. cerevisiae*) are three additional homologs encoding small molecule transporters including quinidine and bile acid efflux protein genes *QDR2* and *YBT1*. Additional lipid metabolism genes include *ARE1* whose disruption in *S. cerevisiae* confers azole hypersensitivity (T. Edlind, unpublished data) and *ATF2* involved in fatty acid and steroid detoxification. A third group of well-represented genes are involved in the cell stress response, including membrane protein gene *HSP12*, and *YML131W-YNL134C*; the latter two are unrelated by blast but share an ADH_zinc_N domain (identified by CD-search; (128)) characteristic of zinc-dependent alcohol dehydrogenases-oxidoreductases. *YML131W-YNL134C* are also coordinately upregulated in *S. cerevisiae* in response to diverse stresses including heat, oxidizing agents, ethanol, nitrogen depletion and stationary phase ((70); see Expression Connection at <http://www.yeastgenome.org>). A similarly regulated *S. cerevisiae* gene is *GRE2*, also encoding an oxidoreductase and among the genes upregulated in Pdr1p–Pdr3p gain-of-function mutants (52, 53). This provides an example of analogous but non-homologous genes upregulated in *C. glabrata* F15 and *S. cerevisiae* Pdr1p–Pdr3p mutants.

Notable among the remaining upregulated genes with significant expression levels are: *SUT1* encoding a transcription factor involved in sterol uptake and hypoxic gene expression, *YIM1* implicated in DNA damage response, *OCH1* and *GSF2* involved in Golgi-ER functions, proteasome-related genes *UFD1* and *RPN8*, putative mitochondrial protein kinase gene *FMP48*, a quinone reductase-like gene curiously lacking in other fungal genomes but present in many bacteria and vertebrates, and the uncharacterized *YIL077C* whose product has been mitochondria-localized but interacts with a nuclear transcriptional complex.

Microarray Analysis: Downregulated Genes

There were 31 genes downregulated $\geq$ twofold in F15 relative to parent 66032 (Table 2-3). Only one of these was also downregulated in *S. cerevisiae* Pdr1p–Pdr3p gain-of-function mutants: membrane transporter gene *PDR12* involved in efflux of weak organic acids such as sorbate. Additional genes with significantly downregulated expression include zinc transporter gene *ZRT1*, major facilitator genes including *FLR1* implicated in fluconazole efflux, and homologs of cell surface protein genes *MUC1-EPA2* and *MKC7* implicated in adhesion and aspartic protease activity respectively. Finally, a gene was downregulated whose product has clear homology to the WRY family of putative membrane-anchored proteins previously identified only in *C. albicans* (unpublished annotation in NCBI database). This family has nine paralogs in *C. albicans* and seven in *C. glabrata* but none, surprisingly, in *S. cerevisiae*, suggesting a possible role in mammalian colonization.

Table 2-3. *C. glabrata* genes downregulated $\geq$ twofold in fluconazole-resistant mutant F15 compared with parent 66032.

Group	Systematic name	<i>S. cerevisiae</i> homolog ^a	<i>C. glabrata</i> designation	Description	Expression ^b		PDRE
					F15	F15/66032	
Small molecule transport	IPF5505	<i>ZRT1</i>	CAGL0E01353g	High-affinity zinc transporter	10	0.5	–
	IPF4392	<i>FLR1</i>	CAGL0H06017g	Multidrug efflux pump of major facilitator superfamily	6.1	0.4	–
	IPF6218	<i>^dPDR12</i>	CAGL0M07293g	ABC transporter of weak organic acids	4.5	0.2	–
	IPF7808	<i>YHR048W</i>	CAGL0J00363g	Uncharacterized; major facilitator superfamily	2.2	0.4	–
	IPF867	<i>ATR1</i>	CAGL0B02343g	Multidrug efflux pump of major facilitator superfamily	1.8	0.4	–
Cell stress	IPF1568	<i>SNG1</i>	CAGL0G09273g	Nitrosoguanidine resistance; putative transporter	1.3	0.1	–
	IPF3249	<i>CTA1</i>	CAGL0K10868g	Catalase A	8.8	0.4	–
	IPF11021	<i>CRS5</i>	CAGL0H04257g	Copper-binding metallothionein-like protein	3.4	0.4	–
	IPF9018	<i>LTV1</i>	CAGL0J00891g	Required for growth at low temperature	1.0	0.4	656
	IPF9641	<i>SLG1</i>	CAGL0F01507g	Sensor of stress-activated Pkc1-Slt2 pathway	0.8	0.5	–
	IPF5937	<i>CUP2(AMT1)</i>	CAGL0I04180g	Metal-activated transcriptional factor	0.9	0.2	–
	IPF986	<i>SDH2</i>	CAGL0C03223g	Succinate dehydrogenase iron-sulphur protein subunit	4.9	0.4	–
Carbohydrate metabolism	IPF6117	<i>DOG2</i>	CAGL0M12925g	2-deoxyglucose-6-phosphate phosphatase	1.6	0.3	–
	IPF290	<i>PCK1</i>	CAGL0H06633g	Phosphoenolpyruvate carboxykinase	1.2	0.3	–
Cell cycle control	IPF2555	<i>SDS22</i>	CAGL0D00264g	Nuclear regulatory subunit of glc7 phosphatase	0.3	0.2	–
RNA processing	IPF8796	<i>PNO1</i>	CAGL0K09460g	Nucleolar protein required for pre-rRNA processing	3.0	0.4	–
	IPF1130	<i>AAR2</i>	CAGL0A04543g	Component of the U5 snRNP	1.0	0.4	–
	IPF9324	<i>DIM1</i>	CAGL0L07678g	Essential 18S rRNA dimethylase	1.0	0.5	–
Cell surface and cytoskeleton	IPF5272	<i>NOP8</i>	CAGL0B01397g	Nucleolar protein required for ribosome biogenesis	0.7	0.4	–
	IPF9500	<i>MKC7</i>	CAGL0J01793g	Muc1/Epa2-like putative cell surface protein	4.2	0.3	–
	IPF8398		CAGL0E01771g	GPI-anchored aspartyl protease	3.6	0.3	–
	IPF807	<i>MNT3</i>	CAGL0B03003g	α -1,3-mannosyltransferase involved in O-glycosylation	3.1	0.4	–
	IPF3539	<i>ECM4</i>	CAGL0G02101g	Promoter insertion mutant is calcofluor-hypersensitive	1.6	0.3	–
	IPF810	<i>MNT3</i>	CAGL0B02992g	α -1,3-mannosyltransferase involved in O-glycosylation	1.4	0.3	–
	IPF821		CAGL0B02882g	<i>C. albicans</i> WRY family; transmembrane domain	0.5	0.1	–
	IPF5228	<i>ECM18</i>	CAGL0B01969g	Insertion mutant is calcofluor hypersensitive	0.3	0.3	–
	IPF7581	<i>TPM2</i>	CAGL0L08338g	Minor isoform of tropomyosin	0.3	0.4	–
	IPF493	<i>YIR035C</i>	CAGL0F06897g	Uncharacterized; alcohol dehydrogenase domain	3.3	0.4	–
Uncharacterized	IPF3428	<i>YNL095C</i>	CAGL0G06468g	Uncharacterized; related to <i>ECM3</i>	2.7	0.4	–
	IPF7665	<i>SIP5</i>	CAGL0L06864g	Uncharacterized; interacts with glc7 and Snf1	0.4	0.3	–
	IPF5688	<i>FMP16</i>	CAGL0G05269g	Uncharacterized; mitochondrial	0.3	0.3	–

^aParentheses indicate a previously named *C. glabrata* gene.

^bExpression in *C. glabrata* 66032 is represented in arbitrary microarray units (for comparison, actin and β -tubulin gene homologs ACT1 and TUB2 had average expression levels of 66 and 8.6 respectively). F15/66032 represents the ratio of expression in the fluconazole-resistant mutant vs. its parent (for comparison, *ACT1* and *TUB2* had ratios of 0.6 and 0.8 respectively).

^cPromoter regions (900 bp) were searched for matches to the *S. cerevisiae* PDRE consensus TCCRYGSR. Numbers indicate the distance (in bp) upstream of the ATG start codon of the PDRE; hyphens (–) indicate the absence of a PDRE.

^dGenes similarly downregulated in *S. cerevisiae* Pdr1–Pdr3 gain-of-function mutants (52, 53).

Confirmation of Microarray Results

As our studies represent the first application of these *C. glabrata* microarrays, it was important to validate the results by independent methods. RNA blots or real-time reverse transcription (RT)-PCR were used to examine the expression of selected genes identified as upregulated in the microarray (other than already confirmed *CgCDR1*, *CgPDH1* and *CgPDR1*). For five of seven genes tested (*YLR346C*, *YOR1*, *YNL134C*, *YML131W* and *RTA1*), RNA blots confirmed F15 upregulation relative to the parent 66032 strain (Figure 2-6A). The two exceptions (*MEC3* and *YJL163C*) represent genes whose expression in both parent and F15 were below the level of detection by RNA blot (not shown).

For all nine genes tested by real-time RT-PCR (*YOR1*, *RTA1*, *RPN4*, *QDR2*, *MET8*, *BAG7*, *CSRI*, *PDR1* and *YBT1*), the upregulation observed by microarray was confirmed (Figure 2-5B). For most of these, the results were quantitatively similar; e.g. *YOR1* was upregulated 16-fold by microarray and 17-fold by RT-PCR and *CSRI* was upregulated 3.8-fold by microarray and 2.9-fold by RT-PCR. Expression of *ERG11* encoding the azole target lanosterol demethylase was essentially unaltered by RT-PCR (not shown), in agreement with microarray analysis ($F15/66032 = 0.6$) and RNA hybridization (216). One anomaly in the microarray analysis was the relatively low upregulation of *CgCDR1* (2.5-fold) compared with its high upregulation (approximately 20-fold) in both RNA blots and RT-PCR (Figure 2-2). Furthermore, CgCdr1p was strongly upregulated on the protein level, as shown by SDS-PAGE of membrane preparations followed by mass spectrometric identification of eluted bands (181). Potential explanations for this anomaly include degradation or masking of the *CgCDR1* mRNA region targeted by the microarray, or a saturation effect due to the relatively high *CgCDR1* basal expression.

Promoter Sequence Analysis

To identify a Candidate *C. glabrata* Pdr1p response element (PDRE), we took advantage of the F15 microarray data, the available genome sequence, and the evolutionary relatedness of this yeast to *S. cerevisiae*. The promoter regions (900 bp upstream of the start codon) for all genes listed in Tables 2-2 and 2-3 were searched for a match to the consensus *S. cerevisiae* PDREs (52, 53): TCC(GA)(CT)G(GC)(AG). At least one match to this sequence was identified in 31 of the 78 genes (40%) upregulated $\geq$ two-fold. Moreover, one or more PDRE were identified in all nine genes upregulated in both *C. glabrata* and *S. cerevisiae* gain-of-function mutants, in 14 of 15 genes in the small molecule transport and lipid metabolism groups, and in 26 of 34 genes with expression level > 3 (arbitrary microarray units). Conversely, only one PDRE was identified among the 31 downregulated gene promoters (Table 2-3); similarly, none was identified in the promoters of representative housekeeping genes (*ACT1*, *TEF1*, *TDH3*) or azole target gene *ERG11*. This analysis therefore identifies TCC(AG)(TC)G(GC)(AG) as a strong Candidate for the *C. glabrata* PDRE. More specifically, we note a clear

Figure 2-6. Confirmation of F15/66032 microarray results for selected genes by RNA hybridization and real-time RT-PCR. A. RNA was isolated from log phase cultures, slot-blotted to membranes, and hybridized to the indicated gene probes; *ACT1* served as loading control. B. Quantitative real-time RT-PCR analysis of relative gene expression in F15 versus 66032. Data are shown as mean $\pm$ SD.

preference for G as the penultimate base (95% of PDREs) and A as the final base (84%), although two of the four PDREs within the *CgCDR1* promoter have G as the final base.

Two exceptions warrant discussion. The promoters of *C. glabrata* upregulated genes *RPN8* and *YOR052C* lack a PDRE but include perfect matches to the *S. cerevisiae* Rpn4p transcription factor-binding site GGTGGCAAA (124); perfect or near-perfect matches are also found in the promoters of their *S. cerevisiae* homologs. As noted above, Rpn4p is upregulated in both *C. glabrata* Pdr1p and *S. cerevisiae* Pdr1p–Pdr3p gain-of-function mutants. Thus, *RPN8*-*YOR052C* upregulation is likely Rpn4p-mediated and only indirectly Pdr1p-mediated.

***Candida glabrata* F15 Exhibits Additional Phenotypes Predicted by Microarray Analysis Which May Alter Virulence**

As coordinate *CgCDR1*-*CgPDH1* upregulation is commonly observed in *C. glabrata* azole-resistant clinical isolates (21, 187, 216), the responsible mutations must have minimal effects on fitness. On the other hand, these mutations could alter *C. glabrata* in subtle ways that affect, for example, its relative virulence in the bloodstream versus mucosa. The F15 microarray results provided us with an opportunity to begin to test this general hypothesis. Specifically, we looked for phenotypes other than azole susceptibility predicted to be associated with altered expression of genes co-regulated with *CgCDR1*-*CgPDH1*.

Upregulation of the *YOR1* transporter 11-fold (Table 2-2) predicts that azole-resistant F15 should be cross-resistant to oligomycin, an inhibitor of mitochondrial F1F₀ ATPase and known *S. cerevisiae* Yor1p substrate. This was confirmed by broth microdilution assay (MIC = 0.5 µg/ml for F15 vs. 0.125 µg/ml for parent 66032), using medium with glycerol as respiratory carbon source. Yor1p also confers tolerance in *S. cerevisiae* to a wide range of organic anions such as leptomycin B and acetic acid, along with cadmium (47). *PDR12*, downregulated fivefold in F15, similarly encodes an efflux pump with specificity for organic acids, in particular sorbic acid (170). Spot assays (Figure 2-7) confirmed sorbate hypersensitivity of F15, although the effects on MIC were modest (4 mM for F15 vs. 8 mM for 66032). With respect to organic acid sensitivity, *PDR12* downregulation may be largely offset by *YOR1* upregulation. There was no detectable change in sensitivity to acetic, boric, or lactic acids (MICs = 64, 16 and 250 mM respectively).

YML131W and *YNL134C* homologs were similarly upregulated approximately nine-fold in F15. As noted above, the products of these uncharacterized genes share a domain characteristic of alcohol dehydrogenases/oxidoreductases; furthermore, they are co-regulated in response to environmental stresses including heat shock and treatment with reactive oxygen species or ethanol (Expression Connection, SGD website). Conversely, catalase gene *CTA1* was downregulated 2.5-fold. Consistent with this, F15 demonstrated hypersensitivity to hydrogen peroxide by spot assay (Figure 2-7) and broth microdilution (MIC = 16 mM vs. 32 mM for 66032). Similarly, F15 demonstrated

Figure 2-7. Spot assays examining sensitivity of 66032 and its azole-resistant mutant F15 to hydrogen peroxide, ethanol and sorbic acid. Approximately 300 cells were spotted on YPD agar with the indicated inhibitor. Plates were incubated for 2–4 days at 35°C. For comparison, 66032Δpdr1, F15Δpdr1 and the 66032Δpdr1-PDR1 replacement strains 66032R and F15R were examined in parallel.

hypersensitivity to ethanol (Figure 2-7; MIC = 2% vs. 4% for 66032). Equivalent results were obtained with F15 *PDR1* replacement clone F15R as compared with wild-type *PDR1* replacement clone 66032R (Figure 2-7), confirming that these altered phenotypes resulted from the *PDR1* gain-of-function mutation. (Note that the *ura3* phenotype of 66032R can account for its variable growth relative to 66032, an effect also observed with the 66032 *ura3* strain; not shown.) Finally, we examined sensitivity to heat shock by exposing mid-log or early stationary phase cultures to 50°C for 10 min, following by plating on YPD with incubation at 35°C for 3 days to obtain colony counts. For F15 versus 66032, viability was 6 versus 0.3% and 16 versus 5% in log and stationary phase cultures respectively. Taken together, these data suggest that regulatory mutations conferring azole resistance in *C. glabrata* may have both positive and negative effects on fitness and virulence.

CONCLUSIONS

An important 'virulence factor' for the emerging opportunist *C. glabrata* appears to be its capacity for intrinsic low-level and acquired high-level azole resistance. The studies completed here with laboratory mutant F15, and initial studies with representative clinical isolates, identify the zinc-cluster transcriptional activator CgPdr1p as a key regulator of azole/multidrug transporter genes *CgCDR1* and *CgPDH1*. Constitutive upregulation of these genes is observed in most azole-resistant clinical isolates; furthermore, they are transiently upregulated in sensitive isolates following azole exposure. Consistent with this, in *PDR1* disruptants acquired resistance was reversed and intrinsic resistance was reduced. We have shown that F15 CgPdr1p has a gain-of-function mutation analogous to those previously characterized in *S. cerevisiae* Pdr1p–Pdr3p, and this mutation is sufficient to confer azole resistance. CgPdr1p mutation is not, however, necessary for resistance, because at least one resistant strain analyzed had unchanged *CgPDR1*(216). Azole resistance may potentially arise from mutations in upstream signaling proteins or transcription cofactors, both of which remain to be defined (although histone modifying enzymes represent likely cofactors). Moreover, we observed here that *PDR1* disruptants, although azole hypersensitive, continued to yield spontaneous azole-resistant mutants at reduced frequency. These Pdr1p-independent resistance mechanisms, and their clinical relevance, warrant further study.

Microarray analysis of genome-wide gene expression has become a central tool in molecular genetics, and the arrays developed and tested here should be particularly useful in studies of *C. glabrata* in large part because of its close evolutionary relatedness to *S. cerevisiae*. Most genes with altered expression in F15 had well-characterized *S. cerevisiae* homologs. This allowed us to predict F15 phenotypes, a number of which were tested including sensitivity to organic acids, alcohols and oxidants. Ultimately, these data should help us to understand and possibly exploit the consequences for *C. glabrata* of regulatory mutations leading to azole resistance. F15 hypersensitivity to hydrogen peroxide is of particular interest, because this implies hypersensitivity to immune cells such as neutrophils and environments such as the lactobacillus-colonized vaginal tract in which hydrogen peroxide plays an important role. Although the relatedness of *C.*

glabrata and *S. cerevisiae* is invaluable in terms of predicting gene function, microarray analysis indicated that the Pdr1p and Pdr1p-Pdr3p gain-of-function mutants of these yeasts are more different than similar. This no doubt reflects the very different pressures placed on these organisms by their very different niches; e.g. the skin of a grape versus the human mucosa.

CHAPTER 3. GENOME-WIDE EXPRESSION PROFILE ANALYSIS OF THE *CANDIDA GLABRATA* PDR1P REGULON

INTRODUCTION

Over the past two decades, there has been an increase in *Candida* infections caused by non-*albicans* species with *Candida glabrata* being the second most common cause of mucosal and invasive fungal infections in humans (162). Development of high-level resistance to the azoles, the most widely-used antifungal class for treatment of *Candida* infections, has been reported in *C. glabrata* oral and bloodstream isolates from head and neck radiation patients, stem cell transplant patients, and human immunodeficiency virus (HIV) patients (21, 175, 185). Furthermore, the development of azole resistance has been implicated in the fluconazole treatment failure and death of a patient suffering from *C. glabrata* candidemia (122).

Studies to determine the mechanisms of high level azole resistance in *C. glabrata* have demonstrated frequent constitutive over-expression of the ATP-binding cassette (ABC) transporters, *CgCDR1*, *CgPDH1*, and *CgSNQ2*, all of which contribute to this phenotype (135, 175, 185, 210, 216). These transporters are regulated by the zinc binuclear cluster transcription factor Pdr1p, a single-gene homologue of *Saccharomyces cerevisiae* Pdr1p and Pdr3p heterodimeric transcription factor (215, 216). *CgPDR1*, *CgCDR1*, *CgPDH1*, and *CgSNQ2* all contain at least one pleiotropic drug response element sequence (PDRE) in their promoters suggesting *CgPdr1p* may regulate its own expression, as well as that of the transporters, through binding these regulatory elements (210, 215). Recently it was shown that *C. glabrata* Pdr1p (and *S. cerevisiae* Pdr1p) directly bind to fluconazole resulting in activation of drug efflux pumps, a mechanism similar to regulation of multidrug resistance (MDR) by the PXR nuclear receptor in vertebrates (207). Furthermore, single point mutations in the putative functional domains of *CgPDR1* result in increased transcription of *CgCDR1*, *CgPDH1*, and *CgSNQ2* as well as increased resistance to azoles (68, 212, 215). A recent study demonstrated a high variability in these mutations and showed the Pdr1p gain-of-function mutations not only have an effect on azole susceptibility but also virulence (68). Collectively these studies indicate that Pdr1p is uniquely important to the ability of *C. glabrata* to not only resist antifungal therapy but to also cause disease.

With these aforementioned studies in mind, we endeavored to delineate the core genes of the Pdr1p regulon by first using genome-wide gene expression profiling to identify genes co-regulated with *CgCDR1* among clinical matched azole-susceptible and –resistant *C. glabrata* isolates. We then identified mutations in *CgPDR1* among these clinical isolates that conferred resistance to fluconazole. Gene expression profiling was again used to identify genes that were differentially expressed in the presence of these mutations. Our results identified a more comprehensive picture of the molecular effects of Pdr1p mutations in azole-resistant isolates and suggest other cellular functions in *C. glabrata*.

MATERIALS AND METHODS

Antifungal Agents

Fluconazole was obtained from MP Biomedicals (Solon, Ohio). Stock solutions of 10 mg/ml were made in dimethyl sulfoxide (DMSO, Sigma; St. Louis, Missouri) and stored at -20°C.

Strains and Growth Media

The isolates and strains used in this study are listed in Table 3-1. Three of these matched fluconazole-susceptible and -resistant clinical isolate sets have been described previously (122, 175, 209). Clinical isolates 62-16 and 62-17 were kindly provided by Dr. John Mohr at the University of Texas Health Science Center, Houston, TX. All isolates/strains used in these studies were grown in YPD (1% yeast extract, 2% peptone, and 1% dextrose) broth at 30°C and stored as 20% glycerol stocks at -80°C. One Shot® *Escherichia coli* TOP10 chemically competent cells (Invitrogen; Carlsbad, California) were used as a host for plasmid construction and propagation. These strains were grown in Luria-Bertani (LB) broth or on LB plates supplemented with 100 µg/ml ampicillin (Sigma) or 50 µg /ml kanamycin (Fisher BioReagents; Fair Lawn, New Jersey) when required.

