DEVELOPMENT OF RESISTANCE TO ANTIFUNGAL AGENTS IN COMMON YEAST SPECIES

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COMMON YEAST SPECIES

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ABSTRACT

Pathogenic fungi are responsible for several serious diseases in both plants and animals with severe consequences in both medicine and agriculture. This makes treatment of fungal infections a prime concern for both economic and humanitarian concerns. Treatment for pathogenic fungus infections is hindered by the lack of development of new drugs that inhibit growth of fungi, as well as the mechanisms by which fungi develop resistance to existing drugs.

The development of new antifungal drugs is slow and requires a great deal of testing to ensure the drug is both safe and effective. It is therefore important that current drugs are used in a manner to prevent the development of resistance phenotypes. The growing number of pathogenic yeast infections has resulted in the greater use of antifungal drugs in patient treatment. With this increase in use, several strains have arisen with resistance to commonly used antifungal agents such as azoles and polyenes. Therefore, the characterization and study of mechanisms of drug resistance is of the utmost concern. The purpose of this study was to characterize how several species respond to drug challenge as well as the mechanisms guiding their responses.

ABC transporters are known for increasing drug resistance phenotype through increased efflux of drugs from the cell. In this thesis, two studies have been performed to examine the ways in which the expression of ABC transporters may lead to drug resistance. First, a commercially-available *Saccharomyces cerevisiae* strain exhibiting an azole resistance phenotype was determined to possess an overexpression of *PDR5*. Subsequently that strain was deleted of the efflux pump *PDR5* and examined for the loss of the azole resistance phenotype. The resulting deletion strain exhibited a decrease in all drugs but retained some resistance to fluconazole and ketoconazole.

Second, several putative ABC efflux transporters from *Aspergillus fumigatus* (AF) were heterologously expressed in a hyper-susceptible *S. cerevisiae* strain. The resulting strains exhibited differences in substrate specificity efflux that may shape the treatment of AF strains that express these transporters. Third, the further study of pathogenic yeasts was facilitated by the development of a table of Minimum Inhibitory Concentration (MIC) values for several common species against a variety of drugs and media. This table was created with the intent of provide basal MIC values in response to common environmental conditions to reduce time needed to establish testing conditions and basic growth conditions for the listed species.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Biological Sciences have examined a dissertation titled “Development of Resistance to Antifungal Agents in Common Yeast Species,” presented by Jacob Parker Shreeve, candidate for the Master of Science degree, and certify that in their opinion it is worthy of acceptance.

Supervisory Committee

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CHAPTER 1

THE ROLE OF *PDR5* IN THE DEVELOPMENT OF SC0000 AZOLE RESISTANCE

Introduction

Human infection by opportunistic fungal pathogens such as *Candida*, *Aspergillus*, and *Cryptococcus*, are on the rise (Brown et al., 2012; White et al., 1998). Patients receiving organ transplants or chemotherapy, and other vulnerable populations are increasing in number with a corresponding increase in infections associated with a fungal opportunistic pathogen. Symptoms of these infections are typically mild but may progress to a life-threatening systemic infection in immunocompromised patients. Common opportunistic pathogens cause hundreds of thousands of systemic infections with ~400,000 and ~200,000 cases per year attributed to *Candida* and *Aspergillus* species respectively (Brown et al., 2012). The most common treatment for these kinds of infections are drugs from the azole family. These compounds inhibit ergosterol biosynthesis, the most common sterol in yeast cell membranes, by targeting enzymes in the ergosterol biosynthesis pathway. The most common target is the *ERG11* gene product. This enzyme, a cytochrome p450 enzyme known as lanosterol 14α-demethylase, catalyzes the C14 demethylation of lanosterol, an important step in the biosynthesis of ergosterol. These drugs have proven to be safe and effective for the treatment of most opportunistic fungal pathogens. Therefore, the emergence of strains and species with resistance to azoles must be studied to prevent the development of multiple azole resistant strains.

Other common non-azole antifungals are used regularly in research and medical practice (Table 2). Polyenes such as amphotericin B (AMB) bind to ergosterol and increase leakage of ions and small molecules. Echinocandins such as caspofungin (CSG) interfere with 1, 3 beta-glucan synthase and inhibit cell wall formation. 5-flucytosine (5FC) inhibits DNA synthesis and

cycloheximide (CH) inhibits protein synthesis. The use of drugs which target many different pathways is a crucial tool to a medical practitioner in the treatment of patients with strains resistant to certain classes of drug. The full list of examined drugs and abbreviation is available in Table 2.

Multiple mechanisms have been previously described for the development of azole resistant strains. Overexpression of *ERG11,* and *ERG11* mutations associated with non-synonymous changes to binding sites have been shown to confer resistance to azole exposure (Flowers et al., 2015; MacPherson et al., 2005; Ming-Jie et al., 2013). Inactivation of the *Erg3* gene prevents the production of the toxic sterol 14α-methylergosta-8,24(28)-dien-3β,6α-diol from methylated lanosterol. This *ERG3* mutation prevents buildup of toxic byproducts associated with cells in which *ERG11* is nonfunctional (Morio et al., 2012). Finally, the overexpression of drug efflux transporters belonging to families such as ATP Binding Cassette transporters (ABC Transporters) and Major Facilitator transporters (MFS Transporters) can actively transport drug molecules out of the cell, decreasing intracellular drug concentration (Prasad et al., 2015).

Many of these efflux transporters include azoles among their target substrates. Of note is the multidrug transporter *PDR5* in *S. cerevisiae*. *PDR5* is a member of the ABC transporter drug efflux pump super family. This family is ubiquitous across all forms of life (Jones and George, 2004). ABC Transporters are characterized by a varying number of conserved transmembrane domains and nucleotide binding domains. Members of this family are involved in a diverse array of biological processes with a similarly broad range of target substrates. This particular transporter is implicated in the successful maintenance of the exponential growth state as well as resistance to many common antifungal agents including azoles (M. et al., 2004; Miranda et al., 2010). It is therefore a gene of interest when considering the development of resistant strains in yeast. This work *in S. cerevisiae* sought to determine the extent to which changes in *PDR5* expression could establish a drug resistant phenotype.

PDR5 was selected for further study based upon previous work by a lab member who sought to determine drug susceptibilities of multiple commercially available *S. cerevisiae* strains (Table 1). One of these strains, SC0000, was found to have an azole-specific drug resistance phenotype (Figure 4). Exploration of the mechanism behind this resistance phenotype could provide insight into how other yeast strains may produce equivalent resistance phenotypes. Each of four commercially-available strains were compared at the genotypic and phenotypic level to identify potential causal agents of the resistance phenotype. qRT-PCR of these strains established that SC0000 greatly overexpresses *PDR5* relative to its parent strain W303. The genetic relationships of the four strains is shown in Figure 1. Thus, *PDR5* was chosen for further study with regard to the azole resistance phenotype noted in SC0000.

Table 1: Genotypes of Examined Strains

S288C	MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6
CKY8	MATα ura3-52 leu2-3,112
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0
W303	MATa/MATα {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15} [phi+]
SC0000	MAT, ade2, arg4, leu2-3,112, trp1-289, ura3-52

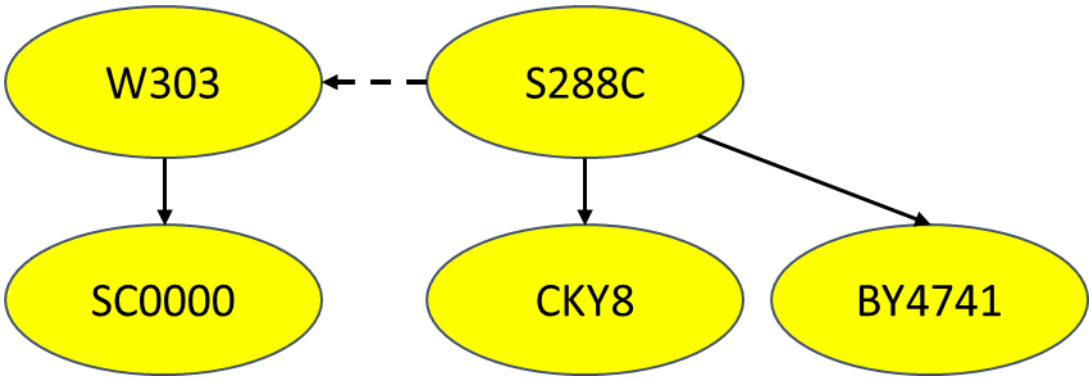

Figure 1. Genetic relations among tested strains.

