Role of zinc cluster proteins in the biology of *Candida glabrata*, a human fungal pathogen

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August 2021

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Masters of Science in Experimental Medicine

Preface

Hetaben Patel contributed to all the technical work in this thesis. Dr. Bernard Turcotte contributed to the preparation of this thesis and the construction of several expression vectors and reporters. Natalia Klimova provided pGRB.PDR1, an expression vector for *PDR1* with a mutation in its coding region. Dr. Martin Olivier provided mice macrophage cells.

Common Abbreviations

PCR = polymerase chain reaction

PDR = pleiotropic drug resistance

PDRE = pleiotropic drug resistance element

SD = minimal synthetic defined medium

YPD = yeast peptone dextrose yeast medium

OD = optical density

µg/ml = microgram per millilitre

ABC = ATP- binding cassette

bp = base pair

Mb = mega base pairs

ODcase = orotidine 5'-phosphate decarboxylase

ROS = reactive oxygen species

HOG = high osmolarity glycerol pathway

ER = endoplasmic reticulum

HLP = hemolysin like protein

PKC = protein kinase C

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Abstract

Candida glabrata is an emerging nosocomial pathogen. The *C. glabrata* genome contains approximately 5300 coding gene and it is closely related to *Saccharomyces cerevisiae*, a non-pathogenic yeast. Zinc cluster proteins form a large family of transcriptional regulators. They are involved in regulating numerous processes, such as controlling the metabolism of sugars, fatty acids, amino acids, and drug resistance. *PDR1* encodes a zinc cluster protein that controls the expression of genes encoding drug efflux pumps that are important for resistance to azoles, a class of antifungal drugs. Pdr1 was shown to positively control its own expression by binding to its promoter, an auto-regulatory loop. One aim of this study was to determine the effects of different *PDR1* mutations on resistance to echinocandins (another class of antifungal drugs) and test if the auto-regulatory loop is necessary for resistance to these drugs. A mutant carrying a mutation in the coding region of *PDR1* (Y208C) behaved as a hyperactive form of Pdr1 resulting in increased resistance to echinocandins. The auto-regulatory loop was necessary for resistance of the mutant. Mitochondrial defects in some clinical strains showed decreased susceptibility to echinocandins. This study also determined that some *C. glabrata* mutant strains, lacking specific zinc cluster factor genes, had altered survival and replication in macrophage cells, as compared to a wild-type strain. A better understanding of the regulation of Pdr1 provides a new avenue of interfering with azole and echinocandin resistance in *C. glabrata*. Our study also provides information that will help better to understand the biological role of these transcription factors with regards to survival in macrophages.

Résumé

Candida glabrata est un pathogène nosocomial émergent. Le génome de *C. glabrata* contient environ 5300 gènes codants et il est étroitement lié à *Saccharomyces cerevisiae*, une levure non pathogène. Les protéines de la grappe de zinc forment une grande famille de régulateurs transcriptionnels. Ils participent à la régulation de nombreux processus, comme le contrôle du métabolisme des sucres, des acides gras, des acides aminés et de la résistance aux médicaments. *PDR1* encode une protéine de groupe de zinc qui contrôle l'expression des gènes codant pour les pompes d'efflux de médicament qui sont importantes pour la résistance aux azoles, une classe de médicaments antifongiques. Il a été démontré que Pdr1 régule sa propre expression en se fixant sur son promoteur, une boucle autorégulatrice. Un but de cette étude était de déterminer les effets de différentes mutations de *PDR1* sur la résistance aux échinocandines (une autre classe de médicaments antifongiques) et de tester si la boucle autorégulatrice est nécessaire à la résistance à ces médicaments. Un mutant comportant une mutation dans la région codante de *PDR1* (Y208C) se comporte comme un facteur hyperactif et confère la résistance aux échinocandines. Cette boucle autorégulatrice est nécessaire pour la résistance du mutant. Les défauts mitochondriaux de certaines souches cliniques ont résulté en une sensibilité réduite aux échinocandines. Cette étude a également permis de déterminer que certaines souches mutantes de *C. glabrata*, dépourvues de gènes spécifiques de facteurs d'amas de zinc, ont une survie et une réplication altérées dans les cellules de macrophages par rapport à une souche de type sauvage. Une meilleure compréhension de la régulation du Pdr1 offre une nouvelle façon d'interférer avec la résistance aux azoles et aux échinocandines chez *C. glabrata*. Notre étude fournit des

informations qui aideront à mieux comprendre le rôle biologique de ces facteurs de transcription en ce qui concerne la survie dans les macrophages.

Section 1: Literature Review

1.1 Fungal infections and *Candida glabrata*

The fungal kingdom contains a vast diversity of taxa with different life-cycle strategies, varied ecological niches and morphologies. However, there is little information about the true biodiversity of the fungal kingdom. From the 1.5 million species estimated to this kingdom, only 5% were classified (1). Plant pathogenic fungi such as the rice blast fungus and the chestnut blight fungus cause damage to crops and trees, respectively. Some other fungi can cause severe disease in humans. These include histoplasmosis, cryptococcosis, aspergillosis and candidiasis (1). Infection by highly opportunistic fungal pathogens has become a significant problem due to the increasing number of immunocompromised patients. They include individuals who are infected with the human immunodeficiency virus, bone marrow and organ recipient patients under immunosuppressive therapy or cancer patients treated with cytotoxic drugs (2).

Candidiasis is the most frequent fungal disease affecting the population in the world (3-5). There are several types of candidiasis such as mucosal candidiasis, cutaneous candidiasis, systemic candidiasis, onychomycosis candidiasis and candidemia (6, 7). Candidemia is the most persistent nosocomial infection, and *Candida* species are the main causative agents in 50 to 70 % of systematic fungal infections (8-10). The involvement of *Candida* in bloodstream infections depends on the patient's condition, age and geographic region (11). Many species recovered from human samples belong to the genus *Candida,* with almost half showing serious infections. *C. albicans* remains the most prevalent species. However, the prevalence of other *Candida* species like *C. parapsilosis*,

C. tropicalis, C. krusei, *C. glabrata*, *C. famata*, *C. lusitaniae*, *C. orthopsilosis* has also increased (12-14).

C. glabrata is an opportunistic pathogen that accounts for up to 29 % of *Candida* bloodstream infections (15, 16). It is the second most common cause of bloodstream infection after *C. albicans* (16-18). Recent epidemiological data suggest a decrease in the frequency of *C. albicans* and an increase in the prevalence of non-*albicans Candida* (17-19). *C. glabrata* can also cause infection in the vagina and urinary tract (20, 21).

1.2 Biology and genetics of *Candida glabrata*

C. glabrata belongs to the kingdom fungi, order Saccharomycetales and the family Saccharomycetaceae. *C. glabrata* is a species of the genus *Candida,* previously known as *Torulopsis glabrata*. *C. glabrata* forms shining, smooth and creamy coloured colonies, which are indistinguishable from other *Candida* species, except their relatively small size on Sbouraud Dextrose Agar (SDA). *C. glabrata* cells are 1-4 μm in size remarkably smaller than *C. albicans* cells (4-6 μm) (22). *C. glabrata* colonies appear white and pink to purple on chromogenic medium. Regarding biochemical reactions, it ferments and assimilates glucose and trehalose (23).

In contrast to the diploid genome of *C. albicans*, *C. glabrata* is haploid. The genome of *C. glabrata* was been sequenced (24). The annotated genome contains 13 chromosomes, named chromosome A to M. The genome is 12.3 Mb in size. Chromosome lengths differ: chromosome A (491 328 bp) and L (1 455 689 bp) being the smallest and largest, respectively (25). From 5293 open reading frames (ORFs) in the *C. glabrata*

genome, approximately 500 ORFs are verified (www.candidagenome.org) with some experimental evidence for their gene products.

The *C. glabrata* genome displays robust linkage with the genome of the non-pathogenic yeast *Saccharomyces cerevisiae.* The same genes were found to be present in 88% of the genome of these two yeasts (24, 26). *C. glabrata* comprises orthologs of 4870 *S. cerevisiae* genes. Additionally, compared to *S. cerevisiae*, a greater degree of gene loss has occurred in *C. glabrata*. The *C. glabrata* genome steadily exhibits lower global redundancy (24). *C. glabrata* contains a small 20 kb circular mitochondrial genome that contains eleven ORFs (27).

1.3 Virulence factors in *C. glabrata*

Fungi dynamically participate in the pathophysiology of the disease process via mechanisms of aggression called virulence factors (28, 29). The pathogenicity of *Candida* species is credited to certain virulence factors, such as the biofilm formation capacity, the production of tissue-damaging hydrolytic enzymes, such as phospholipases, hemolysins, lipases, adherence ability, ability to evade host defenses by filamentous form (28, 29). The non-pathogenic nature of *C. glabrata* in animal models suggest that it has few virulence attributes. It is known that *C. glabrata* possesses some factors tangled in the development of infection (23).