Plasmid Constructions

All primer sequences are listed in Table 3-2. The *CgPDR1* deletion cassette was constructed as follows: The upstream flanking region, containing ~900 bp upstream as well as the first 50 nucleotides of the *PDR1* coding region, was amplified by PCR from *C. glabrata* isolate SM1 genomic DNA with the primers CgPDR1A and CgPDR1B. Likewise, the downstream flanking region of the *PDR1* gene containing the last 50 nucleotides of the *PDR1* coding region as well as ~900 bp downstream, was amplified by PCR from genomic DNA from *C. glabrata* isolate SM1 using the primers CgPDR1C and CgPDR1D. Each PCR product was cloned using the TA cloning® kit (Invitrogen) as per the manufacturer's instructions. The resulting downstream plasmid DNA was digested at the introduced *SacI* and *SacII* restriction sites and cloned downstream of the SAT1-flipper cassette in the corresponding *SacI* and *SacII* sites in plasmid pSFS2A (176) to generate pPDR1CDS2. The upstream flanking sequence was digested at the introduced *ApaI* and *XhoI* restriction sites and cloned upstream of the SAT1 flipper cassette in the corresponding *ApaI* and *XhoI* sites in plasmid pPDR1CDS2 to generate pPDR1S2. The *PDR1* replacement cassette was constructed in the following manner: The *PDR1* ORF plus ~500 bp upstream and ~500 bp downstream of the *PDR1* coding sequence was amplified by PCR from genomic DNA from each *C. glabrata* clinical isolate using *Pfu* DNA polymerase (Stratagene; La Jolla, California) with the primers CgPDR1E and CgPDR1F. The resulting PCR product was cloned using the ZeroBlunt® TOPO®

Table 3-1. *C. glabrata* isolates/strains used in this study.

Strain/Isolate	Parent	Relevant characteristics or genotype	Reference
SM1	--	Clinical isolate, fluconazole susceptible	(122)
SM3	SM1	Clinical isolate, fluconazole resistant	
6856	--	Clinical isolate, fluconazole susceptible	(175)
6955	6856	Clinical isolate, fluconazole resistant	
7754	--	Clinical isolate, fluconazole susceptible	(209)
7755	7754	Clinical isolate, fluconazole resistant	
62-16	--	Clinical isolate, fluconazole susceptible	This study
62-17	62-16	Clinical isolate, fluconazole resistant	This study
SM1 Δ <i>pdrl</i>	SM1	<i>pdrl</i> : : FRT	This study
SM1 Δ PDR1 ^{SM1}	SM1 Δ <i>pdrl</i>	<i>pdrl</i> Δ /PDR1 ^{SM1} -caSAT1	This study
SM1 Δ <i>pdrl</i> / <i>PDR1</i> ^{T2837C}	SM1 Δ <i>pdrl</i>	<i>pdrl</i> Δ /PDR1 ^{T2837C} -caSAT1	This study
SM1 Δ PDR1 ⁶⁸⁵⁶	SM1 Δ <i>pdrl</i>	<i>pdrl</i> Δ /PDR1 ⁶⁸⁵⁶ -caSAT1	This study
SM1 Δ <i>pdrl</i> / <i>PDR1</i> ^{G822T}	SM1 Δ <i>pdrl</i>	<i>pdrl</i> Δ /PDR1 ^{G822T} -caSAT1	This study

Table 3-2. Primers used in this study.

Primer/Gene	Primer pair ^{a,b}	Amplicon size (bp)
Primers used for plasmid construction		
CgPDR1A	F, 5'-AAACATTTTCCTCATAG <u>GggCcc</u> ATCTTTTAC-3'	1080
CgPDR1B	R, 5'-ATTACATTTTATTTTCCTC <u>gag</u> CTACAGC-3'	
CgPDR1C	F, 5'-CACTTATAA <u>ccgcGGA</u> CTTTAGATGAGT-3'	1000
CgPDR1D	R, 5'-AACCCGATACT <u>GagcTc</u> TTAAGTAAGGA-3'	
CgPDR1E	F, 5'-ATAATTGTGGGT <u>Acc</u> ACTAGGTATTAGG-3'	4295
CgPDR1F	R, 5'-TATAATAAAT <u>GggCcc</u> ATCAACGAAGGT-3'	
Primers used for quantitative real-time PCR expression analysis		
18S rRNA	F, 5'-TCGGCACCTTACGAGAAATCA-3' R, 5'-CGACCATACTCCCCCAGA-3'	52
<i>CgPDR1</i>	F, 5'-TTTGACTCTGTTATGAGCGATTACG-3' R, 5'-TTCGGATTTTCTGTGACAATGG-3'	51
<i>CgCDR1</i>	F, 5'-CATAACAAGAAACACCAAAGTCGGT-3' R, 5'-GAGACACGCTAACGTTCCACCAC -3'	71
<i>CgPDH1</i>	F, 5'-ACGAGGAGGAAGACGACTACGA-3' R, 5'-CTTTACTGGAGAACTCATCGCTGT-3'	63
<i>CgSNQ2</i>	F, 5'-CGTCCTATGTCTTCCTTACACCATT-3' R, 5'-TTTGAACCGCTTTTGTCTCTGA-3'	57
Primers used for sequencing <i>CgPDR1</i>		
CgPDR1seq	F, 5'-GGTAAAGTCATTCTTTAGCTACG-3' R, 5'-TACAGGCTATGCACACTGTCT-3'	3393
CgPDR1seqA1	F, 5'-GAAACTCCTATGTCCTCAACA-3'	
CgPDR1seqA2	F, 5'-AAACTGATGAAAGAGACCT-3'	
CgPDR1seqA3	F, 5'-ACATCTGTTGCTTCTTCTTGA-3'	
CgPDR1seqA4	F, 5'-TAAAGAAGATACAGCAGCAGC-3'	
CgPDR1seqA5	F, 5'-TCTACCTCCGATGCTTCTA-3'	
CgPDR1seqB1	R, 5'-TACAGGAAGGCATTGACTTG-3'	
CgPDR1seqB2	R, 5'-TCTGCCATATTGATTAGCCT-3'	
CgPDR1seqB3	R, 5'-TTCAAAACCATAGTATTCGTCG-3'	
CgPDR1seqB4	R, 5'-TGGCTCATAAAGGTGAACAC-3'	
CgPDR1seqB5	R, 5'-CAGAAAGACCTCTATGGCAT-3'	

^aF, Forward; R, Reverse^bRestriction sites introduced into primers are underlined.

cloning kit (Invitrogen) as per manufacturer's instructions. The resulting plasmid DNA was digested at the introduced *KpnI* and *ApaI* sites and cloned upstream of the SAT1 flipper cassette in the corresponding *KpnI* and *ApaI* sites of plasmid pPDR1ABS2 to generate pPDR1SM1S2 (containing the *CgPDR1* gene from isolate SM1), pPDR1SM3S2 (containing the *CgPDR1* gene from isolate SM3), and pPDR16955S2 (containing the *CgPDR1* gene from isolate 6955).

***PDR1* Gene Disruption and Replacement by the SAT1-Flipper Method**

Gene disruption and replacement was performed via the SAT1-flipper method described by Reuss *et al.* (2004). *C. glabrata* cells were transformed by the lithium acetate method (12) using about 1 µg DNA. Transformants were selected on YPD agar containing 200 µg/ml nourseothricin (Nou₂₀₀) as described previously (176). The correct genomic integration was confirmed by Southern hybridization. Subsequently, correct integrants were grown in YPD for 6-24 hours in order to induce expression of the flipper recombinase (FLP gene). Individual colonies were replica plated on both YPD agar and YPD + Nou₂₀₀ agar plates to select for cells in which successful excision of the SAT1-flipper cassette had taken place; further confirmation of cassette excision was performed by Southern hybridization.

Isolation of Genomic DNA and Southern Hybridization

Genomic DNA from *C. glabrata* was isolated as previously described (134). For confirmation by Southern hybridization, approximately 10 µg of genomic DNA was digested with the appropriate restriction enzymes, separated on a 1% agarose gel containing ethidium bromide, transferred by vacuum blotting onto a nylon membrane and fixed by UV-crosslinking. Hybridization was performed with the Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare; Little Chalfont Buckinghamshire, UK) as per manufacturer's instructions.

Drug Susceptibility Tests

Microdilution broth susceptibility testing was performed in duplicate according to the CLSI M27-A3 method in RPMI 1640 (Sigma) growth medium buffered with MOPS (Sigma). Microtiter trays containing serial dilutions of fluconazole were inoculated and incubated at 37°C, and the minimum inhibitory concentration (MIC) was read at 24 and 48 hours. The MIC was defined as the lowest concentration of drug that causes a significant decrease in turbidity (> 50%) compared to growth control. *Candida krusei* ATCC 6258 served as the quality control isolate.

Culture Growth for Microarray Experiments

For microarray performed on clinical isolates, one colony was grown overnight in YPD. Cells were then grown to mid-log phase ($OD_{600} = 0.6-1.0$) in 200 ml YPD and were harvested by centrifugation at $3,500 \times g$ at 4°C for 5 minutes. The medium was completely removed by aspiration and the cell pellets were stored at -80°C until RNA preparation.

RNA Preparation for Microarrays

Total RNA was isolated using the hot phenol method (190). Cells were suspended in 12 ml AE buffer (50 mM sodium acetate (pH 5.2), 10 mM EDTA), 800 μl 25% sodium dodecyl sulphate, and 12 ml acid phenol (Fisher Scientific) was added. This mixture was incubated for 10 min at 65°C , vortexing every minute, stored on ice for 5 min, and subjected to centrifugation for 15 minutes at $12,000 \times g$. Supernatants were transferred to new tubes containing 15 ml chloroform, mixed, and subjected to centrifugation for 10 minutes at $200 \times g$. The aqueous layer was transferred to a new tube containing 0.1 volume 2M sodium acetate and 1X volume room temperature isopropanol and mixed. Total RNA was then collected by centrifugation at $17,000 \times g$ for 40 minutes at which point the supernatant was discarded. The RNA pellet was washed with 5 ml 70% ethanol, and the resulting RNA was suspended in diethyl pyrocarbonate (DEPC)-treated water.

Microarray cRNA Synthesis and Hybridization

C. glabrata microarrays were constructed as described previously (215). These custom Affymetrix NimbleExpress Arrays were manufactured by NimbleGen Systems per our specification (4). Two independent sets of RNA were used in these studies to prepare two independent cDNA samples. The purity and concentration of the RNA was examined by capillary electrophoresis using the RNA 6000 Nano laboratory-on-a-Chip kit and Bioanalyzer 2100 (Agilent Technologies; Waldbronn, Germany) as per manufacturer's instructions. Fifteen μg total RNA was used for first- and second-strand cDNA synthesis using the SuperScript Double-Stranded cDNA synthesis kit (Invitrogen) and an oligo-dT24-T7 primer (Sigma) according to the manufacturer's instructions. Using the T7 promoter-coupled double stranded cDNA as a template and the Bioarray High Yield RNA Transcript labeling kit (ENZO Diagnostics; Farmingdale, NY), cRNA was synthesized and labeled with biotinylated-UTP and -CTP by in vitro transcription. The synthesized double stranded cDNA was washed twice with 70% ethanol and suspended in 22 μl of DEPC-treated water. Reaction buffer, biotin-labeled ribonucleotides, dithiothreitol, RNase inhibitor mix and T7 RNA polymerase were added to the cDNA and incubated for 5 hours at 37°C . The labeled cRNA was purified using a CHROMA SPIN-100 column (Clontech; Mountain View, CA) and precipitated with ethanol overnight at -20°C .

After the cRNA pellet was suspended in 10 μ l of RNase-free water, 10 μ g was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 100 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, 150 mM magnesium acetate. Using the Affymetrix Fluidics Station 450 and its standard EukGEWS2v5 protocol, the fragmented cRNA was hybridized to the *C. glabrata* NimbleExpress GeneChip arrays for 16 hours at 45°C and then washed at 25°C with 6X SSPE (1X SSPE: 0.18M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA (pH 7.7)) + 0.01% Tween-20 followed by a stringent wash at 50°C with 100 mM MES (2-(N-morpholino)ethanesulfonic acid), 0.1 M NaCl, 0.01% Tween-20. The arrays were stained with phycoerythrin-conjugated streptavidin (Molecular Probes; Eugene, OR) and the fluorescence intensities were determined using the GCS 3000 high-resolution confocal laser scanner (Affymetrix, Santa Clara, CA). These images were analyzed using software resident in GeneChip Operating System v2.0 (GCOS; Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity of 250. The signal intensity for each gene was calculated as the average intensity difference, represented by $[\Sigma(\text{PM} - \text{MM})/\text{number of probe pairs}]$, where PM and MM denote perfect-match and mismatch probes.

Microarray Data Analysis

The scaled gene expression values from GCOS software were processed and analyzed using GeneSpring 7.2 software (Agilent Technologies). Probe sets were removed from analysis if they were called absent by the Affymetrix criterion and displayed an absolute value below 20 in all experiments. The expression value of each gene was normalized to the median expression of all genes in each chip as well as the median expression of that gene across all chips in the study. Pairwise comparison of gene expression was performed for each matched experiment.

Quantitative Real-Time RT-PCR

An aliquot of RNA samples used in the microarray experiments was saved for follow-up quantitative RT-PCR studies along with a set of independently-obtained RNA samples from the same strains analyzed by microarray. First strand cDNAs were synthesized from 2 μ g total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer's instructions. All quantitative real-time PCR experiments were performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, Inc.; Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and 18S rRNA using Primer Express software (Applied Biosystems). The PCR conditions were as follows: AmpliTaq Gold activation at 95°C for 10 minutes, 40 cycles of a combination of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each cycle to verify that a single product was amplified using the software provided with the 7000 Sequence

Detection System. The system software monitored the change in fluorescence of SYBR Green I dye in every cycle, and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain ΔC_T value. The ΔC_T of an arbitrary calibrator (e.g. fluconazole-susceptible isolate or untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$. Statistical analysis was performed using Microsoft Office Excel 2007. Relative changes were compared using the Student's t-test. The statistical significance threshold was fixed at α -0.05.

Sequence Analysis of *C. glabrata* *CgPDR1*

PDR1 coding sequences were amplified by PCR (*Pfu* DNA polymerase; Stratagene) of *C. glabrata* genomic DNA using the primers PDR1seqF and PDR1seqR (Table 3-2). Products were cloned into pCR-BLUNTII-TOPO® using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen) and transferred into *E. coli* TOP10 cells with selection on LB agar plates containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAprep®; Qiagen; Germantown, MD) and sequenced on the ABI Model 3130XL Genetic Analyzer using the universal primers M13R and T7 and a set of ten primers (Table 3-2) that span both strands of the *PDR1* coding sequence. The sequencing was performed using three sets of clones derived from three independent PCRs for each strain/isolate sequenced.

RESULTS

Genes Coregulated with *CgCDR1* among Matched Sets of Clinical Isolates

As over-expression of *CgPDR1* and several efflux pump genes regulated by Pdr1p (*CgPDR1*, *CgCDR1*, *CgPDH1*, and *CgSNQ2*) have previously been shown to be a mechanism of high-level azole resistance (21, 185, 210, 215), we first examined expression of these genes by real-time RT-PCR in four azole-resistant *C. glabrata* isolates as compared to their azole-susceptible parents. Genes were considered to be differently expressed if the expression of that gene changed by at least ≥ 1.5 in the resistant isolate when compared to the matched susceptible isolate in three independent experiments. We observed distinctly different expression patterns for *CgPDR1*, *CgCDR1*, *CgPDH1*, and *CgSNQ2* in each of the azole-resistant isolates we examined (Figure 3-1). For example, *CgPDR1* and *CgPDH1* were upregulated in isolates SM3, 62-17, 7755, but not 6955, *CgCDR1* was upregulated in all four of the clinical azole-resistant isolates, and *CgSNQ2* was only upregulated in isolate SM3.

Because *CgCDR1* was upregulated in all four azole-resistant isolates, we analyzed the genome-wide gene expression profiles of each isolate pair using microarray analysis in order to identify genes co-regulated with this transporter gene. Two independent RNA

Figure 3-1. Quantitative real-time RT-PCR analysis of *CgPDR1*, *CgCDR1*, *CgPDH1*, and *CgSNQ2* in SM3 versus SM1 (Set 1), 62-17 versus 62-16 (Set 2), 7755 versus 7754 (Set 3), and 6955 versus 6856 (Set 4). Data are shown as mean $\pm$ standard error. Relative changes were compared using the Student's t-test. Gene expression values with an asterisk indicate statistical significance ($P < 0.05$) in all three experiments.

samples per isolate were hybridized to custom *C. glabrata* Affymetrix microarrays and analyzed as detailed in the Materials and Methods section. Genes were considered to be differentially expressed if the expression of that gene changed by at least ≥ 1.5 in the resistant isolate when compared to the matched susceptible isolate in both independent experiments. There were 495 upregulated genes and 477 down-regulated genes in isolate SM3 compared to isolate SM1, 463 upregulated genes and 386 down-regulated genes in isolate 6955 compared to isolate 6856, 599 upregulated genes and 592 down-regulated genes in isolate 7755 compared to isolate 7754, and 181 upregulated genes and 152 down-regulated genes in isolate 62-17 compared to isolate 62-16 (Figure 3-2). Genes that were upregulated and down-regulated in at least three of the four clinical isolate sets are listed in Tables 3-3 and 3-4, respectively.

Of all the genes identified in the gene expression profiles for these four matched pairs, there were nine genes that were commonly coordinately upregulated with *CgCDR1*: *YOR1*, *LCB5*, *RTA1*, *YIM1*, *YIL077c*, *POG1*, *HFD1*, *GLK1*, and *FMS1*. One of these genes encodes the homolog of *S. cerevisiae* Yor1p, which like *CgCDR1* is a plasma membrane ABC transporter. Another encodes the homolog of *S. cerevisiae* Lcb5p which is a minor sphingoid long-chain base kinase involved in synthesis of long-chain base phosphates which function as signaling molecules (144). *RTA1*, *YIM1*, and *YIL077c* are homologs of *S. cerevisiae* genes and encode molecules involved in stress response. For example, the *S. cerevisiae* homolog of *RTA1* is involved in 7-aminosterol resistance (201). In addition to the aforementioned upregulated genes, there were three down-regulated genes common to all four sets of clinical isolates. These include the uncharacterized gene *CAGL0A03608g* and homologs of *S. cerevisiae* *SNG1* and *SUR7*.

There were 43 genes upregulated in at least three of the clinical matched sets of isolates. Among these genes were *CgPDR1* and the ABC transporter, *CgPDH1*, both consistent with our real-time RT-PCR results. In addition to *CgPDH1*, there were two other upregulated genes encoding transporters, namely, *YBT1* and *QDR2*. Also, upregulated in this set were *RPN4* and *YMR102c*, genes whose *S. cerevisiae* homologs were previously shown to be ScPdr1p and ScPdr3p targets (8, 157). Other notable genes upregulated in at least three sets included *MNT3* and *MKC7*, whose *S. cerevisiae* homologs are involved in cell wall function, and *YMR102c* and *YML131w*, each encoding putative alcohol dehydrogenases involved in stress responses. There were 16 genes down-regulated in at least three of the matched sets; among these are genes encoding the major facilitator transporters *HXT3* and *FLR1*, genes encoding cell wall organization molecules *UTR1* and *ZEO1*, the cytochrome c oxidase gene *COX12*, and the iron-regulating gene *FRA2*.

Identification of *PDR1* Mutations in Azole Resistant Clinical Isolates

The findings that the expression of *CgCDR1* and *CgPDH1* as well as homologs of other known *S. cerevisiae* Pdr1p and Pdr3p targets were increased in many of the fluconazole-resistant clinical isolates suggested that the resistant isolate in each set may contain *CgPDR1* gain-of-function mutations. We therefore sequenced the *CgPDR1* gene

A

B

Figure 3-2. Venn Diagram showing the number of genes that are upregulated (A) or down-regulated (B) in the fluconazole-resistant isolates SM3, 62-17, 7755, and 6955 as compared to their matched fluconazole-susceptible isolates SM1, 62-16, 7754, and 6856, respectively.

Table 3-3. Genes upregulated in all four of the clinical matched isolate sets (A) and in at least three of the clinical matched sets (B, C, D, and E).

Letter designation ^c	Systematic name	<i>S. cerevisiae</i> homolog ^a	<i>C. glabrata</i> designation	Description ^b
A	IPF1617	<i>POG1</i>	CAGL0G08646g	Putative transcriptional activator
A	IPF2180	<i>HFD1</i>	CAGL0K03509g	Putative fatty aldehyde dehydrogenase
A	IPF3783	<i>GLK1</i>	CAGL0F00605g	Glucokinase
A	IPF5152	<i>RTA1</i>	CAGL0K00715g	Protein involved in 7-amincholesterol resistance
A	IPF6116	<i>YIL077c</i>	CAGL0M12947g	Putative protein of unknown function
A	IPF6352	<i>PDR5 (CDR1)</i>	CAGL0M01760g	Plasma membrane ATP-binding cassette (ABC) transporter
A	IPF8922	<i>YOR1</i>	CAGL0G00242g	Plasma membrane ATP-binding cassette (ABC) transporter
A	IPF9036	<i>YIM1</i>	CAGL0M09713g	Protein of unknown function
A	IPF9610	<i>FMS1</i>	CAGL0M07612g	Polyamine oxidase
A	IPF8678	<i>LCB5</i>	CAGL0K05995g	Minor sphingoid long-chain base kinase
B	IPF2441	<i>IMH1</i>	CAGL0E03454g	Protein involved in vesicular transport
B	IPF2464	<i>ECM29</i>	CAGL0E03828g	Major component of the proteasome
B	IPF3052	<i>YCR061w</i>	CAGL0K09218g	Protein of unknown function
B	IPF5973	<i>FLO10 (EPA1)</i>	CAGL0E06644g	Lectin-like protein with similarity to Flo1p, thought to be involved in flocculation
B	IPF7416	<i>RSE1</i>	CAGL0L01507g	Protein involved in pre-mRNA splicing
B	IPF926	<i>MNT3</i>	CAGL0C03916g	Alpha-1,3-mannosyltransferase
B	IPF3655	<i>YLR177w</i>	CAGL0B01078g	Putative protein of unknown function
B	IPF8402	<i>MKC7</i>	CAGL0E01837g	GPI-anchored aspartyl protease (yapsin) involved in protein processing
C	IPF8399	<i>YAP3</i>	CAGL0E01793g	Aspartic protease
D	IPF1455	<i>MAG1</i>	CAGL0I06809g	3-methyl-adenine DNA glycosylase
D	IPF4638	<i>CAK1</i>	CAGL0K12496g	Cyclin-dependent kinase-activating kinase
D	IPF8257	<i>YPT52</i>	CAGL0G07689g	GTPase
D	IPF4699	<i>DOG2</i>	CAGL0D04092g	2-deoxyglucose-6-phosphate phosphatase
D	IPF6780	<i>YNL305c</i>	CAGL0M03839g	Putative protein of unknown function
E	IPF119	<i>DDR48</i>	CAGL0H08844g	DNA damage-responsive protein
E	IPF1620	<i>QDR2</i>	CAGL0G08624g	Multidrug transporter of the major facilitator superfamily
E	IPF1748	<i>YML018c</i>	CAGL0F03641g	Putative protein of unknown function

Table 3-3 (continued).