Examined strains are shown with genetic relations to related strains. Solid lines indicate parent and derived strains. Dotted lines indicate presumed genetic relations.

Table 2: List of Tested Antifungals

Drug Name	Abbreviations	Mode of Action
Fluconazole	FLC	Inhibit Ergosterol Biosynthesis
Ketoconazole	KTC	Inhibit Ergosterol Biosynthesis
Itraconazole	ITC	Inhibit Ergosterol Biosynthesis
Clotrimazole	CLT	Inhibit Ergosterol Biosynthesis
Posaconazole	POS	Inhibit Ergosterol Biosynthesis
Voriconazole	VRC	Inhibit Ergosterol Biosynthesis
Miconazole	MC	Inhibit Ergosterol Biosynthesis
Metconazole	MTC	Inhibit Ergosterol Biosynthesis
G418	G418	Inhibit polypeptide synthesis
5-Flucytosine	5-FC	Inhibits DNA synthesis
Amphotericin B	AMB	Causes ionic leakage across membrane
Caspofungin	CFG	Inhibits cell wall formation
Cerulenin	CER	Inhibits fatty acid and sterol synthesis
Cycloheximide	CH	Inhibits polypeptide synthesis

Materials and Methods

Minimum Inhibitory Concentration

Susceptibility to antifungal agents and growth media was determined by CLSI approved stepwise broth microdilution protocol (CLSI, 2015). Susceptibility was measured as the Minimum Inhibitory Concentration (MIC) of the examined strains. This value was determined as the drug concentration of the well corresponding to an 80% reduction in growth from a drug-free growth control. Cells were grown in a CSM Complete (1.7 g yeast nitrogen base WO amino acids and ammonium sulfate, 0.77 Complete Supplement Mixture, and 5 grams of ammonium sulfate per liter) media with 2% glucose overnight at 30°C and diluted to an OD_{600} of 0.1. The inoculum was prepared by dilution of the cells 1:2000. A 96 well plate was prepared with two-fold serial dilutions of drug. A column with no drug served as a positive growth control while another receiving no cells served as a negative growth control. Cell growth was measured at 48 or 72 hours as necessary to achieve an OD_{600} growth control value of 0.4 or higher. Growth was measured by resuspension of cells followed by determination of the OD_{600}.

Quantitative Real Time PCR

The cells used for RNA isolation were grown in CSM-Complete media overnight at 30°C. The OD_{600} of each culture was used to determine the concentration of cells within the liquid culture. A volume corresponding to 2E7 cells were used. RNA was extracted using a Qiagen RNeasy mini purification kit. Work surfaces and instruments were treated with Ambion RNAseZAP. Yeast cell lysates were created by addition of 300 µl of acid washed glass beads followed by 10 minutes of rapid shaking and a 5-minute rest on ice. RNA concentrations and purity were determined via a multi-mode plate reader (Biotek, Winooski, VT) measurement of 260/280 values. cDNA was synthesized from 650 ng of RNA by a SuperScript III First-Strand kit (Invitrogen, Waltham, MA). Quantitative Real Time PCR (7500 Real-Time PCR, Applied Biosystems) was performed according to

manufacturer's instructions (iTaq Universal SYBR Green Supermix). Gene expression of several genes in sterol biosynthesis and drug resistance were examined in each strain and normalized against the *ACT1* expression data for that strain. Expression levels for each gene were reported following normalization to the W303 expression results. Twofold or greater changes compared to the W303 control was considered significant. All qRT experiments were performed in biological triplicate.

Preparation of PDR5 Deletion Strains

Deletion strains were prepared by transformation of target *S. cerevisiae* strains (S288C, BY4741, W303, SC0000) using a linear deletion cassette in which the genomic *PDR5* was deleted by homologous recombination. This cassette replaced *PDR5* with a DNA fragment containing a selectable marker with a constitutive promoter and terminator. For W303 and BY4741, PDR5 was replaced with a kanamycin resistance gene using a deletion cassette with a Kan selectable marker. However, deletion cassettes against multiple regions of *PDR5* were ineffective in developing S288C and SC0000 *PDR5* deletion strains. For SC0000 and S288C, *PDR5* was replaced with *URA3* using a deletion cassette with a -Ura selectable marker. Deletion cassette oligos are listed in Table 3. A *URA3*$^{-}$ variant of S288C, CKY8, was used to allow selection for transformants. Transformation was performed via a TRAFO derived high efficiency lithium acetate transformation procedure (Gietz and Woods, 2006). Prior to transformation, yeast was grown overnight in YPD (10 g of yeast extract and 20 g peptone per liter) with 2% glucose at 30°C. The cells were then diluted to 0.25 OD_{600} and grown to an OD_{600} of 1 to ensure most cells are in mid-log phase growth. Cells were then washed and resuspended in a 0.1M lithium acetate (0.1M LiOAc, 10mM Tris-Cl (pH 7.5), 1mM Na_2EDTA) solution. After incubation for 1 hour at 30°C, the cells were treated with 1 µg of the deletion cassette DNA per 0.1ml of cells and 5 µl of 10 mg/ml denatured salmon sperm carrier DNA. The tubes were incubated for 30 min at 30°C and mixed with 700 µl of a 40% PEG3300, LiAc, TE (40% PEG3300, 0.1M LiOAc, 0.1M Tris-Cl (pH 7.5), 10 mM Na_2EDTA) solution. After incubation for 1 hour at 30°C, the cells were

heat shocked for 5 min in a 42°C water bath. The cells were then collected by centrifugation, resuspended in 200 µl of water, and plated on appropriate media. CKY8 and SC0000 were plated initially on YPD media followed by transfer to selective media. Potential transformants were passaged three times across selective media, either 200 µg/ml G418 or ura dropout media. Genomic DNA was isolated from these transformants and primers corresponding to *PDR5* were used for PCR verification of the deletion.

Genomic DNA Isolation

Genomic DNA was prepared from cultures of strains grown overnight in YPD. The isolation protocol followed guidelines in Methods in Molecular Biology (Hanna and Xiao, 2006). Cells were pelleted then resuspended in 200 µl of a lysis solution (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris pH 8, 1mM EDTA). Resuspended cells were mixed with 200 µl of a Phenol:Chloroform:Isoamyl alcohol solution (25:24:1) then lysed by the addition of 300 µl of acid washed glass beads followed by 10 minutes of rapid shaking and a 5 minute ice bath. 200 µl of TE (10mM Tris-Cl (pH 7.5), 1mM Na_2EDTA) was added to each tube and then centrifuged to separate aqueous and organic fractions. The aqueous fraction was transferred to a fresh tube and mixed with 1 ml of ice cold ethanol followed by incubation for 30 min in a -80°C freezer. The tubes were then pelleted by centrifugation. The pellet was resuspended in 400 µl of TE and 3 µl of 10 mg/ml RNase A was added followed by incubation for 10 minutes at 37°C. The solution was treated with 40 µl of 3M sodium acetate and mixed with 1 ml of Ice cold 95% ethanol followed by another 20 min incubation at -80°C. The DNA was then pelleted by microcentrifugation and the tubes inverted to be air dried overnight followed by resuspension in TE for further use.

Genomic DNA Sequencing

DNA was sequenced by University of Missouri-Columbia DNA Core and followed supplied procedures.