1.3.1 Adherence

Adherence to the host tissue is an essential factor that leads to colonization and the establishment of successful infections (25). The adhesion ability of *Candida* species is due to the presence of specific proteins on their cell wall, namely, adhesins (23). A significant group of adhesins is encoded by *EPA* (epithelial adhesin) gene family in *C. glabrata* (30). *C. glabrata* has a total of 67 putative adhesins (31). Epa1 is a calcium-dependent lectin and aids adhesion to epithelial cells and macrophages (32, 33). The multidrug resistance transcription factor Cg*PDR1* has been involved in regulating the *EPA1* gene expression (34). The Epa6 and Epa7 adhesins have been shown to possess adherence ability to endothelial and epithelial cells (35-37). Another adhesin subfamily contains the N- terminal PA14 domain, referred to as the Pwp family, comprising seven proteins (25).

Many adhesin encoding genes contain several tandemly repeated sequences, called megasatellites. The number of repeats may govern the function of adhesins. The presence of large number of adhesin genes and their environment-dependent regulations are likely to help *C. glabrata* colonize different host niches and increase the capacity of biofilm formation on a wide-range of surfaces (31, 38, 39).

1.3.2 Biofilm formation

Biofilms are complex and multilayered structures that are formed by microbe-microbe and microbe-surface interactions (40). Biofilm forming ability may offer several advantages to *C. glabrata*. The benefits include aiding survival as commensal and pathogen to human, resistance to antifungal treatment, and withstand competitive

pressure from other organisms (41). In addition, biofilm formation in *C. glabrata* allows good adaption to colonize the tissues and indwelling medical devices (23). The extracellular matrix of biofilm is composed of high level of protein and carbohydrate, including β-1,3 glucan (29, 41, 42).

High-cell density and biofilm conditions are inducers of adhesion. A study involving mutants strains allowed the identification of four genes involved in biofilm formation: telomere-binding (*RIF1*), silent information regulator (*SIR4*) and, *YAK1* and *EPA6* encoding serine threonine kinases (32). In addition, the transcription factor Bcr1 is a dominant regulator of biofilm development (43).

1.3.3 Enzyme production

In the *Candida* genus, in addition to adherence and biofilm formation, another virulence factor is the ability to destroy host tissues, which may be eased by production of hydrolytic enzymes and their release into the local environment. These enzymes such as hemolysin, phospholipase, proteases, and lipases help *Candida* to survive and replicate within macrophages. In addition, extracellular membrane damaging phospholipases hydrolyze phospholipids into fatty acids; their production results in cell membrane damage. The strains which produce this enzyme adhere more strongly to epithelial cells (23).

C. glabrata produces lipases that destroy the host mucosae and facilitate the invasion of the tissues (44-46). In *C. albicans,* ten genes encoding for lipases have been identified and it has been determined that mutants were less virulent in a murine model (44-48). Some researchers reported that *C. glabrata* could degrade haemoglobin using

the enzyme hemolysin in order to obtain iron (49, 50). Some studies revealed that a hemolysin–like protein (HLP) gene is linked to hemolytic activity of *C. glabrata*. *C. glabrata* can produce hemolysins *in vitro,* leading to total or partial erythrocyte lysis (51).

1.3.4 Stress response mechanisms

C. glabrata is able to tolerate oxidative stress, osmotic stress, endoplasmic reticulum (ER) stress and cell wall stress (52). Msn2 and Msn4 regulate the response to environmental stress (53). Response to oxidative stress is regulated by Cta1, Sod1, Sod2, Gsh1 and Gsh2 (54-57). The expression of *CTA1*, encoding an enzyme involved in breaking down hydrogen peroxide, is regulated by Yap1, Msn2, Msn4 and Skn7 (36, 55, 58, 59). Furthermore, tryptophan-based pigment production is linked to survival in ROS stress (60).

The high osmolarity glycerol pathway (HOG) is known to be activated by sorbic acid, and its terminal MAPK Hog1 aids for survival in osmotic stress (61, 62). In response to the ER stress, the Ire1 endoribonuclease activates the non-canonical unfolded protein response pathway (63). The transcriptional co-activator Ada2 is linked to developing resistance to the ER stressor in immunocompromised mice (64). The protein kinase C (PKC)-mediated cell wall integrity and calcineurin signaling pathway have been implicated in regulating the response to ER stress. This signaling pathway is also required to combat antifungal stress (28, 65, 66).

1.4 Antifungal agent and mechanism of action

Since fungi and humans are both eukaryotes, there are only few available targets specific to fungi. A number of antifungal drugs also have side effects in patients. Since 1990, there has been limited but increasing discovery of antifungal agents (67, 68). Antifungal drugs include azoles (fluconazole, itraconazole, ketoconazole, miconazole and clotrimazole), polyenes (amphotericin B and nystatin), 5-flucytosine and echinocandins (caspofungin, micafungin and anidulafungin). Antifungal drugs of other classes, allylamines and morpholines, are used only as topical agents because of either adverse effects or low efficacy (69, 70).

1.4.1 Echinocandins

The echinocandins have been shown to have fungicidal effects in all *Candida* species (71). Micafungin, caspofungin and anidulafungin are examples of echinocandins (1). Echinocandins inhibit (1,3) *β*-D -glucan synthase, an enzyme that catalyzes the polymerization of uridine diphosphate-glucose into *β* (1,3) Glucan. *β* (1,3) Glucan is the component responsible for the maintenance of fungal cell wall rigidity and integrity and it is present in the cell membrane of fungi. Depletion in *β*-D -glucan synthase leads to an abnormal cell wall that is weak and unable to resist osmotic stress (72, 73). This also leads to destabilization of the cell wall and leakage of intracellular components, resulting in fungal cell lysis (74). *β*-D-glucan synthase possesses a catalytic subunit encoded by the *FKS* genes and a regulatory subunit encoded by *RHO1*. In almost all fungi, two *FKS*

genes are found within the genome, *FKS1* and *FKS2*, and echinocandins can inhibit both isoforms (75, 76).

Due to the fact that these drugs are absorbed poorly in the gastrointestinal tract, they are used intravenously. Echinocandins are active against most fungi. Thus, echinocandins are excellent drug to fight fungal infection. When the treatment with azoles for fungal infection fails, the infection is normally successfully managed by echinocandins. Moreover, they offer some advantages; one of them is a lower risk of side effects since animal cells do not have a cell wall (77).

1.4.2 Azoles

Azoles are commonly used antifungal drugs. Azoles are composed of a five-member azole ring containing two (imidazole) or three (triazole) nitrogen atoms attached to a complex side-chain (78, 79). The family of imidazole compounds includes ketoconazole, miconazole, econazole and clotrimazole. The family of triazole compounds includes itraconazole, fluconazole, voriconazole and posaconazole (80, 81). Azoles inhibit ergosterol biosynthesis by interfering with the enzyme lanosterol 14-α demethylase, encoded by the *ERG11* gene, involved in the transformation of lanosterol to zymosterol, a precursor of ergosterol synthesis. This inhibition process occurs in the ER of fungal cell. Ergosterol is an essential constituent of the plasma membrane fungi. The accumulation of 14-α methyl-3, 6- diol, a toxic compound, inhibits fungal growth. The cell membrane structure is altered as the ergosterol concentration is reduced, thereby inhibiting the fungal growth (82).

Scientists studied azoles for their pharmacological properties, mode of action and the resistance mechanisms. Pharmaceutical companies studied azole antifungals in order to enhance azole drugs efficacy and to develop better antifungals (1). In 1990, for example, fluconazole became available for clinical use. Fluconazole is hydrosoluble and therefore it can be quickly injected intravenously. It is recommended to treat the invasive candidiasis with patients who have not previously been medicated with an azole (67, 83). However, azole antifungal drugs have certain limitations. It includes the emergence of resistance and side effects such as hepatotoxicity (84). Therefore, there is a need for improving this class of drug for better treatment of fungal infections or to develop other antifungals (71).

1.4.3 Polyenes

Polyenes are cyclic amphiphilic organic molecules. More than 200 molecules belong to the class of polyenes and possess antifungal activity, most of them being produced by *Streptomyces* bacteria. The chemical class of polyenes include amphotericin B (AmB), nystatin and natamycin. Polyene drugs target ergosterol. Ergosterol is the principal sterol component of the fungal membrane. Their amphiphilic structure lets them bind the lipid bilayer, and it creates pores. Pores promote destabilization and channels that will enable leakage of intracellular components, inhibit aerobic and anaerobic respiration, and induce cell wall lysis and death (1). Alternatively, one group of scientist found that AmB primarily forms large extramembranous aggregates that extract ergosterol from lipid bilayers and thereby kill yeast (85).

Polyene has a lower but non-negligible affinity for cholesterol, the human counterpart of ergosterol. This non-negligible affinity leads to high toxicity associated with polyene, and it is a concerning matter for side effects (86). Nystatin and natamycin absorption through the gastrointestinal mucosa is almost minimal (87, 88). AmB is the most commonly used polyene antifungal for systemic infections. AmB is administered intravenously (86). AmB is the primary drug for treating systemic infections due to the low occurrence of acquired and innate resistance to this drug. This drug is recommended for the treatment of infections by *Candida*, *Aspergillus*, *Fusarium* and many more (86).