Letter designation ^a	Systematic name	<i>S. cerevisiae</i> homolog ^b	<i>C. glabrata</i> designation	Description ^c
E	IPF2196	<i>YMR102c</i>	CAGL0K03377g	Protein of unknown function
E	IPF2382	<i>YNL234w</i>	CAGL0J07502g	Similar to globins and has a functional heme-binding domain
E	IPF2392	<i>ZWF1</i>	CAGL0J07612g	Glucose-6-phosphate dehydrogenase (G6PD)
E	IPF3019	<i>N/A</i>	CAGL0A01650g	Uncharacterized
E	IPF3173	<i>YNL134c</i>	CAGL0K09702g	Putative protein of unknown function
E	IPF3325	<i>PDR1</i>	CAGL0A00451g	Zinc-cluster transcription factor
E	IPF3846	<i>NCE3</i>	CAGL0G01540g	Carbonic anhydrase
E	IPF3875	<i>N/A</i>	CAGL0G01122g	Uncharacterized
E	IPF4064	<i>AIM6</i>	CAGL0C05533g	Putative protein of unknown function
E	IPF4550	<i>UTH1</i>	CAGL0L05434g	Mitochondrial outer membrane and cell wall localized SUN family member
E	IPF4605	<i>YML131w</i>	CAGL0K12958g	Putative protein of unknown function
E	IPF4884	<i>ATF2</i>	CAGL0D05918g	Alcohol acetyltransferase
E	IPF5076	<i>RPN4</i>	CAGL0K01727g	Transcription factor that stimulates expression of proteasome genes
E	IPF6034	<i>ADH6</i>	CAGL0M14047g	NADPH-dependent medium chain alcohol dehydrogenase
E	IPF6722	<i>MET2</i>	CAGL0J08316g	L-homoserine-O-acetyltransferase
E	IPF734	<i>MET3</i>	CAGL0B03839g	ATP sulfurylase
E	IPF7414	<i>EFF2</i>	CAGL0L01485g	ER localized integral membrane protein
E	IPF7952	<i>YNL144c</i>	CAGL0J05830g	Putative protein of unknown function
E	IPF7953	<i>IRC24</i>	CAGL0J05852g	Putative benzil reductase
E	IPF8009	<i>YJL163c</i>	CAGL0M08426g	Putative protein of unknown function
E	IPF9549	<i>FLO1</i>	CAGL0E00209g	Lectin-like protein involved in flocculation
E	IPF9719	<i>PDR15 (PDH1)</i>	CAGL0F02717g	Plasma membrane ABC transporter
E	IPF982	<i>YBT1</i>	CAGL0C03289g	Transporter of the ABC family involved in bile acid transport
E	IPF558	<i>EAR1</i>	CAGL0F07799g	Specificity factor required for Rsp5p-dependent ubiquitination
E	IPF6631	<i>CPS1</i>	CAGL0C00319g	Vacuolar carboxypeptidase yscS
E	IPF6862	<i>WTM2</i>	CAGL0J03960g	Transcriptional modulator

^aLetters correspond to Figure 3-2A.

^bParentheses indicate a previously named *C. glabrata* gene.

^cDescriptions of *S. cerevisiae* homologs (see SGD website <http://www.yeastgenome.org>).

Table 3-4. Genes down-regulated in all four of the clinical matched isolate sets (A) and in at least three of the clinical matched sets (C, D, and E).

Letter designation ^a	Systematic name	<i>S. cerevisiae</i> homolog	<i>C. glabrata</i> designation	Description ^b
A	IPF1222	N/A	CAGL0A03608g	Uncharacterized
A	IPF1568	SNG1	CAGL0G09273g	Protein involved in nitrosoguanidine (MNNG) resistance
A	IPF7418	SUR7	CAGL0L01551g	Putative integral membrane protein
C	IPF14492	N/A	CAGL0I00550g	Uncharacterized
D	IPF1070	UTR2	CAGL0C02211g	Cell wall protein that functions in the transfer of chitin to beta(1-6)glucan
D	IPF14989	COX12	CAGL0A03542g	Subunit VIb of cytochrome c oxidase
D	IPF1884	CIT1	CAGL0H03993g	Citrate synthase
D	IPF2023	NDH1	CAGL0I00748g	Mitochondrial external NADH dehydrogenase
D	IPF2970	HXT3	CAGL0A02321g	Low affinity glucose transporter of the major facilitator superfamily
D	IPF3326	PUF4	CAGL0A00473g	Member of the PUF protein family
D	IPF4392	FLR1	CAGL0H06017g	Plasma membrane multidrug transporter of the major facilitator superfamily
D	IPF453	FET3	CAGL0F06413g	Ferro-O ₂ -oxidoreductase
D	IPF8122	PNS1	CAGL0L04378g	Protein of unknown function
D	IPF126	PUF2	CAGL0H08778g	Member of the PUF protein family
D	IPF3165	SHE2	CAGL0K07612g	RNA-binding protein that binds specific mRNAs and interacts with She3p
E	IPF14693	FRA2	CAGL0I06985g	Protein involved in negative regulation of transcription of iron regulon
E	IPF3506	FMP45	CAGL0G05566g	Integral membrane protein localized to mitochondria (untagged protein)
E	IPF9174	ZEO1	CAGL0E02255g	Peripheral membrane protein of the plasma membrane that interacts with Mid2p
E	IPF9228	BUD22	CAGL0M07678g	Protein involved in bud-site selection

^aLetters correspond to Figure 3-2B.

^bDescriptions of *S. cerevisiae* homologs (see SGD website <http://www.yeastgenome.org>).

from each of the susceptible and resistant isolates. Of the four resistant isolates, two contained nucleotide changes in *CgPDR1* as compared to their corresponding susceptible parent isolates. Isolate SM3 contained a T2837C mutation which is predicted to result in the amino acid substitution L946S, located in the putative transcriptional activation domain of Pdr1p as assumed by similarity with ScPdr1p and ScPdr3p (216). Isolate 6955 contained a G822T mutation which is predicted to result in the amino acid substitution K274N, located near the putative inhibitory domain (Figure 3-3). Although no mutation was found in the resistant isolate 62-17, the susceptible isolate 62-16 was found to contain a nucleotide deletion, resulting in a frameshift which would presumably lead to a truncated Pdr1p in this isolate. We also aligned the sequences from all published *CgPDR1* sequences from fluconazole-susceptible isolates and identified two mutations unique to CgPdr1p in 62-16 and 62-17 that resulted in N768D and S888L amino acid substitutions (68, 210, 212). Surprisingly, no *PDR1* mutation was found in isolate 7755 when compared to the susceptible matched isolate 7754.

T2837C and G822T Are Gain-of-Function Mutations in *CgPDR1* and Each Confer Increased Resistance to Fluconazole

Single point mutations in *CgPDR1* in *C. glabrata* have previously been shown to play a role in azole resistance (68, 215). Therefore, to test whether the L946S and K274N substitutions in CgPdr1p are responsible for the increase in fluconazole MIC in isolates SM3 and 6955, respectively, we expressed the mutated *CgPDR1* from SM3 and 6955 in the isolate SM1 background. For this purpose, we deleted *CgPDR1* from isolate SM1 using the SAT1-flipping method strategy to generate strain SM1 Δ *pdr1* (176). The mutated *CgPDR1* alleles, *PDR1*^{T2837C} and *PDR1*^{G822T}, were then introduced individually into the *CgPDR1* locus in strain SM1 Δ *pdr1* to generate strains SM1 Δ *pdr1*/ *PDR1*^{L946S} and SM1 Δ *pdr1*/ *PDR1*^{K274N}. We also created the corresponding control strains by introducing *CgPDR1* alleles from the susceptible isolates SM1 and 6856 to generate strains SM1 Δ *pdr1*/ *PDR1*^{SM1} and SM1 Δ *pdr1*/ *PDR1*⁶⁸⁵⁶. Replacement of the different *CgPDR1* mutations was verified by sequencing the cloned *CgPDR1* ORF PCR product as detailed in the Materials and Methods section.

Introduction of the two *PDR1* mutations into the susceptible background (SM1 Δ *pdr1*) resulted in fluconazole MICs equivalent to those of the strains from which each *CgPDR1* was originally identified (Table 3-5). Specifically, introduction of *PDR1*^{L946S} resulted in a fluconazole MIC of 64 μ g/ml, and introduction of *PDR1*^{K274N} resulted in a fluconazole MIC of 16 μ g/ml; whereas, the fluconazole MICs of SM1 Δ *pdr1*/ *PDR1*^{SM1} and SM1 Δ *pdr1*/ *PDR1*⁶⁸⁵⁶ were measured as 2 μ g/ml and 1 μ g/ml, respectively (Table 3-5). As introduction of the *CgPDR1* mutated alleles in the SM1 isolate background resulted in increased fluconazole MICs as compared to introduction of the alleles from isolates SM1 and 6856 in the same background, we have demonstrated that these *CgPDR1* gain-of-function mutations are responsible for conferring decreased susceptibility to fluconazole in these *C. glabrata* isolates. Expression of the 62-17

Figure 3-3. Location of gain-of-function mutations found in previous studies (63, 197, 199, 202) and in the present study (bolded). Putative DBD (DNA binding domain) and MHR (Middle homology region) were previously found by Pfam analysis (63). Putative AD (transcriptional activation domain) and ID (inhibitory domain) were previously inferred by similarity with Pdr1p and Pdr3p from *S. cerevisiae* (202). The region with diagonal lines corresponds to the xenobiotics binding domain (XBD) (194).

Table 3-5. Fluconazole MICs for each of the revertant strains as compared to the parent strains.

Isolate set	Isolate names	WT MIC (µg/ml)	SM1Δ <i>pdr1</i> / <i>PDR1</i> MIC (µg/ml)
1	SM1	2	
	SM1Δ <i>pdr1</i>	0.5	
	SM3	64	64
2	62-16	0.5	
	62-17	16	16
3	7254	16	
	7255	> 64	N/A
4	6856	1	
	6955	16	16

CgPDR1 allele in the susceptible background also showed an increase in fluconazole resistance suggesting that this also represents a gain-of-function mutation (Table 3-5).

Identification of Putative *CgPDR1* Target Genes

In order to identify putative *CgPDR1* target genes whose expression is affected by these gain-of-function mutations, we compared gene expression profiles of SM1 $\Delta pdr1/PDR1^{T2837C}$ and SM1 $\Delta pdr1/PDR1^{G822T}$ to SM1 $\Delta pdr1/PDR1^{SM1}$ and SM1 $\Delta pdr1/PDR1^{6856}$, respectively. Again, two independent RNA samples per isolate were hybridized to custom *C. glabrata* Affymetrix microarrays and analyzed as detailed in the Methods section. Genes were considered to be differentially expressed if the expression of that gene changed by at least ≥ 1.5 when compared to the strains expressing the wild-type *CgPDR1* allele in the two independent experiments. There were 142 genes were upregulated in the reconstituted strain expressing *PDR1*^{T2837C} and of these genes 39 were also upregulated in SM3 compared to SM1. Thirty-four genes are upregulated in the reconstituted strain expressing *PDR1*^{G822T}, and of these genes only six were also upregulated in 6955 compared to 6856. 626 genes were down-regulated in the transformants expressing *PDR1*^{T2837C}, and of these genes 58 are also down-regulated in SM3 compared to SM1. Seventy-two genes are down-regulated in the transformants expressing *PDR1*^{G822T}, and of these, ten genes are also down-regulated in 6955 compared to 6856. We defined putative core *CgPdr1p* target genes to be the genes that were not only upregulated in the transformants but also in the clinical isolate sets. These are summarized in Tables 3-6 and 3-7. As seen for SM3 compared to SM1, real-time RT-PCR confirmed upregulation of *CgCDR1*, *CgPDH1*, and *CgSNQ2* in SM1 $\Delta pdr1/PDR1^{T2837C}$ compared to SM1 $\Delta pdr1/PDR1^{SM1}$. Also, as expected, real-time RT-PCR confirmed upregulation of *CgCDR1* but not *CgPDH1* and *CgSNQ2* in SM1 $\Delta pdr1/PDR1^{G822T}$ compared to SM1 $\Delta pdr1/PDR1^{6856}$ (Figure 3-4).

In order to determine which genes' promoters contain a putative PDRE, the promoter region (defined for this purpose as 1000 bp immediately upstream from the start codon of the ORF) of each gene listed in Tables 3-6 and 3-7 was examined for a match to the consensus *S. cerevisiae* PDRE. Twenty-two of the 39 genes in Table 3-6 and four of the six genes in Table 3-7 contained at least one consensus sequence.

DISCUSSION

C. glabrata Pdr1p is the single-gene homolog of two *S. cerevisiae* zinc cluster transcription factors ScPdr1p and ScPdr3p, which regulate the expression of genes involved in multidrug resistance. ScPdr1p and ScPdr3p act by binding to PDREs in the promoters of target genes (95). Furthermore, it has been found that ScPdr1p is constitutively bound to its target DNA (65). In *S. cerevisiae*, gain-of-function mutations in Pdr1p and Pdr3p can result in decreased susceptibility to fluconazole by up-regulation of multidrug efflux pumps. In *C. glabrata*, *CgPdr1p* has been shown to regulate to varying degrees the expression of the ABC transporter genes *CgCDR1*, *CgPDH1*, and

Table 3-6. Genes upregulated in SM1 Δ *pdrl*/ *PDR1*^{L946S} compared to SM1 Δ *pdrl*/ *PDR1*^{SM1} as well as SM3 compared to SM1.

Group	<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Expression		PDRE
				SM3 vs. SM1	<i>PDR1</i> ^{SM3} vs. <i>PDR1</i> ^{SM1}	
Transport	CAGL0G08624g	<i>QDR2</i>	MFS transporter	3.5	2.7	848
	CAGL0I04862g	<i>SNQ2</i>	ABC transporter	3.7	5.4	218, 792
	CAGL0M01760g	<i>PDR5</i>	ABC transporter	10.2	8.6	134, 227, 387, 515, 969
	CAGL0G00242g	<i>YOR1</i>	ABC transporter	10.5	5.1	648
	CAGL0J09900g	<i>POR1</i>	Mitochondrial porin	1.6	2.6	-
	CAGL0F02717g	<i>PDR15</i>	ABC transporter	10.5	7.4	521, 557
Transcription	CAGL0C03289g	<i>YBT1</i>	ABC transporter	8.1	3.9	450
	CAGL0A00451g	<i>PDR1</i>	Transcription factor	5.2	3.6	557, 701
	CAGL0L05434g	<i>UTH1</i>	Mitochondrial outer membrane and cell wall protein	2.9	2.3	-
Response to stress	CAGL0K01727g	<i>RPN4</i>	Transcription	3.1	3.4	378, 394, 552
	CAGL0H08844g	<i>DDR48</i>	DNA damage-responsive protein	5.1	1.7	-
	CAGL0I05874g	<i>RHR2</i>	DL-glycerol-3-phosphatase	2.0	1.8	-
	CAGL0J04202g	<i>HSP12</i>	Plasma membrane localized protein	4.1	16.4	849
	CAGL0M09713g	<i>YIM1</i>	Protein of unknown function	11.5	11.8	127, 179
	CAGL0E00209g	<i>FLO1</i>	Lectin-like protein	10.0	1.9	292, 427
	CAGL0D05918g	<i>ATF2</i>	Alcohol acetyltransferase	7.2	6.8	30, 195, 560, 722
	CAGL0K00715g	<i>RTA1</i>	Protein involved in 7-amincholesterol resistance	5.0	3.6	300, 379
	CAGL0M12947g	<i>YIL077c</i>	Putative protein of unknown function	23.5	26.0	472, 502
	CAGL0I10120g	<i>AGA1</i>	Anchorage subunit of a-agglutinin of a-cells	4.7	1.8	-
Lipid metabolism	CAGL0H04653g	<i>ERG6</i>	Delta(24)-sterol C-methyltransferase	1.6	2.1	-
	CAGL0E04334g	<i>ERG11</i>	Lanosterol 14-alpha-demethylase	1.9	1.9	-
	CAGL0J11770g	<i>PLB1</i>	Phospholipase B	2.3	2.8	754
	CAGL0A00429g	<i>ERG4</i>	C-24(28) sterol reductase	2.0	1.8	-
	CAGL0F01793g	<i>ERG3</i>	C-5 sterol desaturase	1.9	3.6	-
	CAGL0I02970g	<i>ERG24</i>	C-14 sterol reductase	1.9	2.0	226
	CAGL0M10219g	<i>LAC1</i>	Ceramide synthase component	2.3	3.1	531
	CAGL0K12100g	<i>HEM13</i>	Coproporphyrinogen III oxidase	2.9	1.9	-
Heterocycle metabolism	CAGL0M14047g	<i>ADH6</i>	NADPH-dependent medium chain alcohol dehydrogenase	3.0	5.4	-
Other	CAGL0K03509g	<i>HFD1</i>	Putative fatty aldehyde dehydrogenase	4.4	6.2	218
	CAGL0K10164g	<i>SED1</i>	Major stress-induced structural GPI-cell wall glycoprotein	2.6	1.9	1046
	CAGL0M07942g	<i>FRE8</i>	Protein with sequence similarity to iron/copper reductases	2.0	1.6	-

Table 3-6 (continued).

Group	<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Expression		PDRE
				SM3 vs. SM1	<i>PDR1</i> ^{SM3} vs. <i>PDR1</i> ^{SM1}	
Biological process unknown	CAGL0K09702g	<i>YNL134c</i>	Putative protein of unknown function	13.0	7.6	549
	CAGL0G02717g	<i>SGA1</i>	uncharacterized	2.5	2.6	-
	CAGL0F01287g	<i>GAS5</i>	1,3-beta-glucanosyltransferase	1.7	5.8	-
	CAGL0G01122g	<i>N/A</i>	uncharacterized	4.2	3.9	803, 793
	CAGL0M08426g	<i>YJL163c</i>	Putative protein of unknown function	9.1	3.3	450
	CAGL0G07667g	<i>PRY1</i>	Protein of unknown function	2.2	2.2	-
	CAGL0D06402g	<i>MET25</i>	Methionine and cysteine synthase	4.9	1.7	-
	CAGL0A04906g	<i>STA1</i>	GPI-anchored cell surface glycoprotein (flocculin)	25.9	11.7	-

Table 3-7. Genes upregulated in SM1 Δ *pdrl*/ *PDR1*^{L946S} compared to SM1 Δ *pdrl*/ *PDR1*⁶⁸⁵⁶ as well as 6955 compared to 6856.

Group	<i>S. cerevisiae</i> homolog	<i>C. glabrata</i> designation	Description	Expression		PDRE
				6955 vs. 6856	<i>PDR1</i> ⁶⁹⁵⁵ vs. <i>PDR1</i> ⁶⁸⁵⁶	
Transport	<i>PDR5</i>	CAGL0M01760g	Plasma membrane ABC transporter	2.1	6.8	969, 515, 387, 227, 134
	<i>YOR1</i>	CAGL0G00242g	ABC transporter	1.8	2.4	648
Response to stress	<i>YIM1</i>	CAGL0M09713g	Protein of unknown function	2.8	3.0	179, 127
RNA metabolism	<i>PRP39</i>	CAGL0L01441g	U1 snRNP protein involved in splicing	1.7	1.6	-
	<i>FLO10</i>	CAGL0E06644g	Lectin-like protein	1.9	3.3	571
Other	<i>FLO5</i>	CAGL0M14069g	Lectin-like cell wall protein (flocculin)	2.1	1.6	-

Figure 3-4. Quantitative real-time RT-PCR analysis of *CgPDR1*, *CgCDR1*, *CgPDH1*, and *CgSNQ2* in *SM1Δpdr1/ PDR1^{T2837C}* compared to *SM1Δpdr1/ PDR1^{SM1}* (Set 1), *SM1Δpdr1/ PDR1⁶²⁻¹⁷* compared to *SM1Δpdr1/ PDR1⁶²⁻¹⁶* (Set 2), *SM1Δpdr1/ PDR1^{G822T}* compared to *SM1Δpdr1/ PDR1⁶⁸⁵⁶* (Set 4). Data are shown as mean +/- standard error. Relative changes were compared using the Student's t-test. Gene expression values with an asterisk indicate statistical significance ($P < 0.05$) in all three experiments.

CgSNQ2 and has been shown to be responsible for azole resistance in this organism. Recently, strains with *CgPdr1p* gain-of-function mutations were shown to not only result in fluconazole treatment failure in a mouse model but also to result in increased virulence in mice as compared to isolates with wild type alleles (68).

Previously, we examined the gene expression profile of a set of fluconazole-susceptible and laboratory-derived, fluconazole-resistant *C. glabrata* strains (215). In that analysis, we identified genes co-regulated with *CgCDR1* in the F15 fluconazole-resistant strain which carries a *CgPdr1p* allele with a P927L amino acid substitution. In the present study we delineated core genes of the *Pdr1p* regulon by first using genome-wide gene expression profiling to identify genes co-regulated with *CgCDR1* among four clinical matched fluconazole-susceptible and –resistant *C. glabrata* isolate pairs. We identified nine genes co-regulated with *CgCDR1* in all four of the matched sets of clinical isolates. Of these nine genes, six of these, *HFD1*, *RTA1*, *YIL077c*, *LCB5*, *YOR1*, and *YIM1*, were also co-regulated with *CgCDR1* in the laboratory-derived, fluconazole-resistant *C. glabrata* strain. Likewise, of the three genes found to be down-regulated in all four of the matched sets of isolates in this study, only one, *SNG1*, was found to be down-regulated in strain F15. Also in this study we identified 43 genes upregulated in at least three sets of clinical isolates. Of these, 13 genes, *YLR177w*, *YPT52*, *PDR1*, *FLO1*, *YMR102c*, *RPN4*, *QDR2*, *YBT1*, *ATF2*, *CgPDH1*, *YNL134c*, *YJL163c*, and *NCE3* were also upregulated in strain F15. Likewise, of the 16 genes down-regulated in at least three sets, only one, *FLR1*, was also down-regulated in strain F15. These findings suggest a broad repertoire of genes regulated by *Pdr1p*, but with subsets of these genes being differentially regulated possibly depending on how *Pdr1p* is activated.