Table 3: Deletion Cassette Recombination Oligoes

	Kan Deletion Cassette Oligoes
Oligo	Sequence Uppercase PDR5 Lowercase Kan
Kan Pro F	TCGCTTTTATTATCATACCTTAGAATGAAATCCAAAAGAAcgacatggaggcccagaata
Kan Start F	ATGCCCGAGGCCAAGCTTAACAATAACGTCAACGACGTTAcgacatggaggcccagaata
Kan Mid R	CTAACAGAGAAGAAAATGCATTGAATAGAATTGCAAAAAAcagtatagcgaccagcattc
Kan End R	TCTTGGTAAGTTTCTTTTCTTAACCAAATTCAAAATTCTAcagtatagcgaccagcattc
Kan Term R	TAAAATTCTCGGAATTCTTTCGGACATTGAACTTTGATTTcagtatagcgaccagcattc

	URA3 Deletion Cassette Oligoes
Oligo	Sequence Uppercase PDR5 Lowercase URA3
Ura Start F	ATGCCCGAGGCCAAGCTTAACAATAACGTCAACGACGTTAcgaagataaatcatgtcgaa
Ura End R	TCTTGGTAAGTTTCTTTTCTTAACCAAATTCAAAATTCTAataatacagttttttagttt

Results

Establishing the SC0000 Azole Resistance Phenotype

Several MICs were conducted to confirm that the SC0000 strain possesses an azole specific drug resistance phenotype (Table 4). SC0000, its parent strain W303, and two other common laboratory strains were tested for their MIC for several azole and non-azole antifungal agents. The resulting MIC_{80} values indicated that relative to W303, SC0000 possessed at least four fold higher resistance in all examined azoles (Table 4) except for KTC to which SC0000 was equally susceptible. SC0000 was found to be more susceptible than W303 to several non-azole drugs. SC0000 was at least four fold more susceptible to the antimicrobials G418 (MIC_{80} W303 50 μg/ml vs SC0000 12.5 μg/ml) and 5-FC (MIC_{80} W303 3.6 μg/ml vs SC0000 0.9 μg/ml). SC0000 exhibited reduced susceptibility to AMB, CER, and CH (2, 8, and 4 fold increase compared to W303 respectively). SC0000 exhibited no difference in susceptibility to CFG.

Sequencing Erg11

To determine if mutations in *ERG11* were the cause of the noted SC0000 azoles resistance phenotype, *Erg11* in SC0000 and W303 was sequenced and aligned with the *Saccharomyces* genome Database (SGD) S288C reference sequence for *Erg11* (data not shown). The resulting alignments displayed no differences between the sequenced strains and the reference strain. A BLASTn of the SC0000 *Erg11* sequence produced an alignment corresponding to a read of the coding region of *Saccharomyces cerevisiae* lanosterol 14-alpha-demethylase with a 100% identity. These alignments suggest that the sequence of Erg11 was unlikely to be the cause of SC0000's drug resistance.

qRT-PCR of Resistance Genes

qRT-PCR was performed with oligoes corresponding to efflux transporters, members of the ergosterol synthesis pathway, associated transcription factors as well as *ACT1* as a control. Expression data is summarized in Figure 2. The resulting expression data indicated increased

expression of *ERG11*, *UPC2,* and *PDR5* in SC0000 relative to W303 (1.69, 1.62, and 3.75 fold increases respectively). *PDR5*'s high expression along with multidrug efflux activity placed it as the most likely candidate for causing SC0000 drug resistance.

MICs of Wild Type Strains

To determine if *PDR5* is the primary cause of the noted resistance, deletion strains for *PDR5* were created from each of the previously described wild type strains. A second series of MICs against azole and non-azole antifungal agents for all resulting wild type and deletion strains were conducted (Table 5). This set of wild type MICS demonstrated that relative to W303, SC0000 was eight fold more resistant against all tested azoles except for VRC (two fold increase). SC0000 displayed a twofold relative decrease in resistance against CFG and a fourfold increase in resistance to CH.

MICs of *PDR5* Deletion Strains

All deletion strains demonstrated increased susceptibility to all drugs relative to the wild type with the exception of CFG (Table 5 and Figure 4). Despite this, SC0000^{-pdr5} continued to display elevated resistance to some azoles relative to the other deletion strains. MIC_{80} of SC0000^{-pdr5} for FLC and KTC displayed a fourfold increase in resistance relative to W303^{-pdr5}. On the other hand, the CLT, ITC, and VRC MIC_{80} values showed a loss of the resistance phenotype for SC0000^{-pdr5}, with values comparable to W303^{-pdr5} (No difference for CLT, no difference for ITC, and twofold decrease for VRC when compared to W303^{-pdr5}). MIC_{80} for the two non-azoles demonstrated no difference in resistance between SC0000^{-pdr5} and W303^{-pdr5}. Accordingly, loss of *PDR5* from all strains appears to have resulted in SC0000 losing its resistance to all azoles tested except for FLC and KTC as well as the non-azole CH relative to W303. However, the loss of *PDR5* may have resulted in a slight relative increase in CFG resistance (change from twofold relative susceptibility to equivalent susceptibility).

Table 4: MIC80 Values for Common Laboratory S. cerevisiae strains

Azole Antifungal MIC_{80} in μg/ml

Strains	W303	SC0000	S288C	BY4741
Fluconazole	16	128	32	8
Ketoconazole	32	32	64	16
Itraconazole	4	16	8	4
Clotrimazole	2	>8	2	1
Posaconazole	0.5	>1	1	0.25
Voriconazole	0.0313	0.5	0.0313	0.0156
Miconazole	0.0625	1	0.125	0.125
Metconazole	0.00195	0.25	0.03125	0.00195

Non-Azole Antifungal MIC_{80} in μg/ml

Strains	W303	SC0000	S288C	BY4741
G418	50	12.5	50	100
5-Flucytosine	3.6	0.9	7.2	1.8
Amphotericin B	4	8	8	2
Caspofungin	1	1	1	0.5
Cerulenin	0.1875	1.5	0.75	0.75
Cycloheximide	0.0625	0.25	0.0625	0.0625

≥4X Increase	≥4X Decrease	Relative to W303
2X Increase	2X Decrease	

Figure 2: Sequencing of SC0000 Erg11 Gene.

A BLASTn was performed on the SC0000 Erg11 sequence. The predicted alignments included coding region of S. cerevisiae Erg11 with 100% identity match.

***Saccharomyces cerevisiae* lanosterol 14-alpha-demethylase (14DM) gene, complete cds**

Score: 2874 bits(1556) Expect: 0 Identities: 1556/1556(100%) Gaps: 0/1556(0%) Strand: Plus/Plus

