Characterization of the Paradoxical Growth Effect of *Candida albicans* Exposed to Caspofungin

by

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Abstract

Candida albicans is an opportunistic pathogen and major cause of invasive fungal infections. Choice of antifungal therapy is complicated by the underlying associated diseases of patients infected, other drug interactions, and *in vitro* susceptibility of the isolate. Echinocandins are emerging as a preferred first line therapy in candidiasis, as they have few drug interactions or patient side effects, and have a fungal specific mode of action. However, *in vitro* susceptibility testing of caspofungin by broth microdilution has revealed an unexplained paradoxical growth (PG) effect in which there is noticeable growth at concentrations above the minimum inhibitory concentration (MIC) of susceptible isolates. This effect has not been fully characterized, but is believed to be a strictly *in vitro* phenomenon. The incidence of the PG effect varies between *Candida* strains, species, and growth forms and is affected my growth medium composition. My objectives were to more fully understand this effect by evaluating factors that affect *in vitro* growth with the echinocandin caspofungin (CASPO), including inoculum density and medium carbon source. I demonstrated that all *C. albicans* demonstrate the PG effect while *C. glabrata*, suggesting an intrinsic difference between species that demonstrate PG. Sequence and phylogenetic evaluation of the echinocandin target, glucan synthase, does not correlate with MIC or PG. Further *in vitro* evaluation by time kill analysis determined that medium carbon source modulates the PG effect. My research findings contribute to the growing body of evidence that suggests the action of echinocandins is not entirely concentration dependent and highlights the

significant physiological differences between yeast grown at PG and inhibitory CASPO concentrations.

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Abbreviations

^{o}C	degrees Celsius
mg/L	milligram per litre
mM	millimolar
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
C	Cytosine
CASPO	Caspofungin
CAZY	Carbohydrate Active enZYmes
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
CWI	Cell wall integrity
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FKS1	FK506 Sensitivity protein, GS catalytic subunit
G	Guanine
GAP	GTPase activating protein
GC	Growth control
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GS	1,3-β-Glucan synthase
GT	Glycosyltransferase
GTP	Guanosine triphosphate
HOG	High osmolarity glycerol
IFI	Invasive fungal infection
MAPK	Mitogen activated protein
MIC	Minimum inhibitory concentration

MLST	Multilocus sequence typing
OD_{600}	Optical density at 600 nanometers
PAFE	Post-antifungal effects
PCR	Polymerase chain reaction
PG	Paradoxical growth
RHO1	Ras Homolog protein, GS regulatory subunit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SAAMF	Synthetic amino acid medium fungi
SDA	Sabouraud dextrose agar
SSD	Small scale duplication
TCA	Tricarboxylic acid cycle
UDP-Glc	uridine diphosphate glucose
WGD	Whole genome duplication
YNB	Yeast nitrogen broth

Chapter 1: Introduction

1.1. *Candida albicans*

1.1.1. Epidemiology of *Candida* infections

Opportunistic fungal infections are a significant cause of severe human disease due to an increasing population at risk. Invasive fungal infections (IFI) affect normally sterile body fluids and deep tissues; infections occur most often from opportunistic organisms that are present in the body as part of the normal flora or in the environment that become pathogenic when immune defenses are unable to fight infection. Increased risk for opportunistic infections occur with disruptions to the normal flora, immune status, and skin or mucosal barriers. The most common fungi related to opportunistic infections are *Candida* spp., *Aspergillus* spp., and *Cryptococcus neoformans*.(1) The list of medically important fungi is ever increasing, but *Candida albicans* remains the most significant opportunistic pathogen worldwide.(2)

Candidemia is the fourth most common nosocomial bloodstream infection in the USA, affecting 10 cases per 100,000 population.(3) There are more than 20 *Candida* species associated with human disease, with the majority of infections (≥50%) attributable to *Candida albicans*.(4,5) Species distribution differs by geographic region(3), and underlying condition of the patient.(5) Infections caused by *Candida* spp., are associated with increased length of hospital stay, a 14.5% increase in mortality among hospitalized patients, and substantial financial cost to healthcare.(6-9)

C. albicans is a highly adaptable organism, utilizing a number of strategies to evolve to suit changing host and environmental conditions. This is

demonstrated by multilocus sequence typing (MLST), which differentiates 70% of *C. albicans* isolates into four closely related clades (strain types), each demonstrating distinct evolutionary histories.(10) While there are no absolute phenotypic associations attributable to any one clade, there are strong associations among clades for geographic regions, antifungal resistance, and phenotypic characteristics including acid phosphatase activity and growth in 2 M sodium chloride.(11,12) However, no significant associations can be made between virulence and sequence type(13), likely do to the multitude of genetic traits and environmental factors that contribute to virulence.

1.1.2. Colonization and infection

C. albicans colonization of skin, genital mucosa, and/or intestinal mucosa is highly prevalent, affecting 30-70% of healthy individuals.(14,15) Inhibition of growth by nutrient competition and secondary metabolite production of healthy gut bacteria and appropriate host immune modulation of colonized yeast prevent overgrowth and infections.(16) A disruption of the mucosal barrier, normal bacterial flora, and/or host immune system can allow colonized yeast to invade host tissue. Yeast adhere to host cells and undergo the morphological switch to hyphae, which penetrate tissue and disseminate into vasculature and other tissues.(14) *C. albicans* has a number of traits that contribute to the progression from colonization to disease that enhance its ability to adhere to host cells, switch morphological forms, and evade host immune cells.

C. albicans has specialized cell wall and secreted proteins that are essential for cell adhesion, nutrient uptake, and evasion of the host immune system. Adhesins, such as agglutinin-like sequence proteins, allow adhesion to other yeast cells, host tissues, and medical devices.(14) Hydrolytic enzymes, including lipases, phospholipases, and aspartyl proteases, are secreted to degrade host tissues and immune proteins, thereby enhancing nutrient acquisition and evasion of host immune cells.(17)

C. albicans grows as yeast (blastospores), pseudohyphae, and hyphae depending on environmental cues such as pH, cell density, and nutrient availability.(18) Morphological transitions are required for infection, as hyphal forms are required for tissue invasion while yeast forms are required for dissemination.(19) *C. albicans* mutants locked in either yeast or hyphal forms are avirulent in animal models, suggesting the ability to switch morphologies, rather than any one form, significantly contributes to virulence.(20-22)

The ability to switch morphological forms also contributes to biofilm formation. Biofilms consist of layered organized communities of heterogeneous cell types embedded within an extracellular matrix.(23,24) *C. albicans* yeast adhere to host tissue or medical devices, transition to hyphal growth, and produce an extracellular matrix composed of carbohydrates, proteins, phosphorous, and hexosamine.(25,26) Biofilms are more resistant to host defenses and antifungal therapy, as the biofilm structure provides defense against phagocytic cells, limits diffusion of antifungal drugs, and alters the transcription of cellular processes, including upregulation of adhesins and secreted proteins.(26,27)

1.1.3. Diagnosis of fungal infections

Accurate and timely diagnosis of fungal infections is necessary for appropriate and effective treatment. Identification of the organism to the species level helps evaluate the isolate's clinical significance, as certain species from specific specimen types correlate to more severe disease outcomes, and species-specific susceptibilities dictate choice of antifungal therapy.(28) Standard methods for diagnosis of fungal infections rely on the integration of microbiology cultures, histological identification, and clinical presentation. However, diagnosis of fungal infections remains difficult: culture techniques may not support organisms requiring specific media supplementation, and may be difficult to interpret as colonized opportunistic organisms can be isolated from non-sterile sites or from improper collection from sterile sites; histology samples require invasive procedures which may be contraindicated for immunocompromised patients; and the clinical presentation of fungal infections is often non-specific and easily mistaken for symptoms of any underlying disease.(28-30)

New methods to enhance diagnosis include amplification of fungal deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) and detection of fungal cell wall 1,3-β-glucans. These methods are not yet a standard for clinical practice as there is limited data on the efficacy in diagnosis of fungal infections in large-scale prospective clinical studies.(30,31) More research is needed to improve the sensitivity and accuracy of fungal diagnostics, as any delay in treatment significantly reduces prognosis and increases mortality.(32)

1.1.4. Antifungal therapy and resistance

Antifungal therapy currently includes azoles, polyenes, pyrimidine analogues, and echinocandins.(Table 1.1) The type and duration of antifungal treatment is dependent on patient age, immune status, severity of infection, underlying conditions, concomitant medications, fungal species, and antifungal susceptibility profiling. The current guidelines for treatment of candidemia or invasive candidiasis include fluconazole or echinocandins as preferred first line therapy for non-neutropenic patients, and lipid formulations of amphotericin B or caspofungin for neutropenic patients.(33,34) Treatment is usually continued for two weeks after the last positive blood culture and the signs and symptoms of candidemia have resolved.(35,36) The duration of antifungal therapy is debatable, as it must be sufficient to resolve the infection while limiting exposure to antifungals to reduce unwanted side effects and the potential for development of antifungal resistance.

Antifungal resistance can be intrinsic, occurring naturally in an organism regardless of prior antifungal exposure, or acquired, occurring after exposure to antifungals. Resistance generally occurs through four mechanisms: upregulation of drug transporters, antifungal target alterations, compensatory regulation of metabolic pathways, and biofilm formation.(37) *In vitro* susceptibility testing of antifungals aims to predict *in vivo* clinical response by identifying organisms that are susceptible or resistant to a given therapy. While *in vitro* susceptibility profiles are an indicator of therapy success, outcome of antifungal therapy is

affected by the type and severity of underlying disease, drug interactions, and drug metabolism.(38) No one antifungal therapy is without limitations; the toxicities associated with broad spectrum amphotericin B and fluconazole are undesirable side effects that can further complicate patient outcome while other therapies have a limited spectrum of activity. Emerging resistance and the limited treatment options for many fungal species highlights the importance of evaluating new and existing antifungal therapies.

An ideal antifungal would be fungicidal to a broad spectrum of fungal pathogens, have a fungal-specific target, and no drug interactions. While no one antifungal developed to date is ideal, the echinocandins are the most promising prospect for management of fungal infections, as they have a fungal specific target, no significant drug interactions or side effects, and are effective against the most common *Candida* spp. associated with disease.

1.2. Echinocandins

1.2.1. Pharmacology

The echinocandins caspofungin (CASPO, Cancidas®, Merk Inc.), micafungin (Mycamine®, Astellas Pharmaceuticals Inc.), and anidulafungin (Eraxis®, Pfizer Inc.) are semisynthetic lipopeptides derived from the natural fungal products of *Glarea lozoyensis*, *Coleophoma empedri*, and *Aspergillus nidulans*, respectively.(39) They share a similar cyclic hexapeptide core with differing *N*-linked acyl lipid side chains (Figure 1.1).(40,41) Efficacy of these drugs over other derivatives is dependent on the hydroxy proline residue in the

core and the side chain interactions with the target.(42,43) The echinocandins are the newest class of antifungals developed, with caspofungin first approved for clinical use in the United States (2001), followed by micafungin (2005) and anidulafungin (2006).(44-46) All three are currently approved for invasive and esophageal *Candida* infections and treatment of candidemia in non-neutropenic patients, while CASPO is approved for use with invasive *Aspergillus* infections that are unresponsive to other treatment options.

Echinocandins inhibit the incorporation of 1,3-β-glucan into the fungal cell wall by noncompetitive binding of 1,3-β-glucan synthase (GS) in a dose-dependent manner.(47) 1,3-β-glucan is a major structural component of the cell wall and its depletion causes osmotic fragility and cell lysis in actively dividing yeast cells.(48) *In vitro* studies with osmotic stabilizers have shown that they reduce the inhibitory effects of echinocandins(49), indicating altered cell walls by these agents. The cell wall glucans are absent from mammalian cells, resulting in a drug target that is specific for fungal cells and limits undesirable side effects present with other antifungal therapies.

All three echinocandins have similar properties that make them desirable for antifungal therapy of invasive infections, although they have a low (<10%) bioavailability and require intravenous administration.(50) Adverse effects occur infrequently (<2%) but can include infusion-related reactions, nausea, vomiting, fever, leucopenia, and elevation of liver enzymes.(51) The echinocandins have few drug interactions as they are not substantial substrates, inhibitors, or inducers of the cytochrome P450 enzymes or P-glycoprotein transporters.(52) They are

degraded by hydrolysis, *N*-acetylation, and slow chemical degradation to inactive metabolites in the liver and bile.(53,54)

Echinocandins rapidly distribute into tissues such as lung, liver, kidney, spleen, and white blood cells, but have little distribution in cerebrospinal or vitreous fluids.(55-57) Plasma concentrations quickly decline following administration but these agents are excreted essentially unchanged.(54,55,58) With an initial loading dose of 70 mg of CASPO, peak plasma concentrations reach 11-12 mg/L; plasma levels and clearance of CASPO are determined by the rate of re-distribution from tissues.(54) Echinocandins are highly protein bound (>99%), and a portion of the drug bound to proteins may be irreversible; the percent of unbound CASPO concentrations is only 3.5% of the peak plasma concentration.(54)

Echinocandins have long half lives and can persist in tissue long after plasma levels decline.(59) The effect of drug persisting in tissues is still under investigation, as organs seem to act as drug release reservoirs, which may affect efficacy and dosing requirements. The effect of extensive protein binding is also unknown; while it may limit the amount of available active drug, there are other antifungal drugs that are highly protein bound, such as amphotericin B and itraconazole, that are effective in treating fungal infections.(39) *In vitro* studies have demonstrated extensive concentration dependent post-antifungal effects (PAFE) where growth inhibition is maintained after short exposure and removal of drug.(60-63) These effects are likely due to prolonged direct interactions with GS.(64) The effect of extensive protein binding on drug efficacy is unknown, and

it will be important to determine the drug-GS and drug-tissue affinity to fully understand the *in vivo* consequences of protein binding on echinocandin efficacy.

1.2.2. Glucan Synthase

1.2.2.1. The yeast cell wall

The cell wall is the outermost layer of fungi that provides structural support and environmental protection, and functions in cell morphogenesis and cell-cell recognition.(65) The cell wall provides a mechanical barrier against excessive stretch of the plasma membrane, preventing cell lysis from an ever-changing environment. The cell wall is freely permeable to small molecules, allowing interaction of nutrients and signaling molecules to interact with receptors and transporters at the plasma membrane.(66) Adaptation of the fungal cell wall contributes to the persistence of these organisms as infectious agents.

The major components of fungal cell walls vary by species but consist mainly of glucans, chitin, and mannoproteins.(Figure 1.2) Electron micrographs of yeast cell walls demonstrated two distinct layers: an inner electron transparent layer consisting mainly of glucans, and an outer electron dense layer consisting mainly of mannoproteins.(67) 1,3-β-glucans are an integral part of the yeast cell wall, making up 35% of the dry cell weight; it consists of long linear fibrils averaging 60 glucose residues that are only soluble in alkali.(68,69) A smaller fraction of 1,3-β-glucan is branched with 1,6-β-glucans or chitin; this glucan network provides the framework for attachment of glycosylphosphatidylinsitol (GPI) and Pir proteins (proteins with internal repeats) to the cell wall.(70,71) *O*-

and *N*-linked manno-oligosaccharides (mannoproteins) make up the majority of the external cell wall layer attached to either the branched glucans or the plasma membrane.(67) Most of the cell wall proteins are hydrolytic enzymes, agglutinins, or are structural components of the cell wall.(72)

The composition of the cell wall varies between fungal species and reproductive states. For example, 1,3-β-glucans are only present in the sporangiospore of zygomycetes and *Schizosaccharomyces pombe* lacks chitin in vegetative cell walls.(73-75) However, the general structural components of glucans, chitin, and mannoproteins are consistent among medically important fungi, making the fungal cell wall a desirable target for antifungal drugs.

1.2.2.2. Glucan synthase is the target of echinocandins

Actively dividing cells treated with echinocandins are unable to incorporate 1,3-β-glucans into the cell wall, resulting in altered cell wall mechanical properties with increased susceptibility to osmotic forces(76,77) The GS enzyme complex was discovered to consist of a membrane bound putative catalytic subunit (Fks1p) and a soluble regulatory component (Rho1p) through partial purification by product entrapment.(78) Due to the size and complexity of the enzyme complex, the requirement of other proteins in the enzyme complex cannot be excluded, as GS has only been partially purified from crude membrane preparations.

Echinocandins bind to and inhibit GS, demonstrated by direct interaction of photoactivated echinocandin analogs with GS in crude plasma membrane

preparations of *Saccharomyces cerevisiae*.(79) The drug's interaction with GS requires both the hexapeptide core structure and the lipophilic side chain(80); variation in these components between echinocandins and echinocandin derivatives accounts for the differences in potency.(81) Topological modeling of Fks1p suggests a model where the hexapeptide core binds exterior to the cell membrane to a single domain comprised of the three "hot-spot" regions, while the lipid tail interacts with the plasma membrane.(Figure 1.3)(82-84)

1.2.2.3. Fks1p

The catalytic subunit Fks1p (FK506 sensitivity protein) was first discovered through mutant studies with altered sensitivities to FK506 (tacrolimus), cyclosporine A, calcofluor white, echinocandin B, and papulacandin B in the model yeast *S. cerevisiae*; FKS1 is also known as calcineurin dependent protein 1 (CND1), calcofluor white hypersensitivity protein 53 (CWH53), echinocandin target protein 1 (ETG1), glucan synthase of cerevisiae protein 1 (GSC1), and papulacandin B resistance protein 1 (PBR1).(85,86) *S. cerevisiae* has three highly similar FKS genes involved in glucan synthesis that are differentially expressed: FKS1 expression is induced during cell wall remodeling and is cell cycle regulated during vegetative growth;(47,87) FKS2 expression is calcineurin dependent and is induced during sporulation, starvation, and in response to mating pheromones;(88) and FKS3 expression is required for regulation of spore wall formation.(89) Fks1p and Fks2p are two distinct subunits with some overlapping function, as null mutants in either gene are not lethal and over-expression of either

can modulate the mutation effects. The function of Fks1p and Fks2p is essential, as *Δfks1Δfks2* mutants are not viable.(88) The function of Fks3p is unknown but appears to be distinct from Fks1p and Fks2p; deletion of FKS3 results in abnormal spore formation that is not rescued by over expression of either FKS1 or FKS2.(89) Most yeast, including *C. albicans*, contain multiple FKS genes, while most filamentous fungi contain only a single FKS gene.(90,91) All single copy FKS genes appear to be essential for viability.(92-96) The significance of multiple versus single copy FKS genes in GS function and regulation is unknown.

S. cerevisiae Fks1p is a large plasma membrane protein consisting of 1876 amino acids with 16 predicted and 13 experimentally confirmed transmembrane regions.(82) Based on sequence similarities and enzyme kinetics, Fks1p is classified as glycosyltransferase (GT) family 48 in the Carbohydrate Active enZYmes (CAZY) database.(97) Its systematic name (UDP-glucose:(1→3)-β-D-glucan 3-β-D-glucosyltransferase, EC 2.4.1.34) is descriptive of its catalytic mechanism for the addition of a donor uridine diphosphate glucose (UDP-Glc) to an acceptor glucose.(98) Direct binding of UDP-Glc as the donor substrate to Fks1p was demonstrated in *Neurospora crassa*(95); Fks1p is tightly bound to its 1,3-β-glucan products in the presence of UDP-Glc and rapidly dissociates from it in the absence of UDP-Glc, thus allowing partial purification through product entrapment.(99) Fks1p is unique among GTs as it lacks any known UDP-Glc binding domains that are conserved in other UDP-Glc utilizing GTs, including cellulose, chitin, and glycogen synthases.(100,101) Large scale analysis of protein interactions demonstrates that GS does not associate with any known transporters

of sugars(102), and it is assumed that Fks1p is responsible for both the production of 1,3-β-glucans and its transport through the plasma membrane.(103,104)

The fungal cell wall is exposed to a variety of constantly changing environmental stimuli and stressors that have led fungal cell wall proteins to evolve faster than proteins involved in other cell processes, such as metabolism.(105) The few highly conserved cell wall proteins are related to cell wall biogenesis, including Fks1p, which is indicative of the importance of cell wall construction in the evolution of fungi. A phylogenetic review of fungal cell wall proteins conducted by Ruiz-Herrera et al. described the acquisition of the FKS genes by horizontal gene transfer from an ancestor common to plants before the separation of Ascomycota and Basidiomycota from the other phyla in kingdom Fungi.(73) However, the high conservation of cell wall components and sequence similarity of GS across phyla suggests that a single evolutionary gain in a universal common ancestor is more likely to explain the evolution of GS.

GS localizes to bud sites and areas of active cell wall remodeling to maintain a uniform cell wall, which is important for maintenance of cell size, shape, and morphogenesis.(106) In addition to production of cell wall 1,3-β-glucan, Fks1p may be involved in other cellular functions: deletion of FKS1 results in multiple cellular effects, including alterations of 1,6-β-glucan, mannoprotein, and chitin contents of the cell wall(104); and mutation of Fks1p domains results in alterations to cell wall construction, endocytosis, and regulation of cell polarity.(107) It is unknown whether Fks1p plays an active role in the formation of other cell wall components or in other cellular pathways, as

the effects of deletion and mutational studies could result from protein interactions, unknown regulatory mechanisms, or a general stress response.

The significance of potential additional functions of Fks1p is unknown, but the different effects of GS inhibitors in different fungi suggest that these functions, or other unknown functions, significantly contribute to cell viability and drug tolerance. Papulocandin, also an inhibitor of GS, inhibits *in vitro* growth of *S. cerevisiae*, yet has little activity against partially purified GS(108); it is unknown whether papulocandin has some other unknown cellular effect, or if GS has other functions that were not evaluated by these experiments. Echinocandins exhibit an opposite effect in *C. neoformans* as they have very little inhibitory effects on *in vitro* growth yet the catalytic activity of isolated GS is inhibited by concentrations of CASPO considered within the therapeutic range of susceptible organisms.(109) Most studies evaluate echinocandin effects by measuring enzyme kinetics of GS from isolated plasma membranes and inhibition of growth, neither of which identifies other cellular effects. The evaluation of additional Fks1p functions in echinocandin tolerance will be important to fully understand the effects of echinocandins in clinical therapy of fungal infections.

1.2.2.4. Rho1p

The GS subunit Rho1p (Ras homolog 1) was established to be the regulatory component through product entrapment, co-immunoprecipitation, 1,3-β-glucan biosynthesis assays, and the rescue of glucan synthesis deficiencies with recombinant Rho1p.(110,111) It is one of six Rho GTPases found in the model

yeast *S. cerevisiae,* all functioning in establishing cell polarity.(85) Rho proteins are highly conserved in eukaryotes, functioning as pathway regulators through highly conserved protein sequences for guanosine triphosphate (GTP) binding and localization to the plasma membrane.(112)

Rho1p undergoes conformational changes in its transition to an active state; it is inactive when bound to guanosine diphostate (GDP) and activated by binding GTP. The GDP/GTP exchange is regulated by interaction with guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) that stimulate the release of GDP and GTP, respectively.(113) Once activated, Rho1p elicits its cellular effects by activating effector proteins and their subsequent functional pathways; effectors of Rho1p include Pkc1p (Protein Kinase C1), Bni1p (Bud Neck Involved, also known as formin), Sec3p (Secretory), Skn7p (Suppressor of Kre Null), and Fks1p.(85) Rho1p is essential for viability as it is a significant mediator of polarized cell growth through the regulation of glucan synthesis, cell polarity, and the cell wall integrity (CWI) pathways.(Figure 1.4)(112)

Rho1p is the cytoplasmic GS regulatory protein that when associated with GTP regulate the localization of and activates the membrane bound Fks1p, and induces the transcription of FKS1/FKS2 genes.(110,114) Fks1p and Rho1p are transported to the plasma membrane in secretory vesicles, and co-localize to sites of active growth corresponding to active bud sites.(106,115) The involvement of Rho1p in the glucan synthase complex is dependent on the post-translational modification to the C-terminus of Rho1p with a lipophilic geranylgeranyl

isoprene. This modification acts as a lipid anchor to the plasma membrane and is required for both association with and activation of Fks1p.(116) Cell growth, cell wall stress, and growth on nonfermentable carbon sources induces the activation of Rho1p via PAS kinase (Psk1p) signaling causing glucose to be partitioned for cell wall glucan production over other cellular processes utilizing UDP-Glc, such as glycogen synthesis.(117)

Cell polarity is the asymmetric organization of cellular components to facilitate mating, morphogenesis, and proliferation.(118) This tightly regulated process involves the signaling and activation of the cytoskeleton, allowing the asymmetric redistribution of cellular components to allow for growth. In addition to regulating glucan synthesis to accommodate cell wall growth, Rho1p regulates cell polarity by activating proteins involved in the actin cytoskeleton. Bni1p assembles linear actin cables necessary for actin cytoskeleton remodeling; the precise role of Pkc1p in actin organization is unknown, but is implicated in actin and microtubule organization.(119)

Polarized cell growth involves remodeling of the cell wall and plasma membrane, requiring the secretory pathway to transport cell wall digestive enzymes and new membranes to cell surface growth sites.(120) Rho1p is required for the polarized localization of Sec3p and exocyst formation, a protein complex that facilitates vesicle trafficking.(121). While the interaction of Rho1p with Sec3p is independent of the actin cytoskeleton, Rho1p also acts indirectly on the exocyst through interaction with Pck1p and Bni1p, as it requires the intact organization of actin cables for delivery of the exocyst to the cell surface.(122)

Endocytosis is the process by which the plasma membrane is internalized to create a vesicle and internalize material outside of the cell that is unable to pass freely through the plasma membrane. While the direct function of endocytosis in polarized cell growth has yet to be determined, there are strong phenotypic correlations in mutants defective in endocytosis and in actin cortical patch organization.(122) It is suggested cells change the rate of endocytosis to accommodate changes in cell volume during polarized cell growth.(123) Rho1p and Fks1p are implicated in controlling the internalization step of endocytosis through Tor2p (Target of Rapamycin) in *S. cerevisiae*.(124) This role of Rho1p and Fks1p is unaffected by absence of a cell wall or other Rho1p effector pathways, suggesting this function is independent of the other known functions of Rho1p and Fks1p. Rho1p also promotes endocytosis through interactions with Bni1p in an alternative endocytic pathway when the normal endocytic machinery is impaired.(125) It is unknown under which conditions this alternative pathway is utilized, but may be required for as yet undetermined cargo or in response to specific cell stress.

Cell stress from polarized growth, changing environmental stimuli, and mating is managed by the CWI pathway regulated by Rho1p.(126) The CWI pathway is one of four mitogen activated protein kinase (MAPK) pathways in yeast characterized by a phosphorylation cascade that transmits signals from the plasma membrane to the nucleus.(85) Pkc1p is activated by phosphatidylserine when associated with GTP-bound Rho1p.(127) Activated Pkc1p initiates the phosphorylation cascade, stimulating transcription

regulators for cell wall synthesis and cell cycle-dependent cell wall synthesis genes. In addition to the activation of Pkc1p, Rho1p also activates the transcriptional regulator Skn7, which initializes the transcription of genes involved in cell integrity and oxidative stress.(128) While mostly evaluated for maintaining cell wall integrity in response to stress, the CWI pathway has been implicated in oxidative, pH, and DNA stress in *S. cerevisiae*, and in nutritional stress in *S. pombe*.(129-132)

In addition to the well-established functions associated with polarized growth and cell integrity, Rho1p has been implicated in a number of additional cellular processes. Rho1p is involved in cytokinesis by promoting the cytokinetic actomyosin ring assembly, as well as septum formation and dissolution through its regulation of glucan synthesis, secretion, and actin organization.(133) Rho1p has recently been shown to activate Ycf1p (Yeast cadmium factor), a vacuolar transporter involved in oxidative stress.(134) Most recently, Rho1p is implicated as the regulator of a membrane fluidity homeostasis pathway, a potentially new MAPK pathway involving similar components of the CWI pathway.(135) The multitude of cell functions Rho1p participates in highlights its essentiality, but several questions remain regarding how specific Rho1p effectors are targeted.

The regulation of Rho1p effector proteins is determined by the appropriate function, localization, and activation of Rho1p by GEFs and GAPs. The Rho1p GEFs include Tus1p (TOR Unique function Suppressor) and Rom2 (RhO1 Multicopy suppressor); they activate and recruit Rho1p to the plasma

membrane, bud tip, and site of cytokinesis.(113,133) The Rho1p GAPs include Sac7p (Suppressor of Actin), Bag7p (paralog of Sac7) Bem2p (Bud Emergence), and Lrg1p (Lim-Rho Gap homolog).(85) It is unknown how the GEFs and GAPs achieve specificity in their regulation of Rho1p effector pathways, since different GAPs and GEFs affect different Rho1p effectors. For example, Rom2p can be activated by Tor2p in *S. cerevisiae*, affecting actin organization but not glucan synthesis.(136) It may be that the activation or inhibition of specific Rho1p effector pathways is dependent on unknown mechanisms of localizing Rho1p, such as through as yet unknown protein partners.