Two novel gain-of-function mutations in *CgPdr1p* have been described in this study. Not only do these mutated *CgPDR1* alleles cause up-regulation of *CgCDR1* when expressed in a susceptible wild-type isolate, but they also result in the same fluconazole MIC as the original isolate from which the mutated allele was identified. In previous studies, 12 distinct experimentally confirmed activating *CgPdr1p* mutations from fluconazole-resistant isolates have collectively been identified that lead to constitutive up-regulation of ABC transporter genes as well as decreased susceptibility to fluconazole (68, 210, 212, 216). Three of these mutations were found in the putative inhibitory domain, four were found in the xenobiotic binding domain, and five were found in the putative activation domain of *CgPdr1p*. Interestingly, when examined collectively, the gain-of-function mutations identified in previous studies and the present study demonstrate varying patterns of ABC transporter expression. For example, in one of the previous studies, one gain-of-function mutation led to the over-expression of *SNQ2* but not *CgCDR1* and *CgPDH1* (210). In our study, we identified mutations resulting in an amino acid substitution near the putative inhibitory domain (K274N) and in an amino acid substitution in the putative activation domain (L946S) of *Pdr1p* (68, 216). The K274N amino acid substitution resulted in up-regulation of *CgCDR1*, *CgPDH1*, and *CgSNQ2* while the L946S amino acid substitution resulted in up-regulation of only *CgCDR1*. These findings suggest that different amino acid changes in *CgPdr1p* may each result in the activation of a unique subset of genes. Like *Pdr1p* in *S. cerevisiae*, *CgPdr1p* may bind constitutively to the promoters of a repertoire of genes and activate a subset of

them based on the mechanism by which Pdr1p is activated (65). Gain-of-function mutations in the activation domain may result in constitutive activation of CgPdr1p, while mutations in the inhibitory domain may block inhibitory proteins from binding thus allowing transcription of *CgPDR1*. Another possibility for constitutive activation of CgPdr1p is gain-of-function mutations in the xenobiotic binding domain. It has been shown that Pdr1p in *S. cerevisiae* and *C. glabrata* bind directly to ketoconazole resulting in expression of ABC transporters. In the aforementioned studies, four mutations have been identified in this domain; these gain-of-function mutations may allow for constitutive activation of CgPdr1p without the binding of xenobiotics to this domain (68, 212).

Using genome-wide analysis of these different mutations in the same background strain, we were able to identify a core set of Pdr1p activated genes that are co-regulated with *CgCDR1*. *YOR1*, *YIM1*, *CgPDR1*, and *YNL134c* were all co-regulated with *CgCDR1* specifically in the presence of both of these two mutations. In *S. cerevisiae*, *YOR1* encodes an ABC transporter that is involved in oligomycin resistance (96), and *YIM1* and *YNL134c* encode proteins of unknown function; however, both genes may play a role in the stress response to DNA damaging drugs (22, 115). All five of these genes contain at least one PDRE in their promoters suggesting CgPdr1p may regulate their expression through binding these regulatory elements. Of note, both *YOR1* and *YIM1* were also upregulated in all four of the matched sets of clinical isolates. Further studies are needed to determine if these genes contribute to fluconazole resistance.

Table 3-6 summarizes the genes found to be both upregulated due to the L946S amino acid substitution and genes found to be upregulated in the resistant isolate SM3 when compared to the susceptible isolate SM1. We have defined these genes as the core Pdr1p putative target genes activated in this resistant isolate. Interestingly, there were several ABC transporters co-regulated with *CgCDR1* by Pdr1p, including *CgSNQ2*, *CgPDH1*, *YOR1*, and *YBT1*. Also, upregulated was the MFS transporter, *QDR2*. All of these transporters contain at least one PDRE in their promoter region. The role of CgCdr1p, CgPdh1p, and CgSnq2p in azole resistance has been established in *C. glabrata* (90, 210, 215). In the first of these studies, disruption of *CgPDR1* in a resistant *C. glabrata* isolate resulted in fluconazole hypersusceptibility with an MIC of 2 µg/ml, whereas the susceptible isolate had an MIC 16 µg/ml; however, disruption of *CgCDR1* in this same strain resulted in an MIC of 16 µg/ml (215). This suggested CgPdr1p targets other than *CgCDR1* play a role in the fluconazole resistance. Indeed, disruption of *CgSNQ2* in a fluconazole-resistant clinical isolate that does not over-express *CgCDR1* or *CgPDH1* decreased azole resistance (210). Furthermore, disruption of *CgPDH1* in a fluconazole resistant strain had no effect on susceptibility to fluconazole; however, restoring *CgPDH1* expression in a mutant disrupted for both *CgCDR1* and *CgPDH1* decreased susceptibility to fluconazole as compared to the double disruptant. The role of *YBT1* and *QDR2* in fluconazole resistance has yet to be studied in *C. glabrata*.

In *S. cerevisiae*, Upc2p is a zinc-cluster protein known to not only regulate expression of ergosterol biosynthesis genes, including the target of the azoles encoded by the gene *ERG11*, but also genes encoding proteins involved in exogenous sterol uptake, cell wall mannoproteins (DAN/TIR genes), as well Upc2p itself (224). Interestingly,

UPC2 as well as its known *S. cerevisiae* homolog target genes, *ERG11*, *ERG3*, *AUS1*, *DAN4*, *TIR3*, and *TIR4* are upregulated in SM3 (1, 217, 224). Also upregulated in isolate SM3 are the zinc-cluster protein-encoding genes *HAP1* and *STB5*. In *S. cerevisiae*, *HAP1* encodes a transcription factor that regulates expression of the *ERG11* gene, and it has been postulated that this may occur by an interaction with Upc2p (213, 217). In *S. cerevisiae*, *STB5* also encodes a transcription factor that has been identified as a regulator of ergosterol biosynthesis as it is a direct activator of *ERG11* in the presence of oxidative stress (114). Based on the hypothesis that mutations in one of these transcription factors may be responsible for the over-expression of *ERG11* in SM3, we sequenced both *UPC2* *S. cerevisiae* homologs (CAGL0C01199g and CAGL0G07018g), *ERG11* (CAGL0E04334g), *HAP1* (CAGL0K05841g), and *STB5* (CAGL0I02552g) from isolates SM1 and SM3. However, no mutations were found in these genes in SM3 as compared to SM1 (data not shown). Surprisingly, several genes encoding proteins involved in the ergosterol biosynthetic pathway were also found to be CgPdr1p-dependent unique to this gain-of-function mutation including *ERG11* and four enzymes downstream from Erg11p in this biosynthetic pathway encoded by the genes *ERG24*, *ERG6*, *ERG3*, and *ERG4*. Of these only *ERG24* contains a PDRE in its promoter region. Further study will be needed to determine if *ERG11* is directly or indirectly regulated by this hyperactive *CgPDR1* allele.

Other genes found to be Pdr1p-dependent unique to this gain-of-function mutation included the *S. cerevisiae* homolog of the transcription factor *RPN4* which also contains two PDREs in its promoter. In *S. cerevisiae*, *RPN4* is a transcription factor whose expression is dependent on ScPdr1p and ScPdr3p, that stimulates expression of proteasome genes, and is required for normal cell tolerance to various cytotoxic compounds including cycloheximide, a known ABC transporter substrate (157). Together these findings suggest the proteasome may be influenced by Pdr1p via *RPN4* expression; however, its role in azole resistance has not been elucidated. Lastly, other genes found to be CgPdr1p-dependent unique to this gain-of-function mutation included the homologs of the *S. cerevisiae* genes that encode proteins involved in stress response such as *DDR48*, *HSP12*, *YIM1*, *FLO1*, *ATF2*, and *RTA1*. As with *YIM1*, *RTA1* is also upregulated in all four of the matched sets of clinical isolates. In *S. cerevisiae*, *RTA1*, which is also known to be regulated by ScPdr1p and ScPdr3p, encodes a protein member of the fungal lipid-translocating exporter family and is involved in 7-aminosterol resistance; however, the role of *RTA1* in azole resistance has not been studied in *S. cerevisiae* or *C. glabrata*. In *C. albicans*, over-expression of *RTA2*, which encodes a *S. cerevisiae* homolog of *RTA1*, in a *C. albicans* strain with deletions of *CDR1*, *CDR2*, and *MDR1* conferred increased resistance to azoles suggesting a role for this protein family in azole resistance (92).

Recently, it was shown that *C. glabrata* strains expressing hyperactive *CgPDR1* alleles were more virulent in mice than the *C. glabrata* strains expressing wild-type alleles (68). Our study has also allowed for the identification of CgPdr1p targets that may play a role in virulence. Of note, *PLB1*, the homolog of the *S. cerevisiae* gene that encodes phospholipase B, is not only upregulated in the resistant isolate SM3 but also was found to be CgPdr1p-dependent. Moreover, *PLB1* contains a PDRE in its promoter.

In *C. albicans*, phospholipase B activity is involved in the spread of the organism from the gastrointestinal tract as well as in hematogenous dissemination (57, 142). To date, no published studies have analyzed the role of *PLB1* in *C. glabrata*; however, one unpublished study did find an association between phospholipase B activity and persistent *C. glabrata* candidemia (74).

Consistent with the idea that adhesion of *C. glabrata* to host epithelial tissue plays an important role in virulence, the genes *FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11*, were found to be Pdr1p dependent. The *C. glabrata* *FLO* genes are homologs of *S. cerevisiae* genes encoding proteins involved in cell adhesion (80). Only *FLO1* and *FLO10* contain PDREs in their promoters. In *S. cerevisiae*, *FLO1*, *FLO5*, *FLO9*, and *FLO10* are all involved in cell-cell adhesion (flocculation), while *FLO11* is involved in adhesion to substrates. Although *FLO10* in *S. cerevisiae* is involved in flocculation, in *C. glabrata* this gene (named *CgEPA1*) is required for efficient in vitro adherence to human epithelial cells proving a role for *CgEPA1* in adherence to abiotic materials (40). *FLO5* and *FLO9* (*CgEPA13*) have been reported as members of the EPA family of adhesion proteins in *C. glabrata*; however, their role in adherence has not been directly studied. Moreover, the SM3 *CgPDR1* hyperactive allele causes up-regulation of *FLO1*, *FLO9*, and *FLO11*, and the 6955 *CgPDR1* hyperactive allele causes up-regulation of *FLO5* and *FLO10*. Adhesion proteins in *C. albicans* have been shown to confer distinct adhesion profiles towards human proteins and cells (194). This may explain the difference in adhesion gene expression in our study as these isolates were isolated from different human tissues, SM3 was isolated from the blood, and 6955 was isolated from the oral mucosa.

In this study, we also found two fluconazole-resistant isolates that did not contain a *CgPDR1* mutation when compared to its susceptible match; however, both sets over-expressed *CgPDR1*, *CgCDR1*, and *CgSNQ2* when comparing the resistant isolate to its susceptible counterpart. However, the susceptible isolate 62-16 was found to have a nucleotide deletion resulting in a putative truncated CgPdr1p which may lead to its inactivation; this finding is consistent with the fluconazole MIC of this isolate. We compared the coding sequences of *CgPDR1* in this isolate set to all published *CgPDR1* sequences and found two unique mutations whose expression in the susceptible background resulted in an increase in fluconazole MIC as well as over-expression of *CgCDR1*, suggesting that one or both of these represent gain-of-function mutations. Isolate 7755 had no mutation when compared to not only the susceptible isolate 7754 but all the published *CgPDR1* sequences from susceptible isolates. Interestingly, *CgPDR1* is constitutively overexpressed in isolate 7255 suggesting the existence of alternate mechanisms by which *CgPDR1* can be activated.

CHAPTER 4. GENOME-WIDE GENE EXPRESSION PROFILING OF THE RESPONSE TO FLUCONAZOLE IN *CANDIDA GLABRATA*

INTRODUCTION

Azole antifungal resistance has emerged as a significant problem in the management of infections caused by fungal species including *Candida glabrata* (21, 175, 185). Unlike *Candida albicans*, *C. glabrata* exhibits intrinsic reduced susceptibility to the azoles, including fluconazole. Consistent with this observation, multiple studies have shown that fluconazole use is a risk factor for development of invasive candidiasis caused by non-*albicans candida* species, including *C. glabrata* (25, 36, 147, 203). Furthermore, due to the fungistatic nature of fluconazole, *C. glabrata* and other *Candida* species demonstrate tolerance to this antifungal. Tolerance is defined as the capacity of an organism to survive in the presence of a drug at growth-inhibiting concentrations. Azole tolerance may limit the efficacy of this class of antifungals, especially in immunocompromised patients, where the organism is able to survive in the presence of the drug and the immune system less able to clear the fungus. This may also contribute to the development of high level azole resistance in this patient population.

The ability of any organism to alter gene expression allows for adjustment to changes in its environment which is essential for its survival. Genome-wide expression studies of drug responses in *Saccharomyces cerevisiae* and *Candida albicans* have shown that such responses may be specific, reflecting the molecular target of the drug or may represent a more general stress response (2, 118). Moreover, the organism can also cope to changes in the environment with mechanisms that are neither general nor specific to the stressor. For example, not only do *C. albicans* upregulate genes in the ergosterol biosynthesis pathway in response to fluconazole, an antifungal drug that inhibits the ergosterol biosynthesis pathway, but also induces expression of drug efflux pumps (118). Understanding the initial stress response of *C. glabrata* to fluconazole may facilitate a better understanding of how this organism copes with such stresses to its environment. Moreover, such information may provide insight into the problem of intrinsic low-level resistance as well as azole tolerance in *C. glabrata*.

In an effort to characterize the initial stress responses of *C. glabrata* to fluconazole, we used microarray analysis to examine the gene expression profile of *C. glabrata* after exposure to this drug. As *CgPDR1*, a zinc cluster transcription factor, has been implicated as a key regulator of azole resistance and has been shown to be activated in response to azole exposure, we also identified genes that respond to fluconazole exposure in a *CgPDR1*-dependent fashion. Our results provide a more comprehensive picture of the gene expression changes of *C. glabrata* in response to fluconazole. Genes found to influence both intrinsic reduced susceptibility and tolerance to azoles will ultimately serve as targets for future work toward the development of compounds that will both abrogate resistance and enhance the therapeutic utility of this class of antifungal agent.

MATERIALS AND METHODS

Antifungal Agent

Fluconazole was obtained from MP Biomedicals (Solon, Ohio). Stock solutions of 10 mg/ml were made in dimethyl sulfoxide (DMSO, Sigma; St. Louis, Missouri) and stored at -20°C.

Strains and Growth Media

Strain 200989 (*ura3Δ/his3Δ/trp1Δ*) was obtained from ATCC and strain 200989Δ*pdr1* was kindly provided by Dr. Tom Edlind at Drexel University, Philadelphia, PA (63, 100). All isolates/strains used in these studies were grown in YPD (1% yeast extract, 2% peptone, and 1% dextrose) broth. All isolates/strains were grown at 30°C and stored in 20% glycerol stocks at -80°C.

Cell Culture and Fluconazole Exposure for Microarray Experiments

A total of six 100 ml cultures of YPD medium were inoculated from an overnight (saturated) culture of *C. glabrata* to an initial A_{600} 0.005 and grown at 30°C until the A_{600} reached 1.0 (~ 12 hours). The culture was then diluted to an A_{600} of 0.1 and grown until an A_{600} of 0.2 was reached (~ 2 hours). Fluconazole was then added at a final concentration of 64 µg/ml (two times the MIC) to three of the flasks). An equal volume of DMSO was added to the remaining three flasks to serve as the diluent control. DMSO concentrations were less than 0.05% and did not affect the growth curves of control cultures (data not shown). The cultures were grown for 2.5 hours. All cultures were harvested by centrifugation at 3,500 x g at 4°C for 5 minutes. The medium was completely removed by aspiration and the cell pellets were stored at -80°C until RNA preparation.

Microarray cRNA Synthesis and Hybridization

C. glabrata microarrays were constructed as described previously (215). These custom Affymetrix NimbleExpress Arrays were manufactured by NimbleGen Systems per our specification (4). Two independent sets of RNA were used in these studies to prepare two independent cDNA samples. The purity and concentration of the RNA was examined by capillary electrophoresis using the RNA 6000 Nano laboratory-on-a-Chip kit and Bioanalyzer 2100 (Agilent Technologies; Waldbronn, Germany) as per manufacturer's instructions. Fifteen µg total RNA was used for first- and second-strand cDNA synthesis using the SuperScript Double-Stranded cDNA synthesis kit (Invitrogen) and oligo-dT24-T7 primer (Sigma) according to the manufacturer's instructions. Using the T7 promoter-coupled double stranded cDNA as a template and the Bioarray High

Yield RNA Transcript labeling kit (ENZO Diagnostics; Farmingdale, NY), cRNA was synthesized and labeled with biotinylated-UTP and -CTP by in vitro transcription. The synthesized double stranded cDNA was washed twice with 70% ethanol and suspended in 22 μ l of DEPC-treated water. Reaction buffer, biotin-labeled ribonucleotides, dithiothreitol, RNase inhibitor mix and T7 RNA polymerase were added to the cDNA and incubated for 5 hours at 37°C. The labeled cRNA was purified using a CHROMA SPIN-100 column (Clontech; Mountain View, CA) and precipitated with ethanol overnight at -20°C.

After the cRNA pellet was suspended in 10 μ L of RNase-free water, 10 μ g was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 100 mM Tris-acetate (pH8.1), 500 mM potassium acetate, 150 mM magnesium acetate. Using the Affymetrix Fluidics Station 450 and its standard EukGEWS2v5 protocol, the fragmented cRNA was hybridized to the *C. glabrata* NimbleExpress GeneChip arrays for 16 hours at 45°C and then washed at 25°C with 6X SSPE (1X SSPE: 0.18M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA (pH 7.7)) + 0.01% Tween-20 followed by a stringent wash at 50°C with 100 mM MES (2-(N-morpholino) ethanesulfonic acid), 0.1 M NaCl, 0.01% Tween-20. The arrays were stained with phycoerythrin-conjugated streptavidin (Molecular Probes; Eugene, OR) and the fluorescence intensities were determined using the GCS 3000 high-resolution confocal laser scanner (Affymetrix, Santa Clara, CA). These images were analyzed using software resident in GeneChip Operating System v2.0 (GCOS; Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity of 250. The signal intensity for each gene was calculated as the average intensity difference, represented by $[\Sigma (PM - MM)/\text{number of probe pairs}]$, where PM and MM denote perfect-match and mismatch probes.

Microarray Data Analysis

The scaled gene expression values from GCOS software were processed and analyzed using GeneSpring 7.2 software (Agilent Technologies). Probe sets were removed from analysis if they were called absent by the Affymetrix criterion and displayed an absolute value below 20 in all experiments. The expression value of each gene was normalized to the median expression of all genes in each chip as well as the median expression of that gene across all chips in the study. Pairwise comparison of gene expression was performed for each matched experiment.

Quantitative Real-Time RT-PCR

An aliquot of RNA samples used in the microarray experiments was saved for follow-up quantitative RT-PCR studies along with a set of independently-obtained RNA samples from the same strains analyzed by microarray. First-strand cDNAs were synthesized from 2 μ g total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer's instructions. All quantitative real-time

PCR experiments were performed in triplicate using the 7000 Sequence Detection System (Applied Biosystems, Inc.; Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers were designed for the gene of interest and 18S rRNA using Primer Express software (Applied Biosystems) (Table 4-1). The PCR conditions were as follows: AmpliTaq Gold activation at 95°C for 10 minutes, 40 cycles of a combination of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each cycle to verify that a single product was amplified using the software provided with the 7000 Sequence Detection System. The system software monitored the change in fluorescence of SYBR Green I dye in every cycle, and the threshold cycle (C_T) above background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain ΔC_T value. The ΔC_T of an arbitrary calibrator (e.g. fluconazole-susceptible isolate or untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$. Statistical analysis was performed using Microsoft Office Excel 2007. Relative changes were compared using the Student's t-test. The statistical significance threshold was fixed at $\alpha = 0.05$.

In order to find putative targets of *PDRI*, *UPC2*, *RLM1*, and *CRZ1* we used the Yeastact (Yeast Search for Transcriptional Regulators And Consensus Tracking) program available at <http://www.yeasttract.com/index.php> (137, 206). This program allows for the identification of transcription factor regulatory associations with target genes in *S. cerevisiae* based on more than 1000 bibliographic references. All genes (*S. cerevisiae* homologs) upregulated in response to fluconazole were uploaded to this program and direct or indirect target genes were searched. The promoters of these genes (1000bp upstream from start codon) were also searched for putative *S. cerevisiae* binding motifs (Pleiotropic Drug Response Element (PDRE); SRE (steroid response element); CDRE (Calcineurin Dependent Response Element); RRE (Rlm1p response element)).

RESULTS

Global Changes in Gene Expression in Response to Fluconazole

An understanding of the initial stress responses of *C. glabrata* to fluconazole should allow for a better understanding of intrinsic azole resistance and tolerance in this organism. To identify genes that respond to fluconazole treatment, we used gene expression profiling to examine the initial response of *C. glabrata* in the presence of fluconazole. We exposed ATCC strain 200989 to 64 µg/ml fluconazole (2X MIC) for 2.5 hours. Genes were considered to be differentially expressed if the expression of that gene changed reproducibly by at least ≥ 1.5 in the fluconazole-treated strain compared to the untreated strain. There were 714 genes, approximately 13% of the entire *C. glabrata* genome, was upregulated in response to fluconazole. Among these genes were *S. cerevisiae* homologs that are involved in transport, lipid metabolism, cell wall

Table 4-1. Primers used for quantitative real-time PCR expression analysis.

Primer/Gene	Primer pair ^a	Amplicon size (bp)
18S rRNA	F, 5'-TCGGCACCTTACGAGAAATCA-3' R, 5'-CGACCATACTCCCCCAGA-3'	52
<i>CgPDR1</i>	F, 5'-TTTGACTCTGTTATGAGCGATTACG-3' R, 5'-TTCGGATTTTTCTGTGACAATGG-3'	51
<i>CgCDR1</i>	F, 5'-CATACAAGAAACACCAAAGTCGGT-3' R, 5'-GAGACACGCTAACGTTACACCAC-3'	71
<i>CgYOR1</i>	F, 5'-CCATCGGTGCTTGTGTAATGTTA-3' R, 5'-TTGAGAGGCGTGGA AAAAATG-3'	68
<i>CgYBT1</i>	F, 5'-AAGTGCTTCTTCCGCCTCATT-3' R, 5'-AACAGGAGCTGGTGTAGTACCCA-3'	67
<i>CgFLR1</i>	F, 5'-TGGCCTACTTCGGATTCTGTG-3' R, 5'-CCAAAAGGATGATGTATGCAAAAAT-3'	54
<i>CgUPC2</i>	F, 5'-TTCTCTGGCTGCCGAATCA-3' R, 5'-ATCTTGCCTTTTTGCTCATCATAA-3'	51
<i>CgCSR1</i>	F, 5'-TGGATTTTTTCTCCCATCTGGA-3' R, 5'-ACCACAGGGTCAAGCCATTTT-3'	53
<i>CgSAC7</i>	F, 5'-TCTGGACGCAACGGGTTC-3' R, 5'-ACTCCTCAAGCGAGACACCAA-3'	52
<i>CgRTA1</i>	F, 5'-TCCTGTTTGTTCATTAGGGTTAGGG-3' R, 5'-TGGCAATTTTGTTCCTTATTCCTCAG-3'	51

^aF, Forward; R, Reverse.

maintenance, and response to stress (Figure 4-1). Also upregulated were the transcriptional regulator *PDR1*, and homologs of the *S. cerevisiae* transcriptional regulators *UPC2*, *CRZ1*, *RLM1*, *CIN5*, and *YAP1*. Many targets and binding motifs of these transcription factors have been identified in *S. cerevisiae*. We used the World Wide Web based program, Yeastract, to identify putative targets of these transcriptional regulators (137, 206). The Yeastract program allows for analysis of transcription factor associations based on Chromatin immunoprecipitation (ChiP) as well as the effects of deletion of the transcription factor in *S. cerevisiae*. Furthermore, because of the evolutionary relatedness of *S. cerevisiae* and *C. glabrata* and the fact that binding motifs are usually highly conserved, the promoter region (defined for this purpose as 1000 bp immediately upstream from the start codon of the ORF) of each putative target gene was examined for a match to the consensus *S. cerevisiae* sequences for these transcription factors. Tables 4-2, 4-3, and 4-4 list the genes found to be putative targets of these transcriptional regulators as well as contain at least one putative binding motif.