1.4.4 Fluoropyrimidines

Fluoropyrimidines includes 5-flucytosine (5-FC) and 5-fluorouracil (5-FU) and show fungistatic properties. They are synthetic structural analogues of the DNA nucleotide cytosine (1). 5-FC enters the fungal cell through cytosine permease and inhibits the thymidylate-synthetase enzyme interfering with DNA synthesis. 5-FC can be converted to 5-FU by a cytosine deaminase. The 5-FU can be phosphorylated and turn into 5-flurodeoxyuridine monophosphate. This one is also phosphorylated and incorporated into an RNA molecule, interfering with the translation (70, 81, 89).

5-FC is used as combinational therapy with AmB (90, 91). However, if used alone, 5-FC might lead to several side effects like hepatic impairment, interference with bone marrow, and it also shows the rapid occurrence of resistance. The elevated liver and renal toxicity of AmB is increased in this combination therapy, which adds up to 5-FC

hepatotoxicity, which has led to using the combination therapy of 5-FC with the azole drugs (1).

1.5 Antifungal resistance

Fungi can develop resistance to antifungal drugs using three different mechanisms. First, decreasing the drug's affinity for its target; second, modifying metabolism to counterbalance the drug effect; and third, reducing the drug accumulation within the fungal cell (1).

1.5.1 Echinocandin resistance

Echinocandins are the primary agents against invasive candidiasis; however, echinocandin treatment failures are arising. In addition, many non-*Candida albicans Candida* (NCAC) species are already being less susceptible to caspofungin during prolonged therapy. Reduced susceptibility to echinocandins is related to amino acid substitutions in two highly-conserved hot-spot regions of the *FKS* genes. For *C. glabrata*, a variety of amino acid changes in the hotspot regions of *FKS1* and *FKS2* confer resistance to the echinocandins. For example, studies involving sequencing of hot spots revealed an F659V substitution within *FKS2* confer echinocandin resistance (92).

Strains of *C. glabrata* have also been shown to be resistant to echinocandins and fluconazole. In addition, in these strains, mutations in *FKS1* and *FKS2* were detected. These mutations in *Candida FKS* genes that encode the target for echinocandins result

in elevated minimum inhibitory concentration (MIC) and end in therapeutic failures. Several studies confirmed that *FKS* gene mutations are significant for echinocandin resistance (23). For example, in one of the studies from patients with *C. glabrata* bloodstream infections, the treatment outcome was compared with the MIC results and the presence of *FKS1* and *FKS2* gene mutations. The results showed that from the 78 fluconazole-resistant isolates, nearly 14 % were resistant to one or more echinocandins. In addition, 8% of the isolates had *FKS* mutations, and all of them showed intermediate or resistance to MICs to an echinocandin (93).

1.5.2 Azole resistance

C. glabrata infections increase due to low intrinsic susceptibility to both azoles, including imidazole and triazole (94). As a result of rare mutations, *C. glabrata* acquire azole resistance, which is selected by drug pressure (95). In the presence of azole molecules, genes involved in the ergosterol biosynthesis are upregulated. *ERG* genes are *ERG3*, *ERG6*, *ERG7*, *ERG9* and importantly *ERG11*. As stated above *ERG11* is involved in the conversion of lanosterol to zymosterol (4, 4 dimethylcolesta-8,14,24-trienol) (23).

Azole drugs target the ergosterol biosynthetic pathway by inhibition of the 14-alpha demethylase, encoded by *ERG11* gene. It is known from the scientific data that overexpression of *ERG11* is responsible for the resistance to azole agents. For example, some mutations in the *UPC2* gene, encoding a transcriptional regulator, result in a hyperactive factor and increased expression of the target gene *ERG11* (95, 96).

Mutations in *PDR1* may result in hyperactivation and is linked to azole resistance. The increased expression of the Pdr1 *ta*rget gene *CDR1*, encoding a drug efflux pump, contribute to azole resistance (97). Also, some clinical isolate resistant to azoles have been shown to be “petite” i.e., with mitochondrial defects resulting in activation of *PDR1* (98-100).

C. glabrata has intrinsic azole resistance and it acquires resistance to this class of antifungal very easily. The drug resistance is due to prolonged azole therapy by undergoing mutation either *in vitro* or *in vivo* (101). Many *ABC* transporters, including Cdr1, Snq2, Pdh1 and Yor1, contribute to drug efflux. The transcription factor Pdr1 is a major regulator of ABC transporter gene expression. Moreover, it has been found to be a key factor of pleiotropic drug resistance (PDR) (102, 103).

1.5.3 Polyene resistance

It is yet not well understood how *C. glabrata* clinical isolates show polyene resistance. However, according to a study done by Vandeputte P. *et al.* (104), a *C. glabrata* clinical isolate showed lower ergosterol content in its cell membrane compared to wild-type strain. Moreover, this low content was linked to a mutation in the *ERG6* gene. This mutation in the clinical isolate led to a decrease in ergosterol content. There is one more study that analyzed polyene resistance in clinical isolates (105). This study demonstrated that mutation in the *ERG6* gene leads to subsequent changes in the plasma membrane and reduced susceptibility to polyenes. Furthermore, because of that mutation, an accumulation of late sterol intermediates and ergosterol deficiency was

observed. The complementation of this mutated strain with the wild-type copy of the *ERG6* gene restored susceptibility to polyenes.

1.6 Zinc cluster proteins

Zinc (Zn) is a necessary element for the proper functioning of large numbers of proteins, including various enzymes. A majority of zinc-containing proteins are transcription factors, having the ability to bind to DNA and are known as zinc finger proteins. They are divided into various classes depending on their DNA binding motifs. One class is called zinc cluster proteins and they possess the well-conserved motif $CysX_2CysX_{5\text{-}12}CysX_2CysX_{6\text{-}8}Cys$. The cysteine residues bind to two zinc atoms for the folding of the domain involved in DNA binding (106). The family of zinc cluster protein is well studied in the *S. cerevisiae.* The genome of *S. cerevisiae* encodes more than 50 known or putative zinc cluster proteins (106). A well-studied zinc cluster protein is Gal4 involved in the regulation of genes involved in the catabolism of galactose (107). There are many other zinc cluster proteins involved in various cellular processes, such as the metabolism of carbon, amino acids, pyrimidine and fatty acids. Moreover, they are also involved in drug resistance (108).

1.6.1 Structural and functional domains of zinc cluster proteins

Zinc cluster proteins contain several domains, including a cysteine-rich DNA binding domain, an activation domain, and a regulatory domain. Moreover, the DNA binding domain (DBD) is subdivided into three regions: the zinc finger, the linker and the

dimerization region (106). The DBD of zinc cluster proteins is usually located at the N-terminus, while the acidic activating domain is located at the C-terminus. The middle homology region is located between the DNA binding domain and an activation region and is involved in regulating the transcriptional activity of zinc cluster proteins (109). X-ray crystallography of the DBDs of *S. cerevisiae* Gal4 and Ppr1 showed that these proteins bind as homodimers. These homodimer complexes recognize a pair of CGG nucleotide triplets, interacting via major-groove contacts (110). Though the requirement for zinc in stabilizing protein folding and function is necessary in these zinc cluster proteins, several key experiments illustrate that zinc can be replaced by other metal ions, for example, Cd^{2+} (110). The linker region is situated C-terminally to the zinc cluster motif. The linker region can vary in sequence between different zinc cluster proteins and contributes to the DNA binding specificity of the transcription factor. The linker region provides a rigid scaffold thus preventing binding to alternative sites (111). The last region of the DNA binding domain is the dimerization region. It is made up of heptad repeats which are similar to those found in leucine zippers (112). The heptad repeats form a highly conserved coiled-coiled structure which allows protein-protein interaction and dimerization.

As stated above, the regulatory domain termed the middle homology region regulates the transcription activity of these factors (112). The domain displays lesser homology than the zinc finger domain. It is found in between the DNA binding domain and the C-terminal acidic region. Deletion of the regulatory domain results in a constitutively active factor. The *S. cerevisiae* Pdr1 and Pdr3 mutants that contain gain of function mutations within this region, indicating that it has an inhibitory role. The acidic

domain is also known as the activation domain, and it is located C-terminally. The function and structure of this domain of in the zinc cluster protein are varied and not well- defined (106).

1.6.2 DNA-binding specificity

Zinc cluster proteins bind to DNA by recognizing CGG trinucleotide sequences in single or repeated forms, in either a symmetrical or asymmetrical form. Many factors influence DNA targeting and binding by zinc cluster proteins. Many components of DBD lead significantly in binding to target DNA. Additionally, nucleotides around the CGG triplets also determine DNA-binding affinities (113). There are two important determinants of DNA-binding specificity are the orientation of the CGG triplets and the spacing between these triplets. Binding is observed with CGG triplets that are oriented in everted, inverted or direct repeats. Homodimeric, heterodimeric and monomeric binding to DNA has been reported. The spacing between trinucleotide sequences is critical for specificity. For example, Put3 binds to CGG separated by 10 bp (CGG-N_{10}-CCG) while Gal4 binds to CGG triplets by 11 bp (CGG-N_{11}-CCG) (114-116).