1.2.3. Spectrum of activity

Echinocandins are fungicidal against *Candida* species, including: *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. guilliermondii*, and *C. lusitaniae*.(51,137,138) Ultrastructural analysis of cells treated with inhibitory concentrations of CASPO demonstrated the absence of bud scars, wrinkling, and severe lesions resulting in the release of cellular contents.(139) *In vivo* models show echinocandins to be effective in the treatment of azole resistant *Candida* spp., and treatment of invasive candidiasis with CASPO demonstrated success rates of >70%.(51) CASPO is comparable to treatment with amphotericin B liposomal therapy for empirical therapy in persistent febrile neutropenia.(44,140)

Echinocandins are primarily fungistatic against *Aspergillus* spp. as the active incorporation of 1,3-β-glucans by GS occurs at the apical tips of growing hyphae. *In vitro* growth assays demonstrate reduced growth but incomplete killing of *Aspergillus* cells, which are characterized by swollen, truncated hyphal tips yet unchanged hyphal bases.(141) CASPO demonstrates some fungicidal activity as increased cell lysis is seen with microcolony analysis.(142) *In vivo* data indicates that echinocandins may be as effective as amphotericin B in reducing fungal burdens and increasing survival times, and are effective in the treatment of invasive aspergillosis.(143-145)

Most fungi contain some amount of 1,3-β-glucans in their cell wall, yet despite the presence of 1,3-β-glucans and GS, many fungi remain resistant to echinocandins. They have little *in vitro* activity against *Cryptococcus, Fusarium*, *Rhizopus*, *Paecilomyces*, and *Scedosporium* spp.(146-149) Despite having *in vitro* activity against the filamentous forms of the dimorphic fungi *Histoplasma capsulatum* and *Blastomyces dermatitidis*, echinocandins are ineffective *in vivo*.(150-152)

1.2.4. Echinocandin resistance

1.2.4.1. Intrinsic resistance

The echinocandins have little activity against *Cryptococcus* spp.(148) FKS1 is an essential gene in *C. neoformans* and demonstrates high sequence similarity with fungi susceptible to echinocandins.(92) Analysis of the cell wall demonstrated the presence of both 1,3- and 1,6-β-glucans in the cryptococcal cell

wall and their subsequent depletion with caspofungin treatment.(153) It has also been shown that anti-β-glucan antibodies inhibit *in vitro* growth of *C. neoformans* and may confer protection *in vivo*, demonstrating the essential nature of β-glucans in the cell wall.(154)

The two important virulence factors for *Cryptococcus* spp. are its substantial polysaccharide capsule and melanin production.(155) *C. neoformans* GS isolated from plasma membranes is inhibited by CASPO, while whole cells remain resistant in both capsular and acapsular isolates, which suggests that the capsule is not a barrier to echinocandin penetration to GS.(109) Melanin has been shown to bind CASPO(156), but the effects of melanization and binding of CASPO does not fully resolve the discrepancy with *in vitro* susceptibility profiles between *Cryptococcus* spp. as not all *Cryptococcus* isolates produce melanin yet all isolates are resistant to echinocandins. These data suggest there may be other unknown mechanistic features of GS that render *Cryptococcus* spp. (and other fungi) intrinsically resistant to echinocandins.

1.2.4.2. Acquired Resistance

Even with the increasing use of echinocandins since the introduction of CASPO in 2001, acquired resistance remains rare.(157) Acquired echinocandin resistance is associated with amino acid mutations in three specific "hot-spot" regions of that map to the *S. cerevisiae* Fks1p at amino acids 635-649 (HS1), 1354-1361 (HS2), and 690-700 (HS3). (Figure 1.3) FKS mutants in yeast demonstrate cross-resistance to all echinocandins, reduced *in vitro* susceptibility

profiles, and an increased kinetic inhibition constant of partially purified GS.(158,159) Despite reduced *in vitro* susceptibilities of FKS mutants, different *in vivo* models have demonstrated contrasting results. A significantly higher effective dose was required for spontaneous clinical Fks1p mutants in a murine model of disseminated candidiasis(160), yet laboratory derived Fks1p mutants have been shown to be less virulent than wild type measured in toll-deficient *Drosophila melanogaster* flies and a different murine model of disseminated candidiasis.(161) Monitoring emerging mutant populations and clinical outcomes from echinocandin therapies is needed to determine the significance of acquired Fks1p mutants in clinical and laboratory strains.

Some *Candida* spp., including *C. parapsilosis, C. orthopsilosis, and C. metapsilosis*, demonstrate elevated MIC values and have an intrinsic Fks1p P647A (HS1) mutation resulting in decreased enzyme sensitivity to echinocandins.(162) Despite the increased MICs, clinical outcomes with these *Candida* spp. are comparable to *C. albicans*.(51) This discrepancy between *in vitro* and *in vivo* data challenges the clinical significance of Fks1p mutations in echinocandin therapy.

1.2.5. Susceptibility testing and clinical breakpoints

Evaluation of susceptibility to echinocandins is determined by minimum inhibitory concentration (MIC) by broth microdilution assays as outlined by the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).(163-165) Both reference

methods use serial dilutions of echinocandin concentrations in Roswell Park Memorial Institute (RPMI) 1640 broth as the testing medium, assay interpretation at 24 hours incubation, and define the MIC as the lowest concentration which demonstrates a prominent (50%) decrease in turbidity. The methods differ in inoculum density (10^3 CLSI, 10^5 EUCAST), medium glucose content (0.2% CLSI, 2% EUCAST), microdilution plates (round-bottom with CLSI, flat-bottom with EUCAST), and method of endpoint evaluation (visual reading with CLSI, spectrophotometric with EUCAST). Comparison of the two reference methods with yeast demonstrates >90% essential agreement for most species.(164)

Reports of clinical failures are uncommon and acquired resistance to echinocandins remains rare. However, due to the increasing awareness and reporting of Fks1p mutations, clinical breakpoints to detect non-susceptible strains have been established for the CLSI reference method that incorporate molecular and MIC data.(166) (Table 1.2) Clinical breakpoints from MIC testing are set to define susceptible isolates as those with an increased likelihood of therapeutic success; while the significance of Fks1p mutations are unknown, the current guidelines are set to help detect mutants in MIC testing.

1.3. The paradoxical growth effect

1.3.1. Incidence

In vitro susceptibility testing by broth microdilution of echinocandins has revealed a paradoxical growth (PG) effect in which there is reduced fungal killing at concentrations well above the MIC of susceptible isolates that is not observed

with any other antifungals.(167) The PG effect is distinct from other growth phenotypes demonstrating growth above MIC concentrations, such as the trailing effect or resistance: the PG effect is defined as the re-emergence of strong growth in consecutive wells that is ≥2 drug dilutions above the MIC after 48 hr; the trailing effect is persistent but notably reduced growth in drug concentrations above a defined MIC; and resistance is the persistent growth in drug concentrations resulting in an elevated MIC and designation of the isolate as non-susceptible.(168,169)

The frequency of the PG effect varies within and between different *Candida* species, as well as between the echinocandin drugs.(168) The PG effect has been reported to be as frequent as 14-40% in *Candida* spp., occurring most frequently with *C. dubliensis*, *C. parapsilosis*, and *C. albicans* treated with caspofungin, micafungin, and anidulafungin, respectively.(167,169,170) The PG effect is notably absent from all *C. glabrata* isolates.(169) All isolates demonstrated the PG effect more frequently and over a wider range of drug concentrations when grown as biofilms than when grown as blastospores.(168) The differences in drug structure (and thereby interaction with cells) and the different properties between biofilms and yeast within different species and strains is assumed to play a role in the mechanism of the PG effect.

Animal models of different invasive fungal infections have demonstrated an *in vivo* PG effect, but these reports have been inconsistent and not reproducible in duplicate experiments.(171-173) *In vivo* model outcomes are generally measured by tissue fungal burden and survival rates, and higher doses of

caspofungin have demonstrated increased tissue burdens while not significantly affecting the overall survival rates. The lack of substantial experimental evidence from *in vivo* models has led many to assume the PG effect is a limited *in vitro* phenomenon. However, the complexity and progression of human disease is not always well represented by acute infectious animal models, especially when the outcome of human disease should consider the disease severity, duration, and complications rather than rely solely on survival rates. The potential for and significance of the PG effect during the course of echinocandin therapy warrants further investigation as it could have a significant impact on disease outcome and antifungal therapy.

Different growth media influence the frequency and severity of the PG effect. Stevens et al demonstrated less pronounced PG with Synthetic Amino Acid Medium Fungi (SAAMF), RPMI, and yeast nitrogen broth (YNB), respectively.(167) These media vary in the type and concentration of supplemented nutrients, amino acids, and carbon sources. While it has been determined that different glucose concentrations in RPMI 1640 does not affect MIC testing(174), other media conditions can affect susceptibility of fungi to echinocandins.(175) Growth nutrients have been shown to affect cell wall composition, antifungal susceptibilities, and resistance to oxidative stress.(176,177) The significance of media components on the frequency and severity of the PG effect warrants further investigation.

1.3.2. Cellular characteristics

There are several morphological features of yeast at PG concentrations with echinocandins that may be indicative of its mechanism.(Figure 1.5) Yeast treated with CASPO at PG concentrations demonstrate highly aggregated yeast absent of filamentation, with abnormal budding and often incomplete separation of progeny cells.(178) Composition of PG cell walls demonstrate a significant decrease in 1,3- and 1,6-β-glucans by 81% and 73%, respectively, as well as a significant 8-fold increase in chitin.(178,179)

1.3.3. Potential mechanisms

The morphological features of PG cells are similar to those of resistant cells, which also demonstrate increased levels of cell wall chitin.(180,181) Despite the similar morphological features, there are differences in broth microdilution growth patterns and MICs between resistant and PG yeast. Assuming that increased cell wall chitin is responsible for resistance and PG oversimplifies the cellular processes involved in persistence of growth under a multitude of conditions and treatments.

The first investigations into the mechanisms for PG involved the drug quality and changes to GS. It was determined that the concentrations of CASPO over a 24 hour incubation were stable, and there was no degradation of drug by yeast.(167,182) There is no relationship between MIC and incidence of PG(169), and subsequent testing of PG cell progeny demonstrates a reproducible effect.(167,182) PG cells demonstrate a decrease in GS activity and the significant inhibition of glucan production by echinocandins is evidence that up-

regulation of GS is unlikely to be the underlying cause for the PG. The decrease in GS activity is similar to non-susceptible strains with Fks1p mutations, though no mutations in HS1 or HS2 of Fks1p correlate with the PG effect.(183) The hot spot regions of Fks1p only comprise 150 amino acids of a total of ~1800 amino acids. With a protein of this size and complexity, full sequence analysis may warrant further investigation, as mutations may arise affecting protein function without affecting the echinocandin binding site at HS1 or HS2. Also, the role of Rho1p in the PG effect has not been investigated; given that Rho1p has regulatory roles in a multitude of cellular functions, including stress response pathways, it is highly plausible that it could play an important role in mediating growth above MIC concentrations.

The genetic relatedness of *C. albicans* isolates demonstrating the PG effect was conducted by microsatellite analysis of three loci.(184) The authors concluded that there is no significant population structure associated with the incidence of PG, and the trait allowing such growth was gained or lost too rapidly to detect by the stable genetic markers used in multilocus microsatellite typing. However, the study drew conclusions based on a combination of isolates demonstrating the PG and trailing effects and assumed these traits are found in the same populations. Since the mechanisms for these growth phenotypes are unknown, it cannot be assumed that they are the same. Microsatellite typing is useful for population genetics as it provides fast, reproducible data for high throughput studies; however, microsatellite analysis of these loci has proven to be less accurate than multilocus sequence typing.(185) As a result, this study lacked

an appropriate stratification of populations for analysis, insufficient power due to a low sample size (4 PG isolates), and potentially insufficient discriminatory power of the microsatellite markers used. Further investigation into the population structure by MLST may detect differences in sequence types between echinocandin susceptible and PG isolates.

The relationships of PAFEs and the PG effect were investigated to determine the significance of a brief exposure to CASPO, using concentrations similar to those achieved at peak levels *in vivo*.(64) This study described enhanced killing by PAFEs and the elimination of the PG effect after short exposures (<15 minutes). The authors argued that the PG effect would only be clinically relevant if high concentrations were maintained throughout treatment, which at current doses is unachievable. Although this may be true for plasma levels, the high sequestration of echinocandins in tissues at concentrations in which the PG effect is achievable *in vitro* has not been taken into consideration.

The effect of protein binding on the activity of echinocandins is unknown and has been investigated *in vitro* by adding human serum to susceptibility testing media. Shields et al evaluated the effect of human serum on MIC and PG(186); the addition of 10% human serum had no affect on MIC values but increased the median concentration at which PG occurs varies from 2 to 32 mg/L, while the addition of 50% serum increased MIC values 2-fold and eliminated the PG effect in concentrations up to 64 mg/L. These results are similar to others including 10% serum demonstrating an increase in MIC value up to 4-fold.(167) The changes to MIC and PG concentrations with the addition of serum indicate there may be less

available active drug, thereby requiring more drug to elicit the same effects. It is plausible that the echinocandin concentration range tested was insufficient to determine whether the PG effect was truly eliminated by human serum or whether these concentration ranges are were high enough to detect an increase concetration required to elicit the PG effect, similar to increased MICs with the addition of serum.

Cell wall damage caused by echinocandins induces highly conserved stress response pathways including the CWI, high osmolarity glycerol (HOG) and calcineurin pathways. (Figure 1.6) The heat shock protein 90 (Hsp90p) is a molecular chaperone that stabilizes the structure and function of proteins in the stress pathways including calcineurin and Mkc1p (mitogen activated protein of *C. albicans*) of the CWI pathway.(187) Null mutants of Mkc1p and chemical inhibition of calcineurin or Hsp90p significantly reduced growth of *C. albicans* at PG concentrations of CASPO.(188,189) These pathways are also necessary for survival at elevated echinocandin concentrations(190,191), but it is not known whether these pathways are sufficient to account for the PG effect.

1.4. Clinical significance

The clinical significance of the PG effect has been deemed questionable as PG isolates have clear MIC values that fall within the susceptible range, animal models are inconclusive, and it is debatable whether plasma concentrations are maintained high enough for PG to occur *in vivo*. The PG effect has not been demonstrated clinically, and it is recommended that this effect be ignored in

evaluating echinocandin MICs.(192) However, the possibility of persistent growth at elevated echinocandin concentrations poses a potentially significant drawback to a drug class that is otherwise assumed safe and effective for IFIs, and further *in vitro* study of this phenomenon may help determine its significance to treatment failure.

Due to the low toxicity of echinocandins compared to other antifungals, dose escalation studies are being evaluated for safety and efficacy in the treatment of IFIs. In doses as high as 150 mg daily, no significant differences in therapeutic response are observed between the current standard- and high-dose.(193,194) High doses of CASPO of 150 and 210 mg daily demonstrate higher peak plasma concentrations of 29.4 and 33.5 mg/L(195), concentrations at which PG is known to occur *in vitro.* The higher doses are safe and have similar pharmacokinetics to standard doses(196), so is an attractive option in the hopes of increasing efficacy, but it remains unclear whether dose escalation truly improves clinical outcomes. In addition to the potential for treatment failure if the PG effect occurred *in vivo*, the physiological characteristics of yeast grown at PG concentrations, such as altered cell wall components or increased aggregation, could have significant impacts on infection establishment, host immune recognition, effectiveness of concomitant therapy, and diagnostic or prognostic markers.

The stages of biofilm formation involve fungal attachment to a surface, microcolony formation, and the development of an extracellular matrix.(26) There is a higher incidence of the PG effect tested against fully formed biofilms *in vitro*, but it has not been considered whether the increased cellular aggregation or cell-

cell adhesion associated with PG yeast could increase the likelihood of biofilm development after exposure to PG concentrations of echinocandins. CASPO at or below the MIC decreases adherence of *C. albicans* to plastic surfaces(197), but the adherence properties to abiotic surfaces has not been investigated for PG concentrations.

The host immune response generated by IFIs is largely mediated by the fungal cell wall. Immune cells recognize and bind fungal 1,3-β-glucans by the dectin-1 receptor on the surface of neutrophils and macrophages., resulting in the phagocytosis and elimination from the host.(198) Control of infections via dectin-1 is strain dependent in *C. albicans*(199). Chitin may interfere with immune recognition of fungi by blocking dectin-1 recognition of fungal cells.(200) Sub-therapeutic doses of CASPO enhances the exposure of cell wall β-glucans, thereby enhancing immune recognition of yeast *in vivo* and rendering *C. albicans* more susceptible to the host immune system.(201) However, it has not been investigated how cell wall alterations, such as the decreased glucan and increased chitin in PG cells, affects this influence of echinocandins on host response. Moreover, *C. albicans* isolates with increased cell wall chitin are less susceptible to echinocandins both *in vitro* and *in vivo*.(180)

Fungal cell wall components can adapt to the stress of antifungal treatment, as well as from nutrient availability and other environmental signals. Different host niches often differ in nutrient availability which can modulate the cell wall, potentially altering its response to stress or host immune cells.(202) The difference in the PG frequency in different media could be due to the different

supplemented nutrients. The differences in host niches and potential nutrient influences on the PG effect may also contribute to the inconsistency of the PG effect in animal models. The PG effect has been demonstrated inconsistently in *Candida* spp. blood stream infections(171-173), while the PG is more consistently observed in an invasive pulmonary aspergillosis model by measuring inflammatory responses.(203) It could be that differences in the animal models used between species can account for the incidence discrepancy of the PG effect between *in vivo* and *in vitro* experiments.

Due to the high mortality of *Candida* infections, sensitive and timely diagnostics are required for the most favorable therapeutic outcomes. A newer method for detecting IFIs measures serum 1,3-β-glucan levels; while this method will likely never be used alone in fungal diagnostics, it can give an earlier indication of an IFI before cultures, which often require several days for detection.(204) Glucans may also be a useful prognostic marker in response to echinocandin therapy, as a decrease in 1,3-β-glucan levels strongly correlates with a positive response to therapy.(205) If the PG effect could occur in vivo, the use of 1,3-β-glucan in diagnostics would likely be impair the detection given the characteristic cell wall changes associated with the PG effect.

A major drawback to the current echinocandin research is that the majority of experiments are conducted at sub-inhibitory concentrations and do not consider the PG effect. As such, the data cannot be extrapolated to yeast grown at PG concentrations, as there is likely a physiological difference (as evidenced by morphological differences) that allows yeast to grow at elevated echinocandin

concentrations. Regardless of the potential for PG *in vivo*, further understanding of the influence of media on antifungal response, and cell wall stress responses has the potential to identify possible compensatory mechanisms contributing to persistent growth that could be used to identify new antifungal targets or enhance those drugs already in use.

1.5. Research objectives

There remains several anomalies regarding the activity of echinocandins, most notably, the PG effect in susceptible *Candida* spp. and the intrinsic resistance of many fungi despite high similarity in the target protein GS. There is little evidence for or against a similar cellular mechanism for PG and intrinsic resistance, and there is no consensus among echinocandin researchers as to the significance of the PG effect or its potential to contribute to treatment failure. Further investigation into the cellular effects of echinocandins at elevated concentrations is essential to determine its potential to occur *in vivo*.

1. Identify PG positive *C. albicans* isolates among a clinical collection.
2. Evaluate the influence of inoculum density on the PG effect.
3. Reconstruct the evolutionary history of GS in medically important fungi.
4. Correlate GS sequence with CASPO MIC.
5. Understand the relationship between cellular morphology and PG.
6. Determine the effect of medium carbon source on PG effect.

This research describes my investigation into the cellular effects of *C. albicans* exposed to CASPO. I chose to use CASPO as it was the first echinocandin to be approved for clinical use, thus has been used in the majority of the echinocandin research; likewise, *C. albicans* was utilized as a large portion of the previous research has utilized this clinically significant yeast as a model to study the effects of echinocandins.

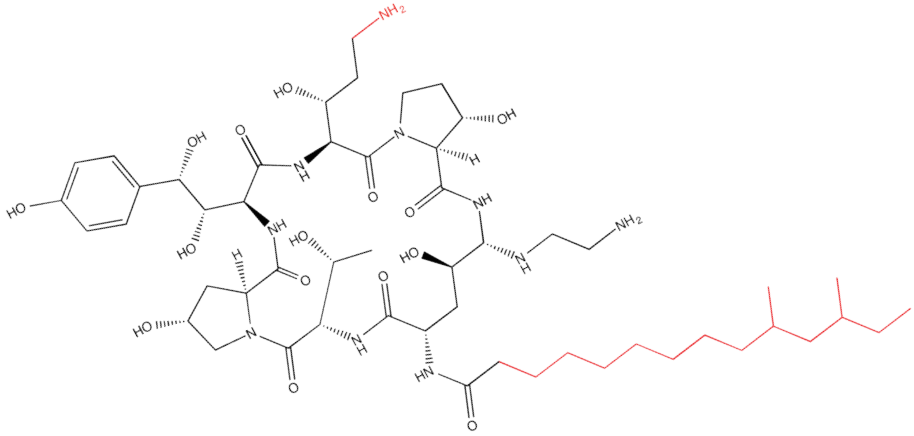
Table 1.1. Antifungal therapy. Summary of mechanisms, efficacy, and resistance of antifungal therapy.(33,206)

Drug class	Polyenes	Azoles	Pyrimidine analogues	Echinocandins
Antifungal	Amphotericin B Nystatin	Fluconazole Itraconazole Voriconazole Ketoconazole Posaconazole	Flucytosine	Caspofungin Micafungin Anidulafungin
Putative mechanism of action	Disrupts plasma membrane stability by directly binding ergosterol, causing cell leakage and lysis	Inhibits lanosterol 1,4-a-demethylase (ERG11) in ergosterol biosynthesis, leads to accumulation of toxic sterol intermediates and affects plasma membrane integrity	Interferes with pyrimidine metabolism (DNA/RNA synthesis) and protein synthesis	Inhibition of 1,3-β-glucan synthesis
Spectrum of activity	*Candida* spp., *Aspergillus* spp., *C. neoformans*, Zygomycetes, dimorphic fungi, some dematiaceous fungi	*Candida* spp., *Aspergillus* spp., *C. neoformans*, dimorphic fungi	*Candida* spp. and *C. neoformans*	*Candida* and *Aspergillus* spp.
Resistance: mechanisms and frequency	Intrinsic resistance: *C. lusitaniae, C. guillermondii*	Increasing resistance with widespread use due to: target (ERG11) mutations and upregulation; upregulation of efflux pumps Little activity against *C. krusei, C. glabrata*	High prevalence of acquired resistance due to: increased synthesis of pyrimidines out-compete flucytosine; decreased cellular uptake.	Target mutations: Glucan synthase (Fks1p, Fks2p) mutations. Resistance is uncommon.
Safety, efficacy	Binds sterols of mammalian cells, resulting in toxicity. Severe side effects: nephrotoxicity, infusional toxicity	Inhibit cytochrome P450, potential for drug interactions.	Limited use in monotherapy due to resistance. Anti-proliferative actions, especially affecting bone marrow and GI.	Effective against azole resistant isolates, and biofilms. Few side effects or drug interactions.

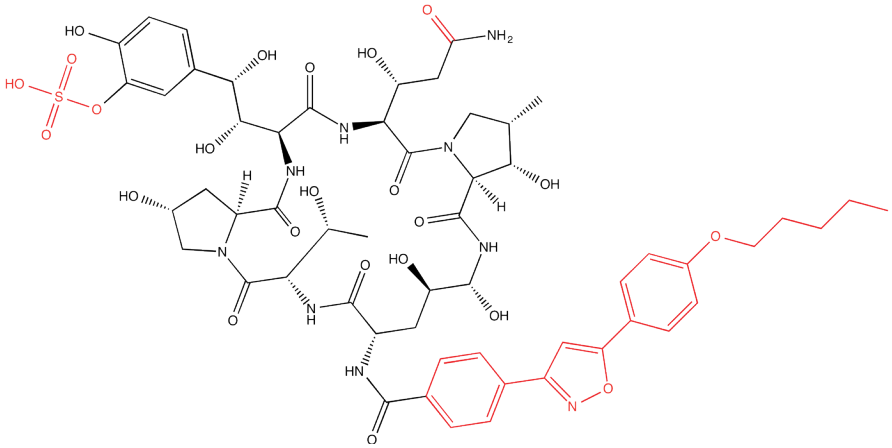
Table 1.2. Echinocandin clinical breakpoints for *Candida* spp. for CLSI broth microdilution methods.(166) MIC interpretive breakpoints (mg/L) incorporate susceptibility data for isolates with confirmed FKS1/FKS2 mutations and clinical data to define the boundaries of susceptible, intermediate or resistant organisms.

Antifungal Agent	Species	Susceptible	Intermediate	Resistant
Anidulafungin	*C. albicans*	≤0.25	0.5	≥1
	C. glabrata	≤0.12	0.25	≥0.5
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8
Caspofungin	*C. albicans*	≤0.25	0.5	≥1
	C. glabrata	≤0.12	0.25	≥0.5
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8
Micafungin	*C. albicans*	≤0.25	0.12	≥1
	C. glabrata	≤0.06	0.25	≥0.25
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8

Caspofungin (Cancidas®, Merck Inc.)



Micafungin (Mycamine®, Astella Pharma Inc.)



Anidulafungin (Eraxis®, Pfizer Inc.)

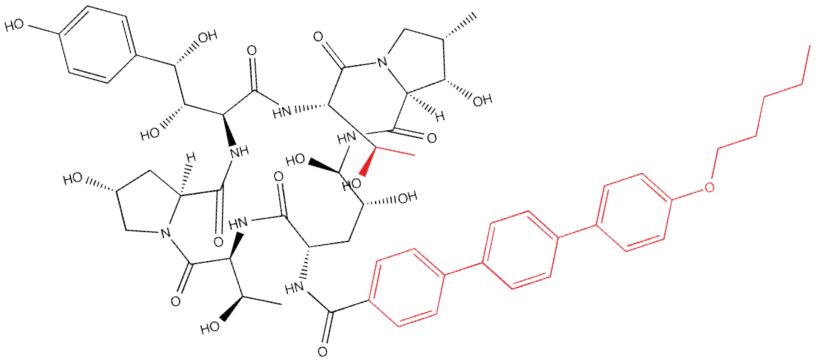


Figure 1.1. Chemical structure of echinocandins. Echinocandins have a similar cyclic hexapeptide core with differing lipid side chains (red). Chemical structures were generated with ChemBioDraw v13.0 (PerkinElmer Inc., Waltham, MA).

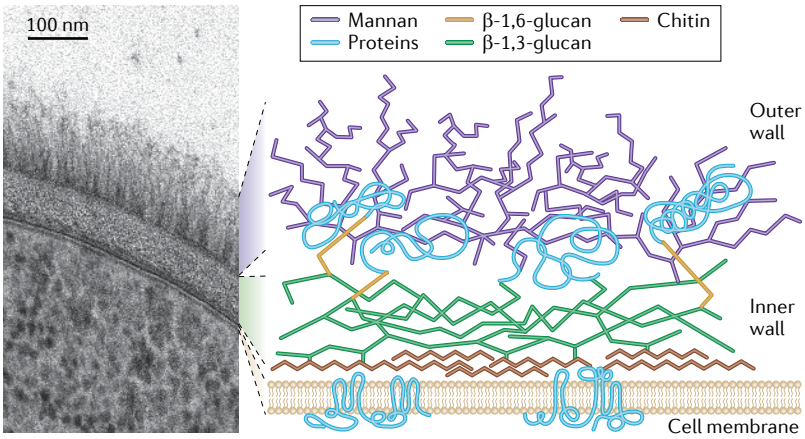


Figure 1.2. The yeast cell wall. The main components of the cell wall include glucans, chitin, and mannoproteins. The electron micrograph (left) demonstrates the cell wall is divided into two distinct layers: an inner electron transparent layer consists mainly of 1,3-β-glucans that branch with 1,6-β-glucans, and an outer electron dense layer of mannoproteins. The 1,3-β-glucans of the inner layer are covalently linked to chitin, providing the structural foundation and shape of the cell. The outer layer of mannoproteins is attached to 1,6-β-glucans of the inner cell wall. Reprinted from Gow et al 2011 with permission.(14)

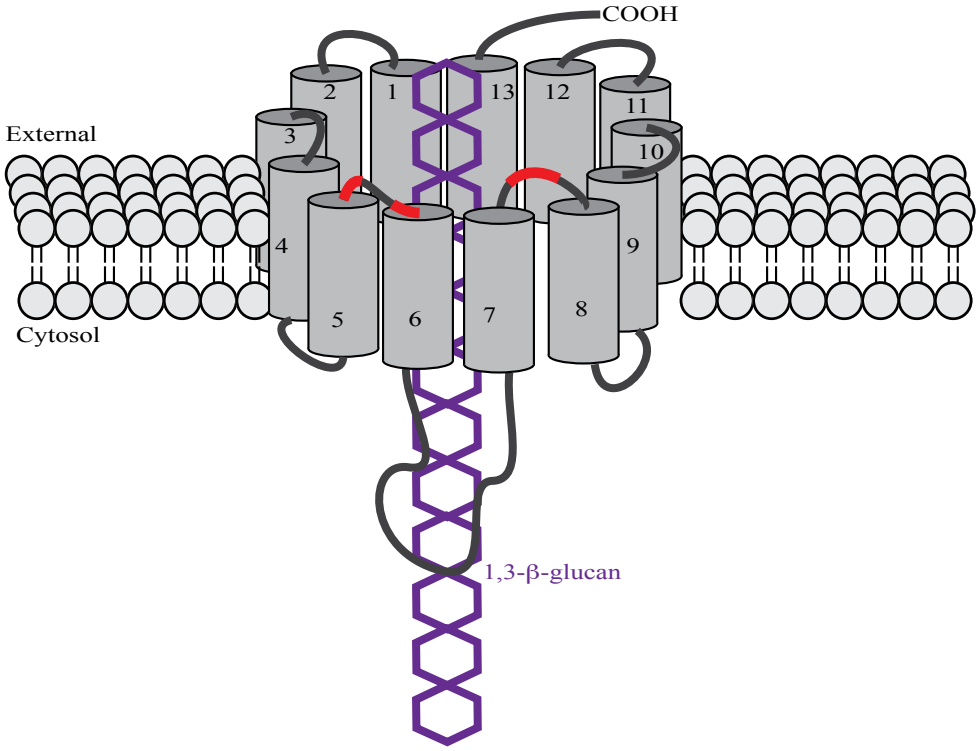


Figure 1.3. Putative Fks1p topology. Similar to the proposed structure of callose synthase in plants(103), my representation of GS incorporates a catalytic and transport function. It is not known how the formed glucan fibrils are transported to the cell's exterior, but it is likely the 13 experimentally confirmed transmembrane regions (numbered cylinders) facilitate its transport through the plasma membrane. As determined by Edlind et al(82), the three "hot-spot" regions (shown in red) determine the susceptibility of *Candida* spp. to echinocandins; the hexapeptide core likely interacts with these regions while the lipid tail interacts with the adjacent plasma membrane.