Other genes of *S. cerevisiae* homologs found to be responsive to fluconazole treatment include genes involved in response to stress such as *DDR48* (DNA damage-responsive protein), and *YIM1* (protein of unknown function). Additionally, several other stress response genes were upregulated in response to fluconazole treatment including genes encoding calcium-regulated signaling proteins *CCH1* (voltage-gated high-affinity calcium channel), *CMP2* (calcineurin A), *PMR1* (Ca²⁺/Mn²⁺ P-type ATPase), *CMK2* (calmodulin-dependent protein kinase) and the aforementioned transcription factor, *CRZ1*. There were 905 genes, approximately 17% of the *C. glabrata* genome, were down-regulated by ≥ 1.5 fold in response to fluconazole (Figure 4-2). Real-time RT-PCR was used to confirm the expression of selected genes (Figure 4-3).

Effects of *CgPDR1* on Global Gene Expression Responses to Fluconazole

It has previously been shown that *CgPDR1* is induced after treatment with fluconazole, suggesting a role for CgPdr1p in the initial stress response to fluconazole (215). To assess the role of Pdr1p on the stress response to fluconazole, we used a previously described strain in which *CgPDR1* had been disrupted (200989 Δ *pdr1*), and we repeated the above experiment (63). Genes were defined as being CgPdr1p-dependent if the expression of that gene decreased by 50% after disruption of *CgPDR1* in the presence of fluconazole in two independent experiments. Surprisingly, only six genes were found to be Pdr1p-dependent (Table 4-5); however, only two of these genes were also upregulated in response to fluconazole. Among these were the aforementioned gene *CgCDR1*.

DISCUSSION

Most *C. glabrata* isolates demonstrate intrinsic resistance to azole antifungals. Moreover, *C. glabrata* exhibits tolerance to azoles which allows this organism to survive in the presence of drug at growth-inhibiting concentrations. In an effort to identify genes

Figure 4-1. Distribution of genes upregulated by ≥ 1.5 fold in response to fluconazole. Genes were annotated and assigned to functional categories based on the gene ontology (GO) annotation of its *S. cerevisiae* homolog.

Table 4-2. *C. glabrata* putative target genes^a of Pdr1p upregulated by ≥ 1.5 fold in response to fluconazole.

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	PDRE ^c
CAGL0F08745g	<i>TMA10</i>	Protein of unknown function that associates with ribosomes	28.3	-644
CAGL0C00275g	<i>HSP31</i>	Possible chaperone and cysteine protease	7.4	-932
CAGL0M08426g	<i>YJL163c</i>	Putative protein of unknown function	7.2	-450
CAGL0K10164g	<i>SED1</i>	Major stress-induced structural GPI-cell wall glycoprotein	4.9	-996
CAGL0K00715g	<i>RTA1</i>	Lipid-translocating exporter (LTE) family of protein	4.3	-379;-300
CAGL0A00451g	<i>PDR1</i>	Zinc cluster transcription factor	3.8	-701;-557
CAGL0M08822g	<i>HSP78</i>	Oligomeric mitochondrial matrix chaperone	3.7	-298
CAGL0G05962g	<i>YHR140w</i>	Putative integral membrane protein of unknown function	3.6	-276
CAGL0M01760g	<i>PDR5</i>	Plasma membrane ATP-binding cassette (ABC) transporter	3.5	-969; -515; -387; -227; -133
CAGL0K09702g	<i>YNL134c</i>	Putative protein of unknown function	3.5	-549
CAGL0K12958g	<i>YML131w</i>	Putative protein of unknown function	3.5	-594
CAGL0G00242g	<i>YOR1</i>	Plasma membrane ATP-binding cassette (ABC) transporter	3.2	-648
CAGL0F02717g	<i>PDR15</i>	Plasma membrane ATP binding cassette (ABC) transporter	2.6	-557;-521
CAGL0A01199g	<i>DIP5</i>	Dicarboxylic amino acid permease	2.5	-737

^aTranscription factor targets identified using Yeastract website (<http://www.yeastract.com/index.php>) (124, 193).

^bFold change defined as the ratio of fluconazole-treated 200989 versus untreated 200989.

^cPDRE (TCC(GA)(CT)G(GC)(AG).

Table 4-3. *C. glabrata* putative target genes^a of Upc2p, Crz1p, Rlm1p, and Cin5p upregulated by ≥ 1.5 fold in response to fluconazole.

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	Binding motif ^c
Putative Upc2p targets				SRE^c
CAGL0K03421g	<i>PGM2</i>	Phosphoglucomutase	3.9	-806
CAGL0I02970g	<i>ERG24</i>	C-14 sterol reductase, acts in ergosterol biosynthesis	1.8	-127
CAGL0L10714g	<i>ERG2</i>	C-8 sterol isomerase, acts in ergosterol biosynthesis	1.7	-926; -738
Putative Crz1p targets				CDRE^c
CAGL0F08745g	<i>TMA10</i>	Protein of unknown function that associates with ribosomes	27.3	-917 [‡]
CAGL0J04158g	<i>WSP1</i>	Protein of unknown function	6.8	-805 [‡]
CAGL0K00715g	<i>RTA1</i>	Lipid-translocating exporter (LTE)	3.3	-659 [‡]
CAGL0A00517g	<i>PMC1</i>	Vacuolar Ca ²⁺ ATPase	2.6	-62 [‡]
CAGL0C04961g	<i>ECM27</i>	Non-essential protein of unknown function	2.6	-659 [‡]
CAGL0J03432g	<i>ARK1</i>	Serine/threonine protein kinase	2.3	-470 [‡]
CAGL0M08206g	<i>YJL171c</i>	GPI-anchored cell wall protein	2.3	-520 [‡]
CAGL0M02211g	<i>PEP4</i>	Vacuolar aspartyl protease	2.0	-321 [‡]
Putative Rlm1p targets				RRE^c
CAGL0F08195g	<i>MGA1</i>	Protein similar to heat shock transcription factor	7.8	-328
CAGL0H02563g	<i>YDR524c</i>	ADP-ribosylation factor	4.5	-144
CAGL0H08844g	<i>DDR48</i>	DNA damage-responsive protein	4.1	-975
CAGL0J04466g	<i>YLR414c</i>	Putative protein of unknown function	3.7	-299
CAGL0M12320g	<i>FLC2</i>	Putative FAD transporter	3.6	-121
CAGL0M04191g	<i>YPS1</i>	Aspartic protease	3.5	-129
CAGL0J00539g	<i>SLT2</i>	Serine/threonine MAP	3.4	-269
CAGL0G09449g	<i>CRH1</i>	Putative chitin transglycosidase	3.4	-406
CAGL0J11506g	<i>CHS1</i>	Chitin synthase I	3.2	-197
CAGL0G08646g	<i>POG1</i>	Putative transcriptional activator	2.9	-295

Table 4-3 (continued).

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	Binding motif ^c
Putative Rlm1p targets, continued				
CAGL0B03619g	<i>PRB1</i>	Vacuolar proteinase B (yscB)	2.7	-391
CAGL0J01463g	<i>CWP1</i>	Cell wall mannoprotein	2.6	-219
CAGL0L00935g	<i>APQ12</i>	Protein involved in nucleocytoplasmic transport of mRNA	2.3	-130
CAGL0M08206g	<i>YJL171c</i>	GPI-anchored cell wall protein	2.3	-60
CAGL0E01969g	<i>SMF1</i>	Divalent metal ion transporter	2.2	-496
CAGL0G04433g	<i>PRM10</i>	Pheromone-regulated protein	1.9	-955
CAGL0M03663g	<i>RIM21</i>	Component of the RIM101 pathway	1.8	-212
CAGL0H05621g	<i>RLM1</i>	MADS-box transcription factor	1.8	-483
Putative Cin5p targets				CRE^c
CAGL0K10164g	<i>SED1</i>	GPI-cell wall glycoprotein	3.9	-143
CAGL0K08162g	<i>GPT2</i>	Glycerol-3-phosphate/dihydroxyacetone phosphate	3.2	-574;2190
CAGL0G02937g	<i>PDC1</i>	Major of three pyruvate decarboxylase isozymes	2.0	-444;-429
CAGL0F07799g	<i>EAR1</i>	Specificity factor required for Rsp5p ubiquitination	2.0	-613
CAGL0J01353g	<i>YTA12</i>	Protein of unknown function	1.8	-318
CAGL0H05621g	<i>RLM1</i>	MADS-box transcription factor	1.8	-393
CAGL0E05610g	<i>CDC19</i>	Pyruvate kinase	1.6	-937
CAGL0K10890g	<i>OYE2</i>	Widely conserved NADPH oxidoreductase	1.6	-517
CAGL0M03377g	<i>GLC3</i>	Glycogen branching enzyme	1.6	-900
CAGL0D05698g	<i>MMM1</i>	Mitochondrial outer membrane protein	1.6	-363

^aTranscription factor targets identified using Yeastract website (<http://www.yeastract.com/index.php>).

^bFold change defined as the ratio of fluconazole-treated 200989 versus untreated 200989.

^cSRE (5'-TCGTATA-3') (217), CDRE (5'-GAGGCTG-3')[†] or (GNGGC(G/T)CA)[‡] (227), RRE (5'-NTA(A/T)TAG-3') (56), CRE (5'-TTACTAA-3')(67).

Table 4-4. *C. glabrata* putative target genes^a of CgAp1p upregulated by ≥ 1.5 fold in response to fluconazole.

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	Binding motif ^c			
				A	B	C	D
CAGL0F08745g	<i>TMA10</i>	Protein of unknown function that associates with ribosomes	27.3	-	-443	-	-
CAGL0K04609g	<i>VHT1</i>	High-affinity plasma membrane H ⁺ -biotin symporter	10.2	-	-864;-474	-	-
CAGL0C02321g	<i>PHM8</i>	Protein of unknown function	7.2	-	-	-	-246
CAGL0I06644g	<i>SED1</i>	GPI-cell wall glycoprotein	6.0	-	-	-452	-708
CAGL0L00759g	<i>HIS1</i>	ATP phosphoribosyltransferase	6.0	-	-173	-	-
CAGL0J00561g	<i>YHI9</i>	Protein of unknown function	5.9	-	-132	-	-723
CAGL0F07029g	<i>MET13</i>	Major isozyme of methylenetetrahydrofolate reductase	5.5	-	-485	-	-
CAGL0A04081g	<i>SRP40</i>	Nucleolar, serine-rich protein	4.7	-	-	-	-482
CAGL0H06721g	<i>UBP7</i>	Ubiquitin-specific protease	4.1	-526	-	-	-558
CAGL0K10164g	<i>SED1</i>	GPI-cell wall glycoprotein	3.9	-143	-	-	-155
CAGL0G08338g	<i>YLR241w</i>	Putative protein of unknown function	3.5	-760;-225	-	-	-
CAGL0M11682g	<i>YLR108c</i>	Protein of unknown function	3.5	-	-	-	-537;-281
CAGL0H07359g	<i>PHO8</i>	Repressible alkaline phosphatase	3.4	-	-	-	-117
CAGL0J03762g	<i>MET7</i>	Folylpolyglutamate synthetase	3.4	-490	-	-	-
CAGL0G09449g	<i>CRH1</i>	Putative chitin transglycosidase	3.4	-	-	-	-285
CAGL0B03883g	<i>YMC1</i>	Mitochondrial protein	3.3	-	-	-95	-920
CAGL0H04983g	<i>PSA1</i>	GDP-mannose pyrophosphorylase	3.2	-377;-59	-	-	-
CAGL0K08162g	<i>GPT2</i>	Glycerol-3-phosphate/dihydroxyacetone phosphate	3.2	-574	-	-	-
CAGL0D00770g	<i>IDP1</i>	Mitochondrial NADP-specific isocitrate dehydrogenase	3.1	-	-243	-	-
CAGL0I03806g	<i>BPL1</i>	Biotin:apoprotein ligase	3.1	-	-	-357	-
CAGL0M02167g	<i>PRM4</i>	Pheromone-regulated protein	3.0	-	-	-408	-
CAGL0M10417g	<i>RCR2</i>	Vacuolar protein	2.9	-	-	-	-39
CAGL0H06699g	<i>GUT2</i>	Mitochondrial glycerol-3-phosphate dehydrogenase	2.8	-47	-	-	-
CAGL0A04477g	<i>UBP13</i>	Putative ubiquitin carboxyl-terminal hydrolase	2.8	-	-	-	-699;-134
CAGL0I10626g	<i>YGR125w</i>	Putative protein of unknown function	2.7	-	-	-469	-
CAGL0E02035g	<i>MCH4</i>	Protein with similarity to monocarboxylate permeases	2.7	-	-102	-	-346
CAGL0M06611g	<i>AVT7</i>	Putative transporter	2.7	-	-	-470	-
CAGL0I01210g	<i>CTM1</i>	Cytochrome c lysine methyltransferase	2.7	-	-	-	-678
CAGL0M13189g	<i>MSN4</i>	Transcriptional activator related to Msn2p	2.6	-	-730	-	-
CAGL0K12034g	<i>ENA1</i>	P-type ATPase sodium pump	2.6	-	-	-	-26
CAGL0E03025g	<i>YGR146c</i>	Putative protein of unknown function	2.6	-	-	-	-607
CAGL0A02574g	<i>TRP4</i>	Anthranilate phosphoribosyl transferase	2.6	-	-170	-	-
CAGL0L12012g	<i>TMT1</i>	Trans-aconitate methyltransferase,	2.6	-	-	-	-785;-211

Table 4-4 (continued).

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	Binding motif ^c			
				A	B	C	D
CAGL0J01463g	<i>CWP1</i>	Cell wall mannoprotein	2.6	-	-	-	-703;-173
CAGL0M13651g	<i>PRC1</i>	Vacuolar carboxypeptidase Y (proteinase C)	2.6	-	-	-	-671
CAGL0L02321g	<i>MET14</i>	Adenylylsulfate kinase	2.6	-	-	-	-741
CAGL0L02607g	<i>YHR202w</i>	Putative protein of unknown function	2.6	-	-	-	-12
CAGL0M01760g	<i>PDR5</i>	Plasma membrane ABC transporter	2.5	-	-	-540	-
CAGL0K12958g	<i>YML131w</i>	Putative protein of unknown function	2.5	-	-	-	-909
CAGL0E02409g	<i>CSI2</i>	Protein of unknown function	2.4	-	-	-	-91
CAGL0H05687g	<i>SSU1</i>	Plasma membrane sulfite pump	2.4	-633;-333	-570	-	-
CAGL0C02167g	<i>YFR039c</i>	Putative protein of unknown function	2.4	-	-	-	-713
CAGL0G05698g	<i>GDH2</i>	NAD(+)-dependent glutamate dehydrogenase	2.4	-259	-952	-	-
CAGL0E01771g	<i>MKC7</i>	GPI-anchored aspartyl protease (yapsin)	2.4	-	-	-	-399
CAGL0D02728g	<i>FMP34</i>	Putative protein of unknown function	2.2	-	-	-	-576
CAGL0E04026g	<i>CBP2</i>	Mitochondrial protein	2.2	-	-304	-	-
CAGL0M08734g	<i>YDR262w</i>	Putative protein of unknown function	2.1	-565;-203	-	-	-
CAGL0L01265g	<i>UTR1</i>	ATP-NADH kinase	2.1	-	-	-	-845
CAGL0L04598g	<i>DCS1</i>	Non-essential hydrolase involved in mRNA decapping,	2.1	-203	-	-	-
CAGL0A00781g	<i>VPS8</i>	Membrane-associated protein that interacts with Vps21p	2.0	-	-	-	-688
CAGL0G02937g	<i>PDC1</i>	Major of three pyruvate decarboxylase isozymes	2.0	-444;-429	-	-616	-
CAGL0M11484g	<i>ARO1</i>	Pentafunctional arom protein	2.0	-	-111	-	-
CAGL0J08118g	<i>IST1</i>	Protein with a role in the multivesicular body sorting pathway	1.9	-319	-	-	-
CAGL0J06402g	<i>LYS21</i>	Homocitrate synthase isozyme	1.9	-	-718	-382	-
CAGL0H08954g	<i>MRM2</i>	Mitochondrial 2' O-ribose methyltransferase	1.9	-	-	-	-447
CAGL0J01353g	<i>YTA12</i>	Protein of unknown function	1.8	-318	-	-	-
CAGL0H05621g	<i>RLM1</i>	MADS-box transcription factor	1.8	-393	-	-	-666;-75
CAGL0F07359g	<i>YGL117w</i>	Putative protein of unknown function	1.8	-	-288	-	-
CAGL0F04697g	<i>YLR257w</i>	Putative protein of unknown function	1.8	-	-	-	-862
CAGL0H04587g	<i>AVT6</i>	Vacuolar amino acid transporter	1.7	-	-	-72	-
CAGL0G05918g	<i>CHS7</i>	Protein of unknown function	1.7	-	-	-	-172
CAGL0G05335g	<i>TPS2</i>	Phosphatase of the trehalose-6-phosphate synthase/phosphatase	1.7	-	-	-712	-
CAGL0G07293g	<i>USA1</i>	Protein involved in ER-associated protein degradation (ERAD)	1.7	-	-	-	-899
CAGL0M04741g	<i>MPD1</i>	Member of the protein disulfide isomerase (PDI) family	1.7	-	-	-	-169
CAGL0H05599g	<i>YDC1</i>	Alkaline dihydroceramidase	1.7	-	-	-588	-
CAGL0C02409g	<i>ZRG8</i>	Protein of unknown function	1.7	-	-	-	-498

Table 4-4 (continued).

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^b	Binding motif ^c			
				A	B	C	D
CAGL0H04103g	<i>UFO1</i>	F-box receptor protein	1.7	-	-	-	-133
CAGL0A01243g	<i>GIT1</i>	Plasma membrane permease	1.6	-	-	-	-795
CAGL0A01826g	<i>HXT5</i>	Hexose transporter with moderate affinity for glucose	1.6	-	-	-	-294
CAGL0F06633g	<i>UBX4</i>	UBX (ubiquitin regulatory X) domain-containing protein	1.6	-	-	-	-34
CAGL0J04752g	<i>TUS1</i>	Guanine nucleotide exchange factor (GEF)	1.6	-400	-	-	-
CAGL0L10912g	<i>TPO4</i>	Polyamine transport protein	1.6	-	-	-	-31
CAGL0M04675g	<i>YOR285w</i>	Protein of unknown function	1.6	-	-	-	-484
CAGL0E05610g	<i>CDC19</i>	Pyruvate kinase	1.6	-937	-	-	-
CAGL0K10890g	<i>OYE2</i>	Widely conserved NADPH	1.6	-517	-	-	-242
CAGL0H03223g	<i>GUP1</i>	Plasma membrane protein involved in remodeling GPI	1.6	-	-	-	-701;-25
CAGL0I01012g	<i>PEP12</i>	Target membrane receptor (t-SNARE)	1.6	-	-	-930	-
CAGL0D01584g	<i>JID1</i>	Probable Hsp40p co-chaperone	1.5	-	-	-	-376

^aTranscription factor targets identified using Yeastract website (<http://www.yeastract.com/index.php>).

^bFold change defined as the ratio of fluconazole-treated 200989 versus untreated 200989.

^cBinding motifs A 5'-TTACTAA-3'(67), B 5'-TGACTCA-3'(67), C 5'-TGACTAA-3'(149), D 5'-T(G/T)ACAAA-3'(84).

Figure 4-2. Distribution of genes downregulated by ≥ 1.5 fold in response to fluconazole. Genes were annotated and assigned to functional categories based on the gene ontology (GO) annotation of its *S. cerevisiae* homolog.

Figure 4-3. Quantitative real-time RT-PCR analysis of relative gene expression in 200989 and 200989Δpdr1 treated with fluconazole versus 200989 treated with DMSO (control). Data are shown as mean $\pm$ standard error. Relative changes were compared using the Student's t-test. Gene expression values with an asterisk indicate statistical significance ($P < 0.05$) in all three experiments.

Table 4-5. *C. glabrata* genes found to be Pdr1p-dependent in response to fluconazole.

<i>C. glabrata</i> designation	<i>S. cerevisiae</i> homolog	Description	Fold change ^a		PDRE ^b
			200989F/ 200989D	200989Δ <i>pdr1</i> F/ 200989D	
CAGL0M01760g	<i>PDR5</i>	Plasma membrane ABC transporter	2.8	0.6	134, 227, 387, 515, 969
CAGL0C02915g	<i>YOR268c</i>	Putative protein of unknown function	2.2	1.0	-
CAGL0D03652g	<i>SPT21</i>	Protein required for normal transcription at several loci including HTA2-HTB2 and HHF2-HHT2	0.7	0.3	-
CAGL0K07700g	<i>BUD27</i>	Protein involved in bud-site selection, nutrient signaling, and gene expression controlled by TOR kinase	0.6	0.1	-
CAGL0E02937g	<i>CGR1</i>	Protein involved in nucleolar integrity and processing of the pre-rRNA for the 60S ribosome subunit	0.3	0.1	-
CAGL0H09064g	<i>FUR1</i>	Uracil phosphoribosyltransferase	0.4	0.2	-

^aFold change defined as the ratio of fluconazole-treated 200989 versus untreated 200989 or 200989Δ*pdr1* versus untreated 200989.

^bPDRE (TCC(GA)(CT)G(GC)(AG).

that may contribute to these phenotypes, we examined the response of *C. glabrata* to fluconazole treatment using genome-wide gene expression profiling.