The presence of monomers and heterodimers of zinc cluster protein suggest that many variations and combinations of this most likely occur. One example is the physiological presence of Pdr1 and Pdr3 homodimers and Pdr1/Pdr3 heterodimers in yeast (117).

1.6.3 Mechanism of action

For many transcriptional regulators, various strategies exist to control their transcriptional activity. These include nuclear-cytoplasmic shuffling, DNA binding, phosphorylation, and unmasking of the activation domain (118, 119). The zinc cluster proteins that could regulate target genes as monomers include the *S. cerevisiae* proteins Upc2 and Ecm22. They activate transcription of the *ERG* genes, which encode enzymes needed for ergosterol biosynthesis. They are acting through DNA response elements that contain the sequence CGTATA (120, 121). Zinc cluster protein can coordinate the transcriptional control of target genes alone or in coordinated networks with other members of this class. They can do this by acting through one or more DNA recognition sites. For instance, three zinc cluster proteins, Pdr1, Pdr3 and Rdr1, regulate the *PDR5* gene, encoding an ATP- binding cassette transporter involved in drug resistance through the same pleiotropic drug response elements (PDREs) (122).

1.6.4 Self-regulation and positive feedback loops

Some members of the zinc cluster proteins regulate the expression of other zinc cluster proteins. Moreover, some are self-regulated and form a positive feedback loop. It has been found that Pdr3 is positively autoregulated, and it also regulated by the Pdr1 zinc cluster protein (106). According to (123), Pdr3 controls its own transcription through two PDREs in its promoter.

1.6.5 Zinc cluster proteins in *C. glabrata*

A phenotypic analysis of zinc cluster proteins has been reported (124). However, only a few zinc cluster proteins have been characterized in more details. Pdr1, which is a homolog of *S. cerevisiae* Pdr1/Pdr3, confers drug resistance by positively regulating the expression of various genes, including *CDR1*, *SNQ2* and *PDH1* encoding ABC transporters. As stated above, these ABC (ATP binding cassette) transporters act as drug efflux pumps (103, 125). These transporters use the binding and hydrolysis of ATP to power the translocation of a diverse variety of substrates, ranging from ions to macromolecules, across membranes. As demonstrated in *S. cerevisiae*, mutations in the *PDR1* gene that result in hyperactivation of the transcription factor, leads to increased expression of genes encoding transporters and, as a result, resistance to various drugs (125). Pdr1 is activated by direct binding to azoles (126). There are two functional homologs of *S. cerevisiae* Upc2/Ecm22, and they are named Upc2A and Upc2B. Upc2A is an activator of ergosterol biosynthetic genes. Upc2A and Upc2B are the positive regulators of the *AUS1* gene encoding sterol transporter (127). *STB5* encodes a repressor of the transporter genes *CDR1*, *YOR1* and *PDH1* (102).

The most prominent cause of azole resistance is mutations in the gene encoding the zinc cluster containing transcription factor, Pdr1 (103, 125, 128). These mutations are typically substitutions and produce a factor that exhibits high-level of transcriptional activation (2, 129). The key Pdr1-regulated gene is *CDR1,* and its elevated expression is required for the observed drug resistance in gain-of-function *PDR1* strains (130). As stated earlier, Pdr1 is a homolog of *S. cerevisiae* Pdr1/Pdr3. Although Pdr3 is absent in

C. glabrata, recent observations suggest that *C. glabrata* Pdr1 is a hybrid molecule between *S. cerevisiae* Pdr1 and Pdr3 (103). Study of these factors in *S. cerevisiae* provided a basis to understand the mechanism of action of *C. glabrata* Pdr1. For example, the PDRE, that is the DNA target site recognized by all these factors was discovered in *S. cerevisiae* and is conserved in *C. glabrata* (125, 131, 132). *PDR1* is autoregulated in *C. glabrata*. Moye-Rowley *et al*. (133) demonstrated that the central domain of this factor confers negative regulation on the activity of Pdr1. A complete understanding of the regulation of Pdr1 provides a new avenue of interfering with azole and echinocandin resistance in *C. glabrata*.

Section 2: Rationale and Objectives

The prevalence of fungal infection is increasing, presenting an enormous challenge to healthcare professionals. This increase is directly related to the rising population of immunocompromised individuals. This increase results from changes in medical practice, such as the use of intensive chemotherapy and immunosuppressive drugs. Drug resistance is an unavoidable consequence of the deployment of antimicrobial drugs. Drug resistance is a severe issue with regards to fungal pathogens.

The major class of antifungal drug used to treat fungal infections are the azole and echinocandins compounds. Azole compounds target a step-in biosynthesis of fungal-specific sterol ergosterol. Echinocandin compounds interfere with β-glucan synthesis. Work from several labs found that the most persistent causes of azole-resistant are mutations in the gene encoding the transcriptional regulator Pdr1 (103, 134). Pdr1 controls the expression of genes encoding drug efflux pumps leading to resistance to azoles. It has been shown that Pdr1 regulates its own expression by binding to PDREs found in its promoter. This autoregulation is necessary for resistance to azoles.

Our lab has found that mitochondrial defects lead to increased levels of *PDR1* mRNA and increased resistance to echinocandins. Moreover, our lab has identified mutations in the coding region of *PDR1* that confer resistance to echinocandins. One objective of this study was to determine if the auto-regulatory loop is necessary for resistance to echinocandins by *PDR1* mutants and to determine the effects of different *PDR1* mutations on drug resistance. To this end, we generated *PDR1* mutants by combining mutations from different regions of the promoter and the coding region of the

PDR1 gene and transform them into a strain lacking the *PDR1* gene. These transformants were tested for micafungin and azole resistance.

Echinocandins inhibit the activity of a two-subunit enzyme involved in the synthesis of the polysaccharide 1,3-β-D-glucan, a major and essential component of the cell wall. *FKS1* and *FKS2* are two redundant genes that encode the subunit with β-glucan synthase activity. Resistance to echinocandins is due to mutations in hot-spot of the *FKS1* and *FKS2* genes. Our study was aimed at determining the effect of combining the rho^- strains (i.e., cells with defects in mitochondrial DNA) with mutations in hot-spot regions of the *FKS1* and *FKS2* on resistance to echinocandins.

Little is known about the possible role of zinc cluster proteins in preventing killing by phagocytes (135). Mouse macrophage cells were used to study the effect of deleting genes encoding zinc cluster genes on *C. glabrata* survival. The *C. glabrata* genome encodes a total of 41 (known or putative) zinc cluster proteins, 39 are encoded by non-essential genes, with a majority of them being uncharacterised. Our lab generated a panel of strains carrying individual deletions of zinc cluster genes (124). Thus, I took advantage of this panel (total of 37 strains) to perform assays for survival in macrophages (objective 2). This work was performed in collaboration with Dr. M. Olivier lab.

Section 3: Materials and Methods

The materials and methods for objective 1A, that is to investigate the role of the zinc cluster transcriptional factor *PDR1* in conferring drug resistance in *C. glabrata,* are as follows.

3.1 Strains and media

The wild-type *C. glabrata* strain 66032ura3 was provided by T. Edlind (Philadelphia PA). A Δ*pdr1* strain derived from this wild-type strain was described in Klimova *et al.* (124). All media were prepared following (136). YPD medium contained 1% yeast extract, 2% peptone and 2% glucose. The SD medium consisted of 2% glucose, 0.67% yeast nitrogen base (devoid of amino acids) and supplemented with the appropriate amino acids.

3.2 Construction of plasmids

We constructed an expression vector (pGRB.PDR1) for Cg*PDR1*. This vector is an episomal, low copy plasmid with a *URA3* marker for selection. *URA3* encodes orotidine 5-phosphate decarboxylase (ODCase), an enzyme involved in the de novo synthesis of pyrimidine ribonucleotides and is used to select colonies carrying the plasmid on SD agar plates lacking uracil.

Construction of an expression vector for Cg*PDR1*:

The pGRB.PDR1 plasmid construction strategy is outlined in Figure 1. Briefly, the plasmids pCgPDR1-C (Klimova, unpublished) and pGRB2.1 (137) were digested using the restriction enzymes EcoRI and SacI and subsequently ligated as described in lab protocol (138). The ligation products were transformed into *E. coli* competent cells as described in the Hanahan method (138). The products were plated on LB agar plate with antibiotic to select for transformants that contained desired plasmids, and incubated at 37°C for 24 h. Colonies were picked and after overnight grown in LB medium, plasmid DNA was purified using the QIAprep Miniprep Kit. Control digests were performed to check for possible candidates that contained the correct constructs. The construct plasmid was sent for sequencing at the Genome Center at McGill.