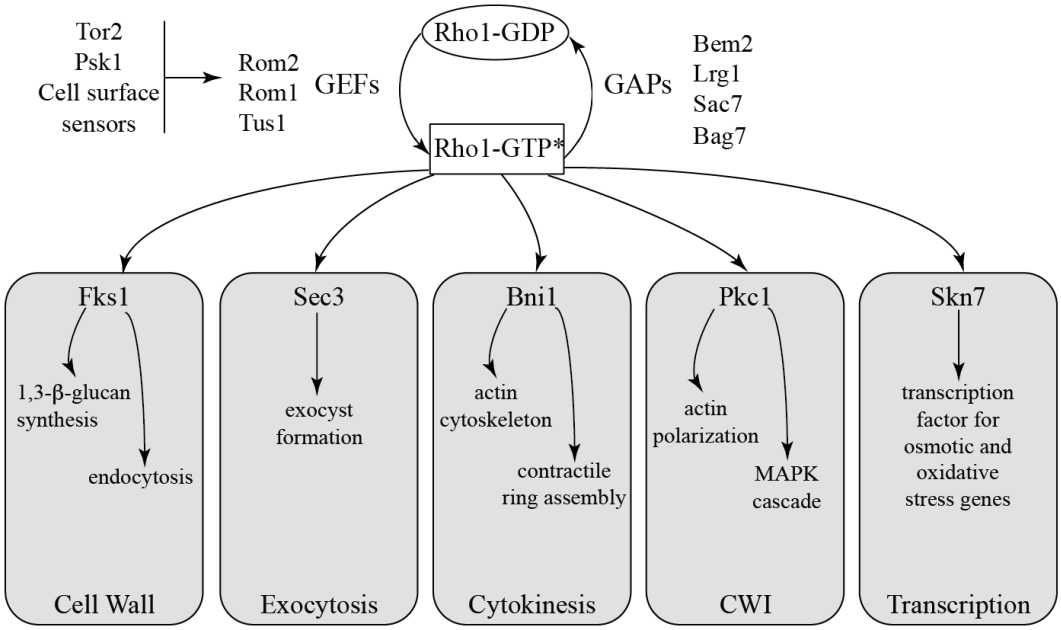


Figure 1.4. Functions of Rho1p. In summary of section 1.2.2.4, Rho1p is involved in the regulation of the cell wall, polarized growth, cytokinesis, and stress responses. Rho1p is activated when bound to GTP (*), activated by GEFs and suppressed by GAPs. GTP bound Rho1p activates effector proteins and their functional pathways: Fks1p, Sec3p, Bni1p, Pkc1p, and Skn7p.

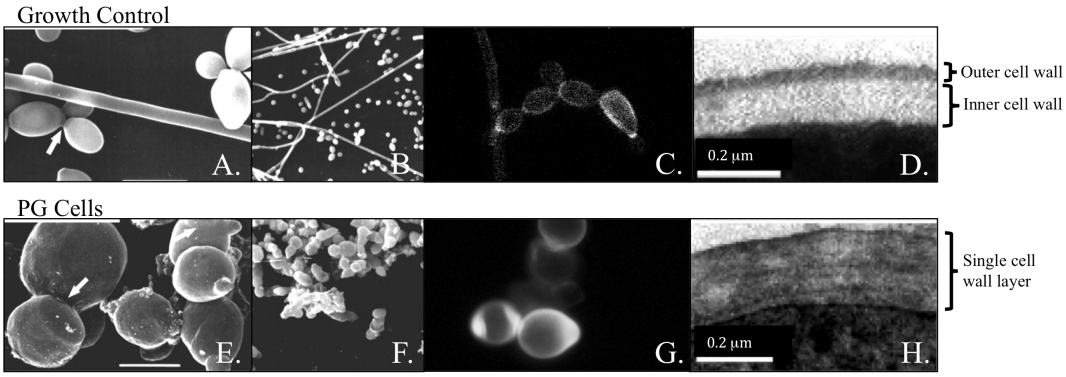


Figure 1.5. Cellular characteristics of PG cells. PG cells of *C. albicans* (E-H) demonstrate distinct morphological features from the growth control cells (A-D). Scanning election microscopy (A, B, E, F) demonstrates the absence of contractile rings at the bud site, absence of filamentation and enlarged unseparated yeasts forming aggregates. PG cells also exhibit increased chitin staining by calcofluor white (C, G), and significant cell wall alterations demonstrated with transmission electron microscopy (D, H). Control cells demonstrate two cell wall layers, as expected (D), while PG cells have a single cell wall layer, largely composed of electron dense chitin. Reprinted from Bizerra et al 2011 with permission.(178)

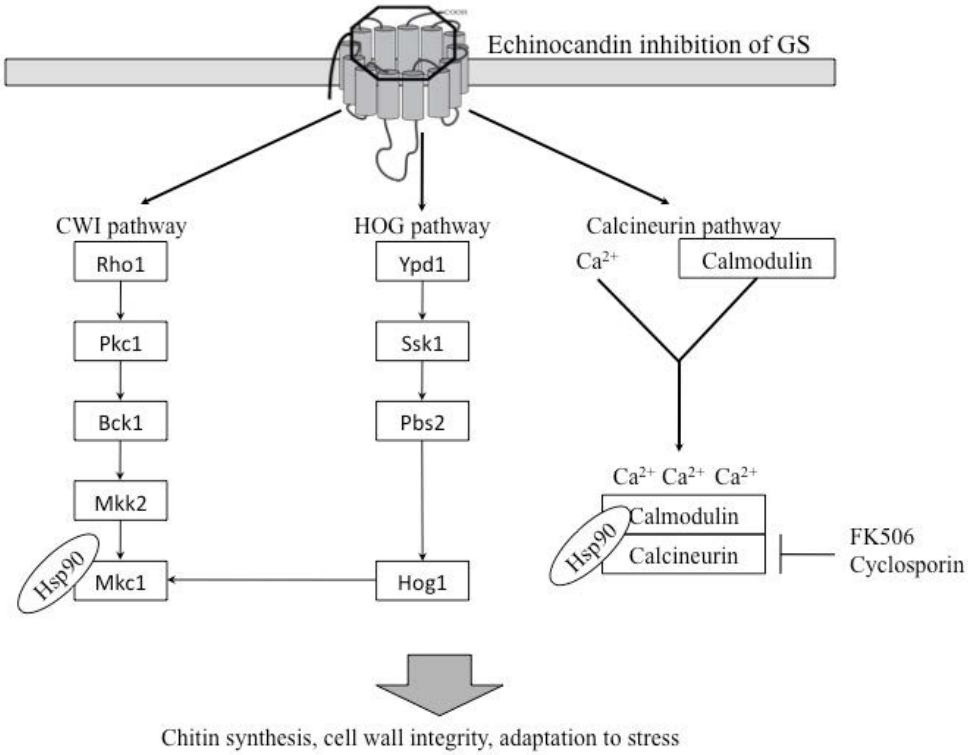


Figure 1.6. Cell wall stress pathways induced by echinocandins. In summary of p.30, The highly conserved stress responses required for the PG effect and resistance to echinocandins include the CWI, HOG, and calcineurin pathways, as well as the Hsp90p. Inhibition of these pathways or Hsp90p significantly reduces *C. albicans* tolerance to elevated echinocandin concentrations and the PG effect.(188-191)

1.6. References

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Chapter 2: Materials and Methods

2.1. PG screening of yeast isolates

2.1.1. Yeast isolates

C. albicans was chosen to test the incidence of the PG effect as it is well documented to demonstrate the PG effect with CASPO, it is the most frequent cause of candidemia, and is used in many experiments as a model organism for *Candida* related diseases.(1) *C. glabrata* is believed to be PG negative and was chosen to test this assumption comparing inoculum densities.

Twenty four *C. albicans* and twelve *C. glabrata* clinical and American Type Culture Collection (ATCC®) yeast isolates differing in patient demographics and source of infection were selected from the Reference Mycology collection, Provincial Laboratory of Public Health, University of Alberta Hospital, Edmonton, Alberta. The isolation, identification, and susceptibility testing of isolates were performed according the CLSI standards by Reference Mycology.(Table 2.1) All isolates were stored at -70^{o}C in skim milk and subcultured twice on Sabouraud Dextrose Agar (SDA, BD Difco™, Sparks, MD) at 35^{o}C before experimental use.

Yeast isolates chosen were susceptible to CASPO and did not demonstrate other *in vitro* phenomena, such as the trailing endpoint. This allowed the evaluation of the PG effect independent of known factors contributing to CASPO resistance or other *in vitro* effects.

2.1.2. Broth microdilution assays

The frequency of the PG effect can vary by species, strain, echinocandin, and is influenced by media composition.(2,3) Consequently, it was important to determine a subset of yeast isolates that reproducibly demonstrate PG. It was also important to evaluate the effects of starting inoculum density on the incidence of PG, as inocula differ between microdilution assays macrodilution assays, such as time kill analysis.

The CLSI broth microdilution method was performed to identify yeast isolates demonstrating the PG effect with CASPO.(4) Reagent grade CASPO was obtained from Merck & Co. (Whitehouse Station, NJ) through Reference Mycology or purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (10,000 mg/L) were prepared in dimethyl sulfoxide (DMSO) and stored at -70^{o}C. Microdilution trays were prepared according to the CLSI document M27-A3.(4) Two times the required volume of the highest CASPO concentration (128 mg/L) was prepared in RPMI 1640 medium (0.2% w/v glucose, with L-glutamine, without bicarbonate) with 0.165 M 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH 7 ±0.1 (Sigma-Aldrich, St. Louis, MO) and serially diluted 1:1 nine times in RPMI1640-MOPS medium to acquire the final dilution (0.25 mg/L). Aliquots of each dilution were dispensed in 100 μl volumes into wells of clear U-shaped bottom 96-well microdilution plates (Grenier CellStar®, Sigma-Aldrich, St. Louis, MO). The dispensed CASPO dilutions were further diluted 1:1 upon addition of yeast suspension resulting in final CASPO concentrations ranging from 0.125 to 64 mg/L after inoculation. The CASPO concentration range tested was modified from the M27-A3 procedure (from 0.0313 – 16 mg/L) to

broaden the upper limit and increase the detection of isolates demonstrating paradoxical growth at elevated CASPO concentrations.

Subcultured isolates were grown for 24 hours at 35°C on SDA. Several isolated colonies were suspended in 0.85% sterile saline and adjusted with a spectrophotometer to a transmittance equal to that of a 1.0 McFarland standard at 530 nm, corresponding to approximately 1 to 5 x 10^7 cells per ml. This stock suspension was diluted 1/10 in RPMI 1640-MOPS medium to achieve a 1 to 5 x 10^6 cells per ml inoculum, then further diluted 1/5 in water followed by 1/200 in RPMI 1640-MOPS medium for a 1 to 5 x 10^3 cells per ml inoculum. Microdilution trays were inoculated with 100 μl of the final yeast dilution, resulting in a final inoculum density of 0.5 to 2.5 x 10^6 and 0.5 to 2.5 x 10^3 cells per ml. Trays were inoculated within 30 minutes of preparation of yeast suspension and the inoculum of one isolate from each tray was plated in triplicate to SDA plates for colony count evaluation to estimate dilutions.

Quality control of the microdilution trays was achieved by evaluating *C. krusei* ATCC® 6258 and *C. parapsilosis* ATCC® 22019 MICs (0.12 – 1.0 mg/L and 0.25 – 1.0 mg/L, respectively) as recommended by the CLSI. Drug free growth and sterility controls assessed the reagent quality and technical competence, containing 100 μl of RPMI 1640-MOPS medium with 100 μl inoculum and 200 μl RPMI 1640-MOPS medium, respectively.

Microdilution trays were incubated at 35°C without agitation for 24 hours and evaluated for growth manually and spectrophotometrically. Growth patterns were first evaluated for the presence or absence of visible growth with the aid of a

reading mirror. After manual reading of plates, pellets were re-suspended by pipetting 5 times with a multiwell pipettor to ensure optimal even cell distributions to read absorbances at 405 nm using an *Opsys* MR™ microplate reader (Dynex Technologies Inc., Chantilly, VA, USA) using Revelation Quicklink v4.25 software (Dynex Technologies, Inc.). The absorbance values of the negative control wells (containing RPMI 1640-MOPS only) were subtracted from test well values. Experiments were replicated three times on separate days.

MIC endpoints were interpreted as the lowest CASPO concentration resulting in a prominent decrease in turbidity compared to the growth control and a ≥50% reduction in absorbance at 405 nm compared to the growth control. Paradoxical growth was defined as re-emergence of growth in the presence of drug concentrations ≥2 drug dilutions above the MIC assessed by visual reading and or a statistically significant increase in absorbance at 405 nm compared to the media control.

2.1.3. Microscopy

Images of microdilution plates were obtained with an Olympus SZ61 stereomicroscope (Olympus American Inc., Centre Valley, PA, USA) equipped with an MDC320 microscope digital camera examined at 1.2X magnification, and processed with ScopeTek ScopePhoto v3.1.312 software (ScopeTek Opto-Electric Co., Ltd., Hangzhou, China). Images were resized, cropped, and enhanced for contrast and exposure in Adobe Photoshop CS5 extended v12.1x64 (Adobe

Systems Inc., San Jose, CA, USA). All adjustments were made equally to each image.

2.1.4. Statistical analysis

The absorbance values at 405 nm for each inoculated well were compared to the absorbance of the negative control (media only) to assess magnitude of growth. This comparison tests the hypothesis that growth in each inoculated well will result in a significant increase in absorbance at 405 nm compared to the negative control:

$$H_O: \mu_{inoc\_Xmg/LCASPO} - \mu_{neg} = 0$$

$$H_A: \mu_{inoc\_Xmg/LCASPO} - \mu_{neg} > 0$$

Where $\mu_{inoc\_Xmg/LCASPO}$ is the mean absorbance readings of the inoculated wells at each CASPO concentration (inoc_Xmg/LCASPO, X = 0—64 mg/L for each isolate at 10^3 and 10^6 cells per ml), and μ_{neg} is the mean absorbance readings of the negative control. Mean absorbance readings from three independent replicates were analyzed by a one-tailed paired t-test, with a p value less than 0.05 considered significant support for increased absorbance readings compared to the negative control and indicative of growth. Graphing and statistics of absorbance readings was executed with GraphPad Prism v6.0d (GraphPad Software Inc., La Jolla, CA, USA).

2.2. Phylogenetic and sequence analysis of GS

2.2.1. Sequence data

Data was obtained for organisms that represent a diverse representation of medically important and related fungal species for which publically available and manually curated data is available. Complete DNA and protein sequences of FKS homologs of 30 medically important fungi were obtained from the Fungal Genome Initiative, the *Candida* Genome Database(5), and the *Saccharomyces* Genome Database.(1,6)(Table 2.2)

2.2.2. Sequence analysis

Protein sequences were aligned with PSI-coffee under default parameters (unweighted with gap open and gap extension penalties of 0).(2,3,7) The protein sequence alignment was used as a guide to align exon sequences by codon using TranslatorX.(4,8) Annotated expressed sequence tag data from each respective genome database was used to identify intron positions and boundaries. The exon sequence alignment was manually adjusted using the intron boundaries to minimize the possibility of intron sliding over an amino acid insertion or deletion as the former event occurs less frequently.(4,9) Intron sequences were extracted and aligned with T-coffee,(5,10) then inserted into the exon sequence alignment within the determined intron boundaries. Intron phases are defined by codon position: splicing occurring after the third, first, or second nucleotide of the codon is a phase 0, 1, and 2 intron, respectively. Extracted intron sequences were evaluated for similarity using the BLASTx plugin in Geneious v6.0.5. Gene

structures were produced using Webscipio(6,11) and analyzed using GenePainter.(7,12)

2.2.3. Phylogenetic analysis

Phylogenetic trees were generated using the nucleotide and protein alignments without partitions and equal weighting of characters and character state transformations. *Rhizopus oryzae* was used as outgroup for tree rooting as it is the earliest known ancestor of the sampled taxa. Trees were generated using maximum likelihood, Bayesian, and maximum parsimony inference. The most appropriate model of evolution for the nucleotide data was the general-time-reversible model with a proportion of invariant sites and gamma distributed rates (GTR+I+Γ), determined by Akaike and Bayesian information criterion in jModelTest v2.1.3.(13,14) The JTT distance model was applied to the protein data. Bayesian analysis was conducted under default priors using MrBayes v3.1.2(15,16) for $1x10^6$ generations sampling every 100 generations for two independent searches, each with four chains. Convergence was assessed by the standard deviation of the split frequencies approaching and stabilizing at 0.005, and the first 3,300 trees were excluded from analysis as burn-in. Maximum likelihood trees were generated in MEGA5 v2.2(17) with a nearest-neighbor-joining heuristic search with 1000 random addition bootstrap replicates. Parsimony analysis was performed in Paup*4.0b10 (Sinauer Associates, Sunderland, MA); most parsimonious trees were generated by a heuristic search with 1000 random addition bootstrap replicates with tree bisection connection

branch swapping. A species tree was generated using the primary FKS protein sequences (Fks1p or Bgs4p for budding and fission yeast, respectively) and used to reconcile the gene tree using Notung v2.6.(18,19) Phylogenetic trees were visualized in TreeViewX v0.5(20) and edited with Adobe Illustrator CS5.1 (Adobe Systems Inc., San Jose, CA).

2.3. Effect of medium carbon source on PG

2.3.1. Yeast isolates

Two *C. albicans* strains were used in all subsequent testing; the clinical yeast isolate M10MY4874 and reference strain ATCC® 90028™ were chosen as both demonstrated prominent growth at PG concentrations of CASPO, and ATCC® 90028™ is the reference strain recommended for CASPO susceptibility testing by the CLSI standards M27-A3.(4) The ATCC® 15126™ *C. glabrata* reference strain was used as a negative control for the PG effect.

2.3.2. Broth microdilution screening.

Broth microdilution assays were performed as described above to screen for the CASPO concentrations PG occurs with each carbon source evaluated. Broth microdilution trays were prepared using RPMI 1640-MOPS (containing 0.2% glucose) or minimal medium containing 2% w/v glucose, galactose, sodium-L-lactate (Sigma-Aldrich, St. Louis, MO), or 3% v/v glycerol (Bio-Rad Laboratories, Inc., Herucles, CA) in 0.67% yeast nitrogen based without amino acids (YNB, BD Difco™, Sparks, MD) pH 5.4 ±0.1.

Inoculation of trays was performed as described above. Fresh (<48 hour) yeast cultures on SDA were suspended in sterile 0.85% saline and adjusted to a 1.0 McFarland standard, and diluted 1/10 in the corresponding carbon source medium to achieve a 1 to 5 x 10^6 cells per ml inoculum. Aliquots of 100 µl were dispensed in the microdilution trays for a final inoculum of 0.5 to 2.5 X 10^6 cells per ml. Microdilution trays were incubated at 35^{o}C without agitation and evaluated for the presence or absence of visible growth with the aid of a reading mirror. Due to the slow growth of yeast in the non-fermentable carbon sources, trays were evaluated for growth at 48 hours.

2.3.3. Time kill analysis.

Time kill analysis was performed on yeast exposed to zero, sub-inhibitory, inhibitory, and PG concentrations of CASPO, the concentrations of which were determined by broth microdilution screening. Yeast isolates were inoculated to minimal media containing 2% carbon source from fresh SDA cultures and incubated overnight at 35^{o}C with agitation (150 rpm). Overnight cultures were diluted to ~1.5 x 10^6 cells per ml in 40 ml of fresh media in a 50 ml conical tube containing the appropriate concentration of CAS and incubated at 35^{o}C with agitation. Growth was monitored by collecting 1.5 ml samples at predetermined time points (0, 3, 6, 9, 12, 24, 48, 72 hours) and evaluated for colony forming units (CFU) and optical density at 600 nm (OD_{600}).

Colony counts (CFU) were obtained by serially diluting samples 10-fold with sterile 0.85% saline and plating 100 µl aliquots in triplicate, streaking for

confluent growth. Plates were incubated at 35^{o}C and the number of colonies per ml was counted at 48 h. Two optical density readings were taken per sample and read at 600 nm with a DU®700 series Beckman Coulter spectrophotometer (Beckman Coulter Inc., Brea, CA). The spectrophotometer was blanked with YNB (with respective carbon sources) before each measurement Experiments were repeated three times on separate days.

2.3.4. Statistical analysis of time kill experiments

Growth curves were used to assess the growth response of yeast to CASPO grown in different carbon sources over time. As described by Klepser et al(21), a fungistatic response was defined as a <99.9% reduction in CFU/ml from the starting inoculum, and a fungistatic response was defined as a ≥99.9% reduction in CFU/ml from the starting inoculum.

The final growth (endpoint) of *C. albicans* exposed to 0, 0.075, 2, and 16 mg/L CASPO in each respective carbon source at 72 hours was evaluated by CFU and OD_{600}. The endpoints for each carbon source were compared against the starting inoculum to test the hypothesis that there is significantly decreased growth at sub-inhibitory and inhibitory concentrations and significantly increased growth at PG concentrations of CASPO and in the growth control (GC):

$$H_O: \mu_{GC}, \mu_{0.075\ mg/L}, \mu_{2\ mg/L,} \mu_{16\ mg/L} = \text{starting inoculum}$$

$$H_A: \mu_{GC}, \mu_{0.075\ mg/L}, \mu_{2\ mg/L,} \mu_{16\ mg/L} < \text{or} > \text{starting inoculum}$$

The endpoints for each carbon source were compared against each other to test the hypothesis that growth of yeast exposed to 16 mg/L (PG) will result in significantly increased growth compared to the subinhibitory and inhibitory CASPO concentrations:

$$H_O: \mu_{16\ mg/L} = \mu_{0.075\ mg/L} = \mu_{2\ mg/L}$$

$$H_A: \mu_{16\ mg/L} < \text{or} > \mu_{0.075\ mg/L} \text{ and or } \mu_{2\ mg/L}$$

The endpoints for each CASPO concentration were compared against each other to test the hypothesis that yeast grown with optimal carbon sources (fermentable) will result in significantly increased growth compared to yeast grown in non-fermentable carbon sources, regardless of the concentration of CASPO:

$$H_O: \mu_{GC\_fermentable} = \mu_{GC\_non\text{-}fermentable}$$

$$\mu_{0.075mg/L\_fermentable} = \mu_{0.075mg/L\_non\text{-}fermentable}$$

$$\mu_{2mg/L\_fermentable} = \mu_{2mg/L\_non\text{-}fermentable}$$

$$\mu_{16mg/L\_fermentable} = \mu_{16mg/L\_non\text{-}fermentable}$$

$$H_A: \mu_{GC\_fermentable} < \text{or} > \mu_{GC\_non\text{-}fermentable}$$

$$\mu_{0.075mg/L\_fermentable} < \text{or} > \mu_{0.075mg/L\_non\text{-}fermentable}$$

$$\mu_{2mg/L\_fermentable} < \text{or} > \mu_{2mg/L\_non\text{-}fermentable}$$

$$\mu_{16mg/L\_fermentable} < \text{or} > \mu_{16mg/L\_non\text{-}fermentable}$$

The mean OD_{600} or CFU endpoint values (μ) of each CASPO concentration for a given carbon source from three experimental replicates were analyzed by a one-way paired analysis of variance (ANOVA) to compare all the means for an endpoint and Tukey's multiple comparisons test to compare every mean with every other mean. A p value less than 0.05 was considered significant support for differences in endpoint growth for a given CASPO concentration. Graphing and statistics of absorbance readings was executed with GraphPad Prism v6.0d (GraphPad Software Inc., La Jolla, CA, USA).

2.4. Cell cycle analysis by flow cytometry

2.4.1. Flow cytometry: sample collection

Yeast isolates were made to a 0.5 McFarland Standard from a fresh (<48 hour) SDA plate in 1ml 0.85% sterile saline, inoculated to 9ml RPMI 1640-MOPS, and incubated overnight (~18 hours) at 35^{o}C with shaking at 150 rpm on a rotary shaker.

Similar to Zhang et al, cells were synchronized in S phase with incubation of 0.2 M hydroxyurea (Sigma-Aldrich, St. Louis, MO) for 1 hour at 35^{o}C with shaking at 150 rpm on a rotary shaker.(22) Cells were washed once with fresh RPMI 1640-MOPS medium and the cell density adjusted to approximately 10^{6} cells per ml. Cells were incubated 1 hour at 35^{o}C with shaking to allow recovery from the hydroxyurea treatment and progression through the cell cycle. At time zero (t0), CASPO was added to the final concentration of 0 (growth control),

0.075 (sub-inhibitory), or 16 mg/L (PG). 1 ml samples were collected in 20-minute intervals from t0 to t120 minutes.

Samples were centrifuged at 13,000 revolutions per minute (rpm) for 10 minutes; the supernatant was decanted and the pellet was fixed in 1 ml 70% ethanol at 4^{o}C for a minimum of 18 hours or until analyzed by flow cytometry. The ethanol fixative was decanted after centrifugation at 13,000 rpm for 10 minutes, and the sample was rehydrated in 750 μl of 1X phosphate buffered saline (PBS) and 1 mg/ml ribonuclease A (RNAse A, Sigma-Aldrich, St. Louis, MO) at 35^{o}C for 1 hour. Samples were stained with 3 μl stock Picogreen (Invitrogen, Carlsbad, CA) for 15 minutes in the dark. Immediately before analysis, cells were sonicated for 10 seconds at low output to limit cell clumping. Experiments were replicated three times on separate days.

2.4.2. Flow cytometry: analysis

A minimum of 10,000 ungated events were analyzed with an LSR-Fortessa flow cytometer (BD Biosciences, Mississauga, ON) with a laser excitation of 488 nm and filter detecting emissions at 530 nm. Results were visualized and analyzed using FlowJo software v10.0.7 (Treestar, Inc., Ashland, OR). Single cells were manually gated from doublets and debris by visualizing with a fluorescent area versus width histogram; the fluorescent area represents the total fluorescence of a cell while the fluorescent width represents the transit time of a cell and correlates to its total fluorescence or DNA content and cell size, respectively.(Figure 2.1) DNA content histograms were generated from the single

cell population was manually gated for the G1/G0, S, and G2/M phases. Histograms images were exported to and edited with Adobe Illustrator CS5.1 (Adobe Systems Inc., San Jose, CA).