As expected, fluconazole treatment induced the expression of *CgPDR1* and many known genes encoding homologs of the *S. cerevisiae* ScPdr1p and ScPdr3p target genes. In *S. cerevisiae*, ScPdr1p and ScPdr3p have been named the master regulators of drug resistance, including resistance to azoles (141). In *C. glabrata*, CgPdr1p has been identified as the single homolog of ScPdr1p and ScPdr3p (215). This zinc cluster transcription factor is known to regulate the ATP binding cassette (ABC) transporters *CgCDR1* and *CgPDH1* which are also upregulated in the present study (215). The role of CgPdr1p in azole resistance has been studied extensively. Gain-of-function mutations in this transcriptional regulator have been shown to be responsible for the up-regulation of these ABC transporters and subsequent decrease in susceptibility to fluconazole (68, 210, 212, 215). Recently it was shown that the regulation of *C. glabrata* Pdr1p is similar to regulation of multidrug resistance (MDR) in vertebrates by the PXR nuclear receptor as Pdr1p directly binds to fluconazole resulting in activation of drug efflux pumps (207). Consistent with this finding, in a previous study expression of *CgPDR1* and its target genes, *CgCDR1* and *CgPDH1* are induced after treatment with fluconazole (215). Furthermore, in this same study disruption of *CgPDR1* in a fluconazole susceptible isolate (fluconazole MIC of 16 µg/ml) resulted in fluconazole hypersusceptibility (fluconazole MIC of 2 µg/ml) suggesting that *CgPDR1* plays an important role in the intrinsic fluconazole resistance of this species. To date, no study has conclusively studied the role of CgPdr1p in azole tolerance.

A recent study identified 22 genes that were essential for *S. cerevisiae* survival in the presence of fluconazole (91). Among the genes identified was the *CgCDR1* *S. cerevisiae* homolog *ScPDR5*. Disruption of *ScPDR5* in *S. cerevisiae* rendered fluconazole fungicidal to this mutant. Alternatively, disruption of *CgCDR1* in *C. glabrata* in a fluconazole susceptible isolate (MIC 16 µg/ml) had no effect on fluconazole MIC; however, trailing growth, a phenomenon typical of *Candida* species, was eliminated (178, 216). The effect of *CgCDR1* on the minimum fungicidal concentration (MFC) has not been measured in *C. glabrata*. Because disruption of *CgPDR1* but not *CgCDR1* results in azole hypersensitivity, these results suggest that other CgPdr1p targets may be playing a role in intrinsic resistance. In order to determine which fluconazole-responsive genes are regulated by Pdr1p, we identified genes that respond to fluconazole in a Pdr1p-dependent fashion by assessing which genes are no longer differentially expressed in response to fluconazole treatment in a *Δpdr1* *C. glabrata* strain. As expected, *CgCDR1* was found to be upregulated in a CgPdr1p-dependent fashion; however, other known ScPdr1p-ScPdr3p target genes were not. This suggests that there are fluconazole-responsive transcriptional factors, in addition to CgPdr1p, are able to regulate CgPdr1p target genes. In fact, of the putative Pdr1p targets listed in Table 4-2, *TMA10* and *RTA1* are also putative Crz1p targets. Also, of all the genes *YOR268c* was also found to be Pdr1p-dependent. The function of this gene is unknown, but a *S. cerevisiae* strain disrupted for the homolog of this gene was found to be selenomethionine resistant (23). Surprisingly, only five other genes were found to be Pdr1p dependent; however, none of these were activated in response to fluconazole.

Fluconazole acts by inhibiting lanosterol demethylase (*ERG11*) in the ergosterol biosynthesis pathway. A *S. cerevisiae* homolog of *UPC2* which is known to induce transcription of both sterol biosynthesis genes and *DAN/TIR* genes was also upregulated in response to fluconazole. In addition, homologs of ScUpc2p target genes were also induced in the present study in response to fluconazole. These included genes encoding proteins involved in the ergosterol biosynthesis pathway (*ERG1*, *ERG2*, and *ERG24*) and heme biosynthesis (*HEM13* and *HEM14*). It has been suggested that in *S. cerevisiae* heme may play an important role in sterol biosynthesis and regulate the transcription of several genes involved in this process (161). Upregulation of genes involved in sterol biosynthesis is consistent with inhibition of this pathway by fluconazole and has also been observed in *S. cerevisiae* and *C. albicans* (2, 118). Also upregulated in response to fluconazole were the homologs of *S. cerevisiae* Upc2p target genes involved in cell wall maintenance (*DAN4*, *TIR1*, and *TIR3*).

Another interesting observation is the up-regulation of homologs of the *S. cerevisiae* genes involved in calcium signaling (*CCH1*, *CMP2*, *CRZ1*, *GSC2*, and *PMR1*). In *S. cerevisiae*, *CCH1* encodes a voltage-gated high-affinity calcium channel involved in calcium influx in response to some environmental stresses. A study in *C. glabrata* found that strains defective in the putative plasma membrane calcium channel (Cch1-Mid1) were not only defective in calcium uptake in response to fluconazole exposure but also showed a significant loss of viability upon prolonged fluconazole exposure (98). The authors concluded that in the absence of calcium signaling, fluconazole has a fungicidal effect on *C. glabrata* suggesting a role for calcium signaling in tolerance to azoles. Calcium influx leads to stimulation of the heterodimer protein phosphatase calcineurin which is encoded by *CMP2* (catalytic A subunit) and *CNB1* (calcium-binding regulatory unit) (Figure 4-4). In *C. glabrata* disruption of *CNB1*, the calcium-binding subunit of calcineurin, resulted in not only increased fluconazole susceptibility but also fungicidal activity to fluconazole in *C. glabrata* (136). The homolog of the *S. cerevisiae* gene *CNB1* is not upregulated in the present study; however, *CMP2* which encodes one isoform of the catalytic subunit A of calcineurin is upregulated. Calcineurin dephosphorylates the transcription factor *CRZ1* which then activates its target genes which include *GSC2* (encodes β -1, 3-glucan synthase) and *PMR1* (encodes a Golgi-localized $\text{Ca}^{2+}/\text{Mn}^{2+}$ importer). In *S. cerevisiae*, disruption of *CRZ1* enhanced azole sensitivity; however, disruption in of the *CRZ1* homolog in *C. glabrata* does not (136). Thus, the precise mechanism calcineurin-dependent azole tolerance in *C. glabrata* remains to be discovered. Of note, of the Crz1p putative targets listed in Table 4-3 one gene encoding the *S. cerevisiae* homolog *RTA1* is also upregulated. In *S. cerevisiae*, *RTA1*, which is also known to be regulated by ScPdr1p and ScPdr3p, encodes a protein member of the fungal lipid-translocating exporter family and is involved in 7-aminosterol resistance. In *C. albicans*, over-expression of *RTA2*, which encodes a *S. cerevisiae* homolog of *RTA1*, in a *C. albicans* strain with deletions of *CDR1*, *CDR2*, and *MDR1* conferred increased resistance to azoles suggesting a role for this protein family in azole resistance (92). However, the role of *RTA1* in azole tolerance has not been studied in *S. cerevisiae* or *C. glabrata*.

Figure 4-4. Proposed model for the calcium-signaling pathway in *C. glabrata*. Calcium influx leads to stimulation of the heterodimer protein phosphatase calcineurin. Calcineurin dephosphorylates the transcription factor *CRZ1* which then activates its target genes which include *GSC2* (encodes β -1, 3-glucan synthase) and *PMR1* (encodes a Golgi-localized $\text{Ca}^{2+}/\text{Mn}^{2+}$ importer).

In *S. cerevisiae*, the calcineurin and cell wall integrity (CWI) pathways function in parallel to induce *FKS2* (*CgGSC2*) function in response to heat (229). The CWI pathway is the major signaling pathway regulated cell wall assembly. Of note, several genes encoding proteins involved in the CWI pathway were differentially expressed in response to fluconazole. Among these genes was the *S. cerevisiae* homologs *MID2* which encodes a sensor for cell wall integrity signaling, *SAC7* which encodes a GTPase activating protein for Rho1p, *RLM1* which encodes a MADS-box transcription factor which is phosphorylated and activated by the MAP-kinase Slt2p, which is also upregulated in response to fluconazole (Figure 4-5). Recently, in *S. cerevisiae* *SLT2* has been found to be essential for survival in the presence of fluconazole. Disruption of *SLT2* in *S. cerevisiae* decreased the MFC suggesting a role for *SLT2* in fluconazole tolerance (91). In *S. cerevisiae*, Rlm1p is the transcription factor that regulates much of the transcriptional change upon Slt2p activation (93). We identified several putative Rlm1p targets involved in cell wall maintenance upregulated in response to fluconazole in this study such as the cell wall proteins *CRH1* (putative chitin transglycosidase), *CHS1* (Chitin synthase I), *CWPI* (Cell wall mannoprotein), and *YJL171c* (GPI-anchored cell wall protein). The activation of the cell wall integrity pathway may be vital in the presence of fluconazole for maintaining the structure of the cell wall to counteract the effects of reduced ergosterol production.

Also upregulated in response to fluconazole treatment were two bZIP transcription factors, *YAP1* (*CgAPI*) and *CIN5*. *CgAPI* and *CIN5* both belong to the YAP (yeast activator protein) family of bZIP trans-activators that have been implicated in various forms of stress response in *S. cerevisiae* (180). In fact, *YAP1* is known as the major regulator of oxidative stress in *S. cerevisiae*. In *C. glabrata*, a homolog of the *S. cerevisiae* *YAP1* has been identified (*CgAPI*) and its function in multidrug resistance has been studied (37). Disruption of *CgAPI* had no effect on fluconazole resistance. Also, a target of *CgAPI* was identified, the major facilitator superfamily transporter *FLR1*. Interestingly, *FLR1* in the present study was found to be down-regulated in response to fluconazole. In *S. cerevisiae*, *FLR1* can be activated by not only Yap1p but also Pdr3p (29). In fact, of all putative Pdr1p direct and indirect target genes identified using Yeastact in this study, 43 genes were identified as also being putative Yap1p direct or indirect targets. Of note, *PDR5*, the *S. cerevisiae* homolog of *CgCDR1* was among these genes. This supports the hypothesis that other fluconazole-responsive transcriptional factors, in addition to CgPdr1p, are able to regulate CgPdr1p target genes. The roles of these other transcriptional regulators (*UPC2* and *RLM1*) in azole tolerance are under investigation.

Figure 4-5. Cell wall integrity pathway in *S. cerevisiae* (116). Signals are initiated at the plasma membrane through cell surface sensors (Wsc1-4p and Mid2p). These sensors stimulate nucleotide exchange on Rho1p which then activates the Pkc1p-MAP kinase cascade. The MAP kinase, Slt2p, is activated by Pkc1p and downregulated by the MAP kinase phosphatases, Msg5p, Ptp2p, and Sdp1p. Slt2p then phosphorylates and activates Rlm1p. *C. glabrata* homologs of these genes that are upregulated in response to fluconazole in the present study are in red and homologs downregulated in response to fluconazole in the present study are in green.

CHAPTER 5. SUMMARY

Over the past two decades, *Candida glabrata* has become the one of the most common causes of fungal infections in humans, only second to *Candida albicans*. The azole class of antifungals is the most widely used antifungal for treatment of *Candida* infections. However, development of high-level resistance to the azoles has been reported in oral and bloodstream isolates from head and neck radiation patients, stem cell transplant patients, and human immunodeficiency virus (HIV) patients (122, 174, 175). *C. glabrata*, in addition, exhibits intrinsic increased susceptibility to the azoles, including fluconazole and can develop tolerance due to the fungistatic nature of azole antifungals. The fungistatic activity of fluconazole not only hampers its efficacy in immunocompromised patient populations but may also contribute to the development of high-level azole resistance.

Azole resistance in *C. glabrata* has been associated with the upregulation of the ABC transporters, *CgCDR1* and *CgPDH1*. We have identified a transcriptional regulator of these transporters, CgPdr1p. Disruption of *CgPDR1* not only resulted in decreased expression of these transporters, but also resulted in azole hypersusceptibility in a laboratory-derived fluconazole resistant *C. glabrata* isolate (Figure 5-1). This phenotype was attributed to a previously identified putative gain-of-function mutation. We next identified novel gain-of-function mutations in fluconazole-susceptible and –resistant matched sets of clinical isolates. These mutations conferred different levels of fluconazole susceptibility. Through genome-wide profiling, we also identified a core set of commonly differentially-expressed genes as well as genes uniquely regulated by specific mutations.

We have also demonstrated that the transcriptional regulator, CgPdr1p, in *C. glabrata* not only influences the development of azole resistance but also influences intrinsic resistance to azoles as disruption of *CgPDR1* in a fluconazole-susceptible isolate rendered the strain hypersusceptible to fluconazole. However, disruption of *CgCDR1* had minimal effect on the fluconazole susceptibility in the same susceptible isolate suggesting other CgPdr1p targets contribute to intrinsic resistance. An understanding of the initial stress response to fluconazole may not only facilitate a better understanding of intrinsic resistance but also how this organism is able to survive at growth inhibiting concentrations of fluconazole (tolerance). As seen previously, our microarray data revealed that *C. glabrata* responds to the exposure to fluconazole by overexpressing *CgPDR1* and two of its known target genes, *CgCDR1* and *CgPDH1*, as well other putative CgPdr1p target genes. However, disruption of *CgPDR1* only decreased the expression of *CgCDR1* in response to fluconazole suggesting other transcriptional activators are able to regulate CgPdr1p target genes. Indeed, several other transcriptional regulatory pathways were also upregulated in response to fluconazole that have redundant Pdr1p targets in *S. cerevisiae*. It is possible that these transcriptional regulatory pathways also contribute to this phenotype that are independent of the regulation of CgPdr1p targets. The roles of these additional transcriptional regulators in azole tolerance are under investigation.

Figure 5-1. Mechanism of *CDR1* induction by Pdr1p. Fluconazole enters the cell by an unknown mechanism. It then inhibits lanosterol demethylase in the ergosterol biosynthesis pathway leading loss of normal sterols in the plasma membrane and a toxic metabolite. CgPdr1p is then activated either by gain-of-function mutations in CgPdr1p itself or by fluconazole directly binding to CgPdr1p thereby activating this transcriptional regulator by an unknown mechanism. CgPdr1p binds to the PDRE in the promoter of *CgCDR1* allowing for transcription of this gene and ultimately efflux of fluconazole out of the cell.

Two of the transcriptional regulators, CgPdr1p and CgUpc2p, identified in these studies belong to a class of fungal specific transcriptional regulators, the zinc-cluster proteins. Development of a drug that inhibit these transcription factors could be used in combination with the azoles that not only overcome intrinsic resistance but also make this class fungicidal which may also attenuate the development of azole resistance in *C. glabrata*. Furthermore, this information can possibly be used for the timely diagnosis of the more azole resistant *Candida* species such as *C. glabrata* and also possibly for identification of resistance mechanisms in this species. Currently the diagnosis of candidiasis relies on blood cultures or tissue biopsy specimens which may require 2-5 days for growth and identification. Furthermore, antifungal susceptibility testing requires another 48 hours for results. Therefore, development of molecular diagnostic tests that can not only quickly identify the *Candida* species, but also predict the susceptibility of the organism to an array of antifungals is needed. Early diagnosis and species identification leads to earlier initiation of optimal treatment which has been shown to improve clinical outcome (69). Clearly, further studies regarding the role of CgPdr1p hyperactive alleles and other azole-responsive transcription regulators on the downstream targets responsible for azole tolerance and development of high level resistance are needed. The hope is that such studies will lead to improved therapies and patient outcomes.

LIST OF REFERENCES

1. **Abramova, N. E., B. D. Cohen, O. Serta, R. Kapok, K. J. Davies, and C. V. Lowry.** 2001. Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in *Saccharomyces cerevisiae*. *Genetics* **157**:1169-1177.
2. **Agawam, A. K., P. D. Rogers, S. R. Baerson, M. R. Jacob, K. S. Barker, J. D. Cleary, L. A. Walker, D. G. Nagle, and A. M. Clark.** 2003. Genome-wide expression profiling of the response to polygene, pyramiding, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *J Boil Chem.* **278**:34998-35015.
3. **Akins, R. A.** 2005. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Med Mycale* **43**:285-318.
4. **Albert, T. J., J. Norton, M. Ott, T. Richmond, K. Nuwaysir, E. F. Nuwaysir, K. P. Stengele, and R. D. Green.** 2003. Light-directed 5'-->3' synthesis of complex oligonucleotide microarrays. *Nucleic Acids Res* **31**:e35.
5. **Ally, R., D. Schurmann, W. Kreisel, G. Carosi, K. Aguirrebengoa, B. Dupont, M. Hodges, P. Troke, and A. J. Romero.** 2001. A randomized, double-blind, double-dummy, multicenter trial of voriconazole and fluconazole in the treatment of esophageal candidiasis in immunocompromised patients. *Clin Infect Dis* **33**:1447-1454.
6. **Alvarez-Lerma, F., J. Nolla-Salas, C. Leon, M. Palomar, R. Jorda, N. Carrasco, and F. Bobillo.** 2003. Candiduria in critically ill patients admitted to intensive care medical units. *Intensive Care Med* **29**:1069-1076.
7. **Anderson, J., L. Cundiff, B. Schnars, M. X. Gao, I. Mackenzie, and D. R. Soll.** 1989. Hypha formation in the white-opaque transition of *Candida albicans*. *Infect Immun* **57**:458-467.
8. **Anderson, J. B., C. Sirjusingh, A. B. Parsons, C. Boone, C. Wickens, L. E. Cowen, and L. M. Kohn.** 2003. Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. *Genetics* **163**:1287-1298.
9. **Ang, B. S., A. Telenti, B. King, J. M. Steckelberg, and W. R. Wilson.** 1993. Candidemia from a urinary tract source: microbiological aspects and clinical significance. *Clin Infect Dis* **17**:662-666.
10. **Arndt, C. A., T. J. Walsh, C. L. McCully, F. M. Balis, P. A. Pizzo, and D. G. Poplack.** 1988. Fluconazole penetration into cerebrospinal fluid: implications for treating fungal infections of the central nervous system. *J Infect Dis* **157**:178-180.
11. **Aun, M. V., M. R. Ribeiro, C. L. Costa Garcia, R. C. Agondi, J. Kalil, and P. Giavina-Bianchi.** 2009. Esophageal candidiasis--an adverse effect of inhaled corticosteroids therapy. *J Asthma* **46**:399-401.
12. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Siedman, J. Smith, and K. Struhl.** 1997. *Current Protocols in Molecular Biology*. Wiley, New York.
13. **Bagshaw, S. M., and K. B. Laupland.** 2006. Epidemiology of intensive care unit-acquired urinary tract infections. *Curr Opin Infect Dis* **19**:67-71.

14. **Bai, Y. L., and G. B. Kohlhaw.** 1991. Manipulation of the 'zinc cluster' region of transcriptional activator LEU3 by site-directed mutagenesis. *Nucleic Acids Res* **19**:5991-5997.
15. **Balkis, M. M., S. D. Leidich, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Mechanisms of fungal resistance: an overview. *Drugs* **62**:1025-1040.
16. **Barns, S. M., D. J. Lane, M. L. Sogin, C. Bibeau, and W. G. Weisburg.** 1991. Evolutionary relationships among pathogenic *Candida* species and relatives. *J Bacteriol* **173**:2250-2255.
17. **Barone, J. A., B. L. Moskovitz, J. Guarnieri, A. E. Hassell, J. L. Colaizzi, R. H. Bierman, and L. Jessen.** 1998. Food interaction and steady-state pharmacokinetics of itraconazole oral solution in healthy volunteers. *Pharmacotherapy* **18**:295-301.
18. **Bartizal, K., C. J. Gill, G. K. Abruzzo, A. M. Flattery, L. Kong, P. M. Scott, J. G. Smith, C. E. Leighton, A. Bouffard, J. F. Dropinski, and J. Balkovec.** 1997. In vitro preclinical evaluation studies with the echinocandin antifungal MK-0991 (L-743,872). *Antimicrob Agents Chemother* **41**:2326-2332.
19. **Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin.** 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **21**:3329-3330.
20. **Ben-Ami, R., R. E. Lewis, and D. P. Kontoyiannis.** 2008. Immunocompromised hosts: immunopharmacology of modern antifungals. *Clin Infect Dis* **47**:226-235.
21. **Bennett, J. E., K. Izumikawa, and K. A. Marr.** 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob Agents Chemother* **48**:1773-1777.
22. **Birrell, G. W., J. A. Brown, H. I. Wu, G. Giaever, A. M. Chu, R. W. Davis, and J. M. Brown.** 2002. Transcriptional response of *Saccharomyces cerevisiae* to DNA-damaging agents does not identify the genes that protect against these agents. *Proc Natl Acad Sci U S A* **99**:8778-8783.
23. **Bockhorn, J., B. Balar, D. He, E. Seitomer, P. R. Copeland, and T. G. Kinzy.** 2008. Genome-wide screen of *Saccharomyces cerevisiae* null allele strains identifies genes involved in selenomethionine resistance. *Proc Natl Acad Sci U S A* **105**:17682-17687.
24. **Bodey, G. P., M. Mardani, H. A. Hanna, M. Boktour, J. Abbas, E. Girgawy, R. Y. Hachem, D. P. Kontoyiannis, and I. Raad.** 2002. The epidemiology of *Candida glabrata* and *Candida albicans* fungemia in immunocompromised patients with cancer. *Am J Med* **112**:380-385.
25. **Borst, A., M. T. Raimer, D. W. Warnock, C. J. Morrison, and B. A. Arthington-Skaggs.** 2005. Rapid acquisition of stable azole resistance by *Candida glabrata* isolates obtained before the clinical introduction of fluconazole. *Antimicrob Agents Chemother* **49**:783-787.
26. **Brammer, K. W., P. R. Farrow, and J. K. Faulkner.** 1990. Pharmacokinetics and tissue penetration of fluconazole in humans. *Rev Infect Dis* **12 Suppl 3**:S318-326.
27. **Branch, R. A.** 1988. Prevention of amphotericin B-induced renal impairment. A review on the use of sodium supplementation. *Arch Intern Med* **148**:2389-2394.