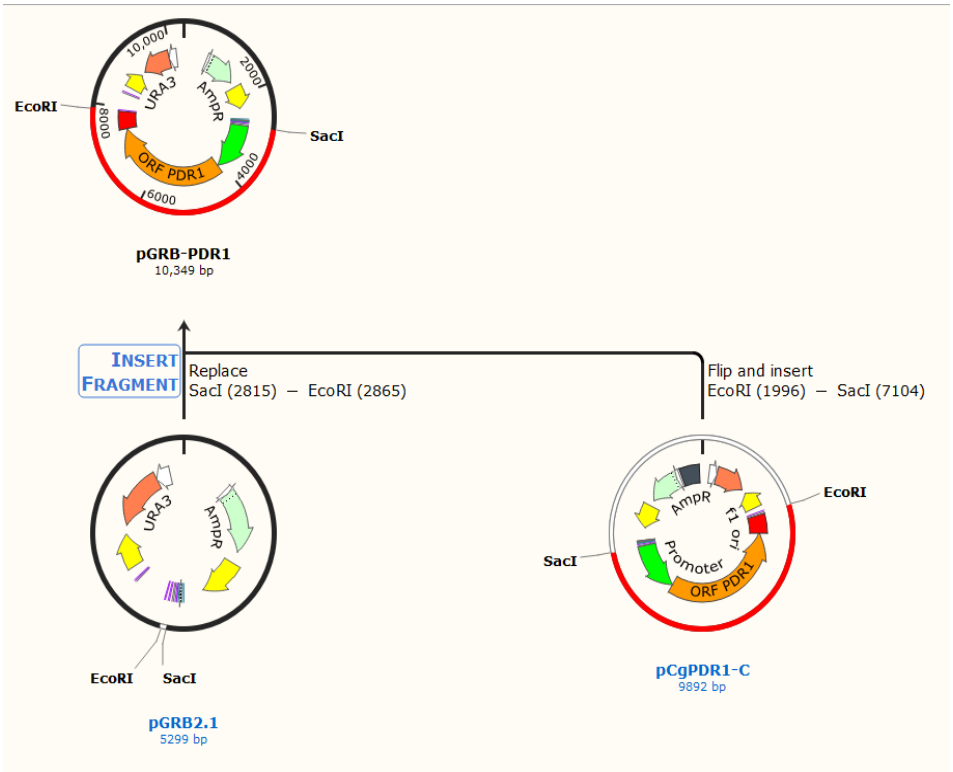


Figure 1. Construction of an expression vector for Cg*PDR1*

Two PDREs are required for autoregulatory induction of *PDR1* and their loss lowers the expression of the gene in *C. glabrata* (133). To study this, we generated the expression vector pGRB.PDR1, where PDREs were mutated to AAAAAA. The oligonucleotides which were used for this plasmid construction are listed in Table 1. We followed the standard Gibson assembly method to construct these plasmids. We also generated *PDR1* mutants by combining mutations from different regions of the promoter and the coding region of the *PDR1* gene and transformed them into the Δ*pdr1* strain.

Table 1. Oligonucleotides used in this study

Oligonucleotide	**Sequence (5' → 3')**
CgPDR1-GIB-E	AGGGAACAAAAGCTGGAGCTCGCATTATCTAGG
GIB-PDR1-10	TCCATATAGCACAATTTGCTTTTTTTAATGAGTCTACAAATACTGGG
GIB-PDR1-20	CCAGTATTTGTAGACTCATTAAAAAAAGCAAATTGTGCTATATGGA
GIB-PDR1-30	CAATAGGAAAAGCCATCTTTAAAAAAAATAGGAGGCTCATCGGGAC
GIB-PDR1-40	GTCCCGATGAGCCTCCTATTTTTTTTAAAGATGGCTTTTCCTATTG
CgPDR1-GIB-F	GCTTTTTCCTTTAAAATCCATTTGG
PCNU-forward	ATGTGGATATCTTGACTGATTTTTCCAGGAAACAGCTATGACCATGATTACG

PCNU-reverse	TAGCGGCTTAACTGTGCCCTCTGTAAAACGACGGCCAGTG AGC
HTT2-reverse	TGCTCACCATGAATTCCTGCAGCCCGG
mcherry-forward	GCAGGAATTCATGGTGAGCAAGGGCGAG
mcherry-reverse	CGACCTGCAGTTACTTGTACAGCTCGTCCATGCCG
HIS3-UTR-forward	GTACAAGTAACTGCAGGTCGACCTCGAG

3.3 Transformation and phenotypic antifungal drug assays

C. glabrata cell transformation was performed using a lithium acetate method as outlined by Gietz *et al.*, 1992 (139). Wild-type and mutant *C. glabrata* strains were grown overnight in selective media for drug sensitivity growth assays. Sensitivity to drugs was assayed on YPD agar plates containing various drugs. Cells were washed, serially diluted, and spotted on rich medium YPD, YPD+ 16 µg/ml fluconazole (FCZ), 32 µg/ml FCZ, 64 µg/ml FCZ, 128 µg/ml FCZ, YPD + 10 ng/ml micafungin (MCF), 20 ng/ml MCF. Plates were incubated at 30°C for 12 to 24 h.

3.4 β-galactosidase assay

The PDR1- LacZ plasmid is a lacz reporter whose expression is driven by the *PDR1* promoter, and it contains a *URA3* marker. We constructed PDR1-LacZ reporter with mutated PDREs and transformed them into wild-type strain and strain with the mutated coding region. All strains were grown in SD medium with MCF (15 ng/ml) or without MCF, and β-galactosidase assays were performed (140).

3.5 Construction of rho$^-$ strains and drug sensitivity assay

Materials and methods for objective 1B (study of the mitochondrial defects result in susceptibility to echinocandins) are as follows. Strains from Table 2 were grown to saturation in minimal medium (0.67 % yeast nitrogen base) containing 2% glucose (SD medium), uracil (0.004%) and 25 µg/ml ethidium bromide to obtain rho$^-$ strains (i.e., cells with defects in mitochondrial DNA) (141). Their rho$^-$ status was verified by their inability to grow on YPE (ethanol containing) plates. Strains were tested for micafungin sensitivity by spotting assays. HS1 and HS2 refer to 'hot spots' regions of the *FKS1* and *FKS2* genes. A number of mutations in these regions result in increased resistance to echinocandins.

Table 2. Description of the *C. glabrata* strains used in this study

Strain	Location of mutations			
	FKS1		*FKS2*	
	HS1	HS2	HS1	HS2
CG321	-	-	-	-
CG323	-	-	-	-
CG325	WT	WT	F695S	-
CG331	D632G	WT	WT	WT

The following are materials and methods for objective 2 to determine the survival rate of the mutant strains lacking zinc cluster gene in macrophages.

3.6 Strains

The wild-type *C. glabrata* strain used to generate the zinc cluster gene deletions is a tight 5-fluoroorotic acid selected *ura3* derivative of 66032 (as referred to in this study

66032-*ura3*), provided by T. Edlind (Philadelphia PA). Strains carrying deletion of zinc cluster genes were described in Klimova *et al.* (124).

3.7 Gene deletion

The gene deletion method has been described in detail in Klimova *et al.* (124). Briefly, via homologous recombination in a *C. glabrata-ura3* strain, a panel of strains carrying deletions of zinc cluster genes was generated. The strains used are listed in Table 3.

Table 3. List of *Candida glabrata* strains used for assays in macrophages

Serial No	**Strain**	**Serial No**	**Strain**	**Serial No**	**Strain**
1	WT 66032	13	CgΔ*zcf14*	25	CgΔ*zcf28*
2	CgΔ*zcf24*	14	CgΔ*zcf15*	26	CgΔ*zcf29*
3	CgΔ*zcf1*	15	CgΔ*zcf16*	27	CgΔ*zcf30*
4	CgΔ*zcf2*	16	CgΔ*zcf17*	28	CgΔ*zcf31*
5	CgΔ*zcf3*	17	CgΔ*zcf18*	29	CgΔ*zcf32*
6	CgΔ*zcf4*	18	CgΔ*zcf19*	30	CgΔ*zcf33*
7	CgΔ*zcf6*	19	CgΔ*zcf20*	31	CgΔ*zcf34*
8	CgΔ*zcf7*	20	CgΔ*zcf21*	32	CgΔ*zcf35*
9	CgΔ*zcf8*	21	CgΔ*zcf22*	33	CgΔ*zcf36*
10	CgΔ*zcf9*	22	CgΔ*zcf25*	34	CgΔ*zcf37*
11	CgΔ*zcf10*	23	CgΔ*zcf26*	35	CgΔ*zcf38*
12	CgΔ*zcf12*	24	CgΔ*zcf27*	36	CgΔ*zcf39*
				37	CgΔ*zcf40*

3.8 Cell culture and macrophage infection assay

We used a wild-type strain and mutant strains to infect murine macrophages (B10R) (142). The infection was done the following way. The murine macrophage cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with

10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Glutamine (PSG) at 37°C under 5% CO_2. For infection assay, 50 µl of overnight grown, 0.1 OD_{600} normalized, PBS washed *C. glabrata* cell suspensions was added to macrophage cells. The multiplicity of infection 1:1 was used for infection assay. At 2 h post-incubation, infected macrophage cells were washed twice with PBS to remove the non-phagocytosed yeast cells. At 2 and 24 h post-ingestion, PBS washed macrophage cells were lysed in water, and the number of recovered yeasts was determined by plate counts of suitable dilutions (CFU, colony forming units assay). The fold replication of *C. glabrata* strains in murine macrophages was calculated by dividing total CFUs at 24 h with those at 2 h.