Table 2.1. Clinical yeast isolates. Isolates were obtained from the National Centre for Mycology, Provincial Laboratory of Public Health, University of Alberta Hospital, Edmonton, Alberta. The isolation, identification, and CASPO MIC testing of isolates were performed according the CLSI standards by the National Centre for Mycology. * Reference strains. Abbreviations: University of Alberta Hosptial (UAH), neonatal intensive care unit (NICU), Royal Alexandra Hospital (RAH), Bronchoalveolar lavage (BAL)

Isolate	MIC	Specimen source	Region
C. albicans			
ATCC® 90028™	*	Blood	Iowa (MA Pfaller)
ATCC® 24433™	*	Nail infection	N/A
M10MY4874	0.25	Urine	Moncton Hospital
M10MY8596	0.25	Intraperitoneal fibrin	UAH NICU
M10MY7506	-	Liver biopsy	UAH
M10MY7837	-	Pleural fluid	RAH
M10MY4875	0.25	Blood	Moncton Hospital
M10MY5059	0.25	Blood	Chinook health region
M10MY2166	0.25	Swab from burn	Moncton Hospital
M10MY1585	-	Bone chip	UAH
M10MY2938	0.12	Blood	$DynaLife_{DX}$
M10MY3029	0.12	Bone chip	UAH
M10MY3028	0.25	Urine	Moncton Hospital
M10MY2978	0.12	Blood	Stanton Territorial Hospital
M10MY4529	0.12	Blood	Red Deer Hospital
M10MY5068	0.12	Peritoneal fluid	Saint John Hospital
M10MY2333	0.12	Blood	UAH
M10MY2222	0.12	Subphrenic abscess	$DynaLife_{DX}$
M10MY2678	0.12	Blood	UAH
M10MY2273	0.12	Unknown source	LifeLabs Medical Lab Services
M10MY2883	0.25	Blood	BC Provincial Laboratory
M10MY2797	0.25	Urine, cystoscopy	$DynaLife_{DX}$
M10MY2168	0.25	Vaginal swab	Moncton Hospital
M10MY2163	0.12	Vaginal swab	LifeLabs Medical Lab Services
C. glabrata			
ATCC® 15126™	*	Unavailable	Takeda Chem. Ind., Ltd.
M06MY000206	-	Bronchial wash	UAH
M06MY000779	0.5	Blood	Victoria General Hospital
M06MY001064	-	Sputum	UAH
M06MY002346	-	Blood	Yukon
M06MY002882	0.5	Ascites fluid	Moncton Hospital
M06MY003156	0.5	Umbilical fluid	Victoria General Hospital
M06MY002192	1.0	Vagina	Victoria General Hospital
M06MY003590	2.0	Urine	Moncton Hospital
M06MY003953	-	BAL	UAH
M06MY004294	-	Peritoneal fluid	UAH
M10MY001407	0.5	Blood	Westlock Healthcare Centre

Table 2.2. GS sequence list. DNA and protein sequences of FKS or orthologous glucan synthase (GS) were obtained from published sequence genomes: *Candida* Genome Database (CGD (5)), *Saccharomyces* Genome Database (SGD (6)), and the Fungal Genome Initiative (FGI).

Organism (isolate)	Gene	Locus	Reference
Candida albicans (SC5314)	Fks1 Fks2 Fks3	orf19.2929 orf19.3269 orf19.2495	CGD
Candida glabrata (CBS138)	Fks1 Fks2 Fks3	CAGL0G01034g CAGL0K04037g CAGL0M13827g	CGD
Candida parapsilosis (CDC317)	Fks1 Fks2 Fks3	CPAR2_106400 CPAR2_804030 CPAR2_109680	CGD
Saccharomyces cerevisiae (S288C)	Fks1 Fks2 Fks3	YLR342W YGR032W YMR306W	SGD
Aspergillus fumigatus	GS	Afu6g12400	FGI
Aspergillus flavus	GS	AFL2G_10481.2	FGI
Aspergillus nidulans	GS	ANID_03729.1	FGI
Fusarium oxysporum (4287)	GS	foxg_0321.2	FGI
Fusarium solani	GS	DQ351540	FGI
Coccidioides immitis (RS)	GS	CNBG_4964.2	FGI
Blastomyces dermatitidis (ATCC 18188)	GS	BDDG_06701.1	FGI
Histoplasma capsulatum (H143)	GS	HCDG_02151.2	FGI
Paracoccidioides brasiliensis (Pb01)	GS	PAAG_05071.1	FGI
Trichophyton rubrum (CBS 118892)	GS	TERG_01127.2	FGI
Neurospora crassa (OR74A)	GS	NCU06871.5	FGI
Schizosaccharomyces pombe	Bgs1 Bgs2 Bgs3 Bgs4	SPBC19G7.05c SPAC24C9.07.c SPAC19B12.03 SPCC1840.02c	FGI FGI FGI FGI
Cryptococcus neoformans var. *grubii* (H99)	GS	CNAG_06508.2	FGI
Cryptococcus gattii (R265)	GS	CNBG_4964.2	FGI
Rhizopus oryzae	GS	RO3G_10349.3	FGI

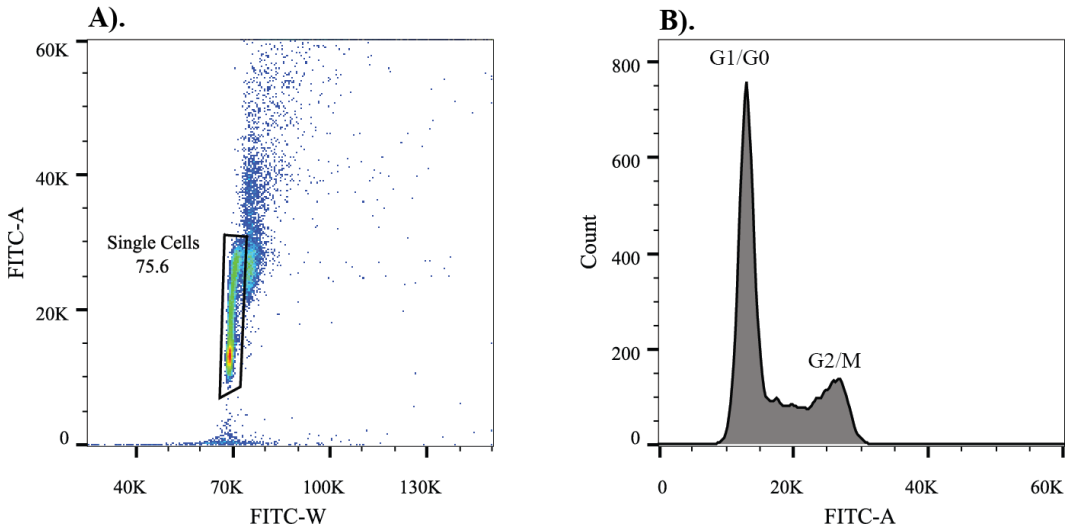

Figure 2.1. Flow cytometry sample analysis. A). Single cells were manually gated by visualizing with a fluorescent area (FITC-A) versus width (FITC-W) dot plot. This allows the evaluation of the total fluorescence (FITC-A) compared to the cell size (FITC-W). Doublets and larger cell morphologies (hyphae, pseudohyphae) have both greater FITC-A and FITC-W, while debris demonstrates lower FITC-A and FITC-W than single cells. Number corresponds to the % of total cells counted included in the single cell gating. **B).** Histograms of total fluorescence (FITC-A) for the corresponding gated population of single cells. G2/M phase corresponds to approximately 2 times the DNA (and fluorescence) of G1/G0.

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Chapter 3: Results

3.1. Effect of inoculum density on PG

Visual assessment of growth compares the degree of yeast exposed to different CASPO concentrations to the growth control. The U-shaped bottom of the 96 well plates allows the yeast to aggregate and pellet towards the bottom. The growth control of the standard (10^3 cells per ml) inoculum appeared as a pellet in the majority of the isolates.(Appendix 1, 2) The growth control of the elevated (10^6 cells per ml) inoculum demonstrated greater variability, with an overall increased dispersed growth across the entire well compared to the standard inoculum growth controls. PG appeared mostly dispersed across the wells, with little pelleting.

Concentrations of CASPO for MICs and PG determined by visual and spectrophotometric methods for standard and elevated starting inocula are listed in Table 3.1. All isolates demonstrated MICs within the susceptible range (≤0.25 mg/L CASPO)(1), regardless of the inoculum used. Due to the lowest CASPO concentration (0.125 mg/L) being several dilutions higher than used in standard CASPO MIC testing, isolates were determined to have an MIC ≤0.125 mg/L when the first well containing CASPO demonstrated a prominent decrease in growth. While not affecting the interpretation of the MIC, there was an overall trend of decreased growth at low CASPO concentrations using the elevated inoculum, observed as a left shift in plotted absorbance profiles (Figure 3.1) and decreased visible growth in one less dilution compared to the standard inoculum (Appendix 1, 2). This data confirms previous analyses demonstrating MICs are not significantly influenced by inoculum density.(2)

The frequency of PG was variable using a 10^3 inoculum (9 of 24 isolates), and only detected visually as the growth was not sufficient to cause a significant increase in absorbance (Figure 3.2). PG using a 10^6 inoculum was sufficient to observe a statistically significant increase in absorbance compared to the media control as observed for 23 of 24 *C. albicans* isolates (Table 3.2, Figure 3.1) and visually confirmed in 24 of 24 *C. albicans* isolates. The PG concentration range was wider with a 10^6 starting inoculum (8—32 mg/L) than with a 10^3 starting inoculum (16 mg/L). PG was not observed with *C. glabrata* (0 of 12 isolates), regardless of inoculum used

3.2. Evolution of GS in fungi.

3.2.1. Optimizing multiple sequence alignment of GS genes

30 GS sequences from 19 medically important fungi (see Table 2.2) were initially aligned with ClustalW2 plugin with Geneious v6.0.5 (Biomatters Ltd., Auckland, NZ) under default conditions (gap open penalty of 15 and gap extension penalty of 6.66).(3) The Clustal software is a fast and reliable method that often gives a good representation of the true sequence alignment. However, this software preferentially down-weights terminal gaps and rarely inserts internal gaps near the end of the alignment, regardless of the optimum criteria used.(4) The nucleotide alignment with ClustalW2 had obvious inaccuracies, largely due to the multiple introns present in the majority of fungi. Given that the FKS genes of fungi are highly conserved, especially within the central domain that putatively contains the catalytic site, gaps are more likely to be within the ends of the N- and

C-termini. The protein alignment generated with ClustalW2 demonstrated gaps that were inappropriate given the domain structure known for *S. cerevisiae* Fks1p.

To resolve the obvious inaccuracies with the nucleotide alignment, FKS protein sequences were aligned with PSI-coffee and reverse translated to aligned nucleotide coding sequences with TranslatorX. The PSI-coffee software incorporates hidden markov models for pairwise comparisons of secondary structure and domain structure predictions from publically available databases.(5) While slower due to the incorporation of protein structure data into the alignment, PSI-coffee provided a more accurate protein alignment that incorporated information of the FKS structure, largely modeled from experimental determinations from Fks1p of *S. cerevisiae*. TranslatorX was used to align exon sequences by codon guided by the PSI-coffee protein alignment. The intron positions were mapped to the alignments to evaluate conservation of intron position. Nucleotide sequences of conserved introns were aligned with T-coffee and inserted into appropriate sites within the exon alignment. Gaps were inserted into exon sequences that did not contain introns or where introns were at non-conserved sites. This stepwise alignment method allowed the final nucleotide alignment to incorporate data from the protein structure using PSI-coffee, more accurately determine non-synonymous or synonymous mutations by codon comparisons with TranslatorX, and compare gene structures and intron diversity.

3.2.2. Phylogenetic analysis

The overall sequence identity for the 30 FKS fungal genes from 19 species is 52.7% and 53.2% for the nucleotide and protein alignments, respectively. The primary domains of the Fks1p in *S. cerevisiae* have recently been identified as the terminal ends, the putative cytosolic central domain and 13 experimentally confirmed transmembrane regions.(6) Sequence diversity of the FKS proteins is largely within the amino- and carboxy-termini, with 38.8% and 12% sequence similarity respectively. The central domain is highly conserved with 72.7% identity.

The "hot-spot" regions corresponding to *S. cerevisiae* Fks1p at amino acids 635-649 (HS1), 690-700 (HS3), and 1354-1361 (HS2) were extracted from the protein alignment to evaluate for correlations in "hot-spot" sequence and intrinsic susceptibilities.(Figure 3.3) The Bayesian tree was used to evaluate protein sequences against organism clustering. Documented acquired mutations associated with decreased echinocandin susceptibility were highlighted to compare "hot-spot" sequences of intrinsically susceptible or resistant organisms. (Table 3.3) The "hot-spot" sequences of the primary fungal GS (Fks1p or Bgs4p) demonstrate high sequence similarity, with 7 of 15 identical sites in HS1, 5 of 11 identical sites in HS3, and 3 of 8 identical sites in HS2. Interestingly, few of the intrinsically resistant fungi have sequence variability at amino acid sites known to confer resistance in intrinsically susceptible fungi. This suggests the intrinsic susceptibility of an organism cannot be predicted by "hot-spot" sequence comparisons.

The protein data produced an alignment of 2,221 total characters with 1,692 variable sites. Parsimony, Bayesian, and likelihood analyses demonstrated similar support for a topology that resembles the accepted species tree for fungi.(7)(Figure 3.4) Parsimony analysis generated 1,346 most parsimonious trees with a length of 10,352 steps. Bayesian analysis generated 12,002 trees with a log likelihood of -62653.81, and maximum likelihood analysis generated an optimum tree with a log likelihood of -27,244.84.

The FKS nucleotide dataset of 30 medically important fungi produced a nucleotide alignment of 7,884 total characters with 5,262 variable sites. Parsimony, Bayesian, and likelihood analyses demonstrated consistent tree topologies and similar branch supports.(Figure 3.5) Parsimony analysis generated 1,096 most parsimonious trees with a length of 33,769 steps. Bayesian analysis generated 13,402 trees with a log likelihood of -129,050.73, and maximum likelihood analysis generated an optimum tree with a log likelihood of -77,880.17.

There are significant differences between the trees of the nucleotide and protein data. The taxa sampled are species from the phyla Zygomycota, Basidiomycota, and Ascomycota. Ascomycota is further sub-divided into the sub-phyla Taphrinomycotina (fission yeast), Saccharomycotina (budding yeast), and Pezizomycotina (filamentous ascomycetes). The phylogenetic tree from the protein sequence alignment is consistent with the known species tree, where Taphrinomycotina are the earliest diverging group of Ascomycota.(7) However, the nucleotide dataset produces a tree incongruent with the accepted species tree that supports the Pezizomycotina as the earliest divergent clade.

3.2.3. FKS gene duplications in yeast.

Gene copy number varied among species, with multiple copies in the budding and fission yeast, but only a single copy in all the other fungi sampled. A reconciled tree for those species with multiple FKS copies is shown in Figure 3.6. Analysis reveals six gene duplications and one loss event: *S. pombe* has four gene copies as a result of three small scale duplications (SSD) events; *C. albicans* and *C. parapsilosis* have three gene copies as a result of two SSD events; and *C. glabrata* and *S. cerevisiae* have three gene copies as a result of two SSD , a single gene loss event, and a whole genome duplication (WGD) event.

3.2.4. FKS gene structures.

The Ascomycete yeasts contain multiple copies of FKS genes but lack introns, while all other fungi have a single copy of FKS with multiple introns.(Figure 3.7) The positions of the two introns in filamentous ascomycetes are conserved at the N- and C- termini, except for *H. capsulatum* in which a large portion of the N-terminus is truncated in comparison to other fungi. *F. oxysporum* contains a unique additional intron in the central domain, the sequence of which aligns with the coding regions of all other fungi, indicating intron acquisition from the coding sequence. *C. neoformans* and *C. gattii* contain seven and eleven introns, respectively; five of these introns share exact positions while the remaining two introns vary in length and affect the alignment of the corresponding amino acid positions. *R. oryzae*, the earliest known ancestor of the

taxa selected, shares no common positions with the other fungi of its five introns. The lack of common introns suggests independent intron acquisitions after the divergence of each subphylum from the common ancestor sequence.

The source of the intron sequences could not be determined by blast search, as no significant sequence similarity excluding self-hits could be determined for any of the introns. However, the sixth and seventh intron of *C. gattii* aligns with the coding region of the other fungi, suggesting the generation of this intron from within the exon sequence.

3.3 Effect of carbon source on the PG.

I performed broth microdilution assays to screen for CASPO concentrations at which PG occurs in medium with fermentable (glucose, galactose) and non-fermentable (lactate, glycerol) carbon sources to use in time kill experiments. For minimal medium with 2% glucose, there was growth in all CASPO concentrations except in 2 mg/L at 48 hours. The PG effect is the re-emergence of growth in the presence of drug concentrations ≥2 drug dilutions above the MIC, and the lack of separation between wells of inhibition and growth prevents the use of 2% glucose in minimal medium for time kill experiments. Minimal medium with 0.2% glucose and RPMI 1640-MOPS, which also contains 0.2% glucose, demonstrated the same MIC and PG values.(see Figure 3.1) Since RPMI 1640-MOPS is the primary medium for CLSI broth microdilution testing and demonstrated the same broth microdilution growth pattern as minimal medium with 0.2% glucose with preliminary screening, I chose to use it as the

representative glucose medium. Media containing galactose, lactate, and glycerol varied in the degree and CASPO concentrations at which PG occurred, but all media demonstrated growth inhibition at 2 mg/L. PG was significantly reduced or questionable in the non-fermentable carbon sources, but 16 mg/L was chosen as PG is the most reproducible in the media tested.

C. glabrata does not utilize galactose as a sole carbon source(8), and grew very poorly in minimal medium with 2% lactate. As this isolate was included as a negative control for the PG effect, I deemed it sufficient to evaluate in RPMI 1640-MOPS (fermentable) and 2% glycerol (non-fermentable).

The growth of yeast in minimal media with 2% galactose, 2% lactate, 3% glycerol, and RPMI 1640-MOPS exposed to 0 (growth control), 0.075 (sub-inhibitory), 2 (inhibitory), and 16 mg/L (PG) CASPO was determined by monitoring CFU and OD_{600}. The assays were conducted over 72 hours to accommodate the slower growth in non-fermentable carbon sources. The effect of carbon source on yeast response to CASPO was evaluated by comparing the final growth (endpoints) for each CASPO concentration for a given carbon source and by comparing the endpoints for a given CASPO concentration for all carbon sources.

The growth of yeast exposed to CASPO grown with different carbon sources was illustrated by plotting the log10 CFU or OD_{600} versus time. The final growth endpoints for each growth condition were compared to evaluate for growth, fungicidal, or fungistatic activity in response to CASPO.(Figures 3.8—3.13)

Growth curves of *C. albicans* ATCC® 90028™ for CFU and OD_{600} determination demonstrated exponential growth for the GC during the initial 12 hours that plateaued at maximum growth for the remaining 60 hours when grown in RPMI, 2% galactose, and 3% glycerol. The GC grown in 2% lactate demonstrated increasing growth over 72 hours by CFU and OD_{600} determination. *C. albicans* ATCC® 90028™ grown at sub-inhibitory CASPO (0.075 mg/L) demonstrated similar exponential growth when grown in RPMI and 2% galactose, but was fungicidal when grown in 2% lactate and 3% glycerol. 2 mg/L CASPO was fungicidal against *C. albicans* ATCC® 90028™ in all media tested. The PG effect was evident at 16 mg/L CASPO in RPMI and 2% galactose by an increase in OD_{600} over 72 hours. There was less PG evident by CFU than OD_{600} (Figure 3.9), likely due to the increased cell growth of large yeast aggregates that grow as a single CFU but increase overall in cell mass.(Figure 3.14) A statistically significant increase ($p<0.05$) in PG OD_{600} was observed in 2% lactate, but given the CFU counts are unchanged from the starting inoculum, the increase in absorbance is likely due to the increase in size of the blastospores and not from active growth.

Growth curves of *C. albicans* M10MY4874 for CFU determination demonstrated exponential growth for the GC during the initial 12 hours that plateaued at maximum growth for the remaining 60 hours when grown in RPMI, 2% galactose, and 2% lactate. The growth control demonstrated an extended lag period, only exhibiting increased CFU counts after 48 hours. The GC demonstrated increased OD_{600} over the 72 hours, with a 12 hour lag period in 2%

lactate and 3% glycerol. 0.075 and 2 mg/L CASPO was fungicidal against *C. albicans* M10MY4874 in all media tested. In lactate and glycerol, a significant increase in OD_{600} compared to the starting inoculum was observed for 2 mg/L CASPO, which is contrary to the significant decrease in CFU observed after 72 hours. This could be due to the presence of non-viable blastospores in the sample, which would contribute to OD_{600} readings but would not grow in culture. Overall these results demonstrate a killing effect of CASPO at sub-inhibitory and inhibitory concentrations. *C. albicans* M10MY4874 grown at 16 mg/L CASPO demonstrates the PG effect, in which there is decreased killing compared to the lower, inhibitory concentrations. No change in growth from the starting inoculum was observed by CFU determination at 16 mg/L CASPO at 72 hours. Growth determination by CFU counts is indicative of a fungistatic effect that is not influenced by carbon source. No significant differences in endpoint OD600 measurements were observed between 2% galactose and RPMI 1640-MOPS , or between 2% lactate and 3% glycerol.

Despite not achieving statistically significant growth or OD600 readings at 16 mg/L CASPO in all carbon sources, the PG concentrations demonstrated greater growth than 2 mg/L CASPO for *C. albicans* ATCC® 90028™ in fermentable carbon sources and greater growth than the 0.075 and 2 mg/L CASPO of *C. albicans* M10MY4874 in all media. The increase in large cell aggregates in fermentable carbon sources not seen in non-fermentable carbon sources correlates with a significant difference in degree of PG that is carbon source dependent.(Figure 3.14)

Growth curves of *C. glabrata* ATCC® 15126™ for CFU and OD_{600} determination demonstrated exponential growth for the GC and 0.075 mg/L CASPO during the initial 12 hours that plateaued at maximum growth for the remaining 60 hours when grown in RPMI.(Figure 3.12, 3.13) In 3% glycerol, similar growth was observed for the GC, but a fungicidal effect was observed. *C. glabrata* ATCC® 15126™ did not demonstrate a PG effect by time kill analysis as a fungistatic effect was seen at 2 and 16 mg/L CASPO. Though 0.075 mg/L was inhibitory to *C. albicans* M10MY4874 regardless of media, this is also suggestive of a carbon source dependent effect of CASPO.

3.4 Cell cycle analysis

3.4.1 Fluorescence of echinocandins affects cell cycle analysis

Initial investigations of the effects of caspofungin on the cell cycle used 4',6-diamidino-2-phenylindole (DAPI, Life Technologies, Carlsbad, CA), as it has been shown to be superior to other dyes, such as propidium idodide, and eliminates the need for RNAse A treatment of *C. albicans* cells in flow cytometry analysis.(9) It is commonly used as a DNA dye, having excitation and emission wavelengths of 350 and 470 nm, respectively. However, the resulting histograms demonstrated little separation of G1 and G2/M peaks typically observed with cell cycle analysis (data not shown).

Certain aromatic or conjugated structures, such as those of echinocandins or other drugs, can exhibit fluorescent properties. Previous investigations with cilofungin, the first echinocandin investigated for clinical use, demonstrated a

blue-shift in emission spectrum when incubated with yeast membranes.(10) While the chemical structures between the echinocandins are slightly different, it is likely that the blue-shift fluorescence could interfere with fluorescence readings in flow cytometry, especially within the blue spectrum (as with DAPI).

Picogreen is a fluorochrome with excitation and emission wavelengths of 502 and 523 nm, and is commonly used in quantifying double stranded DNA. While no reports exist for its use in cell cycle analysis of yeast, it has been used with human cells,(11) and the emission spectrum sufficiently different from the blue spectrum to eliminate the interference from the echinocandin blue-shift. Cell cycle histograms using Picogreen demonstrated well defined G1 and G2/M peaks. The difference in histograms between the DAPI and Picogreen fluorochromes demonstrates that the choice of fluorochrome should be taken into consideration in future fluorescent analyses with echinocandins.

3.4.2 Caspofungin blocks cell cycle at PG concentrations.

C. albicans M10MY4874 and ATCC® 90028™ isolates were analyzed for DNA content by flow cytometry over 140 minutes, sampling every 20 minutes. This time period examined the course of one cell division (approximately 2 hours).(12) The growth control demonstrated an increasing population of cells in G2 (t0—t120) as cells duplicated DNA content.(Figure 3.15) At 140 minutes, the proportion of cells in G1 began to increase as cells completed the cell division with mother and daughter cells separating. Cells treated with 16 mg/L CASPO (PG) demonstrate a increased proportion of cells in G2 compared to the growth

control. The time point (t140) demonstrated almost no PG cells in G1, indicating an inhibition of the cell cycle at G2 or M at 0.075 mg/L, no effects were observed for *C. albicans* ATCC® 90028™, but detrimental cell cycle effects were observed in *C. albicans* M10MY4874. The cell cycle of *C. glabrata* ATCC® 15126™ was not synchronized by hydroxyurea, but no changes to the cell cycle at any concentration were observed.

Table 3.1. Effects of inoculum density on PG. CASPO concentrations (mg/L) for MIC and PG of 24 *C. albicans* and 12 *C. glabrata* isolates evaluated for visible growth and absorbance at 405 nm after 24 hours. Concentrations at which PG resulted in statistically significant increase in absorbance compared to the media control ($p<0.05$), and concentrations in which PG was only observed by manual plate readings are denoted in brackets.

	10^3 starting inoculum		10^6 starting inoculum	
Isolate	MIC	PG	MIC	PG
C. albicans				
M10MY4874	≤0.125	(16)	≤0.125	16
ATCC® 90028™	≤0.125	(16)	≤0.125	16 (8-32)
ATCC® 24433™	≤0.125	-	≤0.125	16-32
M10MY2168	≤0.125	-	≤0.125	8-16 (8-32)
M10MY8596	≤0.125	(16)	≤0.125	16
M10MY7506	0.25	(16)	0.25	8-16
M10MY7837	≤0.125	-	≤0.125	16-32
M10MY4875	≤0.125	-	≤0.125	16 (16-32)
M10MY5059	≤0.125	-	≤0.125	8 (8-16)
M10MY2166	≤0.125	-	≤0.125	8 (8-32)
M10MY1585	≤0.125	(16)	≤0.125	16 (8-32)
M10MY2938	≤0.125	(16)	≤0.125	16-32
M10MY3029	0.25	-	≤0.125	16-32
M10MY3028	0.25	-	≤0.125	8-16 (8-32)
M10MY2978	≤0.125	-	≤0.125	(16)
M10MY4529	≤0.125	-	≤0.125	16 (16-32)
M10MY5068	≤0.125	(16)	≤0.125	16-32
M10MY2333	≤0.125	(16)	≤0.125	16-32
M10MY2222	≤0.125	-	≤0.125	8-16 (8-32)
M10MY2678	0.25	(16)	≤0.125	16 (16-32)
M10MY2273	≤0.125	-	≤0.125	16 (16-32)
M10MY2883	≤0.125	-	≤0.125	16 (8-32)
M10MY2797	0.25	-	≤0.125	8-16 (8-32)
M10MY2163	0.≤0.125	-	≤0.125	16 (8-32)
C. glabrata				
M06MY000206	0.5	-	0.25	-
M06MY000779	0.5	-	0.25	-
M06MY001064	0.5	-	0.5	-
M06MY002192	0.5	-	0.5	-
M06MY002346	0.5	-	0.5	-
M06MY002882	0.5	-	0.5	-
M06MY003156	0.5	-	0.5	-
M06MY003590	1.0	-	1.0	-
M06MY003953	0.5	-	0.5	-
M06MY004294	0.5	-	0.5	-
M10MY001407	0.5	-	0.5	-
ATCC® 15126™	0.5	-	0.5	-

Table 3.2. Significance testing of PG in *C. albicans* with an elevated inoculum. A one tailed paired t test was conducted for the absorbance of each inoculated well compared to the absorbance of the media (negative control). Results displayed demonstrate statistically significant growth ($p<0.05$) above the MIC at 16 mg/L CASPO for isolates using 10^6 CFU. The mean (standard deviation), p value, and 95% confidence intervals for the difference between the absorbance at 16 mg/L and the media control are listed. Isolates of which comparisons are not statistically significant ($p>0.05$) are denoted by *.