28. **Brockert, P. J., S. A. Lachke, T. Srikantha, C. Pujol, R. Galask, and D. R. Soll.** 2003. Phenotypic switching and mating type switching of *Candida glabrata* at sites of colonization. *Infect Immun* **71**:7109-7118.
29. **Broco, N., S. Tenreiro, C. A. Viegas, and I. Sa-Correia.** 1999. FLR1 gene (ORF YBR008c) is required for benomyl and methotrexate resistance in *Saccharomyces cerevisiae* and its benomyl-induced expression is dependent on pdr3 transcriptional regulator. *Yeast* **15**:1595-1608.
30. **Brun, S., F. Dalle, P. Saulnier, G. Renier, A. Bonnin, D. Chabasse, and J. P. Bouchara.** 2005. Biological consequences of petite mutations in *Candida glabrata*. *J Antimicrob Chemother* **56**:307-314.
31. **Budtz-Jorgensen, E., P. Mojon, A. Rentsch, and N. Deslauriers.** 2000. Effects of an oral health program on the occurrence of oral candidosis in a long-term care facility. *Community Dent Oral Epidemiol* **28**:141-149.
32. **Buscemi, L., A. Arechavala, and R. Negroni.** 2004. [Study of acute vulvovaginitis in sexually active adult women, with special reference to candidosis, in patients of the Francisco J. Muniz Infectious Diseases Hospital]. *Rev Iberoam Micol* **21**:177-181.
33. **Calcagno, A. M., E. Bignell, P. Warn, M. D. Jones, D. W. Denning, F. A. Muhlschlegel, T. R. Rogers, and K. Haynes.** 2003. *Candida glabrata* STE12 is required for wild-type levels of virulence and nitrogen starvation induced filamentation. *Mol Microbiol* **50**:1309-1318.
34. **Carvajal, E., H. B. van den Hazel, A. Cybularz-Kolaczowska, E. Balzi, and A. Goffeau.** 1997. Molecular and phenotypic characterization of yeast PDR1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. *Mol Gen Genet* **256**:406-415.
35. **Castano, I., S. J. Pan, M. Zupancic, C. Hennequin, B. Dujon, and B. P. Cormack.** 2005. Telomere length control and transcriptional regulation of subtelomeric adhesins in *Candida glabrata*. *Mol Microbiol* **55**:1246-1258.
36. **Chave, J. P., C. Durussel, M. P. Glauser, and J. Bille.** 1996. Asymptomatic oral yeast carriage in HIV-infected patients: frequency and fluconazole susceptibility profile. *Clin Microbiol Infect* **1**:249-252.
37. **Chen, K. H., T. Miyazaki, H. F. Tsai, and J. E. Bennett.** 2007. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*. *Gene* **386**:63-72.
38. **Chocarro Martinez, A., F. Galindo Tobal, G. Ruiz-Irastorza, A. Gonzalez Lopez, F. Alvarez Navia, C. Ochoa Sangrador, and M. I. Martin Arribas.** 2000. Risk factors for esophageal candidiasis. *Eur J Clin Microbiol Infect Dis* **19**:96-100.
39. **Clark, T. A., and R. A. Hajjeh.** 2002. Recent trends in the epidemiology of invasive mycoses. *Curr Opin Infect Dis* **15**:569-574.
40. **Cormack, B. P., N. Ghori, and S. Falkow.** 1999. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285**:578-582.

41. **Corsello, S., A. Spinillo, G. Osnengo, C. Penna, S. Guaschino, A. Beltrame, N. Blasi, and A. Festa.** 2003. An epidemiological survey of vulvovaginal candidiasis in Italy. *Eur J Obstet Gynecol Reprod Biol* **110**:66-72.
42. **Courtney, R., D. Wexler, E. Radwanski, J. Lim, and M. Laughlin.** 2004. Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. *Br J Clin Pharmacol* **57**:218-222.
43. **Cowen, L. E., and W. J. Steinbach.** 2008. Stress, drugs, and evolution: the role of cellular signaling in fungal drug resistance. *Eukaryot Cell* **7**:747-764.
44. **Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E. Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman.** 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J* **21**:546-559.
45. **Csank, C., and K. Haynes.** 2000. *Candida glabrata* displays pseudohyphal growth. *FEMS Microbiol Lett* **189**:115-120.
46. **Cui, Z., D. Hirata, and T. Miyakawa.** 1999. Functional analysis of the promoter of the yeast SNQ2 gene encoding a multidrug resistance transporter that confers the resistance to 4-nitroquinoline N-oxide. *Biosci Biotechnol Biochem* **63**:162-167.
47. **Cui, Z., D. Hirata, E. Tsuchiya, H. Osada, and T. Miyakawa.** 1996. The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. *J Biol Chem* **271**:14712-14716.
48. **de Wet, N., A. Llanos-Cuentas, J. Suleiman, E. Baraldi, E. F. Krantz, M. Della Negra, and H. Diekmann-Berndt.** 2004. A randomized, double-blind, parallel-group, dose-response study of micafungin compared with fluconazole for the treatment of esophageal candidiasis in HIV-positive patients. *Clin Infect Dis* **39**:842-849.
49. **Decottignies, A., A. M. Grant, J. W. Nichols, H. de Wet, D. B. McIntosh, and A. Goffeau.** 1998. ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. *J Biol Chem* **273**:12612-12622.
50. **Defontaine, A., J. P. Bouchara, P. Declerk, C. Planchenault, D. Chabasse, and J. N. Hallet.** 1999. In-vitro resistance to azoles associated with mitochondrial DNA deficiency in *Candida glabrata*. *J Med Microbiol* **48**:663-670.
51. **Defranoux, N., M. Gaisne, and J. Verdiere.** 1994. Functional analysis of the zinc cluster domain of the CYP1 (HAP1) complex regulator in heme-sufficient and heme-deficient yeast cells. *Mol Gen Genet* **242**:699-707.
52. **DeRisi, J., B. van den Hazel, P. Marc, E. Balzi, P. Brown, C. Jacq, and A. Goffeau.** 2000. Genome microarray analysis of transcriptional activation in multidrug resistance yeast mutants. *FEBS Lett* **470**:156-160.
53. **Devaux, F., P. Marc, C. Bouchoux, T. Delaveau, I. Hikkel, M. C. Potier, and C. Jacq.** 2001. An artificial transcription activator mimics the genome-wide properties of the yeast Pdr1 transcription factor. *EMBO Rep* **2**:493-498.
54. **Diamond, R. D.** 1991. The growing problem of mycoses in patients infected with the human immunodeficiency virus. *Rev Infect Dis* **13**:480-486.

55. **Diekema, D. J., S. A. Messer, A. B. Brueggemann, S. L. Coffman, G. V. Doern, L. A. Herwaldt, and M. A. Pfaller.** 2002. Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. *J Clin Microbiol* **40**:1298-1302.
56. **Dodou, E., and R. Treisman.** 1997. The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. *Mol Cell Biol* **17**:1848-1859.
57. **Dolan, J. W., A. C. Bell, B. Hube, M. Schaller, T. F. Warner, and E. Balish.** 2004. *Candida albicans* PLD I activity is required for full virulence. *Med Mycol* **42**:439-447.
58. **Domergue, R., I. Castano, A. De Las Penas, M. Zupancic, V. Lockatell, J. R. Hebel, D. Johnson, and B. P. Cormack.** 2005. Nicotinic acid limitation regulates silencing of *Candida* adhesins during UTI. *Science* **308**:866-870.
59. **Douglas, L. J.** 2003. *Candida* biofilms and their role in infection. *Trends Microbiol* **11**:30-36.
60. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. *Nature* **430**:35-44.
61. **Dunkel, N., T. T. Liu, K. S. Barker, R. Homayouni, J. Morschhauser, and P. D. Rogers.** 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryot Cell* **7**:1180-1190.
62. **Dupont, B., D. W. Denning, D. Marriott, A. Sugar, M. A. Viviani, and T. Sirisanthana.** 1994. Mycoses in AIDS patients. *J Med Vet Mycol* **32 Suppl 1**:65-77.
63. **Edlind, T. D., K. W. Henry, J. P. Vermitsky, M. P. Edlind, S. Raj, and S. K. Katiyar.** 2005. Promoter-dependent disruption of genes: simple, rapid, and specific PCR-based method with application to three different yeast. *Curr Genet* **48**:117-125.
64. **Espinel-Ingroff, A., F. Barchiesi, M. Cuenca-Estrella, M. A. Pfaller, M. Rinaldi, J. L. Rodriguez-Tudela, and P. E. Verweij.** 2005. International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. *J Clin Microbiol* **43**:3884-3889.

65. **Fardeau, V., G. Lelandais, A. Oldfield, H. Salin, S. Lemoine, M. Garcia, V. Tanty, S. Le Crom, C. Jacq, and F. Devaux.** 2007. The central role of PDR1 in the foundation of yeast drug resistance. *J Biol Chem* **282**:5063-5074.
66. **Feigal, D. W., M. H. Katz, D. Greenspan, J. Westenhause, W. Winkelstein, Jr., W. Lang, M. Samuel, S. P. Buchbinder, N. A. Hessel, A. R. Lifson, *et al.*** 1991. The prevalence of oral lesions in HIV-infected homosexual and bisexual men: three San Francisco epidemiological cohorts. *AIDS* **5**:519-525.
67. **Fernandes, L., C. Rodrigues-Pousada, and K. Struhl.** 1997. Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol Cell Biol* **17**:6982-6993.
68. **Ferrari, S., F. Ischer, D. Calabrese, B. Posteraro, M. Sanguinetti, G. Fadda, B. Rohde, C. Bauser, O. Bader, and D. Sanglard.** 2009. Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog* **5**:e1000268.
69. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin Infect Dis* **43**:25-31.
70. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11**:4241-4257.
71. **Gaur, M., D. Choudhury, and R. Prasad.** 2005. Complete inventory of ABC proteins in human pathogenic yeast, *Candida albicans*. *J Mol Microbiol Biotechnol* **9**:3-15.
72. **Gbelska, Y., J. J. Krijger, and K. D. Breunig.** 2006. Evolution of gene families: the multidrug resistance transporter genes in five related yeast species. *FEMS Yeast Res* **6**:345-355.
73. **Geiger, A. M., B. Foxman, and J. D. Sobel.** 1995. Chronic vulvovaginal candidiasis: characteristics of women with *Candida albicans*, *C. glabrata* and no candida. *Genitourin Med* **71**:304-307.
74. **Ghannoum, M. A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* **13**:122-143.
75. **Gietz, D., A. St Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**:1425.
76. **Goldman, M., G. A. Cloud, K. D. Wade, A. C. Reboli, C. J. Fichtenbaum, R. Hafner, J. D. Sobel, W. G. Powderly, T. F. Patterson, L. J. Wheat, D. K. Stein, W. E. Dismukes, and S. G. Filler.** 2005. A randomized study of the use of fluconazole in continuous versus episodic therapy in patients with advanced HIV infection and a history of oropharyngeal candidiasis: AIDS Clinical Trials Group Study 323/Mycoses Study Group Study 40. *Clin Infect Dis* **41**:1473-1480.
77. **Goswami, D., R. Goswami, U. Banerjee, V. Dadhwal, S. Miglani, A. A. Lattif, and N. Kochupillai.** 2006. Pattern of *Candida* species isolated from patients with diabetes mellitus and vulvovaginal candidiasis and their response to single dose oral fluconazole therapy. *J Infect* **52**:111-117.

78. **Grimoud, A. M., J. P. Lodter, N. Marty, S. Andrieu, H. Bocquet, M. D. Linas, M. Rumeau, and J. C. Cazard.** 2005. Improved oral hygiene and *Candida* species colonization level in geriatric patients. *Oral Dis* **11**:163-169.
79. **Grunstein, M.** 1997. Molecular model for telomeric heterochromatin in yeast. *Curr Opin Cell Biol* **9**:383-387.
80. **Guo, B., C. A. Styles, Q. Feng, and G. R. Fink.** 2000. A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc Natl Acad Sci U S A* **97**:12158-12163.
81. **Hallstrom, T. C., and W. S. Moye-Rowley.** 2000. Multiple signals from dysfunctional mitochondria activate the pleiotropic drug resistance pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **275**:37347-37356.
82. **Hanic-Joyce, P. J., and P. B. Joyce.** 1998. A high-copy-number ADE2-bearing plasmid for transformation of *Candida glabrata*. *Gene* **211**:395-400.
83. **Hazen, K. C.** 1995. New and emerging yeast pathogens. *Clin Microbiol Rev* **8**:462-478.
84. **He, X. J., and J. S. Fassler.** 2005. Identification of novel Yap1p and Skn7p binding sites involved in the oxidative stress response of *Saccharomyces cerevisiae*. *Mol Microbiol* **58**:1454-1467.
85. **Hellauer, K., B. Akache, S. MacPherson, E. Sirard, and B. Turcotte.** 2002. Zinc cluster protein Rdr1p is a transcriptional repressor of the PDR5 gene encoding a multidrug transporter. *J Biol Chem* **277**:17671-17676.
86. **Higgins, C. F.** 2001. ABC transporters: physiology, structure and mechanism--an overview. *Res Microbiol* **152**:205-210.
87. **Hikkel, I., A. Lucau-Danila, T. Delaveau, P. Marc, F. Devaux, and C. Jacq.** 2003. A general strategy to uncover transcription factor properties identifies a new regulator of drug resistance in yeast. *J Biol Chem* **278**:11427-11432.
88. **Hube, B., M. Monod, D. A. Schofield, A. J. Brown, and N. A. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* **14**:87-99.
89. **Hurley, R., and J. De Louvois.** 1979. *Candida* vaginitis. *Postgrad Med J* **55**:645-647.
90. **Izumikawa, K., H. Kakeya, H. F. Tsai, B. Grimberg, and J. E. Bennett.** 2003. Function of *Candida glabrata* ABC transporter gene, PDH1. *Yeast* **20**:249-261.
91. **Jansen, G., A. Y. Lee, E. Epp, A. Fredette, J. Surprenant, D. Marcus, M. Scott, E. Tan, T. Nishimura, M. Whiteway, M. Hallett, and D. Y. Thomas.** 2009. Chemogenomic profiling predicts antifungal synergies. *Mol Syst Biol* **5**:338.
92. **Jia, X. M., Z. P. Ma, Y. Jia, P. H. Gao, J. D. Zhang, Y. Wang, Y. G. Xu, L. Wang, Y. Y. Cao, Y. B. Cao, L. X. Zhang, and Y. Y. Jiang.** 2008. RTA2, a novel gene involved in azole resistance in *Candida albicans*. *Biochem Biophys Res Commun* **373**:631-636.
93. **Jung, U. S., and D. E. Levin.** 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol Microbiol* **34**:1049-1057.

94. **Kantarcioglu, A. S., and A. Yucel.** 2002. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses* **45**:160-165.
95. **Katzmann, D. J., T. C. Hallstrom, Y. Mahe, and W. S. Moye-Rowley.** 1996. Multiple Pdr1p/Pdr3p binding sites are essential for normal expression of the ATP binding cassette transporter protein-encoding gene PDR5. *J Biol Chem* **271**:23049-23054.
96. **Katzmann, D. J., T. C. Hallstrom, M. Voet, W. Wysock, J. Golin, G. Volckaert, and W. S. Moye-Rowley.** 1995. Expression of an ATP-binding cassette transporter-encoding gene (YOR1) is required for oligomycin resistance in *Saccharomyces cerevisiae*. *Mol Cell Biol* **15**:6875-6883.
97. **Kauffman, C. A., J. A. Vazquez, J. D. Sobel, H. A. Gallis, D. S. McKinsey, A. W. Karchmer, A. M. Sugar, P. K. Sharkey, G. J. Wise, R. Mangi, A. Mosher, J. Y. Lee, and W. E. Dismukes.** 2000. Prospective multicenter surveillance study of funguria in hospitalized patients. The National Institute for Allergy and Infectious Diseases (NIAID) Mycoses Study Group. *Clin Infect Dis* **30**:14-18.
98. **Kaur, R., I. Castano, and B. P. Cormack.** 2004. Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. *Antimicrob Agents Chemother* **48**:1600-1613.
99. **Kaur, R., R. Domergue, M. L. Zupancic, and B. P. Cormack.** 2005. A yeast by any other name: *Candida glabrata* and its interaction with the host. *Curr Opin Microbiol* **8**:378-384.
100. **Kitada, K., E. Yamaguchi, and M. Arisawa.** 1995. Cloning of the *Candida glabrata* TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation. *Gene* **165**:203-206.
101. **Kliemann, D. A., A. C. Pasqualotto, M. Falavigna, T. Giaretta, and L. C. Severo.** 2008. *Candida* esophagitis: species distribution and risk factors for infection. *Rev Inst Med Trop Sao Paulo* **50**:261-263.
102. **Klingspor, L., E. Tornqvist, A. Johansson, B. Petrini, U. Forsum, and G. Hedin.** 2004. A prospective epidemiological survey of candidaemia in Sweden. *Scand J Infect Dis* **36**:52-55.
103. **Kolaczowska, A., and A. Goffeau.** 1999. Regulation of pleiotropic drug resistance in yeast. *Drug Resist Updat* **2**:403-414.
104. **Kontoyiannis, D. P., B. T. Reddy, H. Hanna, G. P. Bodey, J. Tarrand, and I. Raad.** 2002. Breakthrough candidemia in patients with cancer differs from de novo candidemia in host factors and *Candida* species but not intensity. *Infect Control Hosp Epidemiol* **23**:542-545.
105. **Krause, D. S., A. E. Simjee, C. van Rensburg, J. Viljoen, T. J. Walsh, B. P. Goldstein, M. Wible, and T. Henkel.** 2004. A randomized, double-blind trial of anidulafungin versus fluconazole for the treatment of esophageal candidiasis. *Clin Infect Dis* **39**:770-775.
106. **Kumamoto, C. A., and M. D. Vences.** 2005. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell Microbiol* **7**:1546-1554.

107. **Kuse, E. R., P. Chetchotisakd, C. A. da Cunha, M. Ruhnke, C. Barrios, D. Raghunadharao, J. S. Sekhon, A. Freire, V. Ramasubramanian, I. Demeyer, M. Nucci, A. Leelarasamee, F. Jacobs, J. Decruyenaere, D. Pittet, A. J. Ullmann, L. Ostrosky-Zeichner, O. Lortholary, S. Koblinger, H. Diekmann-Berndt, and O. A. Cornely.** 2007. Micafungin versus liposomal amphotericin B for candidaemia and invasive candidosis: a phase III randomised double-blind trial. *Lancet* **369**:1519-1527.
108. **Kvaal, C., S. A. Lachke, T. Srikantha, K. Daniels, J. McCoy, and D. R. Soll.** 1999. Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. *Infect Immun* **67**:6652-6662.
109. **Kvaal, C. A., T. Srikantha, and D. R. Soll.** 1997. Misexpression of the white-phase-specific gene WH11 in the opaque phase of *Candida albicans* affects switching and virulence. *Infect Immun* **65**:4468-4475.
110. **Lachke, S. A., S. Joly, K. Daniels, and D. R. Soll.** 2002. Phenotypic switching and filamentation in *Candida glabrata*. *Microbiology* **148**:2661-2674.
111. **Lachke, S. A., S. R. Lockhart, K. J. Daniels, and D. R. Soll.** 2003. Skin facilitates *Candida albicans* mating. *Infect Immun* **71**:4970-4976.
112. **Lachke, S. A., T. Srikantha, L. K. Tsai, K. Daniels, and D. R. Soll.** 2000. Phenotypic switching in *Candida glabrata* involves phase-specific regulation of the metallothionein gene MT-II and the newly discovered hemolysin gene HLP. *Infect Immun* **68**:884-895.
113. **Landers, D. V., H. C. Wiesenfeld, R. P. Heine, M. A. Krohn, and S. L. Hillier.** 2004. Predictive value of the clinical diagnosis of lower genital tract infection in women. *Am J Obstet Gynecol* **190**:1004-1010.
114. **Larochelle, M., S. Drouin, F. Robert, and B. Turcotte.** 2006. Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Mol Cell Biol* **26**:6690-6701.
115. **Lee, M. W., B. J. Kim, H. K. Choi, M. J. Ryu, S. B. Kim, K. M. Kang, E. J. Cho, H. D. Youn, W. K. Huh, and S. T. Kim.** 2007. Global protein expression profiling of budding yeast in response to DNA damage. *Yeast* **24**:145-154.
116. **Levin, D. E.** 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **69**:262-291.
117. **Lewis, R. E.** 2009. Overview of the changing epidemiology of candidemia. *Curr Med Res Opin* **25**:1732-1740.
118. **Liu, T. T., R. E. Lee, K. S. Barker, L. Wei, R. Homayouni, and P. D. Rogers.** 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob Agents Chemother* **49**:2226-2236.
119. **Lorenz, M. C., R. S. Muir, E. Lim, J. McElver, S. C. Weber, and J. Heitman.** 1995. Gene disruption with PCR products in *Saccharomyces cerevisiae*. *Gene* **158**:113-117.
120. **MacPherson, S., M. Larochelle, and B. Turcotte.** 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. *Microbiol Mol Biol Rev* **70**:583-604.

121. **Maesaki, S., P. Marichal, M. A. Hossain, D. Sanglard, H. Vanden Bossche, and S. Kohno.** 1998. Synergic effects of tactolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. *J Antimicrob Chemother* **42**:747-753.
122. **Magill, S. S., C. Shields, C. L. Sears, M. Choti, and W. G. Merz.** 2006. Triazole cross-resistance among *Candida* spp.: case report, occurrence among bloodstream isolates, and implications for antifungal therapy. *J Clin Microbiol* **44**:529-535.
123. **Mamane, Y., K. Hellauer, M. H. Rochon, and B. Turcotte.** 1998. A linker region of the yeast zinc cluster protein *leu3p* specifies binding to everted repeat DNA. *J Biol Chem* **273**:18556-18561.
124. **Mannhaupt, G., R. Schnall, V. Karpov, I. Vetter, and H. Feldmann.** 1999. Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. *FEBS Lett* **450**:27-34.
125. **Manoharlal, R., N. A. Gaur, S. L. Panwar, J. Morschhauser, and R. Prasad.** 2008. Transcriptional activation and increased mRNA stability contribute to overexpression of CDR1 in azole-resistant *Candida albicans*. *Antimicrob Agents Chemother* **52**:1481-1492.
126. **Marchetti, O., J. M. Entenza, D. Sanglard, J. Bille, M. P. Glauser, and P. Moreillon.** 2000. Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans*. *Antimicrob Agents Chemother* **44**:2932-2938.
127. **Marchetti, O., P. Moreillon, M. P. Glauser, J. Bille, and D. Sanglard.** 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob Agents Chemother* **44**:2373-2381.
128. **Marchler-Bauer, A., and S. H. Bryant.** 2004. CD-Search: protein domain annotations on the fly. *Nucleic Acids Res* **32**:W327-331.
129. **Marcos-Arias, C., E. Eraso, L. Madariaga, J. M. Aguirre, and G. Quindos.** 2009. Phospholipase and proteinase activities of *Candida* isolates from denture wearers. *Mycoses*. Epub ahead of print.
130. **Marichal, P., H. Vanden Bossche, F. C. Odds, G. Nobels, D. W. Warnock, V. Timmerman, C. Van Broeckhoven, S. Fay, and P. Mose-Larsen.** 1997. Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob Agents Chemother* **41**:2229-2237.
131. **Martin, G. S., D. M. Mannino, S. Eaton, and M. Moss.** 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* **348**:1546-1554.
132. **Mehra, R. K., J. L. Thorvaldsen, I. G. Macreadie, and D. R. Winge.** 1992. Cloning system for *Candida glabrata* using elements from the metallothionein-IIa-encoding gene that confer autonomous replication. *Gene* **113**:119-124.
133. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110**:293-302.