For phagocytosis assays and microscopic imaging studies, the plasmid pHTT2-GFP-URA and the plasmid pHTT2-mcherry-URA were constructed. Intracellular replication of wild-type strain, transformed with GFP (green fluorescent protein) expressing plasmid and intracellular replication of mutant strains, transformed with RFP (red fluorescent protein) expressing plasmid, in macrophages could be verified with fluorescence microscopy. Oligos used in this study listed in Table 1. The plasmid pHTT2-GFP-URA was constructed as follows: PCR was done with oligos PCNU-forward and PCNU-reverse using pCU-HTT2-GFP as a template and gibson assembly done using plasmid pRS306 cut with NcoI. The plasmid pHTT2-mcherry-URA was constructed as follows: PCR was done with oligos PCNU-forward and HTT2-reverse using pCU-HTT2-GFP as a template, PCR was done with oligos mCherry-forward and mCherry-reverse using hbarr2-PAmCherry as a template, PCR was done using oligos HIS3-UTR-forward and PCNU-reverse using pCU-HTT2-GFP as a template and gibson assembly done using plasmid pRS306 cut with NcoI. It was assumed that pHTT2-GFP-ura plasmid HTT2

should be integrated at the *URA3* locus of deletion strain while RFP at the *URA3* locus of the wild-type strain. However, PCR analysis of colonies selected for the loss of the *URA3* gene due to the integration of the reporters did not show any expected integration.

SECTION 4: Results

4.1. Plasmid construction

The expression vector pGRB.PDR1 is an episomal, low copy plasmid with a *URA3* marker for selection. As shown in Figure 2, the introduction of a pGRB.PDR1 in a *Δpdr1* strain (CG201) restores resistance to fluconazole (FCZ).

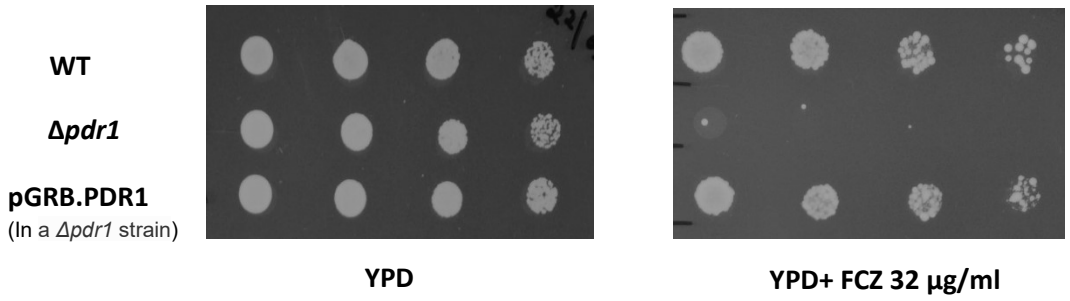


Figure 2. The pGRB.PDR1 plasmid restores FCZ resistance of a strain lacking the *PDR1* gene. Strains grown overnight in YPD, serially diluted and spotted on YPD plates without or with 32 µg/ml FCZ. Introduction of a pGRB.PDR1 in a *Δpdr1* strain restores resistance to fluconazole FCZ.

Figure 3. Schematic representation of the location of the two pleiotropic drug response elements (PDREs) located in the *PDR1* promoter. The numbering is relative to the *PDR1* ATG codon with +1 corresponding to the adenine residue of this codon. WT and mutant sequences of PDRE1 and PDRE2 are indicated below the diagram. Arrows indicate an imperfect everted repeat.

Zinc cluster proteins preferentially bind to CGG triplets. The PDRE is an imperfect everted repeat. Wild-type and mutant sequences of PDRE1 and PDRE2 are indicated in Figure 3. Mutations were introduced in each of these PDREs (Figure 3). The PDREs contain TGG and CGG triplets, which is the recognition site for CgPdr1. We successfully generated the expression vector pGRB.PDR1, where PDREs were mutated to AAAAAA, denoted as M1 mutant. Our lab has generated the pGRB.PDR1 with a mutation in the coding region (mutant Y208C) represented as an M2 mutant. Moreover, we generated *PDR1* mutants by combining mutations from the different regions of the promoter and the coding region of the *PDR1* gene, denoted as M3 (Table 4) and then transformed them into the Δ*pdr1* strain (CG201).

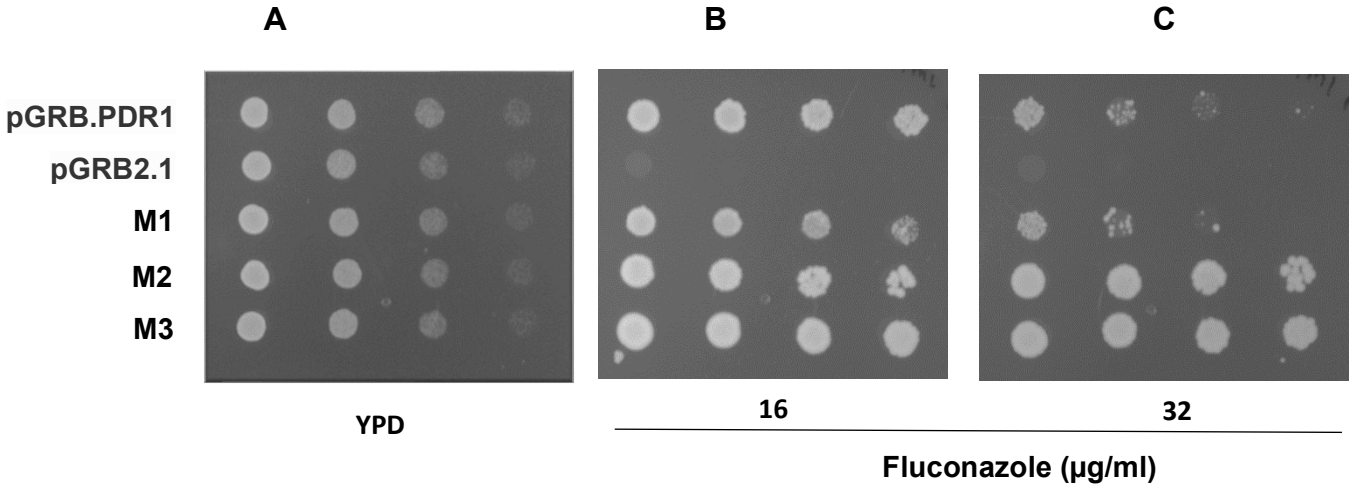
Table 4. List of *Candida glabrata* mutants

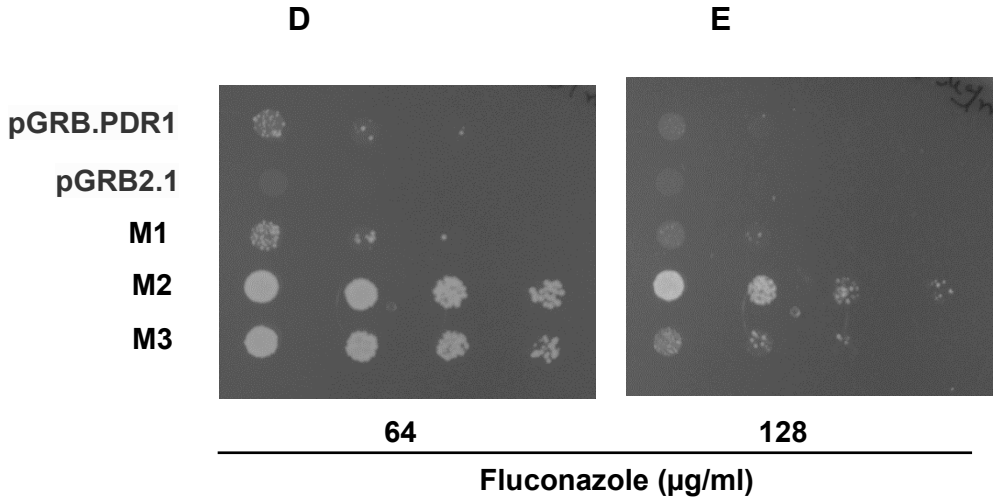
Mutants	**Remark**
M1	PDREs 1 and 2 mutated
M2	Mutant Y208C
M3	PDREs 1 and 2 mutated and combined with mutant Y208C

4.2. Drug sensitivity

For spotting assays, four concentrations of fluconazole were used: 16 µg/ml, 32 µg/ml, 64 µg/ml and 128 µg/ml while two concentrations of micafungin were used: 10 ng/ml, 20 ng/ml. There is similar growth in the absence of the drug (Figure 4A). As expected, there is no growth of a *Δpdr1* strain, transformed with empty vector pGRB2.1 (Figure 4B to 4E). The fluconazole and echinocandin (Figure 4) drug sensitivity assay revealed that mutations in both PDREs (M1) had modest effect on fluconazole resistance

and more prominent effect on echinocandin resistance (Figure 4B and 4G, respectively). Better growth of Y208C mutant strain (M2) in the presence of fluconazole and 20 ng/ml micafungin was observed as compared to other strains (Figure 4B to 4E and 4G, respectively). These findings demonstrate that the PDREs in the *PDR1* promoter are essential for the normal regulation of this gene. The mutant (M2) obtained by randomly mutating coding region and selecting for resistance showed high resistance to both drugs, fluconazole and micafungin (Figure 4). This analysis determines that the auto-regulatory loop is necessary for resistance to echinocandins by the *PDR1* mutant (M3).