A). *C. albicans*

Isolate	Mean difference (SD)	p value	95% CI
ATCC® 90028™	0.154 (0.03189)	0.0002	0.1284 to 0.1796
ATCC® 24433™	0.1143 (0.01967)	0.007	0.08295 to 0.1455
M10MY4874	0.08150 (0.03189)	0.0072	0.03076 to 0.1322
M10MY8596	0.1218 (0.05082)	0.0086	0.04088 to 0.2026
M10MY7506	0.1360 (0.05449)	0.0077	0.04930 to 0.2227
M10MY7837	0.1695 (0.02329)	0.0004	0.1324 to 0.2066
M10MY4875	0.0960 (0.04902)	0.0148	0.01800 to 0.1740
M10MY5059	0.04725 (0.02229)	0.0120	0.01178 to 0.08272
M10MY2166	0.03575 (0.01837)	0.0150	0.006514 to 0.06499
M10MY1585	0.0625 (0.003109)	<0.0001	0.05755 to 0.06745
M10MY2938	0.1013 (0.04117)	0.008	0.03574 to 0.1668
M10MY3029	0.08 (0.03442)	0.0094	0.02523 to 0.1348
M10MY3028	0.05425 (0.01941)	0.0057	0.02336 to 0.08514
M10MY2978*	0.01875 (0.01875)	0.0712	-0.01142 to 0.04892
M10MY4529	0.03175 (0.01916)	0.0226	0.001270 to 0.06223
M10MY5068	0.07375 (0.04124)	0.0187	0.008124 to 0.1394
M10MY2333	0.0975 (0.03558)	0.006	0.04089 to 0.1541
M10MY2222	0.06725 (0.03078)	0.011	0.01827 to 0.1162
M10MY2678	0.1128 (0.07694)	0.0305	0.01199 to 0.1265
M10MY2273	0.0325 (0.01848)	0.0195	0.003087 to 0.06191
M10MY2883	0.0440 (0.01903)	0.0095	0.01372 to 0.07428
M10MY2797	0.0570 (0.03556)	0.0246	0.0004127 to 0.1136
M10MY2168	0.1128 (0.03459)	0.0037	0.05771 to 0.1678
M10MY2163	0.04275 (0.01846)	0.0095	0.01337 to 0.07213

Table 3.2, continued
B). *C. glabrata*

Isolate	Mean difference (SD)	p value	95% CI
M06MY000206*	0.00333 (0.007024)	0.2487	-0.01411 to 0.02078
M06MY000779*	0.0003333 (0.004726)	0.4570	-0.01141 to 0.01207
M06MY001064*	-0.006667 (0.006351)	0.1053	-0.02244 to 0.009110
M06MY002192*	-0.00200 (0.003606)	0.2190	-0.01096 to 0.006957
M06MY002346*	0.004333 (0.003215)	0.0723	-0.003652 to 0.01232
M06MY002882*	0.003313 (0.003215)	0.1072	-0.004652 to 0.01132
M06MY003156*	-0.0006667 (0.003786)	0.3946	-0.01007 to 0.008738
M06MY003590*	0.008667 (0.01779)	0.2438	-0.03552 to 0.05285
M06MY003953*	-0.000000 (0.005292)	0.5	-0.01314 to 0.01314
M06MY004294*	-0.001333 (0.004726)	0.3367	-0.01307 to 0.01041
M10MY001407*	0.0006667 (0.003786)	0.3946	-0.008738 to 0.01007
ATCC® 15126™*	0.01533 (0.01365)	0.0956	-0.01858 to 0.04924

Table 3.3. Fks1p mutations conferring decreased echinocandin susceptibility. Overview of literature describing GS mutants with reduced echinocandin susceptibility. Mutations indicate acquired mutations unless otherwise noted. Mutations written as: intrinsic amino acid – GS sequence number – acquired amino acid mutation.

Organism	Mutation	Reference
C. albicans	Fks1p: S645F,Y,P,C F641S,L L644F D648Y P649H R1361H	(13,14) (15) (16)
C. parapsilosis	Fks1p: Intrinsically occurring P660A	(17)
C. glabrata	Fks1p: F625S S629P R631G D632G,E,Y,H S633P Fks2p: F659del,V,S,Y S663P,F R665G,S D666G,E P667T,H W1375L R1377STOP	(18-21)
S. cerevisiae	Fks1p W695F,C	(22)
F. solani	Intrinsic Y639	(23)

A).

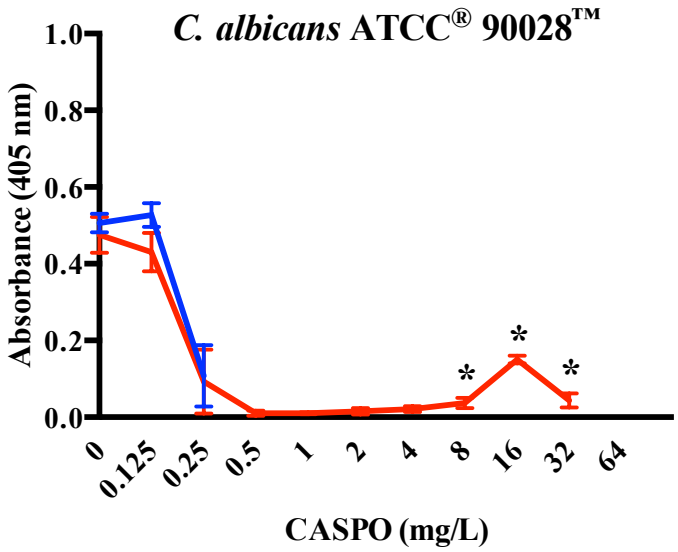
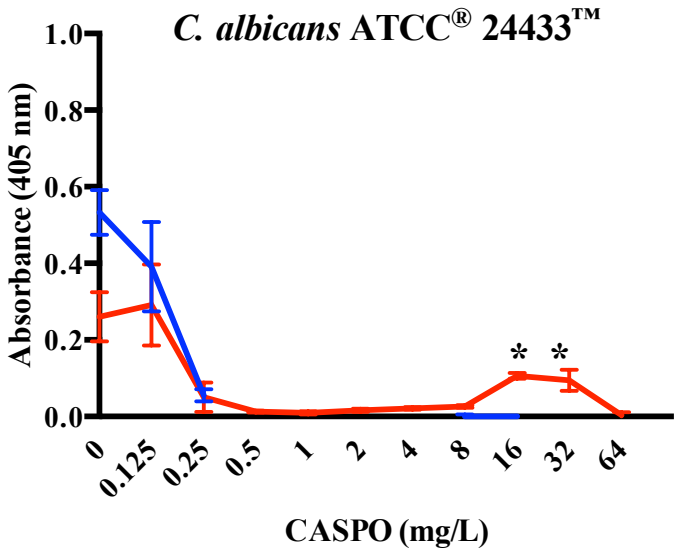


Figure 3.1. Absorbance profiles of CASPO microdilution growth patterns. Absorbance readings of **A).** *C. albicans* ATCC® 90028™, **B).** *C. albicans* ATCC® 24433™, and **C).** *C. glabrata* ATCC® 15126™ exposed to CASPO at 10^3 (blue) and 10^6 (red) starting inocula assessed by broth microdilution at 24 hours. Plotted absorbance readings (405nm) represent the average of three independent replicates after removal of baseline (media control). Error bars represent standard error of measure. *Statistically significant growth above the MIC ($p<0.05$)

B).



C).

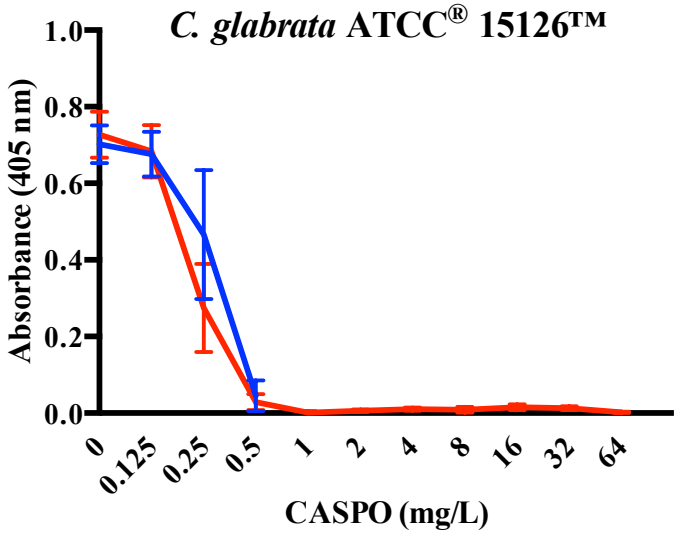


Figure 3.1, continued

(A). *C. albicans* ATCC® 90028™

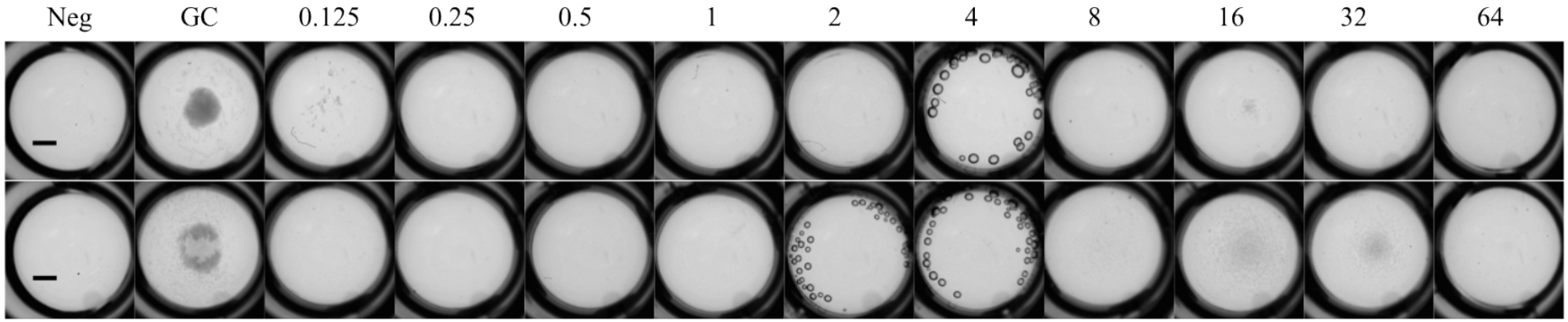


Figure 3.2. Stereomicroscopy of CASPO microdilution growth patterns. Individual wells of 96-well microdilution plates containing *C. albicans* or *C. glabrata* exposed to CASPO for 24 hours were visualized and photographed under a stereomicroscope at 1.2X magnification to compare PG of standard (10^3, upper image) and increased (10^6, lower image) inocula. Reference strains **(A)** *C. albicans* ATCC® 90028™ and **(B)** *C. glabrata* ATCC® 15126™ are shown as representative organisms. Numbers above images indicate CASPO concentrations (mg/L), the growth control (GC), and negative control (Neg). Scale bars represent 1 mm. **(C).** Uncompressed images of *C. albicans* ATCC® 90028™ demonstrate a dense pellet of the growth controls compared to the highly dispersed growth of PG cells. Scale bars represent 0.5 mm.

(B). *C. glabrata* ATCC® 15126™

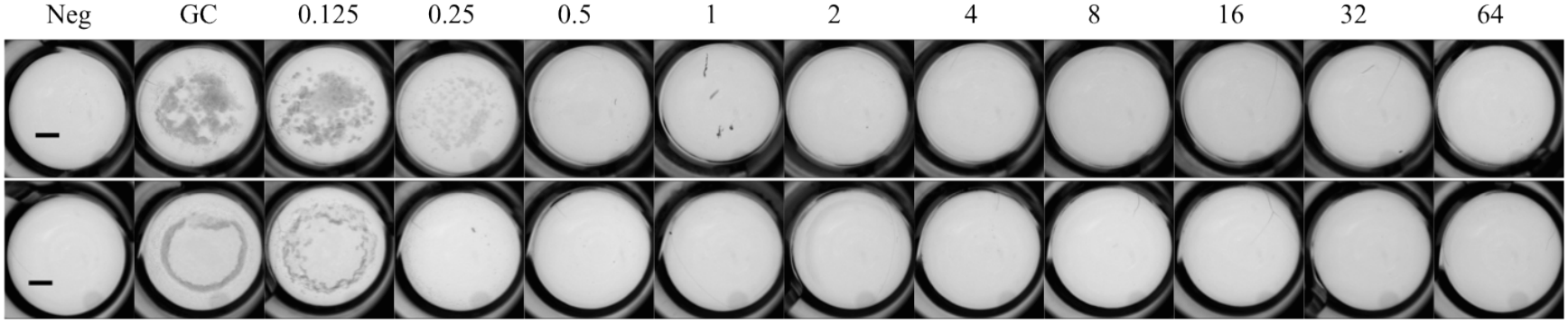


Figure 3.2, continued

(C). *C. albicans* ATCC® 90028™

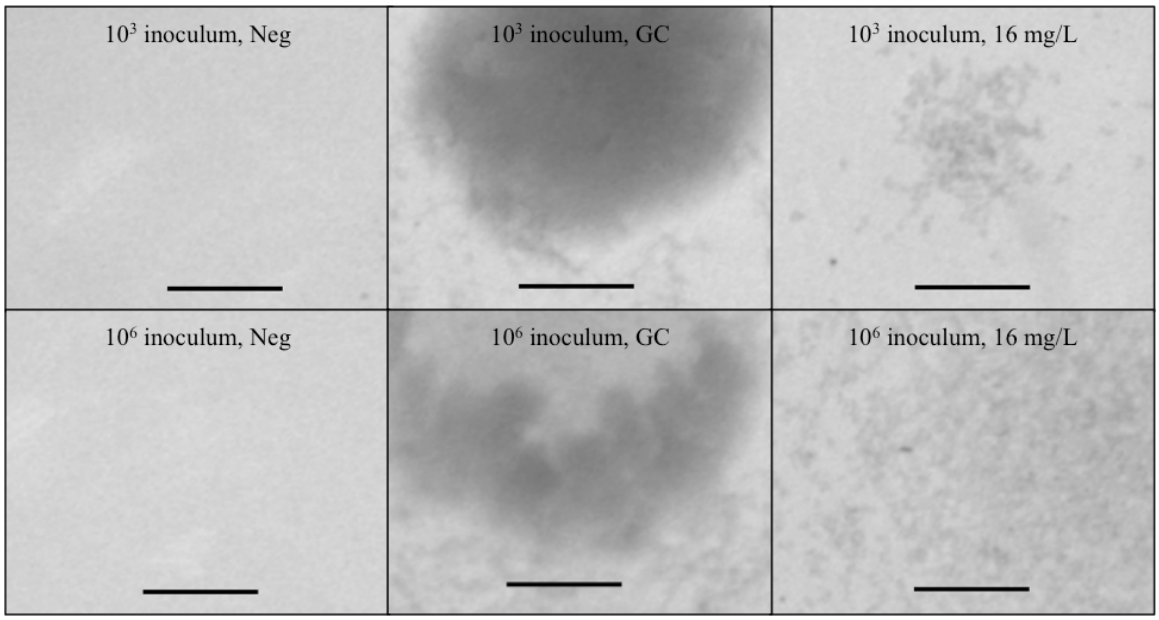


Figure 3.2, continued

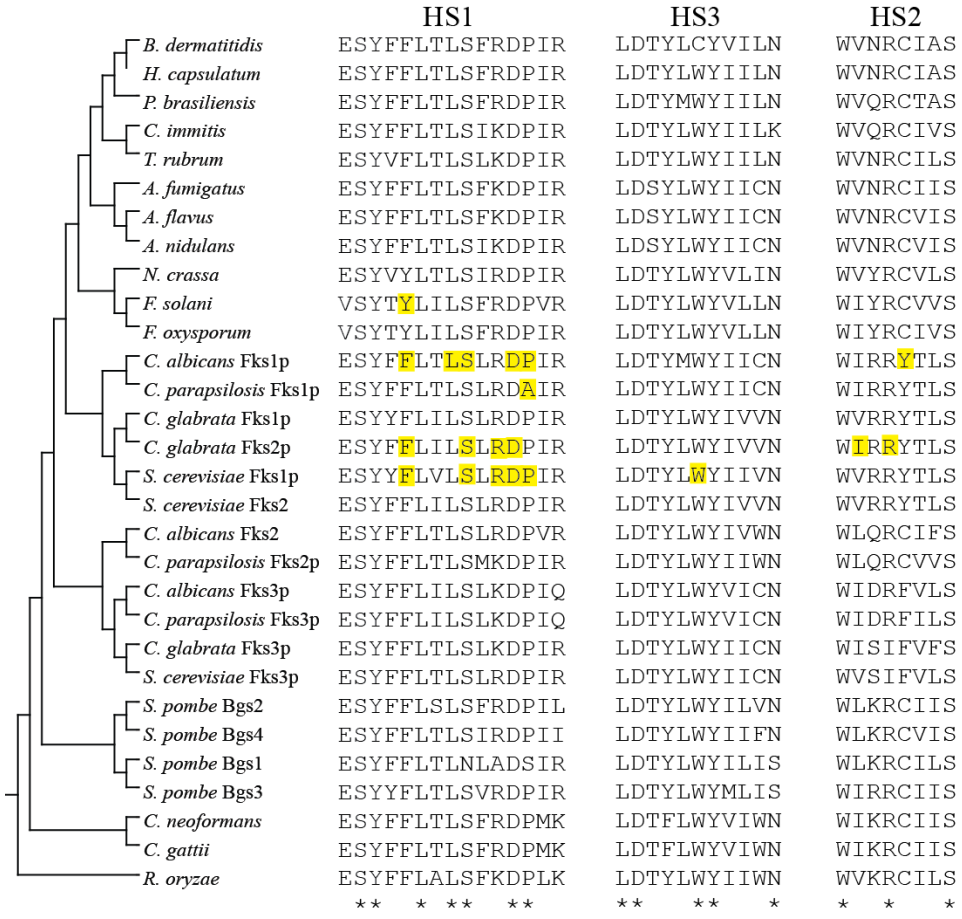


Figure 3.3. "Hot-spot" sequence comparisons. Aligned sequences corresponding to *S. cerevisiae* Fks1p at amino acids 635-649 (HS1), 690-700 (HS3), and 1354-1361 (HS2) were examined to evaluate correlations between hot-spot sequence and response to CASPO. HS1-3 are the putative binding sites of echinocandins with GS and mutations in susceptible organisms correlate with decreased susceptibility; amino acids associated with decreased susceptibility when mutated are highlighted in yellow. Conserved sites in the primary GS (Fks1p or Bgs4p) are denoted by *****.

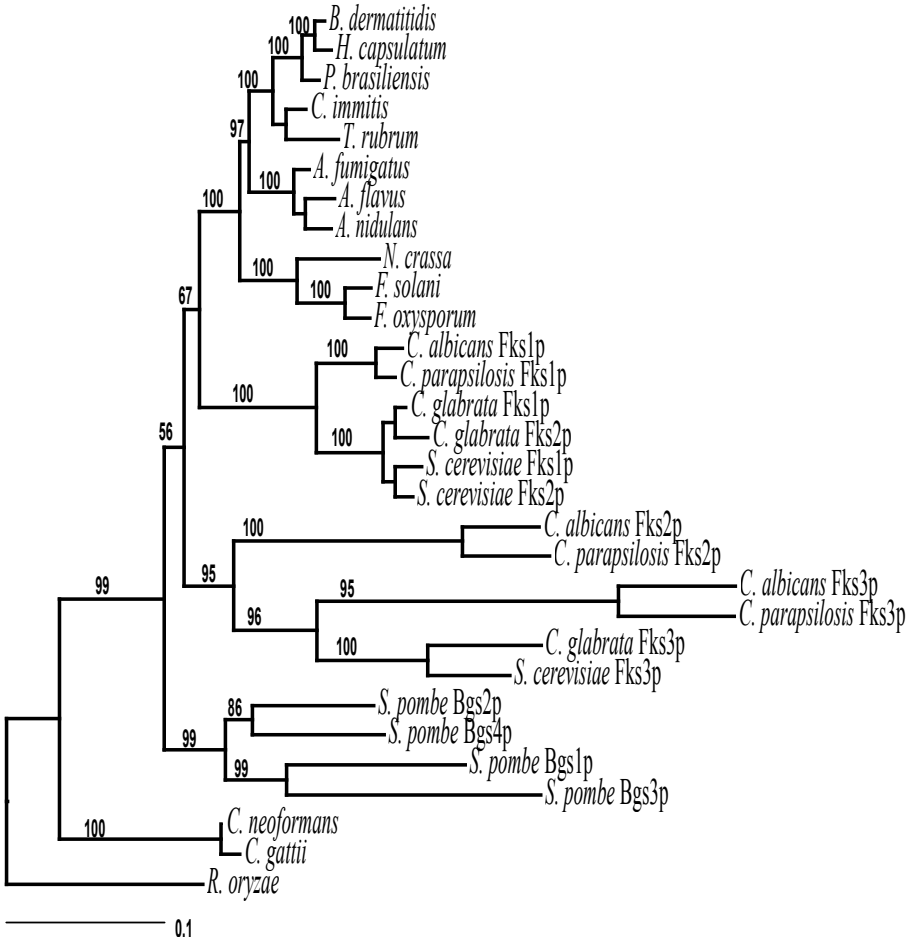


Figure 3.4 Phylogenetic analysis of FKS protein dataset. ML tree from FKS protein data analysis. Numbers above branches represent indices of support (ML bootstrap). Branch lengths are proportional to the number of substitutions per site.

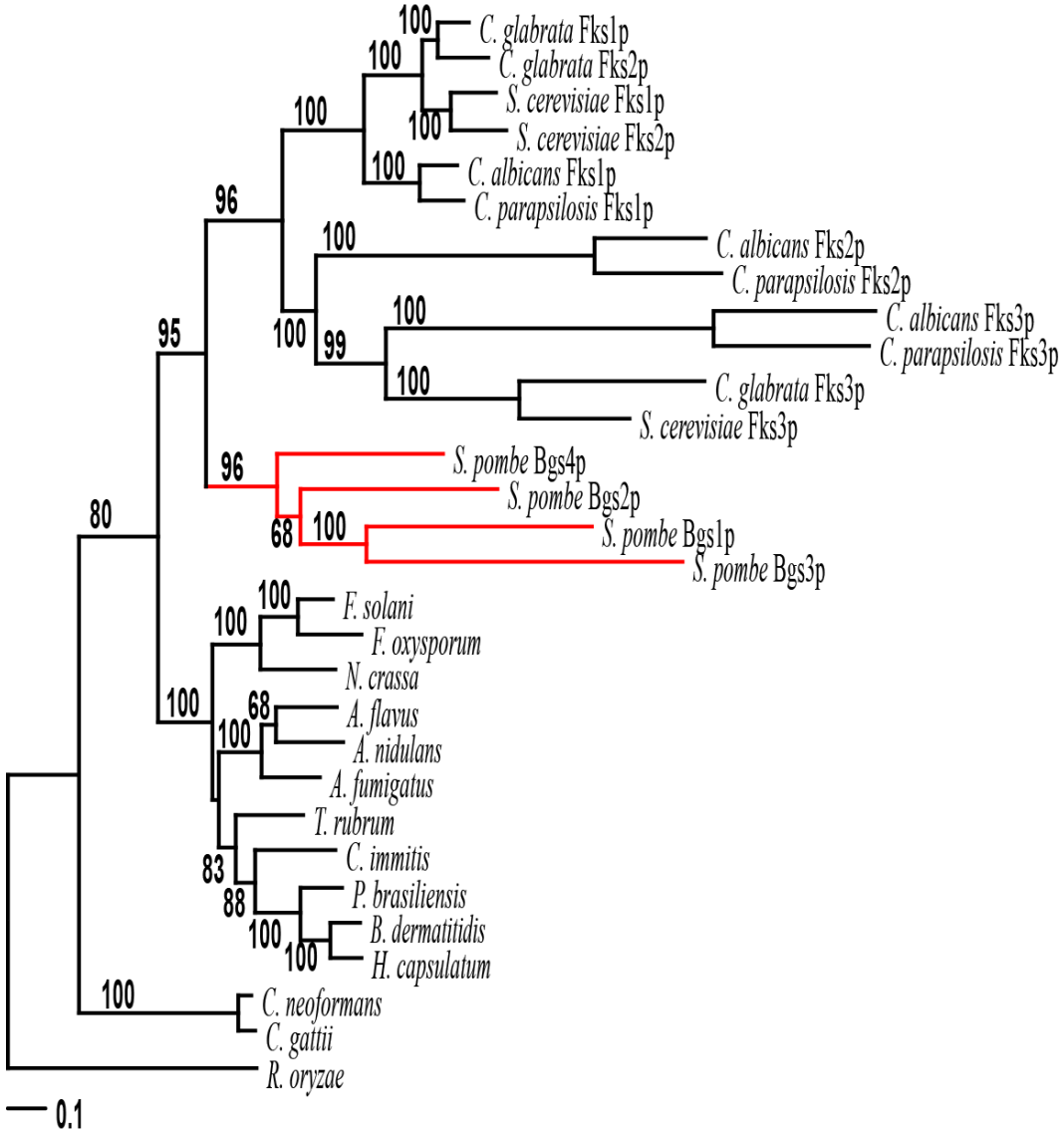


Figure 3.5. Phylogenetic analysis of FKS nucleotide dataset. ML tree from FKS nucleotide data analysis. Numbers above branches represent indices of support (ML bootstrap). Branch lengths are proportional to nucleotide substitutions per site. The red branch (*S. pombe*) is divergent from the known species tree.

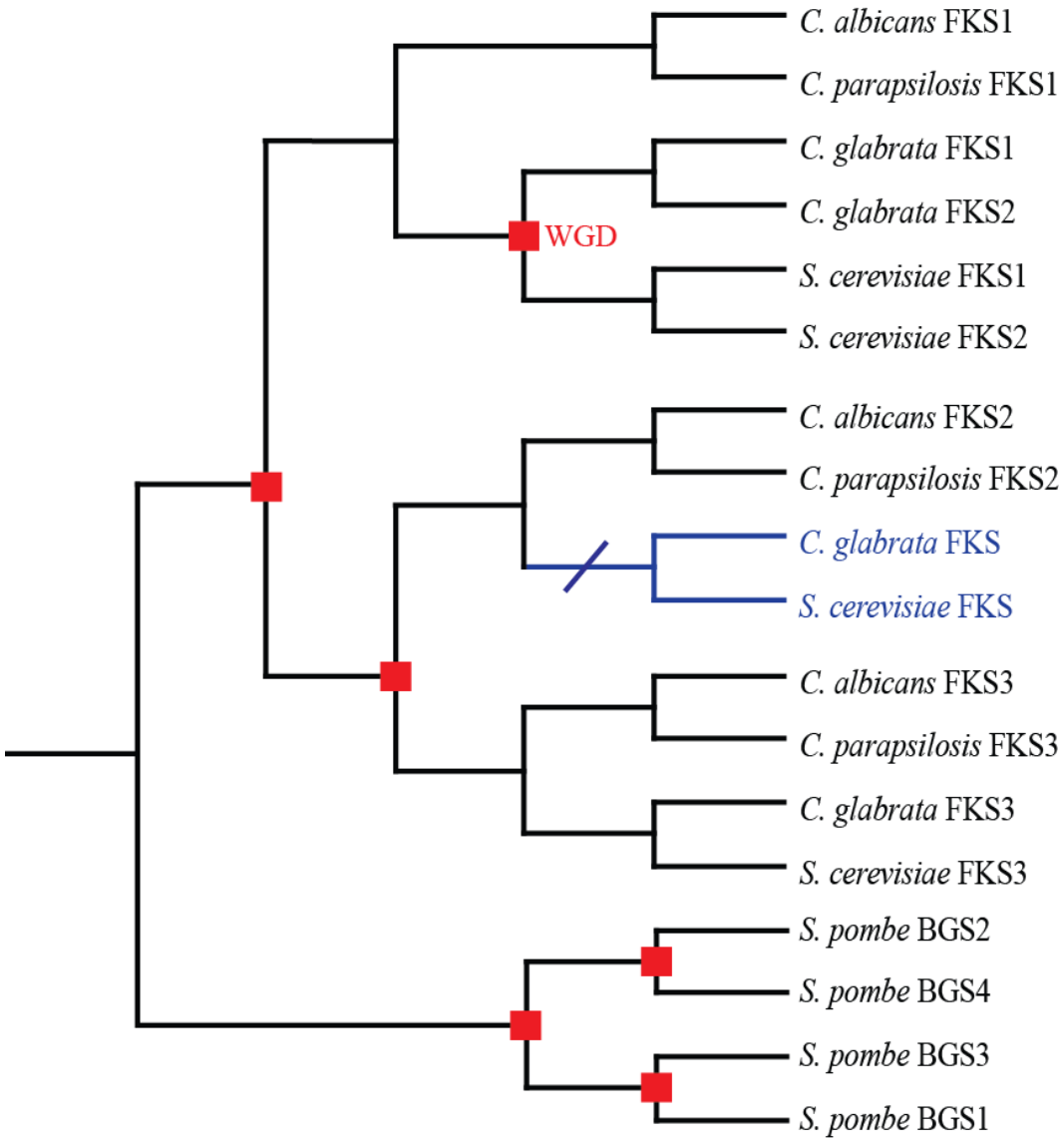


Figure 3.6. FKS gene duplications in yeast. Reconciled FKS gene tree from known species tree generated by Notung v2.6. Red squares indicate gene duplication events; other than the WGD event for *C. glabrata* and *S. cerevisiae*, all duplications are SSD. Blue strike through on blue branches indicates gene loss.