134. **Millon, L., A. Manteaux, G. Reboux, C. Drobacheff, M. Monod, T. Barale, and Y. Michel-Briand.** 1994. Fluconazole-resistant recurrent oral candidiasis in human immunodeficiency virus-positive patients: persistence of *Candida albicans* strains with the same genotype. *J Clin Microbiol* **32**:1115-1118.
135. **Miyazaki, H., Y. Miyazaki, A. Geber, T. Parkinson, C. Hitchcock, D. J. Falconer, D. J. Ward, K. Marsden, and J. E. Bennett.** 1998. Fluconazole resistance associated with drug efflux and increased transcription of a drug transporter gene, PDH1, in *Candida glabrata*. *Antimicrob Agents Chemother* **42**:1695-1701.
136. **Miyazaki, T., S. Yamauchi, T. Inamine, Y. Nagayoshi, T. Saijo, K. Izumikawa, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, Y. Miyazaki, and S. Kohno.** 2010. Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*. *Antimicrob Agents Chemother* **54**:1639-43.
137. **Monteiro, P. T., N. D. Mendes, M. C. Teixeira, S. d'Orey, S. Tenreiro, N. P. Mira, H. Pais, A. P. Francisco, A. M. Carvalho, A. B. Lourenco, I. Sa-Correia, A. L. Oliveira, and A. T. Freitas.** 2008. YEASTRACT-DISCOVERER: new tools to improve the analysis of transcriptional regulatory associations in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **36**:D132-136.
138. **Moore, R. D., and R. E. Chaisson.** 1996. Natural history of opportunistic disease in an HIV-infected urban clinical cohort. *Ann Intern Med* **124**:633-642.
139. **Mora-Duarte, J., R. Betts, C. Rotstein, A. L. Colombo, L. Thompson-Moya, J. Smietana, R. Lupinacci, C. Sable, N. Kartsonis, and J. Perfect.** 2002. Comparison of caspofungin and amphotericin B for invasive candidiasis. *N Engl J Med* **347**:2020-2029.
140. **Morrow, B., T. Srikantha, and D. R. Soll.** 1992. Transcription of the gene for a pepsinogen, PEP1, is regulated by white-opaque switching in *Candida albicans*. *Mol Cell Biol* **12**:2997-3005.
141. **Moye-Rowley, W. S.** 2003. Transcriptional control of multidrug resistance in the yeast *Saccharomyces*. *Prog Nucleic Acid Res Mol Biol* **73**:251-279.
142. **Mukherjee, P. K., K. R. Seshan, S. D. Leidich, J. Chandra, G. T. Cole, and M. A. Ghannoum.** 2001. Reintroduction of the PLB1 gene into *Candida albicans* restores virulence in vivo. *Microbiology* **147**:2585-2597.
143. **Mullaoglu, S., H. Turktas, N. Kokturk, C. Tuncer, A. Kalkanci, and S. Kustimur.** 2007. Esophageal candidiasis and *Candida* colonization in asthma patients on inhaled steroids. *Allergy Asthma Proc* **28**:544-549.
144. **Nagiec, M. M., M. Skrzypek, E. E. Nagiec, R. L. Lester, and R. C. Dickson.** 1998. The LCB4 (YOR171c) and LCB5 (YLR260w) genes of *Saccharomyces* encode sphingoid long chain base kinases. *J Biol Chem* **273**:19437-19442.
145. **National Committee for Clinical Laboratory Standards.** 2002. Reference method of broth dilution antifungal susceptibility testin of yeast; approved standard, M27-A2. National Committee for Clinical Laboratory Standards, Wayne, PA.
146. **National Committee for Clinical Laboratory Standards.** 2004. Methods for antifungal disk diffusion susceptibility testing in yeasts: approved guideline, M44-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.

147. **Nawrot, U., J. Nowicka, K. Juszczak, and B. Gusin.** 2005. Susceptibility to antifungal agents of *Candida* species isolated from paediatric and adult patients with haematological diseases. *Mycoses* **48**:385-390.
148. **Newmann, G., and U. Kaben.** 1975. [Blastomycoid flora of the urogenital tract in nonpregnant and pregnant patients]. *Zentralbl Gynakol* **97**:372-378.
149. **Nguyen, D. T., A. M. Alarco, and M. Raymond.** 2001. Multiple Yap1p-binding sites mediate induction of the yeast major facilitator FLR1 gene in response to drugs, oxidants, and alkylating agents. *J Biol Chem* **276**:1138-1145.
150. **Nikawa, H., H. Egusa, S. Makihira, M. Nishimura, K. Ishida, M. Furukawa, and T. Hamada.** 2003. A novel technique to evaluate the adhesion of *Candida* species to gingival epithelial cells. *Mycoses* **46**:384-389.
151. **Odds, F. C.** 1988. *Ecology and Epidemiology of Candidiasis*. University Park Press, Baltimore, MD.
152. **Odds, F. C., M. G. Rinaldi, C. R. Cooper, Jr., A. Fothergill, L. Pasarell, and M. R. McGinnis.** 1997. *Candida* and *Torulopsis*: a blinded evaluation of use of pseudohypha formation as basis for identification of medically important yeasts. *J Clin Microbiol* **35**:313-316.
153. **Okungbowa, F. I., O. S. Isikhuemhen, and A. P. Dede.** 2003. The distribution frequency of *Candida* species in the genitourinary tract among symptomatic individuals in Nigerian cities. *Rev Iberoam Micol* **20**:60-63.
154. **Onyewu, C., J. R. Blankenship, M. Del Poeta, and J. Heitman.** 2003. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. *Antimicrob Agents Chemother* **47**:956-964.
155. **Ortiz, O., and W. J. Lee.** 1989. Percutaneous nephrostomy in the management of renal candidiasis. *Arch Surg* **124**:739-740.
156. **Ostrosky-Zeichner, L., J. H. Rex, P. G. Pappas, R. J. Hamill, R. A. Larsen, H. W. Horowitz, W. G. Powderly, N. Hyslop, C. A. Kauffman, J. Cleary, J. E. Mangino, and J. Lee.** 2003. Antifungal susceptibility survey of 2,000 bloodstream *Candida* isolates in the United States. *Antimicrob Agents Chemother* **47**:3149-3154.
157. **Owsianik, G., L. Balzi I, and M. Ghislain.** 2002. Control of 26S proteasome expression by transcription factors regulating multidrug resistance in *Saccharomyces cerevisiae*. *Mol Microbiol* **43**:1295-1308.
158. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **48**:503-535.
159. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, and W. E. Dismukes.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis* **37**:634-643.

160. **Pappas, P. G., C. M. Rotstein, R. F. Betts, M. Nucci, D. Talwar, J. J. De Waele, J. A. Vazquez, B. F. Dupont, D. L. Horn, L. Ostrosky-Zeichner, A. C. Reboli, B. Suh, R. Digumarti, C. Wu, L. L. Kovanda, L. J. Arnold, and D. N. Buell.** 2007. Micafungin versus caspofungin for treatment of candidemia and other forms of invasive candidiasis. *Clin Infect Dis* **45**:883-893.
161. **Parks, L. W., and W. M. Casey.** 1995. Physiological implications of sterol biosynthesis in yeast. *Annu Rev Microbiol* **49**:95-116.
162. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* **45**:321-346.
163. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2008. In vitro susceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. *J Clin Microbiol* **46**:150-156.
164. **Pfaller, M. A., and D. J. Diekema.** 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J Clin Microbiol* **42**:4419-4431.
165. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**:133-163.
166. **Pfaller, M. A., D. J. Diekema, and D. J. Sheehan.** 2006. Interpretive breakpoints for fluconazole and *Candida* revisited: a blueprint for the future of antifungal susceptibility testing. *Clin Microbiol Rev* **19**:435-447.
167. **Pfaller, M. A., S. A. Messer, L. Boyken, R. J. Hollis, C. Rice, S. Tendolkar, and D. J. Diekema.** 2004. In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn Microbiol Infect Dis* **48**:201-205.
168. **Pfaller, M. A., S. A. Messer, R. J. Hollis, R. N. Jones, G. V. Doern, M. E. Brandt, and R. A. Hajjeh.** 1999. Trends in species distribution and susceptibility to fluconazole among blood stream isolates of *Candida* species in the United States. *Diagn Microbiol Infect Dis* **33**:217-222.
169. **Pfeifer, K., K. S. Kim, S. Kogan, and L. Guarente.** 1989. Functional dissection and sequence of yeast HAP1 activator. *Cell* **56**:291-301.
170. **Piper, P., Y. Mahe, S. Thompson, R. Pandjaitan, C. Holyoak, R. Egner, M. Muhlbauer, P. Coote, and K. Kuchler.** 1998. The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J* **17**:4257-4265.
171. **Polakova, S., C. Blume, J. A. Zarate, M. Mentel, D. Jorck-Ramberg, J. Stenderup, and J. Piskur.** 2009. Formation of new chromosomes as a virulence mechanism in yeast *Candida glabrata*. *Proc Natl Acad Sci U S A* **106**:2688-2693.
172. **Pryde, F. E., and E. J. Louis.** 1999. Limitations of silencing at native yeast telomeres. *EMBO J* **18**:2538-2550.
173. **Reboli, A. C., C. Rotstein, P. G. Pappas, S. W. Chapman, D. H. Kett, D. Kumar, R. Betts, M. Wible, B. P. Goldstein, J. Schranz, D. S. Krause, and T. J. Walsh.** 2007. Anidulafungin versus fluconazole for invasive candidiasis. *N Engl J Med* **356**:2472-2482.

174. **Redding, S. W., M. C. Dahiya, W. R. Kirkpatrick, B. J. Coco, T. F. Patterson, A. W. Fothergill, M. G. Rinaldi, and C. R. Thomas, Jr.** 2004. *Candida glabrata* is an emerging cause of oropharyngeal candidiasis in patients receiving radiation for head and neck cancer. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **97**:47-52.
175. **Redding, S. W., W. R. Kirkpatrick, S. Saville, B. J. Coco, W. White, A. Fothergill, M. Rinaldi, T. Eng, T. F. Patterson, and J. Lopez-Ribot.** 2003. Multiple patterns of resistance to fluconazole in *Candida glabrata* isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. *J Clin Microbiol* **41**:619-622.
176. **Reuss, O., A. Vik, R. Kolter, and J. Morschhauser.** 2004. The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341**:119-127.
177. **Revankar, S. G., O. P. Dib, W. R. Kirkpatrick, R. K. McAtee, A. W. Fothergill, M. G. Rinaldi, S. W. Redding, and T. F. Patterson.** 1998. Clinical evaluation and microbiology of oropharyngeal infection due to fluconazole-resistant *Candida* in human immunodeficiency virus-infected patients. *Clin Infect Dis* **26**:960-963.
178. **Rex, J. H., M. A. Pfaller, T. J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M. A. Ghannoum, L. L. Gosey, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, and D. W. Warnock.** 2001. Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microbiol Rev* **14**:643-658.
179. **Richter, S. S., R. P. Galask, S. A. Messer, R. J. Hollis, D. J. Diekema, and M. A. Pfaller.** 2005. Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* **43**:2155-2162.
180. **Rodrigues-Pousada, C. A., T. Nevitt, R. Menezes, D. Azevedo, J. Pereira, and C. Amaral.** 2004. Yeast activator proteins and stress response: an overview. *FEBS Lett* **567**:80-85.
181. **Rogers, P. D., J. P. Vermitsky, T. D. Edlind, and G. M. Hilliard.** 2006. Proteomic analysis of experimentally induced azole resistance in *Candida glabrata*. *J Antimicrob Chemother* **58**:434-438.
182. **Safdar, A., D. Armstrong, E. W. Cross, and D. S. Perlin.** 2002. Prospective epidemiologic analysis of triazole-resistant nosocomial *Candida glabrata* isolated from patients at a comprehensive cancer center. *Int J Infect Dis* **6**:198-201.
183. **Safdar, A., V. Chaturvedi, E. W. Cross, S. Park, E. M. Bernard, D. Armstrong, and D. S. Perlin.** 2001. Prospective study of *Candida* species in patients at a comprehensive cancer center. *Antimicrob Agents Chemother* **45**:2129-2133.
184. **Sanglard, D., F. Ischer, and J. Bille.** 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. *Antimicrob Agents Chemother* **45**:1174-1183.
185. **Sanglard, D., F. Ischer, D. Calabrese, P. A. Majcherczyk, and J. Bille.** 1999. The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob Agents Chemother* **43**:2753-2765.

186. **Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille.** 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol Microbiol* **48**:959-976.
187. **Sanguinetti, M., B. Posteraro, B. Fiori, S. Ranno, R. Torelli, and G. Fadda.** 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob Agents Chemother* **49**:668-679.
188. **Schaller, M., C. Borelli, H. C. Korting, and B. Hube.** 2005. Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**:365-377.
189. **Schjerling, P., and S. Holmberg.** 1996. Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. *Nucleic Acids Res* **24**:4599-4607.
190. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. *Nucleic Acids Res* **18**:3091-3092.
191. **Schuman, P., J. D. Sobel, S. E. Ohmit, K. H. Mayer, C. C. Carpenter, A. Rompalo, A. Duerr, D. K. Smith, D. Warren, and R. S. Klein.** 1998. Mucosal candidal colonization and candidiasis in women with or at risk for human immunodeficiency virus infection. HIV Epidemiology Research Study (HERS) Group. *Clin Infect Dis* **27**:1161-1167.
192. **Scully, C., M. el-Kabir, and L. P. Samaranayake.** 1994. *Candida* and oral candidosis: a review. *Crit Rev Oral Biol Med* **5**:125-157.
193. **Shahi, P., K. Gulshan, and W. S. Moye-Rowley.** 2007. Negative transcriptional regulation of multidrug resistance gene expression by an Hsp70 protein. *J Biol Chem* **282**:26822-26831.
194. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J Biol Chem* **279**:30480-30489.
195. **Smith, W. L., and T. D. Edlind.** 2002. Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in CDR and ERG upregulation. *Antimicrob Agents Chemother* **46**:3532-3539.
196. **Sobel, J. D.** 1985. Epidemiology and pathogenesis of recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol* **152**:924-935.
197. **Sobel, J. D.** 1986. Recurrent vulvovaginal candidiasis. A prospective study of the efficacy of maintenance ketoconazole therapy. *N Engl J Med* **315**:1455-1458.
198. **Sobel, J. D., P. S. Kapernick, M. Zervos, B. D. Reed, T. Hooton, D. Soper, P. Nyirjesy, M. W. Heine, J. Willems, H. Panzer, and H. Wittes.** 2001. Treatment of complicated *Candida* vaginitis: comparison of single and sequential doses of fluconazole. *Am J Obstet Gynecol* **185**:363-369.
199. **Sobel, J. D., C. A. Kauffman, D. McKinsey, M. Zervos, J. A. Vazquez, A. W. Karchmer, J. Lee, C. Thomas, H. Panzer, and W. E. Dismukes.** 2000. Candiduria: a randomized, double-blind study of treatment with fluconazole and placebo. The National Institute of Allergy and Infectious Diseases (NIAID) Mycoses Study Group. *Clin Infect Dis* **30**:19-24.

200. **Soll, D. R.** 1997. Gene regulation during high-frequency switching in *Candida albicans*. *Microbiology* **143** (Pt 2):279-288.
201. **Soustre, I., Y. Letourneux, and F. Karst.** 1996. Characterization of the *Saccharomyces cerevisiae* RTA1 gene involved in 7-amincholesterol resistance. *Curr Genet* **30**:121-125.
202. **Srikantha, T., S. A. Lachke, and D. R. Soll.** 2003. Three mating type-like loci in *Candida glabrata*. *Eukaryot Cell* **2**:328-340.
203. **St-Germain, G., M. Laverdiere, R. Pelletier, A. M. Bourgault, M. Libman, C. Lemieux, and G. Noel.** 2001. Prevalence and antifungal susceptibility of 442 *Candida* isolates from blood and other normally sterile sites: results of a 2-year (1996 to 1998) multicenter surveillance study in Quebec, Canada. *J Clin Microbiol* **39**:949-953.
204. **Staab, J. F., and P. Sundstrom.** 2003. URA3 as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. *Trends Microbiol* **11**:69-73.
205. **Sundstrom, P.** 2006. *Molecular Principles of Fungal Pathogenesis*. Columbia University College of Physicians and Surgeons, New York.
206. **Teixeira, M. C., P. Monteiro, P. Jain, S. Tenreiro, A. R. Fernandes, N. P. Mira, M. Alenquer, A. T. Freitas, A. L. Oliveira, and I. Sa-Correia.** 2006. The YEASTRACT database: a tool for the analysis of transcription regulatory associations in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **34**:D446-451.
207. **Thakur, J. K., H. Arthanari, F. Yang, S. J. Pan, X. Fan, J. Breger, D. P. Frueh, K. Gulshan, D. K. Li, E. Mylonakis, K. Struhl, W. S. Moye-Rowley, B. P. Cormack, G. Wagner, and A. M. Naar.** 2008. A nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature* **452**:604-609.
208. **Thompson, G. R., 3rd, P. K. Patel, W. R. Kirkpatrick, S. D. Westbrook, D. Berg, J. Erlandsen, S. W. Redding, and T. F. Patterson.** Oropharyngeal candidiasis in the era of antiretroviral therapy. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **109**: 488-495.
209. **Thompson, G. R., 3rd, N. P. Wiederhold, A. C. Vallor, N. C. Villareal, J. S. Lewis, 2nd, and T. F. Patterson.** 2008. Development of caspofungin resistance following prolonged therapy for invasive candidiasis secondary to *Candida glabrata* infection. *Antimicrob Agents Chemother* **52**:3783-3785.
210. **Torelli, R., B. Posteraro, S. Ferrari, M. La Sorda, G. Fadda, D. Sanglard, and M. Sanguinetti.** 2008. The ATP-binding cassette transporter-encoding gene CgSNQ2 is contributing to the CgPDR1-dependent azole resistance of *Candida glabrata*. *Mol Microbiol* **68**:186-201.
211. **Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, and R. P. Gaynes.** 2002. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. *Clin Infect Dis* **35**:627-630.
212. **Tsai, H. F., A. A. Krol, K. E. Sarti, and J. E. Bennett.** 2006. *Candida glabrata* PDR1, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. *Antimicrob Agents Chemother* **50**:1384-1392.

213. **Turi, T. G., and J. C. Loper.** 1992. Multiple regulatory elements control expression of the gene encoding the *Saccharomyces cerevisiae* cytochrome P450, lanosterol 14 alpha-demethylase (ERG11). *J Biol Chem* **267**:2046-2056.
214. **Varma, A., J. C. Edman, and K. J. Kwon-Chung.** 1992. Molecular and genetic analysis of URA5 transformants of *Cryptococcus neoformans*. *Infect Immun* **60**:1101-1108.
215. **Vermitsky, J. P., K. D. Earhart, W. L. Smith, R. Homayouni, T. D. Edlind, and P. D. Rogers.** 2006. Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Mol Microbiol* **61**:704-722.
216. **Vermitsky, J. P., and T. D. Edlind.** 2004. Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. *Antimicrob Agents Chemother* **48**:3773-3781.
217. **Vik, A., and J. Rine.** 2001. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol Cell Biol* **21**:6395-6405.
218. **Villanueva, A., E. G. Arathoon, E. Gotuzzo, R. S. Berman, M. J. DiNubile, and C. A. Sable.** 2001. A randomized double-blind study of caspofungin versus amphotericin for the treatment of candidal esophagitis. *Clin Infect Dis* **33**:1529-1535.
219. **Villanueva, A., E. Gotuzzo, E. G. Arathoon, L. M. Noriega, N. A. Kartsonis, R. J. Lupinacci, J. M. Smietana, M. J. DiNubile, and C. A. Sable.** 2002. A randomized double-blind study of caspofungin versus fluconazole for the treatment of esophageal candidiasis. *Am J Med* **113**:294-299.
220. **Viscoli, C., C. Girmenia, A. Marinus, L. Collette, P. Martino, B. Vandercam, C. Doyen, B. Lebeau, D. Spence, V. Krcmery, B. De Pauw, and F. Meunier.** 1999. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). *Clin Infect Dis* **28**:1071-1079.
221. **Wada, S., M. Niimi, K. Niimi, A. R. Holmes, B. C. Monk, R. D. Cannon, and Y. Uehara.** 2002. *Candida glabrata* ATP-binding cassette transporters Cdr1p and Pdh1p expressed in a *Saccharomyces cerevisiae* strain deficient in membrane transporters show phosphorylation-dependent pumping properties. *J Biol Chem* **277**:46809-46821.
222. **Wang, E. J., K. Lew, C. N. Casciano, R. P. Clement, and W. W. Johnson.** 2002. Interaction of common azole antifungals with P glycoprotein. *Antimicrob Agents Chemother* **46**:160-165.
223. **White, T. C., S. H. Miyasaki, and N. Agabian.** 1993. Three distinct secreted aspartyl proteinases in *Candida albicans*. *J Bacteriol* **175**:6126-6133.
224. **Wilcox, L. J., D. A. Balderes, B. Wharton, A. H. Tinkelenberg, G. Rao, and S. L. Sturley.** 2002. Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. *J Biol Chem* **277**:32466-32472.
225. **Willins, D. A., G. H. Shimer, Jr., and G. Cottarel.** 2002. A system for deletion and complementation of *Candida glabrata* genes amenable to high-throughput application. *Gene* **292**:141-149.

- 226. **Wong, S., G. Butler, and K. H. Wolfe.** 2002. Gene order evolution and paleopolyploidy in hemiascomycete yeasts. *Proc Natl Acad Sci U S A* **99**:9272-9277.
- 227. **Yoshimoto, H., K. Saltsman, A. P. Gasch, H. X. Li, N. Ogawa, D. Botstein, P. O. Brown, and M. S. Cyert.** 2002. Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **277**:31079-31088.
- 228. **Zhang, X., Z. Cui, T. Miyakawa, and W. S. Moye-Rowley.** 2001. Cross-talk between transcriptional regulators of multidrug resistance in *Saccharomyces cerevisiae*. *J Biol Chem* **276**:8812-8819.
- 229. **Zhao, C., U. S. Jung, P. Garrett-Engele, T. Roe, M. S. Cyert, and D. E. Levin.** 1998. Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. *Mol Cell Biol* **18**:1013-1022.
- 230. **Zhou, P., M. S. Szczypka, R. Young, and D. J. Thiele.** 1994. A system for gene cloning and manipulation in the yeast *Candida glabrata*. *Gene* **142**:135-140.

VITA

Kelly E. Caudle, daughter of Gary and Virginia Earhart, was born in San Diego, California on June 24, 1978. She moved to Germantown, Tennessee in 1980 and enrolled in the Shelby County School District. After graduating from Germantown High School in 1996, she attended Middle Tennessee State University in Murfreesboro, Tennessee, graduating with honors with a Bachelors of Science in Chemistry in 2000. In 2002, she enrolled in the Pharm.D./Ph.D. dual degree program at The University of Tennessee Health Sciences Center in Memphis, Tennessee. After graduating with a Doctor of Pharmacy in 2006, she took a formal leave from the program to complete a Residency in Pediatric Pharmacy Practice at LeBonheur Children's Medical Center. In 2007, she returned to the graduate program and anticipates completing her Doctor of Philosophy in May 2010.