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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2328  GGCATTACCCAGAGGGTAAGACCGTTCCACCTCCTGACTTTACATCTATGGTTACTCTTCCAACCGGTCCAGCCAAGATCATCTGGGAAA  2417
Query  4849  AGAGAAATCCAGAACAAAAGATCTAA  4874
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Sbjct  2418  AGAGAAATCCAGAACAAAAGATCTAA  2443
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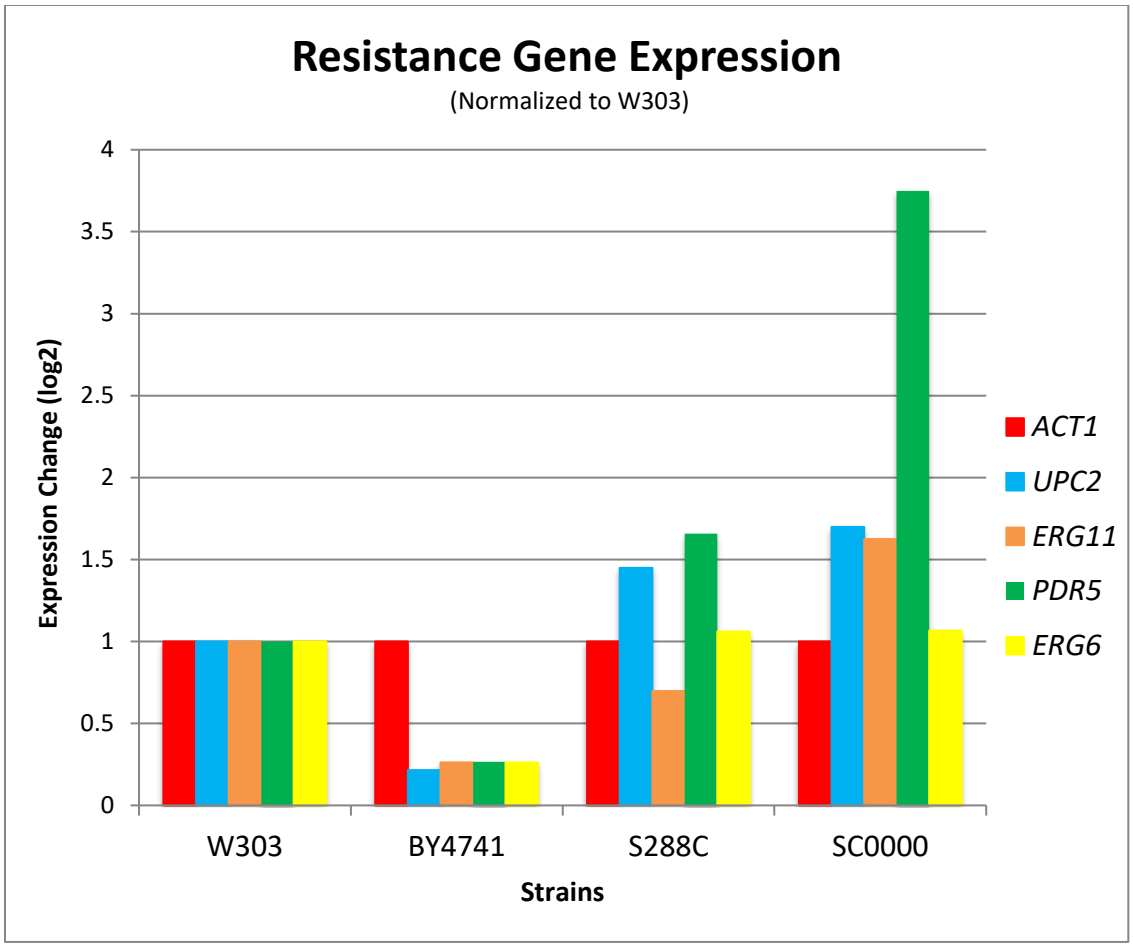

Figure 3: mRNA expression levels for potential resistance genes.
Expression levels of several genes suspected to be involved in the azole resistance phenotype. All data was normalized to W303 as well as *ACT1* as a control.

Table 5: MIC80 Values for Wild Type and Deletion strains.

	Wild Type MIC80 Values in µg/ml			
Strains	**W303**	**SC0000**	**CKY8** (S288C)	**BY4741**
Fluconazole	16	128	16	16
Ketoconazole	16	128	32	16
Clotrimazole	2	16	4	1
Itraconazole	8	64	16	16
Voriconazole	0.125	0.25	0.125	0.125
Caspofungin	0.125	0.0625	0.125	0.125
Cycloheximide	0.01563	0.0625	0.0625	0.0625

	Deletion Strain MIC80 Values in µg/ml			
Strains	**W303^{-PDR5}**	**SC0000^{-PDR5}**	**CKY8^{-PDR5}**	**BY4741^{-PDR5}**
Fluconazole	2	8	2	2
Ketoconazole	2	8	2	2
Clotrimazole	0.125	0.125	0.25	0.125
Itraconazole	4	4	2	2
Voriconazole	0.0625	0.03125	0.03125	0.03125
Caspofungin	0.125	0.125	0.125	0.125
Cycloheximide	0.03125	0.03125	0.0625	0.03125

≥4X Increase	**Relative to W303 WT/Del Strain**
4> Increase	
4> Decrease	
≥4X Decrease	

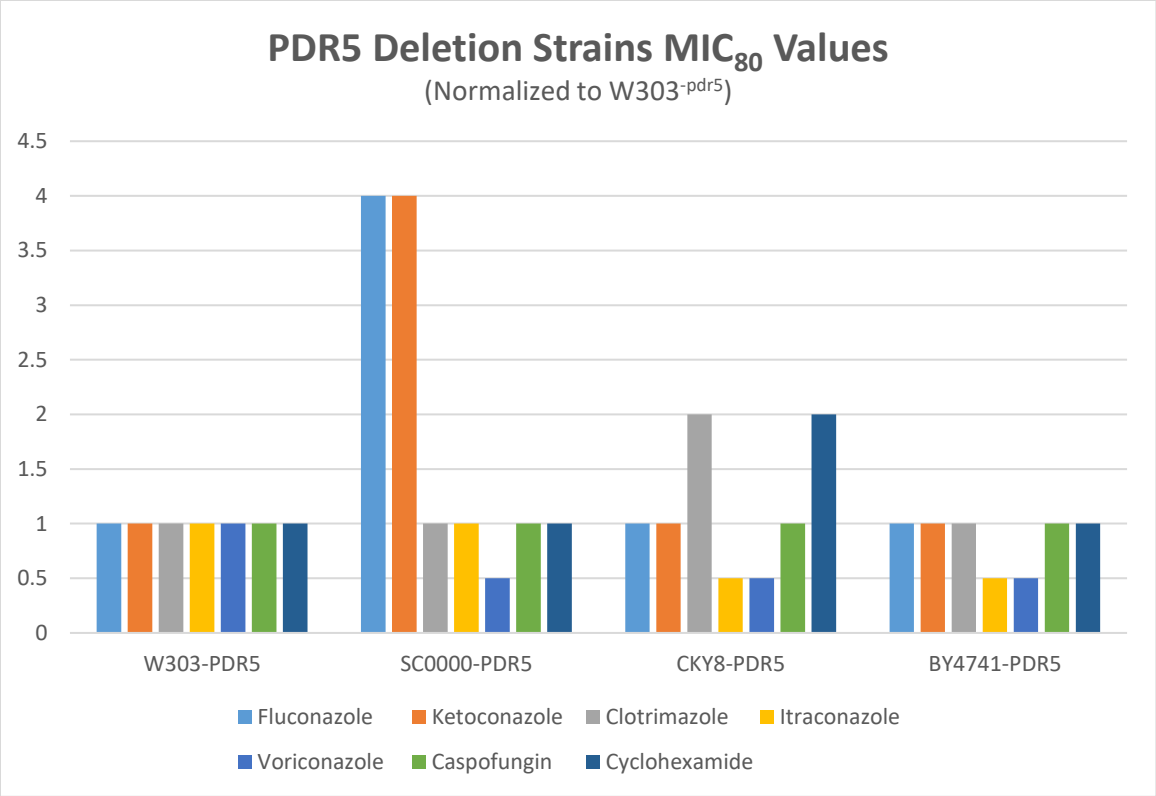

Figure 4: Drug resistance in strains deleted of PDR5.

MIC_{80} values for five azoles and 2 non-azoles were collected for four strains deleted of *PDR5.* The resulting values were then normalized against $W303^{-PDR5}$.

Discussion

These studies were conducted with the intent of determining the causative agent behind the azole-specific resistance phenotype associated with the commercially-available *S. cerevisiae* strain SC0000. Previous studies in the laboratory noted that SC0000 possessed an azole-resistance phenotype that was absent even in its closest genetic relative W303. This in spite of the fact that SC0000 exhibits poor growth under most conditions and frequently requires supplemental methods to achieve sufficient growth for testing.

The study began by characterizing the response of four *S. cerevisiae* strains to several azole and non-azole antifungal agents (Table 4). SC0000 was noted to be resistant to all azoles except KTC when compared to the W303, while having variable resistance to non-azoles. These findings were consistent with previous observations of this strain and thus it was concluded that SC0000 had an azole resistant phenotype. Interestingly, several of the non-azole drugs to which SC0000 was resistant were known substrates of the ABC transporter *PDR5*, including CER and CH (Hiraga et al., 2001). A change in activity of efflux transporters is one of the common mechanisms for drug resistance. This points to *PDR5* or a similar ABC transporter being involved in the development of the SC0000 resistant phenotype. SC0000 proved to be especially susceptible to drugs that inhibited nucleic acid and protein synthesis (5-FC and G418) which may imply a deficiency in the associated cellular machinery or may only be indicative of the overall reduction in growth associated with SC0000. Further testing with a more diverse portfolio of drugs may be a valuable future step.