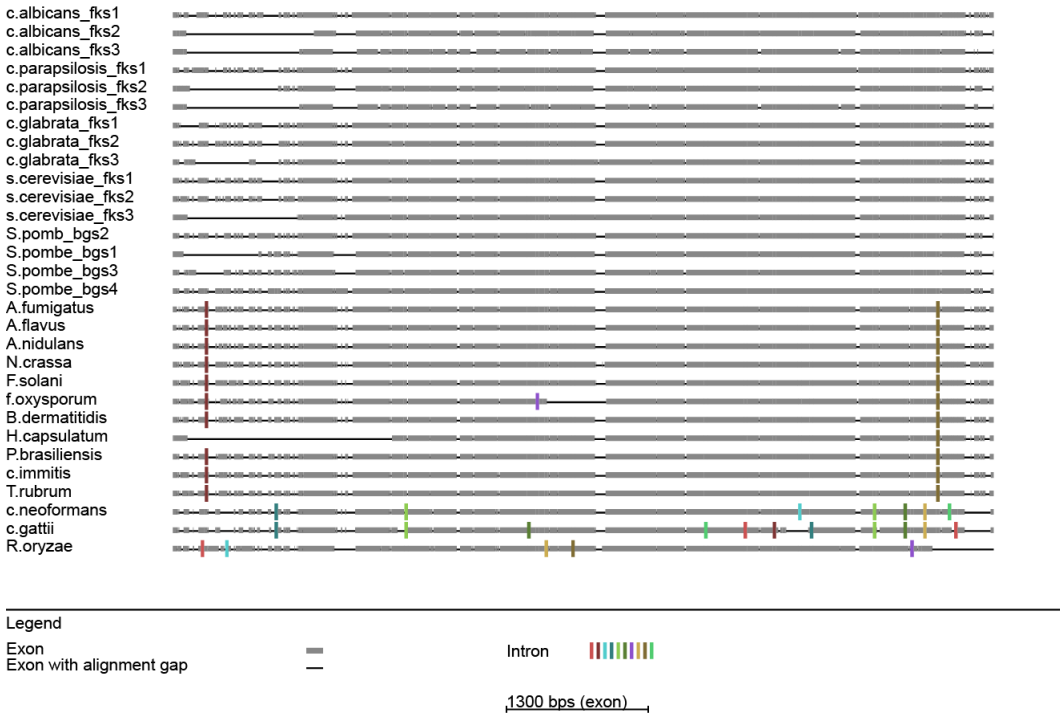


Figure 3.7. FKS gene structures. The protein alignment was used with GenePainter software to generate gene structures with aligned introns. Grey bars represent exons and thin black lines represent alignment gaps, both drawn to scale. Introns are colored vertical bars; shared intron positions are represented by the same color.

A). *C. albicans* ATCC® 90028™ - RPMI

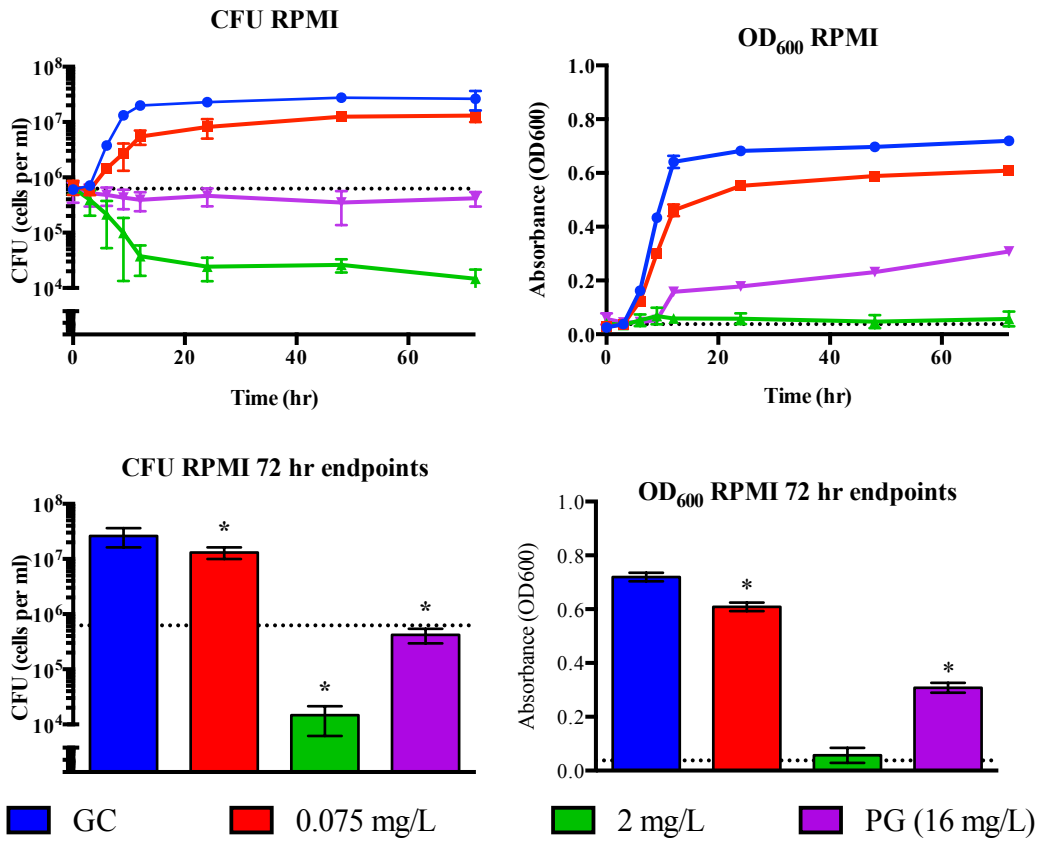


Figure 3.8. Time kill analysis of *C. albicans* ATCC® 90028™ exposed to CASPO grown in different carbon sources. Growth of *C. albicans* ATCC® 90028™ exposed to 0 (blue), 0.075 (red), 2 (green), and 16 mg/L (purple) CASPO in **A).** RPMI, **B).** 2% galactose, **C).** 2% lactate, and **D).** 3% glycerol. Growth was measured over 72 hours by CFU and OD_{600}. Overall growth at 72 hours (endpoint) was evaluated for CFU and OD_{600} by comparing endpoint and starting inoculum by student's t-test. Figures represent three experimental repeats (mean ±SD). Significant inhibition below or growth above the starting inoculum ($p < 0.05$) is indicated by *****. GC for all media tested demonstrated significant growth. Dotted line represents the starting inoculum (average of t0).

B) *C. albicans* ATCC® 90028™ - 2% Galactose

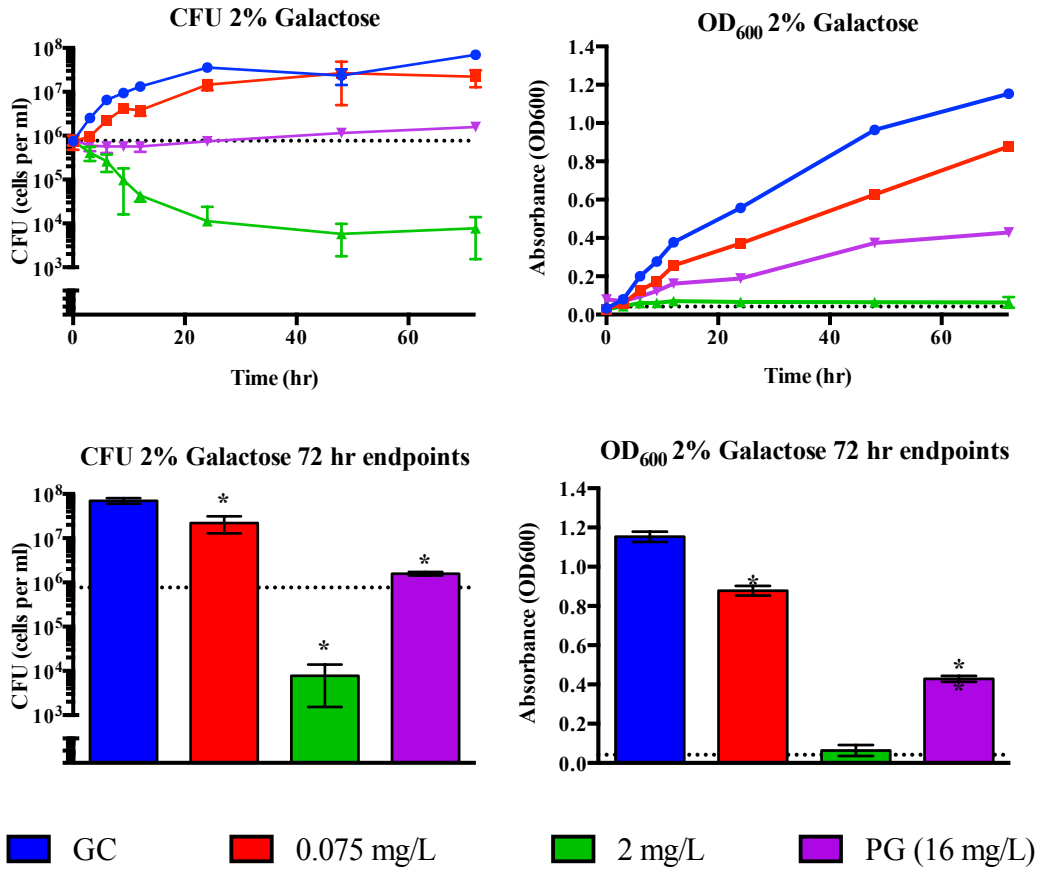


Figure 3.8, continued

C). *C. albicans* ATCC® 90028™ - 2% Lactates

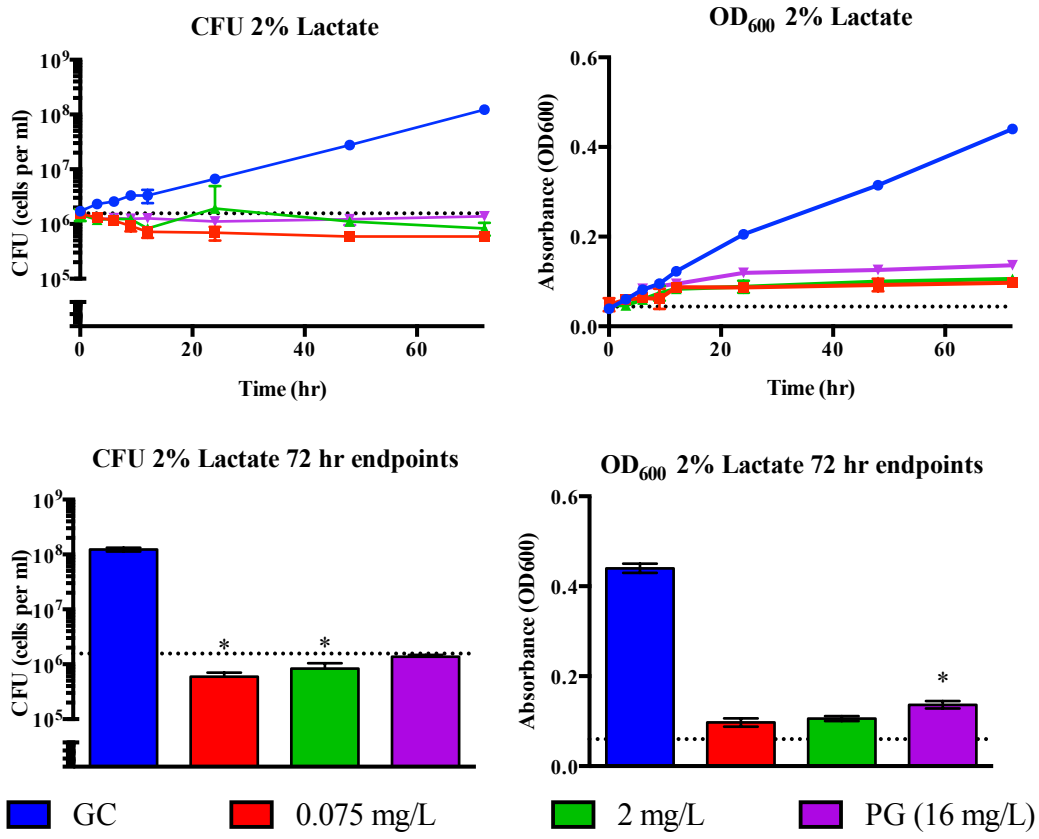


Figure 3.8, continued

D). *C. albicans* ATCC® 90028™ - 3% Glycerol

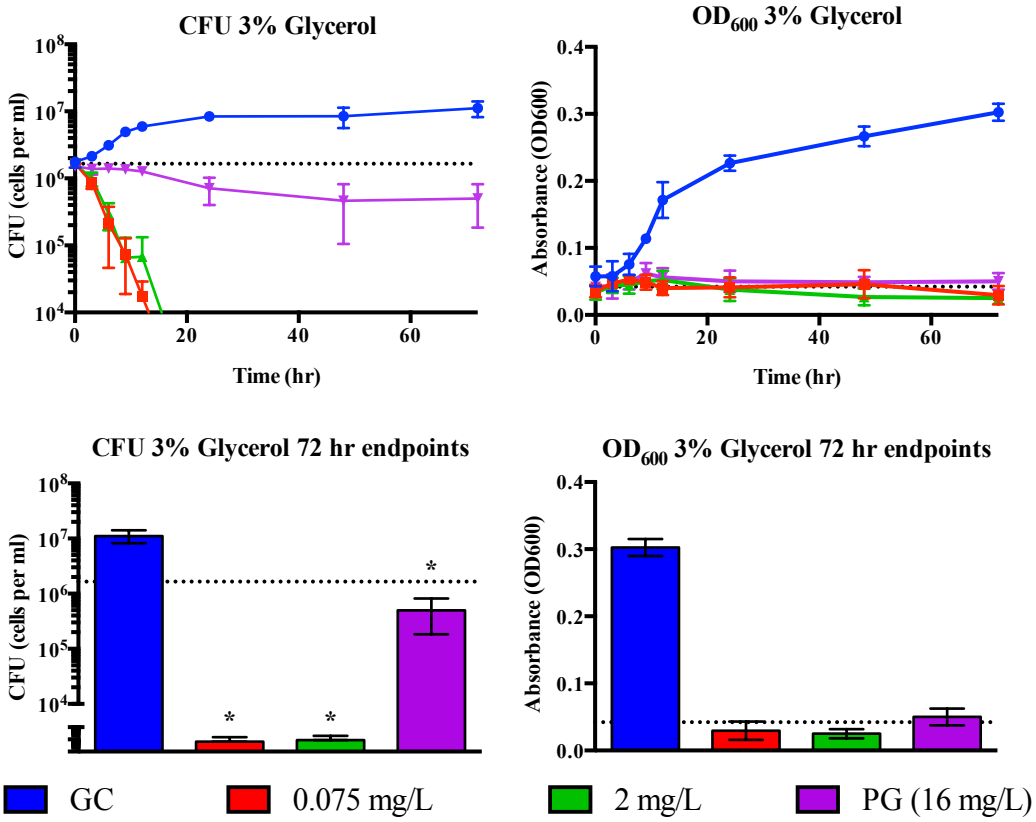


Figure 3.8, continued

A). *C. albicans* ATCC® 90028™ - CFU endpoint comparisons

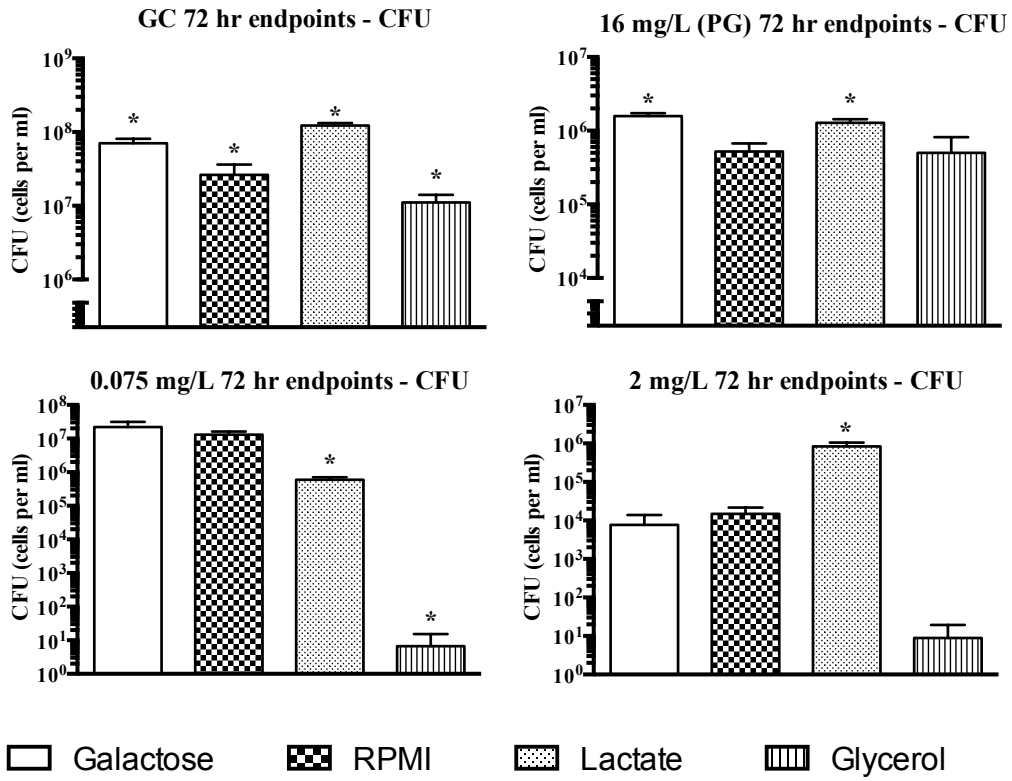


Figure 3.9. Comparative analysis of time kill endpoints: *C. albicans* ATCC® 90028™. Final growth (endpoints) were determined by **A).** CFU and **B).** OD_{600} for *C. albicans* ATCC® 90028™ grown in 2% galactose, RPMI, 2% lactate, or 3% glycerol exposed to 0, 0.075, 2, and 16 mg/L CASPO after 72 hours. Endpoints for a given growth condition were compared by a repeated measures one-way ANOVA with the Greisser-Greenhouse correction. For those comparisons indicating a statistically significant difference between one or more growth conditions by ANOVA, a Tukey's corrected comparison was performed to compare every mean to every other mean. Figures represent three experimental repeats (mean ±SD). Significant differences between endpoints ($p < 0.05$) are indicated by *.

B). *C. albicans* ATCC® 90028™ - OD_{600} endpoint comparisons

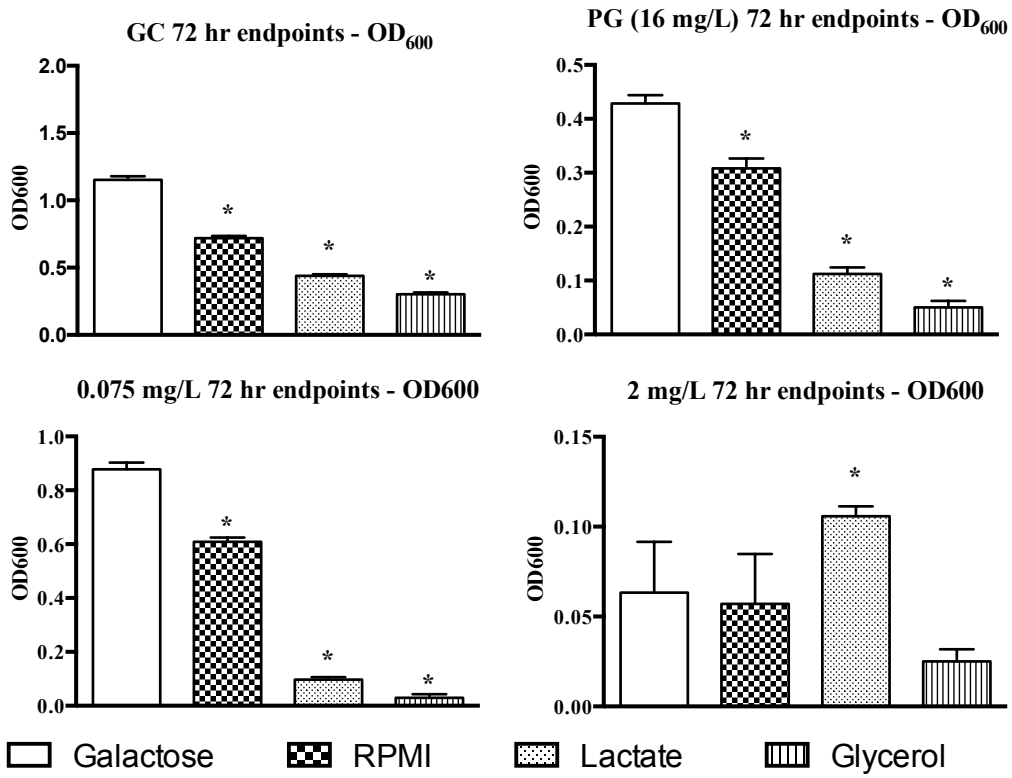


Figure 3.9, continued

A). *C. albicans* M10MY4874 - RPMI

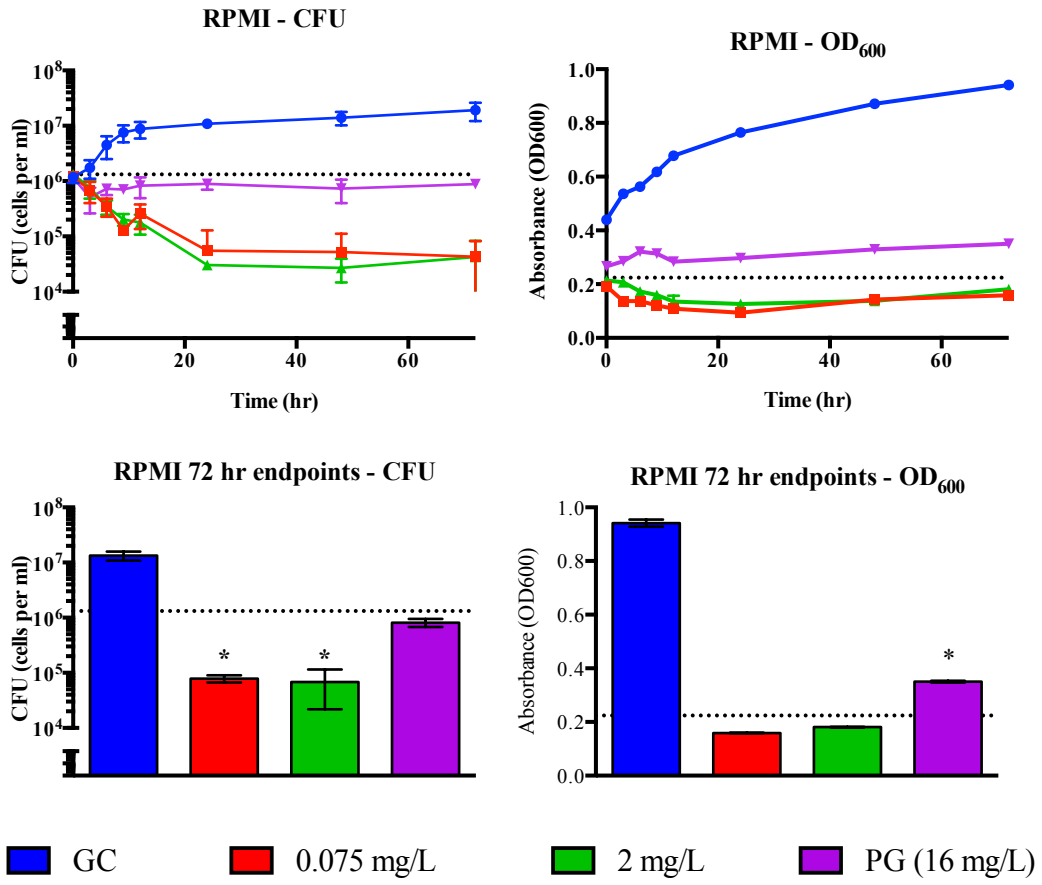


Figure 3.10. Time kill analysis of *C. albicans* M10MY4874 exposed to CASPO grown in different carbon sources. Growth of *C. albicans* M10MY4874 exposed to 0 (blue), 0.075 (red), 2 (green), and 16 mg/L (purple) CASPO in **A).** RPMI, **B).** 2% galactose, **C).** 2% lactate, and **D).** 3% glycerol. Growth was measured over 72 hours by CFU and OD_{600}. Overall growth at 72 hours (endpoint) was evaluated for CFU and OD_{600} by comparing endpoint and starting inoculum by student's t-test. Figures represent 3 experimental repeats (mean ±SD). Significant inhibition below or growth above the starting inoculum ($p < 0.05$) is indicated by *****. GC for all media tested demonstrated significant growth. Dotted line represents the starting inoculum (average of t0).