A
B
C
pGRB.PDR1
pGRB2.1
M1
M2
M3
YPD
16
32
Fluconazole (µg/ml)


D
E
pGRB.PDR1
pGRB2.1
M1
M2
M3
64
128
Fluconazole (µg/ml)

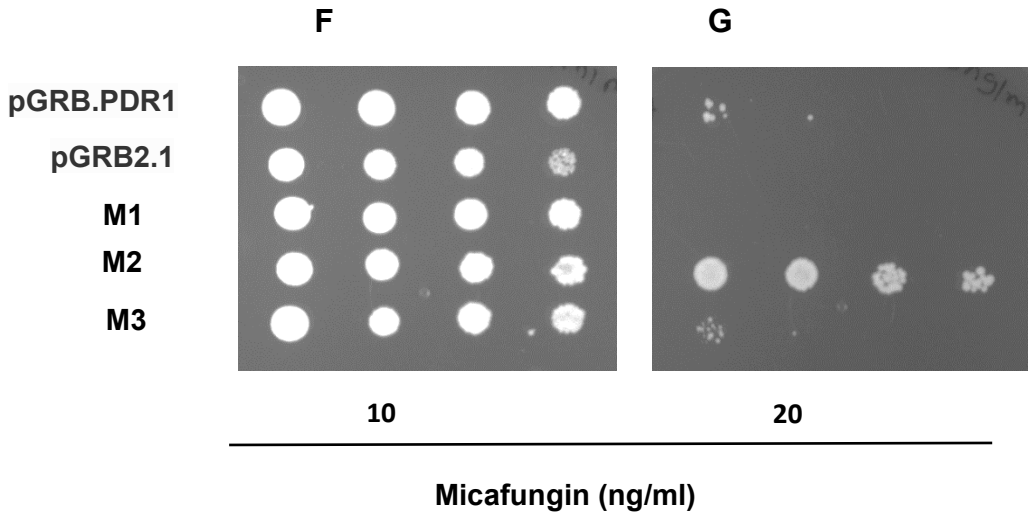

F
G
pGRB.PDR1
pGRB2.1
M1
M2
M3
10
20
Micafungin (ng/ml)

Figure 4. Fluconazole and micafungin sensitivity assay. Fluconazole and micafungin sensitivity assay of the *pdr1Δ* (CG201) strain transformed with, the empty vector pGRB2.1 or the plasmid containing the wild-type *PDR1* gene called pGRB.PDR1 (WT) or either mutant form M1, M2 and M3. All transformants were grown to saturation in selective medium and then plated in 1:5 dilutions on YPD plates without drug or with fluconazole or micafungin.

4.3. β-galactosidase assay

Results from the drug sensitivity assay show that PDREs are required for increased resistance to echinocandins in the mutant Y208C. qPCR analysis showed that *PDR1* mRNA levels are increased in mutant Y208C (M2) (Klimova *et al.,* unpublished). There are two possibilities for this result, stability of the *PDR1* mRNA mutant may be increased or the promoter activity may be increased. To distinguish between these two possibilities, we performed β-galactosidase assays. The β-galactosidase assay measures the levels of active β-galactosidase expressed in cells transformed with plasmids expressing the *lacZ* gene. Data for β-galactosidase (Table 5 and Figure 5) revealed that β-galactosidase activity of ΔPDREs–LacZ in both strains was reduced compared to PDR1-LacZ in both strains. This result shows that the removal of PDREs reduces *PDR1* promoter activity. Thus, an auto-regulatory loop is important for increased resistance of the Y208C mutant.

Table 5. Results of β-galactosidase assays. The strain EF3 carries a mutation in the *PDR1* gene resulting in a change of tyrosine to cysteine at amino acid 208 (mutant Y208C). Cells were grown overnight, diluted to OD660 of 0.5 and treated or not with 15 ng/ml MCF for 4 h.

	β-galactosidase activity	
Strains	PDR1-LacZ	ΔPDREs – LacZ
WT	5	4
WT (MCF treated)	3	2
EF3	44	8
EF3 (MCF treated)	97	20

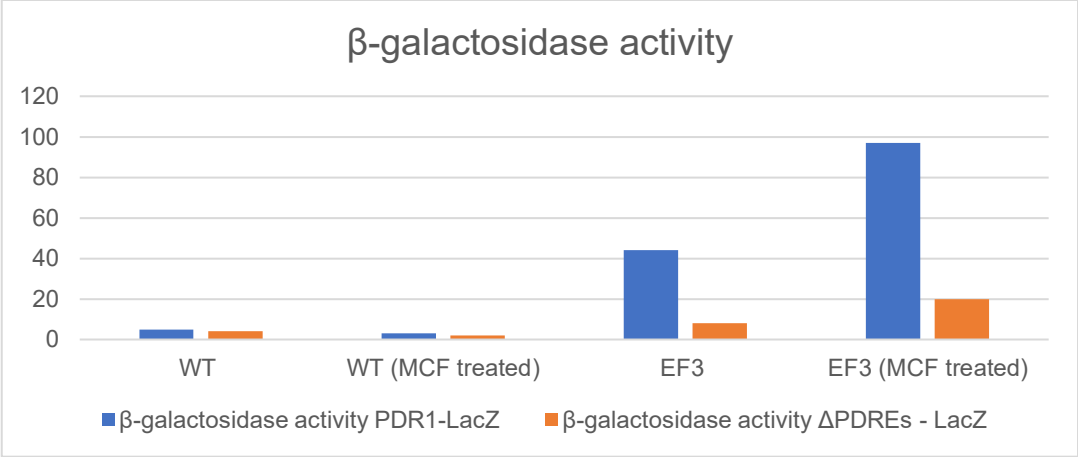


Figure 5. Schematic representation of β-galactosidase activity

4.4. Micafungin sensitivity assay to verify if mitochondrial defects result in decreased susceptibility to echinocandins

As from unpublished results from the lab, rho$^-$ strains are more resistant to echinocandins than rho$^+$ strains. As described in the method section, the CG321 and CG323 strains do not have *FKS* mutation, while CG325 and CG331 strain have mutation in *FKS1* or *FKS2*. Figure 6 shows the CG325 strain is more resistant due to *FKS2* gene mutation than other strains without mutation and the WT strain. Moreover, there were no effects seen with the rho$^-$ strains carrying *FKS1* or *FKS2* mutation. Thus, this study determines that cells lacking mitochondrial DNA (rho$^-$) are resistant to micafungin, but no effect was seen with strains that contained *FKS1* or *FKS2* mutation. This can be explained by the fact that these strains are already highly resistant to micafungin.

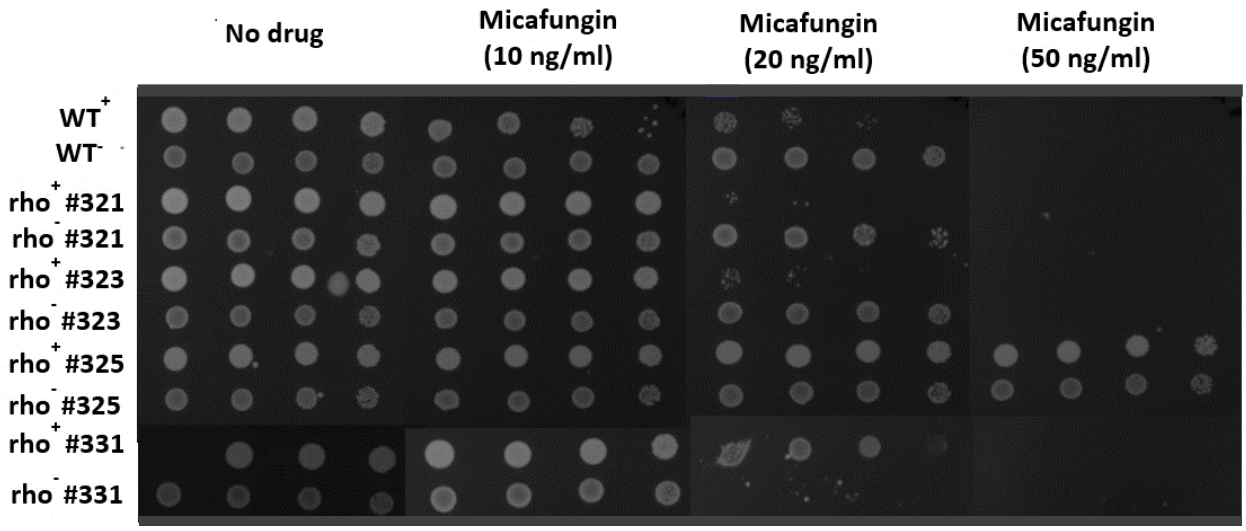


Figure 6. **Micafungin sensitivity assay.** Cells lacking mitochondrial DNA (rho$^-$) are resistant to micafungin but no effect were seen with strains that contained *FKS1* or *FKS2* mutations.