Azoles inhibit growth by binding to the ergosterol biosynthesis intermediate enzyme *ERG11.* Lack of ergosterol prevents development of proper membrane structure and fluidity and is typically lethal. Changes in the *ERG11* sequence or expression of *ERG11* are therefore prime candidates when considering causes of azole resistance. The sequencing reaction for SC0000 was a complete match for the *Erg11* sequence available on the SGD as well as an independent sequence aligned via BLAStn.

The first 38 bases of the SC0000 *Erg11* gene coding region were not successfully sequenced leaving a blank towards the beginning of the sequence. However, this region is not a known site of drug interaction. The lack of divergence between sequences implies that changes in binding affinity resulting from *Erg11* mutations are unlikely to be the cause behind a change in azole resistance. However, the sequencing reactions provided no information on upstream sequences. Changes in the promoter sequence could alter expression enough to result in increased *ERG11* activity in the presence of azoles. This could provide information about the increase in *ERG11* expression detected by qRT-PCR. Further sequencing of the beginning of the gene and upstream sequences may provide further information about the role of *ERG11* in SC0000's resistance.

qRT-PCR was conducted to determine if changes in expression of relevant genes were associated with the SC0000 resistance phenotype. Among the examined genes *ERG11*, *UPC2*, and *PDR5* expression were found to be elevated. *Erg11* elevation results in a greater likelihood of avoiding complete inhibition by azoles and thus improves the likelihood of normal ergosterol biosynthesis. *UPC2* is a sterol sensing transcription factor that may increase expression of several key members of the ergosterol biosynthesis pathway including *ERG11*. *PDR5* is an ABC efflux transporter associated with broad drug efflux activity including several azoles. Expression data indicated that *PDR5* possessed the highest increase in expression among the examined genes relative to its parent strain. Therefore, in consideration of its expression and previous MIC data, *PDR5* was selected as the most likely single candidate for SC0000's azole resistance. However, the other two candidates are also viable targets of investigation that may be of secondary importance in the establishment of the resistant phenotype.

In order to determine if the elevation in *PDR5* levels was responsible for the noted azole resistance, we attempted to delete *PDR5* from SC0000. If the *PDR5* overexpression was sufficient to cause the resistance phenotype, then the deletion of *PDR5* from SC0000 should increase its

susceptibility relative to the other strains. Deletion of *PDR5* can have dramatic effects. *PDR5*$^{-}$ strains exhibit drastically decreased growth potential and hyper susceptibility to many drugs. Therefore, SC0000^{-pdr5} must be compared to other *PDR5*$^{-}$ strains to control for the broader effects of its deletion. *PDR5*$^{-}$ strains were formed by homologous recombination of a deletion cassette containing a selectable marker against *PDR5*. Strains W303^{-pdr5} and BY4741^{-pdr5} were readily produced with a Kan selectable cassette. However, strains SC0000^{-pdr5} and S288C^{-pdr5} were much more difficult to create and required the use of several different selectable markers and refinements of transformation procedures. In the end, strain S288C was replaced by a related strain, CKY8, a –Ura –Leu variant. Additionally, in order to receive sufficient live transformed cells following transformation attempts, these strains were plated on rich media before replating the cells to the selective media. The differences in ease of transformation may indicate that these strains had different degrees of success in compensating for PDR5 with other efflux transporters or with other cellular mechanisms. However, it should also be noted that according to relative genetic distance (Figure 1) BY4741 and CKY8 should both behave similarly to their parent strain S288C, as should SC0000 and its parent W303. Further investigation into activity of other efflux transporters in *PDR5*$^{-}$ strains, as well as full genome sequencing may be useful.

Once all four *PDR5*$^{-}$ strains were assembled, several MICs provided data about their growth in response to the examined antifungal agents. These MICS show that SC0000^{-pdr5} retained some resistance to FLC and KTC relative to W303^{-PDR5}. This may indicate that PDR5 deletion is not sufficient to revert the phenotype and another mechanism may exist that encourages resistance. Interestingly, the two azoles to which resistance was retained are fairly different in terms of physical properties. FLC has two triazole rings. KTC has a single imidazole ring and is substantially larger than FLC. SC0000^{-PDR5} did not retain resistance to several other azoles. Compared to W303^{-pdr5}, SC0000^{-pdr5}

became equally susceptible to CLT and ITC and more susceptible to VRC. This implies that the resistance phenotype in these drugs was largely due to the activity of *PDR5.*

Concurrently with the deletion MICs, a second group of reference wild type MICs was gathered. These MICs showed differences in relation to the MICs which established the resistance phenotype. The absolute values were different but the relative resistance comparing SC0000 to W303 was mostly the same with the exception of KTC, and CFG (Relative resistance change from initial MICs: KTC equivalent to 8 fold increase, CFG equivalent to 2 fold decrease). These changes may be due to differences in media preparation, drug solubility or degradation issues, or environmental changes such as room temperature. The second set of wild type MICs were performed at the same time in the same plates as the deletion strains and thus control for the majority of sources of error attributable to environmental conditions.

Relative to the wild type, there was a universal decrease in absolute resistance for all *PDR5* deletion strains in all drugs. This difference was not entirely consistent however and there are some conclusions worth noting. While all deletions strains exhibited lower MIC_{80} values, $SC0000^{-pdr5}$ resistance decreased more than the genetically similar $W303^{-PDR5}$ relative to their respective wild types in all drugs (Fold Differences: FLC = -2, KTC = -2, CLT = -8, ITC = -8, VRC = -4, CH = -4) except CFG (2 fold increase). This may indicate that other strains had more diverse means of compensating for the loss of *PDR5* or may simply indicate that efflux from PDR5 was proportionally more significant than the other strains.

From the collected data, we can conclude that overexpression of *PDR5* is likely the cause of some of the drug resistant phenotype exhibited by SC0000. However, this may not be the sole contributing factor. qRT-PCR data suggests that *ERG11* and *UPC2* are also overexpressed in SC0000. The overexpression of *ERG11* in particular is known to contribute to the development of a resistant phenotype. However, the overexpression of *UPC2* could have potentially more far-ranging effects.

UPC2 not only promotes transcription of multiple genes in the ergosterol biosynthesis pathway but may also regulate the filamentous growth response. Further analysis of associated pathways should be examined to clarify the roles these genes may have in developing azole resistance in strain SC0000.

While it was shown that *PDR5* was overexpressed relative to its parent strain, the degree to which its whole genome sequence differed from wild type has yet to be investigated. Changes in *PDR5* sequence may be responsible for a portion of the phenotype that is currently attributed to expression levels alone. The reason why it is overexpressed is also worth investigating. Changes in its promoter as well as changes in expression and sequence of related transcription factors *PDR1* and *PDR3* may provide more information on resistant strains. Expression levels and sequences of the *PDR5* paralog *PDR15* may also be worth investigating.

CHAPTER 2

CHARACTERIZATION OF PUTATIVE *ASPERGILLUS FUMIGATUS* ABC EFFLUX TRANSPORTERS IN HYPER-SUSCEPTIBLE *S. CEREVISIAE* STRAINS

Introduction

The recent rise in infections by opportunistic pathogens has led to greater interest in the study of mechanisms of drug resistance in pathogenic yeasts. Some of the most common organisms involved in opportunistic yeast infections are members of the *Aspergillus* genus. The primary fungus responsible for these cases, *A. fumigatus*, is a common soil borne saprophyte that plays an important role in the breakdown of organic matter. This fungus spreads by the release of thousands of tiny airborne conidia. As such, pulmonary exposure to *A. fumigatus* by inhalation of conidia is a ubiquitous threat around the entire world. In healthy individuals, conidia are quickly removed from the lung by mucous entrapment or phagocytosis (Segal, 2009). However, in immune-compromised patients the failure of normal host defenses allows the survival and growth of hyphae followed by systemic infection via dissemination through the circulatory system. Treatment of affected individuals depends upon patient health, any strain specific resistance, and the extent of the infection. Drugs targeting fungal specific pathways have minimal effects on the patient but are more likely to develop resistant strains. Drugs with broader targets are less likely to generate resistance but may have side effects preventing long term use or large doses. Therefore, it is crucial that the development of resistance be carefully monitored.