B). *C. albicans* M10MY4874 - 2% Galactose

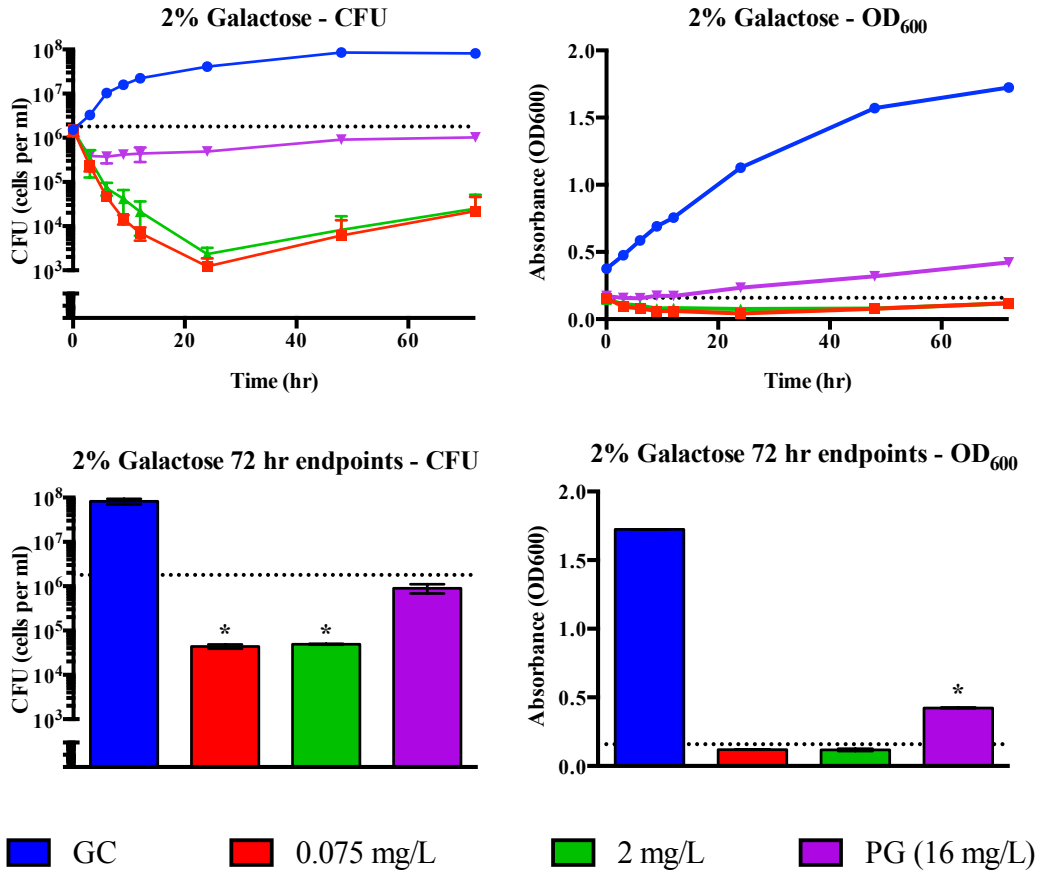


Figure 3.10, continued

C). *C. albicans* M10MY4874 - 2% Lactate

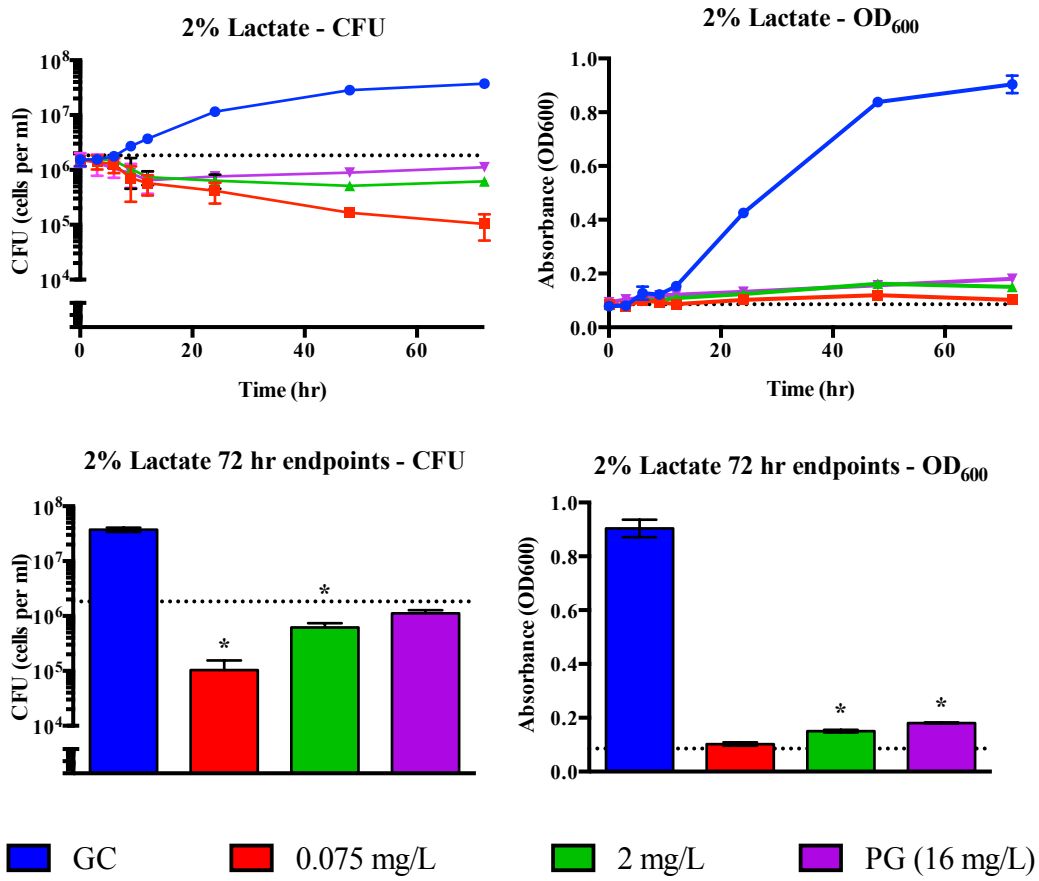


Figure 3.10, continued

D). *C. albicans* M10MY4874 - 3% Glycerol

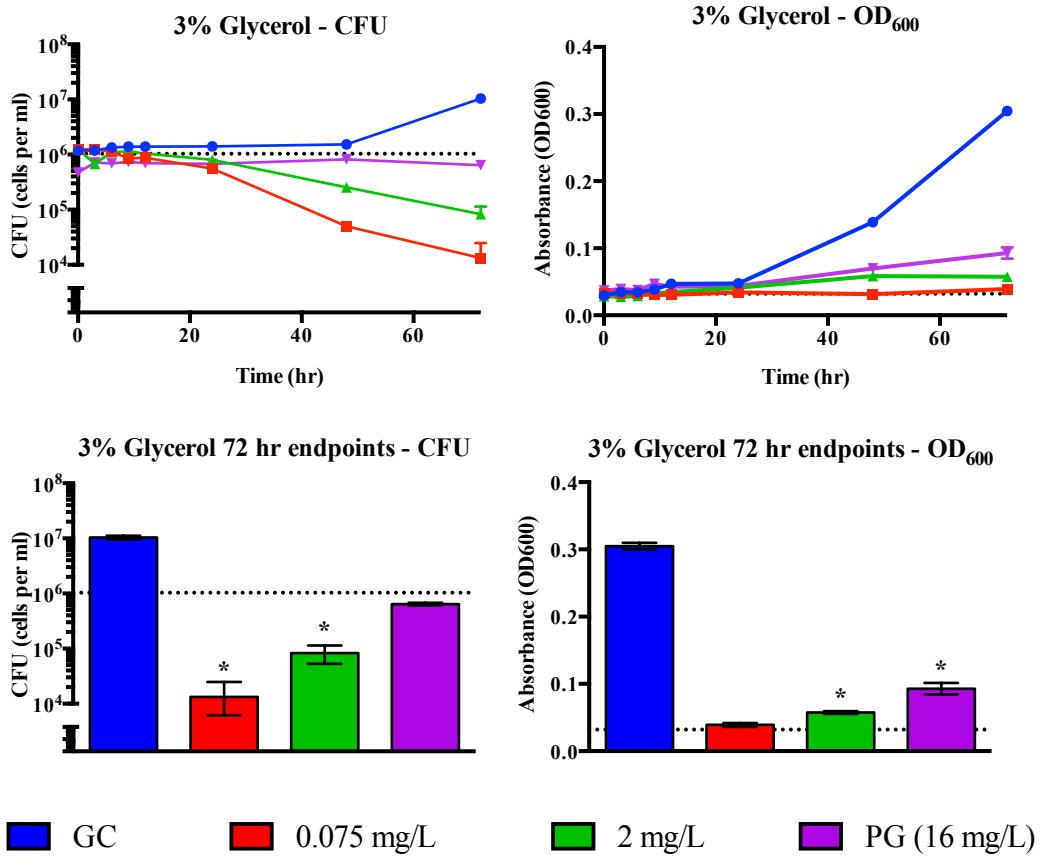


Figure 3.10, continued

A). *C. albicans* M10MY4874 - CFU endpoint comparisons

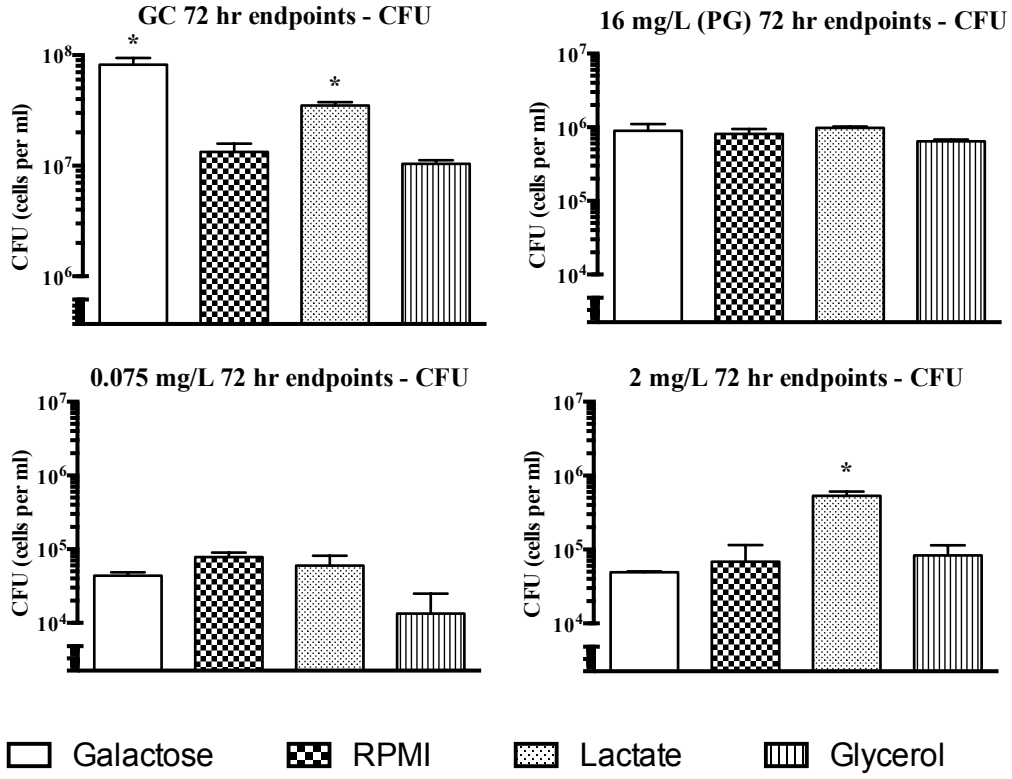


Figure 3.11. Comparative analysis of time kill endpoints:*C. albicans* M10MY4874. Final growth (endpoints) were determined by **A).** CFU and **B).** OD_{600} for *C. albicans* grown in 2% galactose, RPMI, 2% lactate, or 3% glycerol exposed to 0, 0.075, 2, and 16 mg/L CASPO after 72 hours. Endpoints for a given growth condition were compared by a repeated measures one-way ANOVA with the Greisser-Greenhouse correction. For those comparisons indicating a statistically significant difference between one or more growth conditions by ANOVA, a Tukey's corrected comparison was performed to compare every mean to every other mean. Figures represent 3 experimental repeats (mean ±SD). Significant differences between endpoints ($p < 0.05$) are indicated by *.

B). *C. albicans* M10MY4874 - OD_{600} endpoint comparisons

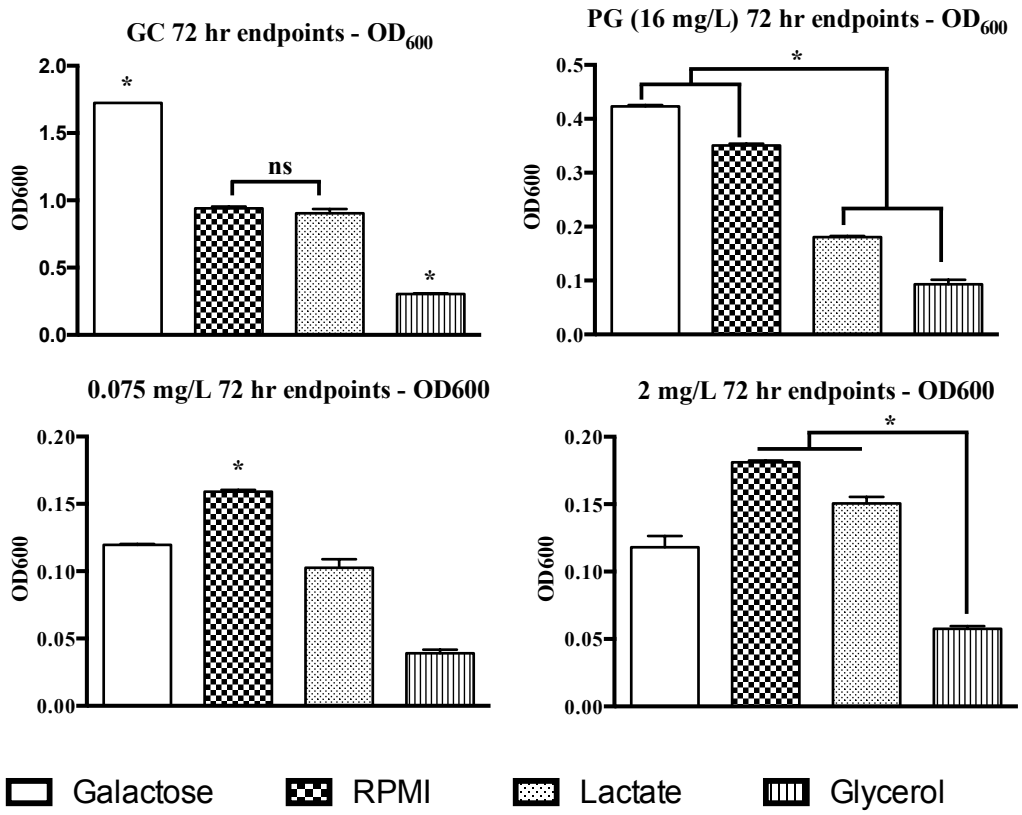


Figure 3.11, continued

A). *C. glabrata* ATCC® 15126™ – RPMI

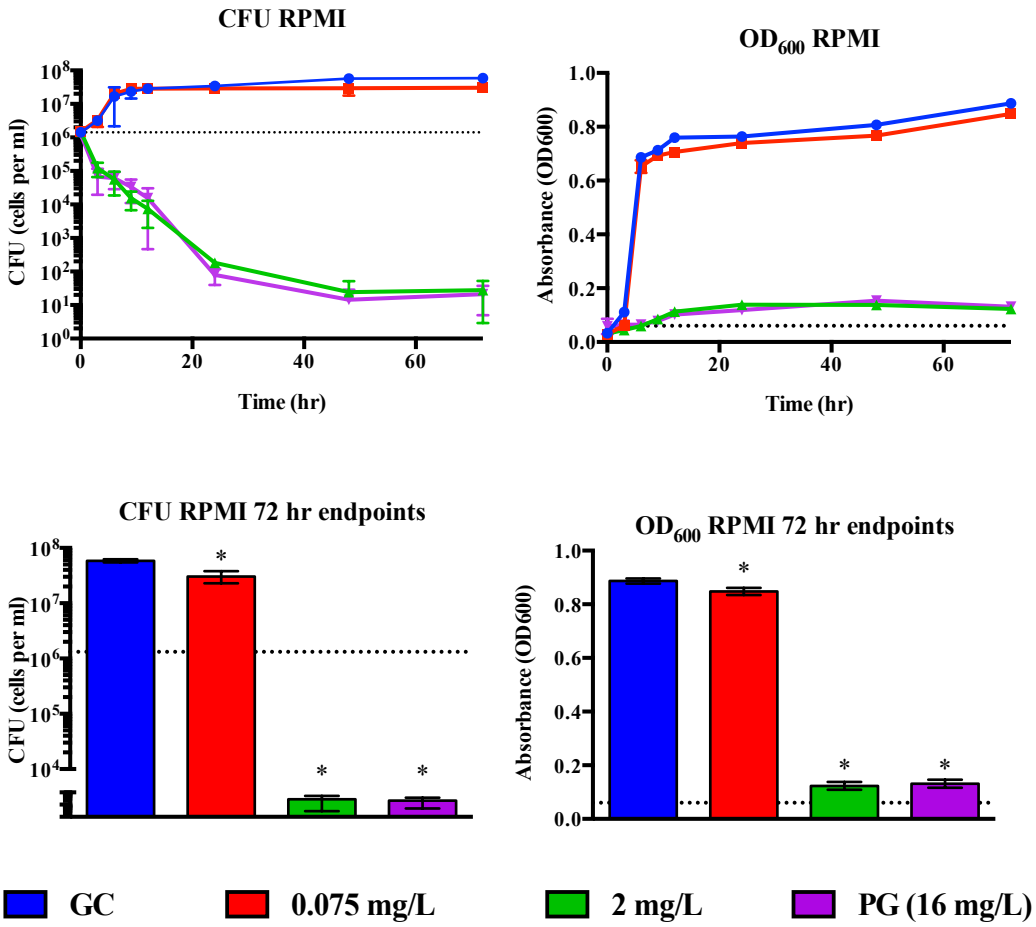


Figure 3.12. Time kill analysis of *C. glabrata* ATCC® 15126™ exposed to CASPO grown in different carbon sources. Growth of *C. glabrata* ATCC® 15126™ exposed to 0 (blue), 0.075 (red), 2 (green), and 16 mg/L (purple) CASPO in **A).** RPMI and **B).** 3% glycerol. Growth was measured over 72 hours by CFU and OD_{600}. Overall growth at 72 hours (endpoint) was evaluated for CFU and OD_{600} by comparing endpoint and starting inoculum by student's t-test. Figures represent 3 experimental repeats (mean ±SD). Significant inhibition below or growth above the starting inoculum ($p < 0.05$) is indicated by *****. Dotted line represents the starting inoculum (average of t0).

B). *C. glabrata* ATCC 15126 – 3% Glycerol

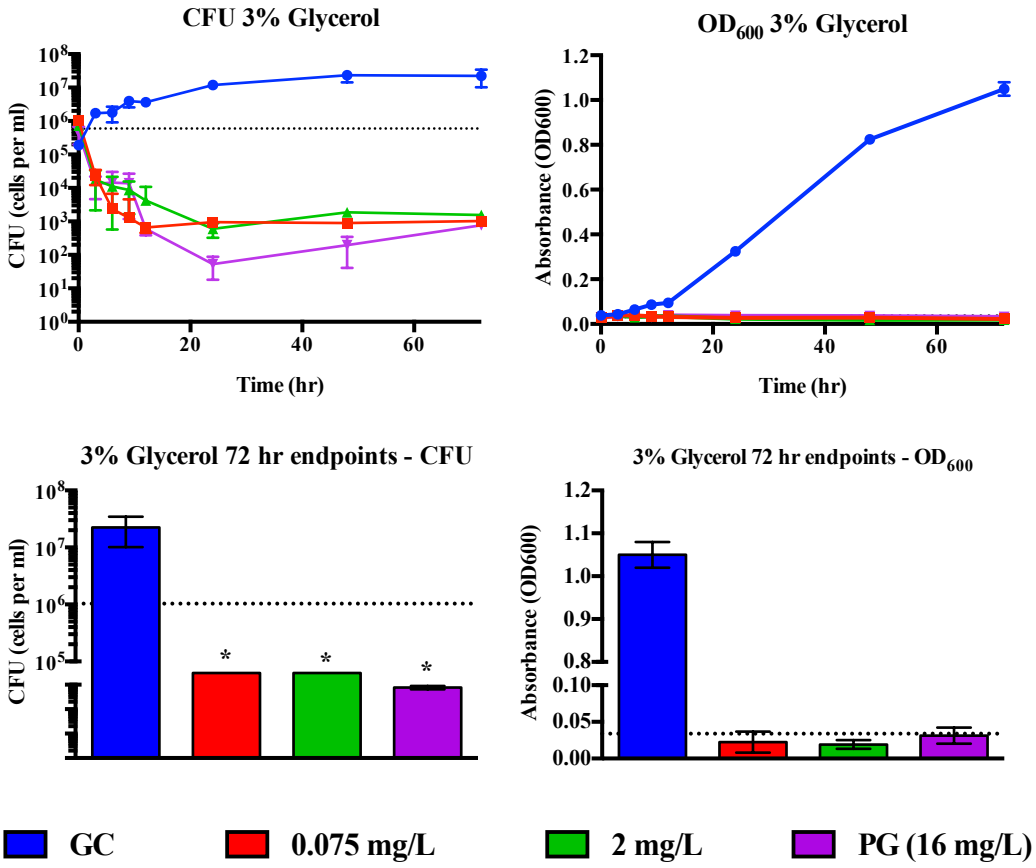


Figure 3.12, continued

A) *C. glabrata* ATCC® 15126™ - CFU endpoint comparisons

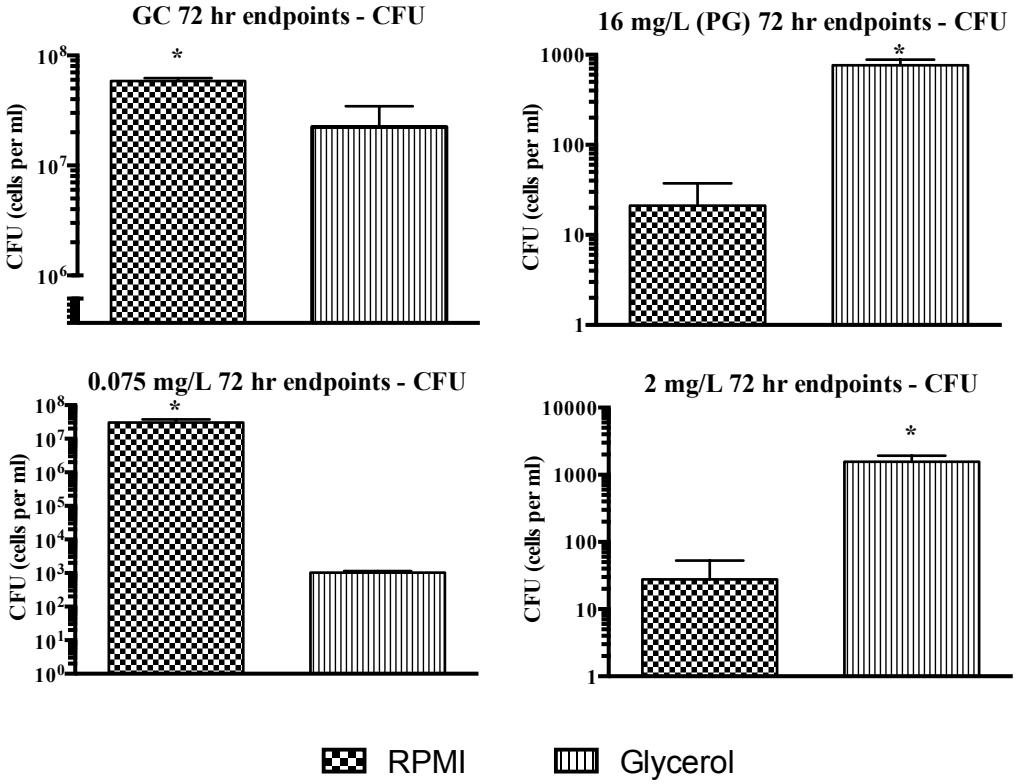


Figure 3.13. Comparative analysis of time kill endpoints: *C. glabrata* ATCC® 15126™ . Final growth (endpoints) were determined by **A).** CFU and **B).** OD_{600} for *C. glabrata* ATCC® 15126™ grown in RPMI or 3% glycerol exposed to 0, 0.075, 2, and 16 mg/L CASPO after 72 hours. Endpoints for a given growth condition were compared by a two-tailed paired student's t-test. Figures represent 3 experimental repeats (mean ±SD). Significant differences between endpoints ($p < 0.05$) are indicated by *.

B). *C. glabrata* ATCC® 15126™ - OD_{600} endpoint comparisons

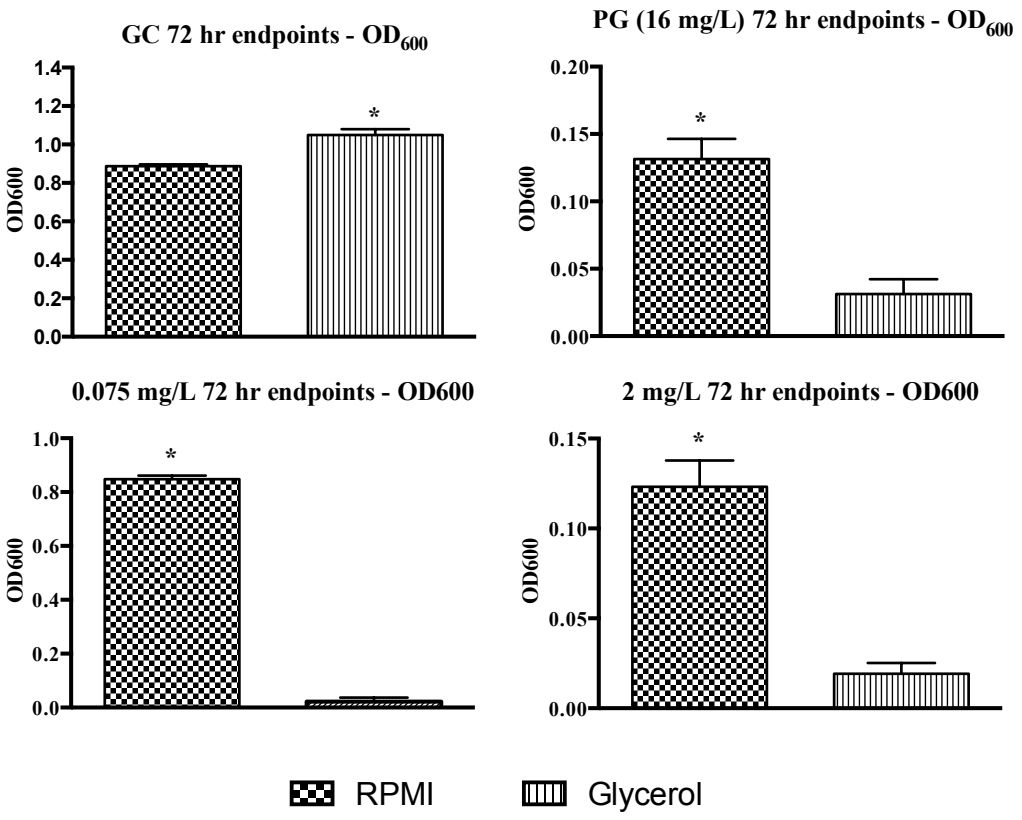


Figure 3.13, continued

A) *C. albicans* ATCC® 90028™

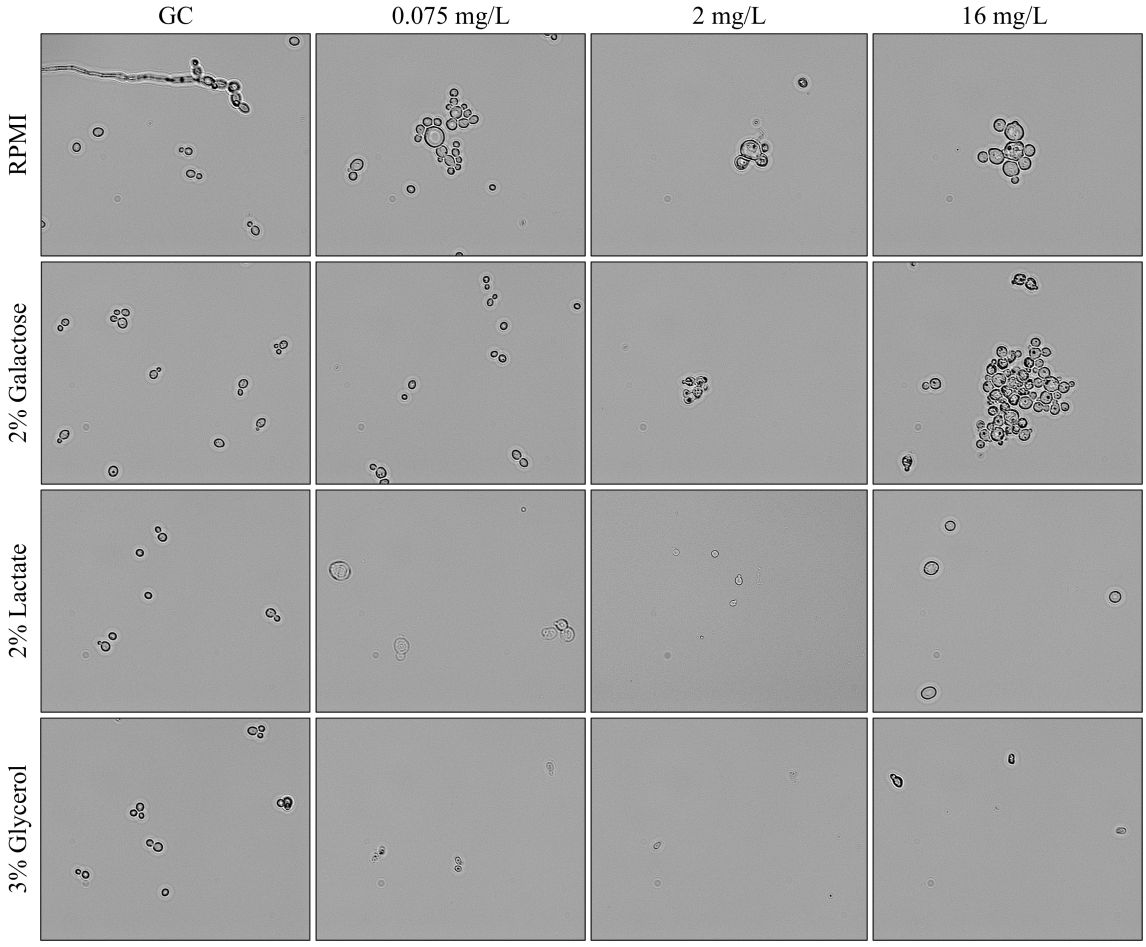
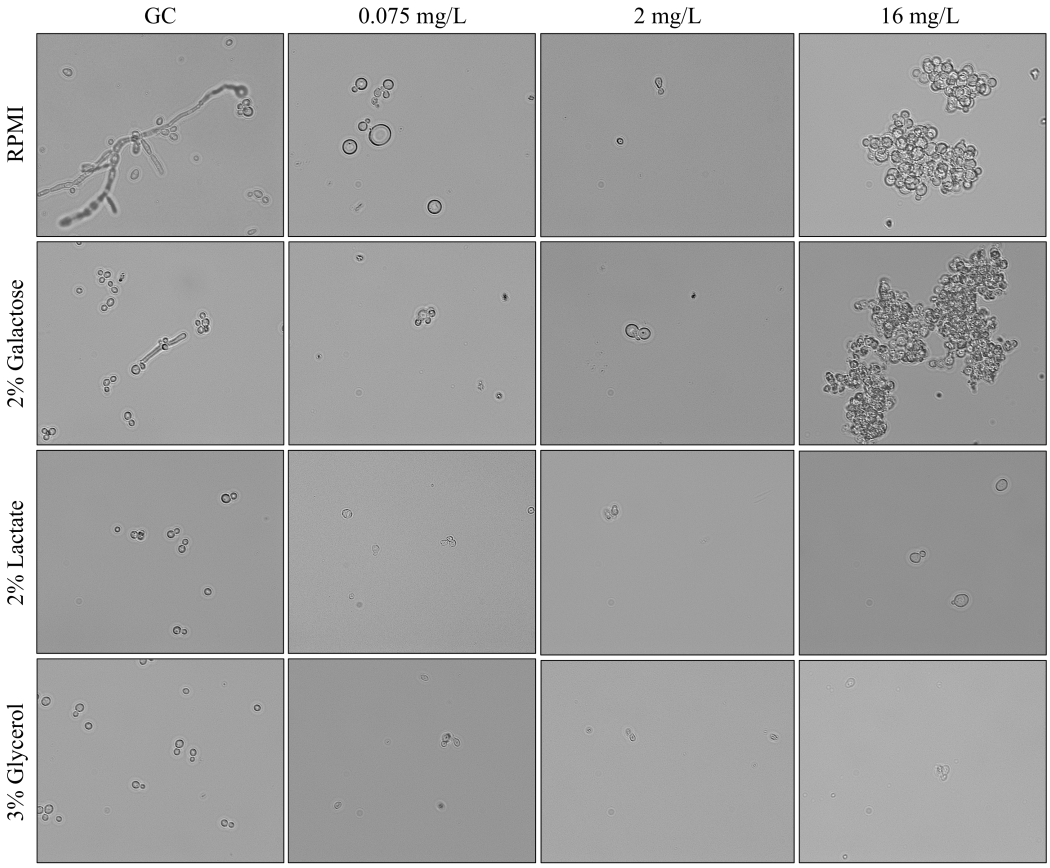


Figure 3.14. Light microscopy of yeast exposed to CASPO in different carbon sources. Samples were photographed by light microscopy (400X) after 72 hours.