4.5. *C. glabrata* survival and replication in macrophage cells

C. glabrata has emerged as the second most common cause of candidiasis. However, our knowledge about the strategies it employs to multiply in host phagocytic cells is minimal. The potential role of zinc cluster protein in this aspect is yet to be determined. To this end, we have tested 37 mutant strains from our panel of deletion strains for survival in macrophages. To study the interaction of *C. glabrata* with macrophages, we first established the infection dynamics of *C. glabrata* cells with murine macrophage cells line B10R. Infection studies of macrophage cells with *C. glabrata* cells at an MOI of 1:1 revealed a moderate and altered fold increase in the wild-type compared to mutant strain (Figure 7).

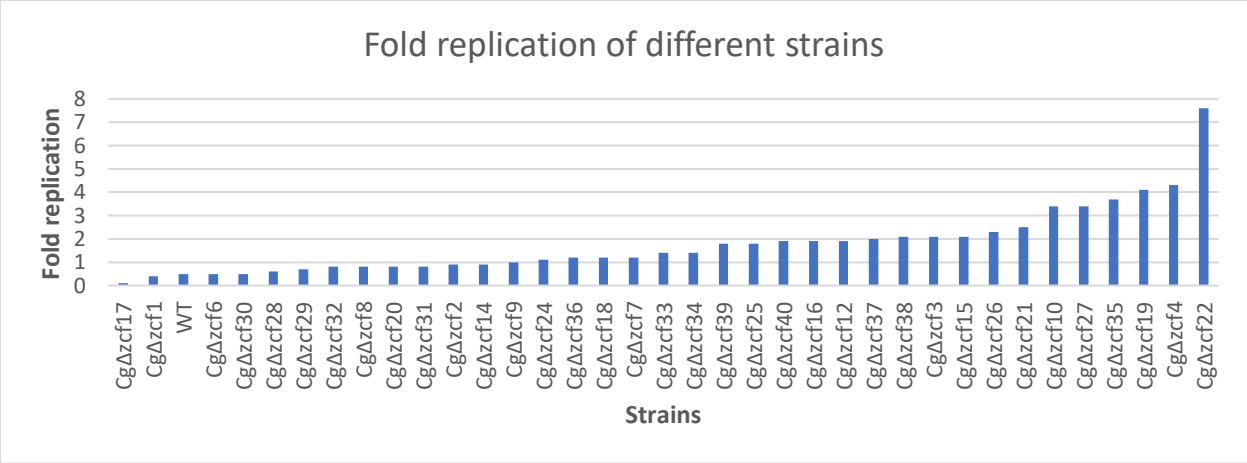


Figure 7. **Schematic representation of the altered survival of *C. glabrata* mutant strains as compared to a WT strain**. Strain Cg∆*zcf1* carries a deletion in the *PDR1* gene.

We observed altered survival of mutant strains as compared to a wild-type strain. Specifically, Strain Cg∆*zcf22* showed a 7.6-fold increase in replication and strains, Cg∆*zcf17* and Cg∆*zcf1* showed a decrease fold of replication. More details about the strains can be found in Klimova *et al*. (124). The *CgZCF22* gene is a homolog of the *S. cerevisiae WAR1* gene (weak acid resistance). War1 is sequence-specific DNA binding transcription factor that is involved in the response to weak acids. For example, War1 activates transcription of organic acid transmembrane transporter *PDR12*. CgZcf4 is homolog of the *S. cerevisiae* Hap1. This factor is involved in regulating gene expression in response to levels of heme and oxygen. CgZcf24 is highly homologous to *S. cerevisiae* Stb5. Klimova *et al*. and Noble *et al*. (102, 124) found that the Cg*Δzcf24* strain was sensitive to oxidative stress. Stb5 is an activator to genes of the pentose phosphate pathway and other genes involved in the production of NADPH, a cofactor involved in conferring resistance to oxidative stress (143). However, CgZcf24 does not control the expression of genes involved in the production of NADPH (102). In addition, a strain carrying a deletion of Cg*ZCF24* does not show altered replication in

macrophages. It is not clear why sensitivity of this strain to oxidative stress does not result in decreased fitness in macrophages. Cg*ZCF17* gene is a homolog of the *S. cerevisiae ASG1* gene. Asg1 is involved in the stress response. The Cg*ZCF1* gene is homolog of the *S. cerevisiae PDR1/PDR3* gene. Pdr1 and Pdr3 are transcriptional activators of the pleiotropic drug resistance network. This study determined the *C. glabrata* mutant strains survival and replication in macrophage cells compared to a wild-type strain.

Section 5: Discussion

A better understanding of *C. glabrata* Pdr1 regulation is important. This understanding is significant to develop the interventions to reverse the frequent azole or echinocandin resistance in *C. glabrata* caused by the mutant forms of this protein. Pdr1 represents a blend of the properties of ScPdr1 and ScPdr3 (133). Pdr1 shares autoregulation and mitochondrial control with ScPdr3 and also shares the highest sequence similarity with ScPdr1 (125, 128). *S. cerevisiae* seems to have split its zinc cluster-containing Pdr factor functions between two paralogs while *C. glabrata* reserved all of these in its single *PDR1* gene. We confirm that autoregulation is critical feature of transcriptional regulation of *PDR1* through PDREs for the mutant Y208C and for high doses of antifungals. There is more prominent effect seen for autoregulation of transcriptional regulation of *PDR1* through PDREs compared to our data in the study done by Khakhina S. *et al*., (133). We also confirm that the mutant (Y208C) obtained by randomly mutating the *PDR1* coding region and selecting for resistance mutation showed high resistance to both, fluconazole and micafungin. Analysis of the *PDR1* promoter confirms the predicted important roles of the PDREs present in this DNA region.

We also confirm that cells lacking mitochondrial DNA (rho^-) are resistant to micafungin, but no effect was seen with cells that contained *FKS1* or *FKS2* mutations with or without mitochondrial defects. This observation could be explained by the fact that strains bearing mutations in *FKS1* or *FKS2* already show a high degree of resistance to micafungin. We determined that *C. glabrata* mutant strains carrying deletions of zinc cluster genes altered survival and replication in macrophage cells compared to a wild-type strain. We found that most mutants except with the exception of two mutants showed

increased survival in macrophage. These results will need to be confirmed by the use of an internal control. For example, a wild-type strain expressing an RFP reporter and deletion strains expressing a GFP reporter could be constructed. Infection of macrophages would then be performed by mixing wild-type cells expressing RFP and deletion strains expressing GFP. Measuring the signal obtained with GFP as compared to RFP would provide an accurate way of measuring survival of deletion strains in macrophages. An important goal of future work is to study *C. glabrata* mutant strains with altered survival and replication in macrophage cells compared to a wild-type strain, *in vivo* in mice model and try to understand possible role of zinc cluster protein in *C. glabrata*.

Section 6: Conclusion

The incidence of fungal infections has increased drastically in the past few decades. This incidence is mainly due to resistance to antifungal drugs. Therefore, a better understanding of the drug resistance in *C. glabrata*, the second leading cause of candiasis, is needed. The focus of this project was to better understand the role of zinc cluster protein, Pdr1, in transcriptional regulation of antifungal resistance in *C. glabrata* and to study survival of strains carrying deletion of zinc cluster genes in *C. glabrata* in macrophages.

Drug sensitivity analysis in this study indicated that the auto-regulatory loop is necessary for resistance to echinocandins by *PDR1* mutants. Moreover, it is observed altered survival of mutant strain as compared to wild-type strain in macrophage cells.

In summary, this study provides understanding of regulation of *C. glabrata* Pdr1. In the future, an increased understanding of the role of zinc cluster protein in survival in *C. glabrata* within macrophages and understanding of network that exist among different zinc cluster proteins involve in antifungal resistance will provide a new possible target in the development of drugs against this pathogenic yeast.

Section 7: Acknowledgements

I am grateful to Dr. Bernard Turcotte for providing guidance. I am thankful to Natalia Klimova for providing technical knowledge. I am also gratified to Dr. Martin Olivier and Caroline Martel for providing their technical expertise for the phagocytosis experiment. I am also grateful to the lab member Karla Paez. I am also thankful to Brendan Cormack for providing several plasmids. I am also grateful to my other committee members Dr. Malcolm Whiteway, Dr. David Labbé and Dr. Gregory Marczynski, for their tremendous guidance. This work is supported by a grant from the Fonds de Recherche Québec-Nature et Technologies (FRQNT).

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