ATP Binding Cassette transporters belong to a family of ATPase associated transmembrane proteins that are found in nearly all extant organisms. Determination of drug resistance mechanisms in other pathogens has demonstrated that overexpression of ABC transporters is a common mechanism for the development of drug resistance. However, many genes identified as putative

ABC transporters have not been well characterized. While some of these genes may not function as transporters, it is likely that several efflux transporters that contribute to drug resistance can be identified based on phylogeny. Previously, a White laboratory graduate student/postdoctoral fellow inserted several of these putative transporters into a pYES plasmid and transformed the transporter-carrying plasmids separately into a *PDR5* deficient *S. cerevisiae* strain. Not all of the putative transporters produced an observable phenotype in wild type strain. Therefore, this study was designed to transform the putative transporters into a hyper-susceptible strain of *S. cerevisiae* deficient for several major efflux transporters. The relatively low background efflux activity should allow small efflux activities to be observed in the absence of endogenous pumps.

Materials and Methods

Minimum Inhibitory Concentration

Susceptibility to antifungal agents and growth media was determined by CLSI approved stepwise broth microdilution protocol (CLSI, 2015). Susceptibility was measured as the Minimum Inhibitory Concentration (MIC) of the examined strains. This value was determined as the drug concentration of the well corresponding to an 80% reduction in growth from a drug-free growth control. Cells were grown in CSM -Ura (1.7 g yeast nitrogen base WO amino acids and ammonium sulfate, 0.77 g CSM-Ura powder, and 5 grams of ammonium sulfate per liter) media with 2% glucose overnight at 30°C and diluted to an OD_{600} of 0.1. The inoculum was prepared by dilution of the cells by 1:100. A 96 well plate was prepared with two-fold serial dilutions of drug in CSM-Ura media with 2% galactose. A column without drug served as a positive growth control. Another column of wells received no cells serving as a negative growth control. Cell growth was measured at 72 or 96 hours as necessary to achieve an OD_{600} growth control value of 0.4 or higher. Growth was measured by resuspension of cells followed by determination of the OD_{600}.

Preparation of *Aspergillus* Pump Strains

S. cerevisiae strains expressing the putative transporters were prepared by transformation of an efflux deficient *S. cerevisiae* strain, Scadkan, with one of 12 plasmids based upon the pYES plasmid (Table 4). Each plasmid contained a single *Aspergillus* putative drug efflux transporter, synthesized by PCR amplification from cDNA, linked to a *GAL1* promoter as well a *URA3* selectable marker. Scadkan transformed with an empty pYES plasmid served as a reference for drug susceptibilities. Transformation was performed via a TRAFO derived high efficiency lithium acetate transformation procedure (Gietz and Woods, 2006). Prior to transformation, yeast was grown overnight in YPD (10 g of yeast extract and 20 g peptone per liter) with 2% glucose at 30°C. The cells were then diluted to 0.25 OD_{600} and grown to an OD_{600} of 1.0 to ensure most cells are in mid-log phase growth. Cells were then washed and resuspended in a 0.1M lithium acetate (0.1M LiOAc, 10mM Tris-Cl(pH 7.5), 1mM Na_2EDTA) solution. After incubation for 1 hour at 30°C, the cells were treated with 1 µg plasmid DNA per 0.1 ml of cells and 5 µl of 10 mg/ml denatured salmon sperm carrier DNA. The tubes were incubated for 30 min at 30°C and mixed with a 700 µl 40% PEG3300, LiAc, TE (40% PEG3300, 0.1M LiOAc, 0.1M Tris-Cl (pH 7.5), 10 mM Na_2EDTA) solution. After 1 hour at 30°C the cells were heat shocked for 5 min in a 42°C water bath. The cells were then collected by centrifugation, resuspended in 200 µl of water, and plated on appropriate selective media. Potential transformants were passaged three times on Ura dropout media.

Plasmid DNA Isolation

Plasmid DNA intended for transformation was isolated from overnight cultures of E. coli grown in LB broth (10 g Bacto-tryptone, 5g yeast extract, 10 g NaCl per liter). Plasmid DNA was extracted using a Thermo Scientific GeneJET Plasmid Miniprep Kit. Plasmid DNA isolated from yeast was isolated from overnight cultures of yeast grown in YPD (10g peptone and 5 g yeast extract per liter). After

resuspension in Resuspension Buffer, ~300 µl of acid washed glass bead were added to each tube and shaken for 10 minutes followed by a five minute ice bath. A needle was used to pierce the microcentrifuge tubes and drain the supernatant by centrifugation. 250 µl of lysis solution were added to the supernatant and mixed by inversion. 350 µl of neutralization solution was added and centrifuged to pellet cell debris. Supernatant was added to a spin column and washed twice with 500 µl of wash solution. Elution buffer was added to the spin column and incubated for two minutes prior to elution via centrifugation. The resulting plasmid solution was stored at -20°C.

Table 6: Putative Transporters List

Strain	Transporter	Gene Accession Number
SK0	Empty pYES	N/A
SK3	AF3	Afu2g14020
SK4	AF4	Afu6g05080
SK5	AF5	Afu500790
SK6	AF6	Afu2g15130
SK7	AF7	Afu6g08020
SK8	AF8	Afu4g01050
SK9	AF9	Afu01400
SK10	AF10	Afu5g09460
SK14	AF14	Afu1g14330
SK15	AF15	Afu6g07280
SK17	AF17	Afu1g17440

Results

Aspergillus fumigatus Efflux Pump Transformed Scadkan Resistance

Previously, attempts were made to characterize several putative ABC transporters found in the *Aspergillus* genome. These putative transporters were expressed separately in a *S. cerevisiae* strain deleted for *PDR5.* To further characterize these putative efflux transporters, this study performed MICs for several drugs on these putative transporters, overexpressed in the hyper-susceptible Scadkan strain. The resulting values were normalized to the empty transformation vector and shown in Table 2. Scadkan expressing AF3, AF6, and AF10 (SK3, SK6, SK10) putative efflux transporters exhibited an increase in resistance relative to the control for all tested drugs. AF3 had the largest overall effect in azoles with 96 fold average increase in resistance. AF6 conferred the greatest resistance to non-azoles at 4 fold average increase. AF10 displayed resistance to all drugs but conferred especially high resistance to FLC (SK10: 256 fold increase). AF7 Increased susceptibility to all drugs except FLC with an average 4 fold decrease in resistance. AF4, 5, and 14 exhibited increased resistance to all azoles (average respective fold increase: SK4 = 32, SK5 = 28, SK14 = 48) while decreasing resistance to examined non-azoles (average respective fold decrease in AMB and CFG: SK4 = 5, SK5 = 5, SK14 = 6). Of the examined drugs to which transporters conferred resistance, FLC was the largest average increase in resistance (58.2 average fold increase in resistance).

Table 7: Change in MIC80 relative to SK0

Fold Change in MIC_{80}

Normalized to SK0

Strains	FLC	KTC	CLT	ITC	VRC	AMB	CFG
SK 0	1	1	1	1	1	1	1
SK 3	128	32	128	64	128	2	2
SK 4	32	32	64	32	64	0.125	0.5
SK 5	32	32	64	32	32	0.125	0.5
SK 6	16	32	8	16	32	4	4
SK 7	1	0.25	0.5	0.5	0.5	0.0625	0.5
SK 8	16	0.5	2	1	1	4	1
SK 9	32	8	8	8	16	2	1
SK 10	256	2	8	8	32	4	2
SK 14	64	16	64	32	64	0.125	0.25
SK 15	64	16	2	4	16	0.25	1
SK 17	1	0.5	4	1	1	2	1

>0 Increase
≥4 Increase
≥16 Increase
≥64 Increase
≥256 Increase
<0 Decrease
≤4 Decrease
≤16 Decrease
≤64 Decrease
≤256 Decrease

Discussion

For the purposes of this study, several putative *Aspergillus* ABC efflux transporters were identified and expressed in a model *Saccharomyces* background. Strains with and without the known ABC transporter *PDR5* had received a plasmid containing one of the putative transporters. Efflux activity was noted and some degree of substrate specificity was identified. However, it was hoped that a strain exhibiting even further hyper-susceptibility could expand what is known about these specificities and activities. Scadkan is a S288C derived strain with 7 common transporters deleted. Use of this strain would allow detection of drug susceptibility phenotypes that may be obscured by the action of endogenous efflux transporters. MIC_{80} values for strains expressing these transporters were used to determine substrate specificity of the AF pumps, as well displaying any role the putative transporters have in the development of drug resistance.

In most of the generated strains, expression of one of the putative transporters was sufficient to raise resistance to all tested azole drugs. However, expression of AF7 increased susceptibility to almost all tested drugs except that there was no change to FLC. For expression of a transporter to lead to a decrease in resistance may mean one of several things. AF7's near universal increase in susceptibility may indicate that this predicted ABC transporter is an influx pump which may lead to enhanced accumulation of antifungal agents. Alternatively, this pump may be specific to certain drug types not tested in this study. Expression of this transporter would not raise resistance but may alter activity of certain compensatory endogenous transporters. Finally, the pump may simply be inactive, reducing survival of the cell through constitutive production of a non-functional product which may divert cellular resources or even trigger an unfolded protein response. Further testing of overall efflux and influx activity through an R6G assay could provide insight into the unusual phenotype associated with this transporter. A qRT-PCR for known endogenous efflux transporters in the presence and absence of expressed AF transporter genes would demonstrate

how other transporters may compensate for the presence or absence of these putative AF transporters.

There also are many transporters which display significant substrate-specificity for a few of the examined drugs. AF8 and AF17 exhibit increased resistance only in FLC and CLT respectively. Tentatively, it can be predicted that AF8 and AF17 are efflux transporters with high specificity for certain azole substrates. AF10 may also exhibit high substrate specificity. This transporter provides reduced susceptibility to all drugs but this effect is substantially higher in FLC. This indicates that this transporter has a much higher specificity for FLC than other azoles. While it is helpful to identify substrates to which these transporters confer resistance, further study must be done on these drugs and their activities against these pumps to determine if the substrate-specificity can be linked to certain physical features of the drug.

AF4, AF5, and AF14 exhibit reduced susceptibility to all azoles while increasing susceptibility to the tested non-azoles. This may indicate that any remaining transporters in the Scadkan strain are compensating to a degree for the overall lack of efflux activity. When the AF efflux transporters are expressed, other endogenous transporters may be downregulated such that resistance to transporter specific substrates goes up, while resistance to other compounds goes down.

The degree to which these efflux transporters shape the resistance phenotype of their host is still unknown. Further work remains to be done on the characterization of the efflux activity as well as the degree to which other mechanisms can compensate. MIC_{80} for more drugs, both azole and non-azole, would help determine which specific transporters confer resistance to any given drug and to what degree. A R6G assay for AF genes expressed in Scadkan would provide information about the overall degree to which the visible results are due to compensatory changes in activity of other pumps. If SK0 exhibits similar pump activity to the other strains, there may still be

background endogenous pumps influencing the detected phenotype. Finally, qRT-PCR would be helpful in identifying further efflux transporters that may influence susceptibilities

CHAPTER 3

PRODUCTION OF MIC TABLE FOR COMMON LABORATORY YEAST SPECIES

Introduction

Cryptococcus neoformans is an encapsulated fungal opportunistic pathogen. The primary route of infection is inhalation of spores. *C. neoformans* is characterized by the presence of a thick polysaccharide capsule that helps the cell to survive environmental conditions such as desiccation while also diminishing the efficacy of any immune response (O'Meara and Alspaugh, 2012). Despite this, immune-competent patients typically exhibit only mild symptoms before the yeast is cleared from their system. However, immune-compromised patients such as those infected by HIV lack the ability to phagocytize the growing cells leading the rapid spread of infection through the lungs and nervous system. An increase in the total number of immune-compromised individuals has led to higher rates of infection by opportunistic pathogens. For this and other reasons *C. neoformans* has become a focus of research as the study of its properties both pathogenic and experimental has become useful.

Whenever a researcher seeks to begin a project with yeast it is necessary for them to learn its characteristics. The temperature, type of media, and base resistance to drugs and peptides may all need to be examined to control for effects beyond that being considered by their study. With this in mind, a table was constructed for several common yeast species with an aim to establish baseline data. MICs corresponding to different, drugs, media, and growth temperatures were all collected. This information will be of use to both in the laboratory as a reference point, as well as in medical use to understand its pathogenic properties. For the purposes of this study, I participated in this study by conducting MICs corresponding to *Cryptococcus neoformans* as part of a more general survey conducted by the White laboratory.

Materials and Methods

Minimum Inhibitory Concentration

Susceptibility to antifungal agents and growth media was determined by CLSI approved stepwise broth microdilution protocol (CLSI, 2015). Susceptibility was measured as the Minimum Inhibitory Concentration (MIC) of the examined strains. This value was determined as the drug concentration of the well corresponding to an 80% reduction in growth from a drug-free growth control. Drug susceptibility was tested at 30°C and 35°C and in three different media (YAD (1.7 g yeast nitrogen base WO amino acids and ammonium sulfate and 5 g ammonium sulfate per liter), CSM Complete (1.7 g yeast nitrogen base WO amino acids and ammonium sulfate, 0.77 Complete Supplement Mixture, and 5 grams of ammonium sulfate per liter), and YPD (10g peptone and 5 g yeast extract per liter)). All three media were supplemented with 2% glucose. Cells were grown in a CSM Complete media with 2% glucose overnight at 30°C and diluted to an OD_{600} of 0.1. The inoculum was prepared by dilution of the cells by 1:2000 in each of the three media. Two 96 well plates per examined drug were prepared with two-fold serial dilutions of drug in each of the three media (YPD, CSM Complete, and YAD). One column received inoculum and no drug to serve as a positive growth control. One column received no inoculum and only the lowest concentration of drug to serve as a negative growth control. Cell growth was measured at 72 hours. Growth was measured by resuspension of cells followed by determination of the OD_{600}.

Results

Base MICs for Common Laboratory Strains (*C. neoformans*)

In order to generate data about the basal responses of several common laboratory strains, MICs analysis was conducted with a variety of drugs, media, and temperatures. *Cryptococcus neoformans* exhibited the highest resistance in CSM for all drugs and temperatures tested. It had the least resistance in YPD with all drugs except 5-FC where it had poor growth in YAD. *C. neoformans* growth and resistance is slightly restricted at temperatures higher than 30°C. MIC 35°C GC values were on average 20% lower than 30°C GC values (Data not shown). *C. neoformans* exhibits elevated resistance to CFG relative to other tested species as well as a slight resistance to 5-FC. The information gathered is summarized in Table 8.

Table 8: Table of MIC_{80} values for common laboratory species

Drug	Media	Temp	CA	CN	SC	CG
FLC	YPD	30	8* TRAIL	4	8* TRAIL	16
		35	*TRAIL	4	16* TRAIL	16
	CSM	30	16	16	16	128
		35	16	4	16	64
	YAD	30	8	16	8	128
		35	16	4	16	64
KTC	YPD	30	0.06	2	0.5	1
		35	0.06	1	1	0.5
	CSM	30	2	>128	64	64
		35	2	64	64	64
	YAD	30	0.5	128	32	64
		35	0.5	128	32	64
VRC	YPD	30	0.02	0.063	0.25	1
		35	0.02	0.063	0.25	X
	CSM	30	0.016	8	0.125	8
		35	0.016	8	0.125	2
	YAD	30	0.008	8	0.125	8
		35	0.008	8	0.25	4
AMB	YPD	30	>128	8	16	
		35	>128	8	16	
	CSM	30	>128	32	64	
		35	>128	32	32	
	YAD	30	>128	16	16	
		35	>128	16	16	
5FC	YPD	30	.2*4	>0.8	>0.2	>0.2
		35	>0.2	>0.8	>0.2	>0.2
	CSM	30	>0.2	>0.8	>0.2	>0.2
		35	>0.2	>0.8	>0.2	>0.2
	YAD	30	0.006	0.2	0.0008	0.006
		35	0.006	0.2	0.0008	0.003

CFG	YPD	30	0.063	8	0.0625	
		35	0.063	8	0.0625	
	CSM	30	0.5	8	0.25	
		35	0.063	8	0.25	
	YAD	30	0.25	8	0.25	
		35	0.25	8	0.25	
R6G	YPD	30	100	200	>400	
		35	100	200	>400	
	CSM	30	200	>400	>400	
		35	100	>400	>400	
	YAD	30	50	>400	400	
		35	50	>400	400	

Discussion

The associated table for this project was built with the intent of establishing initial testing parameters for several common laboratory species of yeast. *Cryptococcus neoformans* was selected as one of these species and was tested against several types of media, drug, and growth temperature. From these initial findings, several tentative conclusions can be drawn about *C. neoformans* which may be of use to researchers using this species.

C. neoformans was consistently more resistant to antifungal agents in CSM rather than YPD or YAD. This may indicate a response to some chemical constituent of these media that reduces growth under tested conditions. YPD is a rich media that should possess required nutrients in excess. That YPD MICs are lower relative to CSM may indicate some constituent of YPD is reducing resistance. Yeast extract and peptone are both largely undefined constituents of this media. One or both could contain some undefined peptide or signaling molecule that may be lowering the growth rate or gene expression rates of *C. neoformans*. Alternatively, the difference could be in the physical characteristics of the media such as pH or osmotic pressure. YAD is largely identical to CSM but contains no added amino acids. While this is probably sufficient to lower resistance there is still the potential that other factors may reduce resistance. Further study of the effect media constituents on resistance development may prove valuable.

C. neoformans was more resistant to CFG than other tested strains (between 16 and 256 times higher). This resistance phenotype has been previously noted and may be related to the polysaccharide envelope limiting access of the drug to the developing cell wall or alternatively an inability of CFG to inhibit the *C. neoformans* copy of 1,3 β-D-glucan synthase (Feldmesser, 2000). A slight increase in resistance to 5-FC is also notable. It has been previously reported that resistant isolates of C. neoformans for 5-FC appears spontaneously. Multiple mechanisms have been

predicted including deficiencies in drug import as well as mutations in enzymes associated with target pathway (Block, 1973).

C. neoformans also exhibits a slight decrease in growth and resistance at higher temperatures. This is interesting in light of *C. neoformans* role as an opportunistic pathogen. Physiological temperatures are something this species must confront regularly if it is to colonize a living host. This may have some implications regarding altered growth patterns in response to the transition from environmental saprophyte to human pathogen. Alternatively, the strains examined may have adapted to laboratory conditions and are no longer optimized for pathogenic activity. Further testing of this fungus in conditions comparable to physiological conditions may be advised.

During the course of study *C. neoformans* was frequently observed to form a sticky globular colony that was difficult to suspend prior to growth reading (data not shown). This trait may be related to *C. neoformans* capsule formation properties which help it invade and thrive in the host body. Further study on environmental conditions resulting in expression of this trait would be advised in light of known effects of envelope on the development of resistance to environmental conditions.

CHAPTER 4

CONCLUSIONS AND FUTURE STEPS

Pathogenic fungi are a ubiquitous threat to both humans as well as other lifeforms upon which our society depends. Treatment of these microorganisms depends on comprehensive knowledge of their unique metabolic pathways as well as the mechanisms by which they resist treatment. The growing number of serious infections has led to an increased reliance upon antifungal agents shown to be safe and effective. This reliance provides treated strains the opportunity to develop resistance to commonly used drug with a resultant increase in treatment difficulty. It is therefore valuable to study the development of resistance in yeasts so that we understand how to prevent the development of resistant strains.

In Chapter 1, we established that a W303 derivative strain, SC0000, exhibited increased resistance to azoles. qRT-PCR data suggested that ABC efflux transporter *PDR5* was a potential cause. MICs for *PDR5* deletion strains confirmed the loss of resistance to several antifungals but failed to completely eliminate resistance to FLC and KTC. Further testing of PDR5 deletion strains may find evidence for a link between retained resistance and chemical specificity. Sequencing of *PDR5* as well as its promoter and associated transcription factors would provide further information on the means by which *PDR5* overexpression affects resistance in SC0000. Finally, overexpression of *ERG11* and *UPC2* should be investigated for a potential role in the SC0000 resistance phenotype.

In Chapter 2, we provided some evidence of substrate specificity and activity of putative AF ABC transporters by expressing them in hyper susceptible *S. cerevisiae* strains. MICs for a wider variety of drugs might provide insight into the substrate specificity of individual efflux transporters. qRT-PCR for strains overexpressing these transporters would provide information about the degree to which endogenous transporters are active within these strains. An R6G assay would provide information about overall efflux activity in strains expressing these transporters.

In Chapter 3, we sought to develop a table of baseline response for several yeast species to drugs, media, and temperature. Future work will expand the table and provide more information about the degree to which currently observed trends are consistent across a broader range of environmental conditions.

The degree to which pathogenic fungi interact with our life cannot be underestimated. As pathogenic strains adapt to our methods of treatment, so to must we shape our response in turn. The study of resistance in these strains provides valuable data about ways current and future strains will develop resistance. This encourages the development of new antifungal drugs as well as ensuring that our current techniques and treatments retain efficacy into the future.

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VITA

Jacob Parker Shreeve was born in Redlands, California in 1993. He moved to Houston, Texas in 1997 and Kansas City, Kansas in 2005. He graduated from The Barstow School in 2011. Jacob was a recipient of The Barstow School Biology Cup, awarded by the School's faculty to the Department's outstanding biology student.

In the fall of 2011, Jacob began his post-high school education at Truman State University, in Kirksville, Missouri. He was awarded the President's Honorary Scholarship for significant academic achievement and cocurricular involvement. During the summer of 2012, Jacob served as an intern at an international non-profit research organization MRI Global and was awarded the Hallmark Cards Internship. This award was given to the intern that best demonstrates a commitment to the fields of science, technology, and engineering. In August of 2014, he became involved in research with the Norimatsu Lab affiliated with A.T. Still University. He studied functional drug-receptor interactions of mutant and wild type TAAR1 and CFTR. In May, 2015 Jacob graduated with a B.S. in Biology and a minor in chemistry with the President's Recognition Award.

In the summer of 2015, Jacob served as an analyst for TechAccel, a technology development organization focusing on identification and investment in new technologies. His duties included researching technologies of interest and feasibility of acquiring said technologies and creating and delivering cogent summaries to associated individuals. In fall, 2015 Jacob began attending the iBIO graduate program at the University of Iowa in Iowa City, Iowa. During this period, he gained experience via rotations in the Fassler, Phillips, and Prahlad labs. There he studied polyglutamine tract expansion in *S. cerevisiae* as well as asymmetric cell division and stress responses in *C. elegans*. In spring of 2016, he served as a graduate teaching assistant and lab instructor.

In fall of 2016, Jacob was accepted into the Graduate Cell and Molecular Biology program at the University of Missouri-Kansas City. In January of 2017, Jacob became affiliated with the Theodore White Lab and began the study of drug resistance in several common yeast including: *S. cerevisiae, Aspergillus fumigatus*, and *Cryptococcus neoformans*.