B). *C. albicans* M10MY4874



C). *C. glabrata* ATCC® 15126™

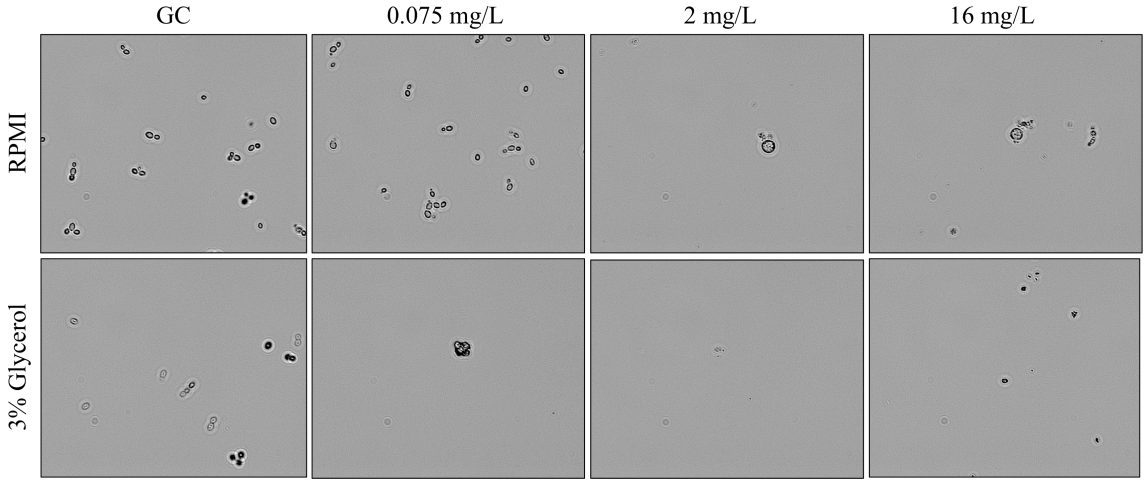
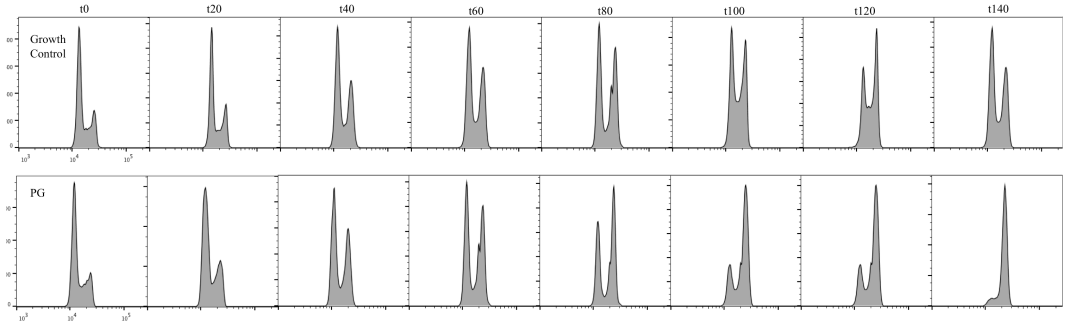


Figure 3.14, continued

A). *C. albicans* ATCC® 90028™



B). *C. albicans* M10MY4874

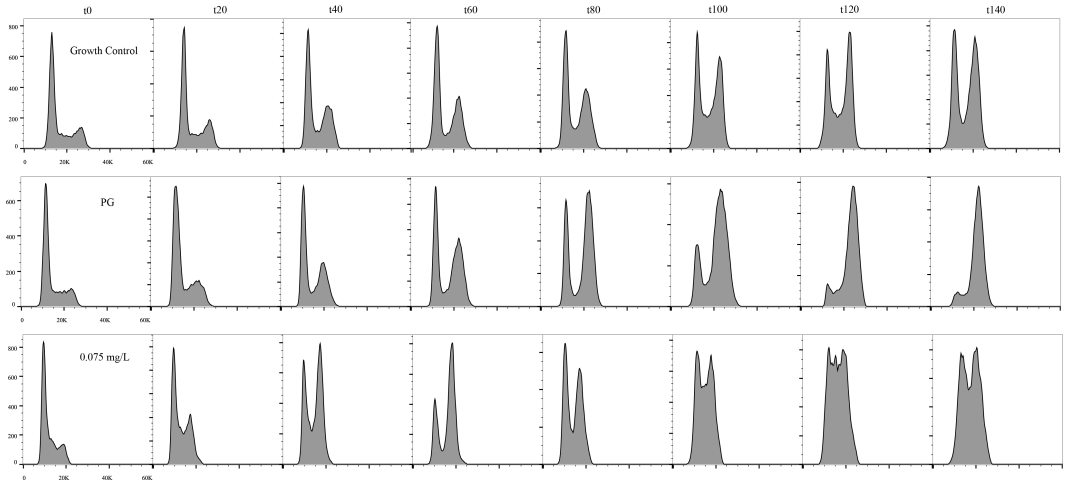


Figure 3.15. Cell cycle analysis by flow cytometry. The cell cycle was evaluated for **A).** *C. albicans* ATCC® 90028™ and **B).** *C. albicans* M10MY4874**.** DNA histograms were generated by manually gating fluorescent area versus fluorescent width dotplots in FlowJo v10.0.7. The histogram (number of cells) versus fluorescent area (DNA content) are shown.

3.5. References

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Chapter 4: Discussion

4.1. General remarks

Severe fungal infections are a major concern in healthcare, especially with an ever-increasing population at risk. The underlying conditions of patients, the high morbidity and mortality of IFIs, and the limitations of treatment options including scope of antifungals, increasing rates of resistance, and nonspecific antifungal targets complicate the management of IFIs. An ideal antifungal would be fungicidal to a broad spectrum of fungal pathogens, have a fungal-specific target, and no other drug interactions. While no one antifungal developed to date is ideal, the echinocandins are the most promising prospect for management of fungal infections, as they have a fungal specific target, no significant drug interactions or side effects, and are effective against the most common *Candida* spp. associated with disease. A limitation of echinocandins is their efficacy profile, as they are fungistatic or ineffective against many species of fungi, despite the high similarity of the drug target in all fungi.(see Introduction, p21-22)

In addition to the unexplained disparities in efficacy among fungi(1), an unexplained PG effect is demonstrated *in vitro* in which there is re-emergence of noticeable growth at concentrations above the MIC of susceptible isolates.(2) The clinical significance of the PG effect is unknown. This effect is reversible, as serial passages of cells collected from PG concentrations repeat the same growth patterns as the parent cell, and is not associated with known resistance phenotypes involving changes to GS in hot-spot regions.(2,3) Current standards recommend disregarding the PG effect in routine clinical testing, as it is not associated with MICs.(4) While the clinical significance of the PG effect is unknown,

understanding the morphological and physiological changes that allow growth at elevated concentrations will contribute to our overall understanding of the cellular effects of echinocandins in fungi, which could be utilized to improve management of IFIs through the design of more effective antifungals or combination drug therapies. This research aimed to better understand the PG effect to advance our overall understanding of echinocandins by evaluating the effects of elevated CASPO concentrations on *C. albicans*.

4.2. Influence of inoculum on the PG effect of *C. albicans* exposed to CASPO

In order to evaluate the characteristics of the PG effect, it was first essential to screen yeast isolates to identify PG positive and negative isolates for use in further testing. Previous reports on the incidence of the PG effect with echinocandins used standard CLSI methods of broth microdilution susceptibility testing and reported different frequencies of PG between different *Candida* spp. and strains.(4) However, the incidence of the PG effect is variable by study and test conditions used.(2,4,5) The most notable difference between assays to assess the PG effect is starting inocula, which differ between international standards, planktonic and biofilm assays, and between broth microdilution screens and larger scale experiments, such as time kill analysis. These assays have been the standard to assess the incidence of the PG effect between strains and physiological states, but should not be directly compared due to differences in growth conditions, particularly in the starting inoculum used. The use of the same starting inoculum would provide more reliable comparisons between studies and eliminate the

possibility of the effects of cell density being a confounder in the interpretation of PG results. This study tested the hypothesis that increased inoculum density influences the incidence and degree of PG in *Candida albicans*. Data from the 24 hour microdilution experiments supports a correlation between increased cell density and paradoxical growth in *C. albicans* exposed to CASPO.

Similar to previous reports, 9 of 24 (38%) *C. albicans* isolates demonstrated PG with a standard starting inoculum of 10^3 cells per ml by visual evaluation but not spectrophotometric assessment of microdilution trays. However, the PG effect was observed in all *C. albicans* isolates with an elevated starting inoculum of 10^6 cells per ml. Stereomicroscope examination showed the degree of PG in many isolates was not sufficient to cause a statistically significant change in absorbance. Nonetheless, observing any growth at CASPO concentrations above which there is complete inhibition is suggestive of a cellular response that permits growth above a susceptible MIC. While the PG effect has been considered “not uncommon”(5,6), this is the first report to demonstrate PG in all *C. albicans* within a selection of isolates differing in patient demographics and source of infection. The PG effect was not observed in any *C. glabrata* strain tested, regardless of starting inoculum used, confirming the previous study that concluded *C. glabrata* does not demonstrate PG with CASPO assessed by broth microdilution testing at 24 hours.(4)

While inoculum density influences the PG effect in *C. albicans*, it does not have a significant affect on MIC.(Table 3.1) Using an elevated starting inoculum resulted in a lower MIC compared to the standard inoculum for several isolates;

these values correlated within one dilution and had no affect on the MIC interpretation as CASPO susceptible. This has been similarly observed in comparing EUCAST and CLSI methods(7-9); while these standardized methods differ in inoculum density, medium glucose concentration, and endpoint evaluation, there is high essential agreement between the two methods, with the main discrepancies resulting from overall lower MICs (≤1 dilution) tested by EUCAST standards.(8) While not significant to the interpretation of MIC, this is also a paradox in CASPO's action, as it would be assumed that higher inocula (and a higher organism or target to drug ratio) would result in higher MICs. Lower MICs with higher inocula, did not alter the interpretation of the MIC as susceptible, which highlights a cellular response to CASPO that is not concentration dependent.

The criteria for the PG effect varies by study, but is typically defined as a significant resurgence of growth, such as ≥50% of that in the drug free control, at caspofungin concentrations ≥2 dilutions above the MIC. While a statistically significant change in growth is desirable to support the presence of the PG effect, the previous criteria defining the PG effect have resulted in isolates deemed negative despite visual confirmation of growth at CASPO concentrations above the MIC.(10) Any resurgence of growth following complete inhibition is suggests a change in the cellular response to CASPO. By defining the PG effect as re-emergence of any growth above which there is complete inhibition, this study is more accurate in determining the incidence of PG.

Many studies utilize the XTT assay to correlate metabolic activity to viability and growth.(5) While this method is sensitive to small changes in metabolic activity, there is significant variability between strains and not necessarily a linear correlation between the metabolic activity and cell number, especially at increased inocula density.(11) The XTT method is an indirect determination of growth and assumes that growth is proportional to the cell's capacity for aerobic respiration. While *C. albicans* primarily uses aerobic respiration to generate ATP, it is capable of adapting to different situations by fluctuating metabolic pathways depending on need.(12) Two methods of evaluation I used to directly assess changes in growth of yeast exposed to CASPO, and avoided the pitfalls of indirect methods of growth assessment, such as with XTT.

The PG effect has been compared for *Candida* spp. grown as planktonic cells and biofilms by Melos et al, with biofilms demonstrating a higher frequency of the PG effect.(5) However, this study failed to account for the methodological differences used to assess PG in the different growth forms, as they assessed planktonic PG using standard broth microdilution assays with a 10^3 starting inoculum, but used 24 hour preformed biofilms from a 10^6 starting inoculum. Biofilms demonstrate increased resistance to antifungals compared to planktonic cells that is not wholly due to inoculum density(13), likely because the biofilm structure limits diffusion of antifungal drugs and alters transcription of cellular processes.(14,15) The physiological differences between planktonic and biofilm cells may contribute to the difference in incidence of the PG effect, but the

significant difference in PG between the 10^3 and 10^6 inocula suggests that cell density, not the growth form, significantly alters the potential for PG at elevated concentrations of CASPO. Inoculum density affects cell growth, affecting morphology and quorum sensing(16), which may impact cell changes correlated with PG cells, including cell wall alterations and cell aggregation.(3) Further investigation into the physiology of PG cells affected by inoculum density should elucidate potential mechanisms for growth of fungi at elevated echinocandin concentrations.

Identifying the PG effect in all *C. albicans* but not in *C. glabrata* provides insight to its mechanism of action. It has been previously hypothesized that the PG mechanism likely involved a genetic component, as the incidence appeared variable among strains and species.(4) However, the data from this study demonstrates this effect is more likely species specific, not strain dependent. This is further supported by the lack of genetic associations of isolates positive for PG by microsatellite typing in *C. albicans* performed by Khilf et al.(17) The authors assumed that the PG effect was only present in certain strains, and concluded that the PG phenotype was "gained or lost too rapidly to be predicted by more stable genomic markers". However, my data suggests the PG effect is likely present in all *C. albicans* and therefore would not be associated with any one genetically related strain or clade.

The use of echinocandins in the management of *Candida* spp. infections is increasing, as they are approved for first line therapy of candidiasis.(18) Despite increasing use since the introduction of CASPO in 2001, isolation of resistant

isolates remains rare(19), and reports of breakthrough infections are limited to case reports.(20) With little evidence of clinical failure, the clinical significance of the PG effect is poorly understood. PG cells are slower growing than control yeast, with several experiments requiring a 48—120 hour evaluation period to demonstrate PG by time kill or broth micro-dilution methods.(2,3) Echinocandins are administered daily (24 hours); if PG requires an extended growth period for measurable *in vitro* effects, it could be assumed that the 24 hour dosing regimen would not support PG *in vivo*. However, given that I have demonstrated PG is achievable at 24 hours *in vitro*, 24 hour daily dosing could result in CASPO concentrations that may support PG *in vivo*.

There is little evidence for PG during echinocandin therapy, and it is controversial whether i*n vivo* mammalian models of candidiasis demonstrate the PG effect.(21,22) It is recommended that this effect be ignored in evaluating echinocandin MICs by CLSI guidelines as it is not associated with MIC or treatment failure.(23) Invasive yeast infections result from dissemination of yeast following a breech in a colonized mucosal barrier (as described, p. 3) Most *in vivo* models for candidiasis involve the induction of disseminated blood stream infections through intravenous or intraperitoneal injections.(24) While this model is comparable to disseminated yeast infections in humans, it may not be representative of the whole infection process from colonization to tissue invasion, as evidenced by different organs infected in intravenous versus colonization and dissemination murine models of candidiasis.(25) In murine models, this may limit the evaluation of echinocandin therapy and the potential for PG with, for example,

prophylatic echinocandin therapy. The potential for PG in an established infection is unknown, as is the potential of PG to influence the infection process from colonization to invasion. Given that animal models may not be representative of human infections via host colonization and that these results show that all tested strains of *C. albicans* have the potential for PG, there may be greater clinical potential for PG than had been previously assumed. Further understanding of the morphological and physiological changes that allow growth at elevated echinocandin concentrations will likely contribute to our overall understanding of *Candida* spp. that contribute to the infection process.

Given that PG occurs in *C. albicans* but not *C. glabrata*, the factors contributing to PG are likely intrinsic to specific species. While most medically important yeast are phylogenetically closely related to *C. albicans*, *C. glabrata* is most closely related to *S. cerevisiae* and differs from other *Candida* spp. in genome evolution and many metabolic pathways.(26) Exploring the differences between *C. glabrata* and *C. albicans* will likely identify mechanisms contributing to the PG effect.

4.3. Evolution of glucan synthase in fungi

4.3.1. Glucan synthase in fungi

C. albicans and *C. glabrata* are the most frequently isolated yeast species associated with invasive disease and are attributable for up to 75% of all cases of candidiasis.(27) Both species colonize the skin and mucosal surfaces of healthy individuals, and are capable of causing severe systemic infections in

immunocompromised populations. While *C. glabrata* is often grouped with the medically important *Candida* spp., it is actually most closely related to *S. cerevisiae*; *S. cerevisiae* is a model organism that has been an important tool used in echinocandin and glucan synthase research, as genetic manipulations are more easily accomplished than in other budding yeast species. However, it is rarely pathogenic and differs in many fundamental cellular processes as a result of its different evolutionary history.

The data from the inoculum density studies indicates the PG effect is intrinsic to specific species. Similarly, the susceptibility of fungi to echinocandins is due to an undetermined species-specific factor. Decreased antifungal susceptibility is most often associated with biofilm formation, compensatory regulation of metabolic pathways, upregulation of drug transporters, and antifungal target alterations.(28) While biofilms may contribute to the decreased susceptibility of some fungi to echinocandins, many fungi are intrinsically resistant regardless of growth form.(29) Upregulation of the HOG, calcineurin, and chitin synthesis pathways are observed in PG and resistant cells(30-32), but cannot fully account for the intrinsic differences between different species. The best proposed mode of action for echinocandins is the inhibition of GS by binding exterior to the cell.(33)(Figure 1.3) Therefore, drug transporters are unlikely to significantly influence the cellular response to echinocandins. Acquired resistance to echinocandins is associated with point mutations in “hot-spot” regions of GS, but it is not clear why many fungi demonstrate intrinsic resistance to echinocandins.

To evaluate the contribution of differences in GS to the cellular response of fungi to echinocandins, sequence analysis of GS homologs was conducted for 19 medically important fungi. The evolutionary history of fungal GS was evaluated in a previous study.(34) However, the resulting reconstruction was represented by an inaccurate phylogenetic topology, due to unrooted trees without consideration of gene duplications. The objective of this analysis was to reconstruct a more accurate evolutionary history of GS to provide further insights into the differences in cellular response of some species to echinocandins.

4.3.2. Phylogenetic and sequence analysis of fungal glucan synthase

Despite the high overall similarity in DNA and protein sequences, phylogenies may be difficult to resolve due to the presence of introns, gene duplication or loss events, and the appropriate selection for rooting. Previous phylogenetic analysis of FKS sequences resulted in unresolved tree topologies that were inconsistent with known species evolution.(34) That analysis acknowledged the presence of gene expansion by duplication in Ascomycota but failed to resolve these relationships, resulting in an inaccurate representation of FKS evolution.

In contrast to the previous study(34), the present analysis optimized the multiple sequence alignment, resolving the inaccuracies of the nucleotide alignment due to introns, resolved the gene tree to assess duplications and loss events, and rooted the trees with *R. oryzae*, the earliest known ancestor of the selected fungi, to better define the context of the FKS lineage. These steps

resolved the topologies of fungal FKS sequences and provided better inference of the GS evolutionary history. The multiple sequence alignments demonstrated the number and sequence of amino acids is highly conserved, which further highlights the conservation of 1,3-β-glucans in the cell walls of medically important fungi. There is evidence for the indispensable function of GS by the essentiality of single copy FKS genes and of particular FKS genes in multicopy organisms.(29,35-37)

The discrepancy in the nucleotide and protein data can be explained by examining the nucleotide composition of each gene. The signal from nucleotide compositional bias in unrelated species can overwhelm the phylogenetic signal of the data to group unrelated species with similar GC content together.(38) The percentage of guanine (G) and cytosine (C) in the FKS genes in the fission and budding yeast is consistent with the genomic averages(39-41), which is much lower than other fungi. Because the protein sequence is so highly conserved, it is less affected by the bias in GC composition and is representative of the true phylogeny.

4.3.3. FKS gene duplications in yeast

Gene and genome duplication events are important mechanisms for genetic innovation. Duplicated genes are more frequently lost than retained, and gene retention is influenced by the partitioning of ancestral functions (subfunctionalization) or by the emergence of new functions (neofunctionalization).(42) This evolution appears through modification of

transcriptional regulation, and posttranslational control (particularly increased phosphorylation).(43) Duplications can occur by small scale duplications (SSD) or whole genome duplication events (WGD), and can differ in functions and mode of evolution.(44) WGD paralogs are more likely to share functions, thus are able to compensate for the loss of the other gene copy, and are more likely to evolve by subfunctionalization; SSD paralogs are more likely to evolve by neofunctionalization and have a greater diversity and number of interaction partners.(42) Not all genes are equally gained or lost, as conserved genes with a greater number or complexity of functional domains are more likely to be retained after duplication events.(45)

The FKS gene products are large transmembrane proteins that provide an essential function, as double FKS1/FKS2 knockouts are non-viable in *S. cerevisiae*. While FKS2 is primarily involved in spore wall assembly and transcriptionally regulated by calcineurin, it can compensate for the loss of FKS1 (as described, p. 12). The different transcriptional regulation and subfunctionalization of FKS2 is consistent with the current knowledge of WGD paralog evolution. Fks1p is likely a multifunction protein, as targeted mutations in *S. cerevisiae* Fks1p affect glucan synthesis, endocytosis, and cell polarity. It is unknown the extent each paralog contributes to these functions, or what other functions may have evolved through altered transcriptional regulation, sub-cellular localization, or protein interaction partners.

Nearly all studies on the structure and function of Fks1p have been conducted in the model yeast *S. cerevisiae*, despite having significant cellular

differences as a result of its whole genome duplication. While it is an ideal model organism as it is more amenable to genetic manipulations, it lacks essential factors in other yeast associated with virulence, such as proteases, lipases, or a true hyphal form.(46) This is likely why it remains a rare cause of IFIs.(47) Given that echinocandins are approved for use in *Candida* spp., it is important to understand the nuances in functional complexity and regulation of *Candida* spp. paralogs.

The three FKS copies in *S. cerevisiae* and *C. glabrata* evolved through two SSD, a single gene loss event, and retention of both WGD paralogs.(Figure 3.6) This is in contrast to other *Candida* spp. in which all three FKS copies evolved from two SSD; given the differences in evolution and functional divergence of SSD and WGD paralogs, it would be expected that there are differences in regulation, localization, and interaction partners of *C. albicans* (and other closely related *Candida* spp.) FKS proteins. Fully understanding species-specific differences in FKS proteins could significantly improve our understanding of GS inhibition by echinocandins.

C. glabrata is an increasingly important cause of invasive candidiasis, and while it retains the "*Candida*" genus, it is more closely related to *S. cerevisiae* than the other *Candida* spp. The difference in the evolutionary history of FKS genes from other *Candida* spp. is apparent in the increasing reporting of *in vitro* resistance due to FKS1 and FKS2 mutations in *C. glabrata*.(48,49) *C. glabrata* is a haploid organism and contains a single allele of FKS1 and FKS2, making it easier to acquire echinocandin resistance through a mutation in either loci.

Acquired Fks1p mutations in other *Candida* spp. contribute to resistance, while mutations in Fks2p or Fks3p have not been reported for changes in echinocandin susceptibility.

Evidence from topological modeling of *S. cerevisiae* Fks1p predicts the binding of echinocandins exterior to the cell, the drug's hexapeptide core interacting with the "hot-spot" regions of the protein.(33)(Figure 1.3) The high conservation of sequence similarity in paralogs suggests a similar structure, and it would be reasonable to assume they would also bind echinocandins. Since SSD paralogs typically evolve through neofunctionalization and changes to sub-cellular localization, it is feasible that intracellular accumulation of echinocandins could exert unknown effects to FKS paralogs. *S. cerevisiae* Fks1p and Fks3p have been isolated from highly purified mitochondria in high-throughput studies(50-52); while echinocandins likely bind Fks1p exterior to the cell, the possibility of intracellular interactions cannot be excluded. Confirmation of paralog sub-cellular localization and functions will likely provide evidence for mechanisms of resistance without Fks1p mutations and or the paradoxical growth effect.

4.3.4. FKS gene structures.

There is a striking difference in the gene structure of single and multiple FKS copy genes. The multicopy FKS genes in the fission and budding yeast do not contain any introns while the single-copy FKS genes contain multiple introns with apparent independent acquisitions along fungal lineages. This is consistent with the known genomic structures of yeast and other fungi, as the evolution of

both fission and budding yeast favored intron loss while the basidiomycetes have remained intron dense. The most striking difference in gene structure is between the *Cryptococcus* spp. and other medically important fungi. Since it is unknown why *Cryptococcus* spp. are resistant to echinocandins, the gene structure may be important to the function of GS and its resistance.

Cryptococcus spp. have a greater number of introns per gene than most fungi (~5.5). For example, *A. fumigatus* contains ~2 introns per gene while *S. cerevisiae* only contains a few hundred introns in the entire genome.(53) The *Cryptococcus* spp. have a higher number of introns in the FKS gene than the genomic average, with 7 and 11 introns in the FKS gene that are predominantly distributed within the last 25% of the coding sequence. Introns may contain regulatory or other functional elements, and can be alternatively spliced to generate transcript and protein diversity.(54) The presence of multiple introns may be significant as alternative splicing is frequent in *Cryptococcus* spp.(55)

The retention of yeast paralogs is indicative of the potential complexity and multifunctionality of the protein, yet most other fungi retain only a single copy. Alternative splicing may account for this difference, as it increases proteomic diversity and results in different mRNA transcripts that may result in alternate functional proteins, differential targeting of protein localization, and altered transcriptional regulation.(56,57) Alternative splicing can result in exon skipping, intron retention, or alternative 5' or 3' splice sites; in fungi, intron retention is most prevalent.(58) Intron retention within the FKS gene in the *Cryptococcus* spp. would most likely produce a premature stop codon, though

most of the introns are towards the C-terminus and could result in a truncated yet functional protein. Introns in *C. neoformans* transcripts affect the abundance of mRNA accumulation, altering rate of translation and transcripts with pre-mature stop codons are regulated and degraded by the nonsense mediated mRNA decay pathway.(59) More information on the structure, function, and regulation of FKS is necessary to understand how alternative splicing could affect the function of GS or its sensitivity to inhibitors.

The two important virulence factors for *Cryptococcus* spp. are its substantial polysaccharide capsule and melanin production.(60) *C. neoformans* GS isolated from plasma membranes is sensitive to CASPO, while whole cells remain resistant in both capsular and acapsular isolates.(61) Melanin has been shown to bind CASPO,(62) but the effects of melanization and binding of CASPO does not fully resolve the discrepancy with *in vitro* susceptibility profiles between *Cryptococcus* spp. and other echinocandin susceptible fungi. Deletion of Ccr4p, a protein involved in the nonsense mediated mRNA decay pathway, results in CASPO susceptibility in *C. neoformans*(63), and is implicated in osmotic stress and cell wall integrity in *S. cerevisiae*.(64,65) Collectively, this suggests there may be other unknown mechanistic features of GS that render *Cryptococcus* spp. intrinsically resistant to echinocandins, such as alternative splicing of FKS.

Analysis of the evolution of FKS homologs in medically important fungi demonstrates: (i) the importance of a fully resolved gene phylogeny to accurately infer gene evolution; (ii) the differences in FKS paralog evolution in yeast and

highlights the pitfalls of extrapolating knowledge from the model yeast *S. cerevisiae* to evolutionary divergent yeast; and (iii) the significance of identifying variance in gene structures to study differences in protein function and regulation. Further characterization of the differences between FKS proteins will identify the potential differences in organism susceptibilities to GS inhibitors, thus providing new insights on optimum treatment options for invasive fungal infections.

4.4. Medium carbon source impacts the degree of PG in *C. albicans*

4.4.1. Effect of carbon source on PG by time kill analysis

The high sequence similarity among fungal GS, especially among budding yeast, suggests that differences in GS do not account for the differences in response to CASPO at elevated concentrations and that other intrinsic differences between these species contribute to PG. Despite being closely related phylogenetically, *C. albicans* and *C. glabrata* evolved differently to utilize nutrients to colonize and invade the host: virulence of *C. albicans* is dependent on its ability to switch between yeast and hyphal forms, and can utilize multiple carbon sources simultaneously predominantly through aerobic respiration; *C. glabrata* does not have a true hyphal form and is a facultative anaerobe, preferentially fermenting sugars even in the presence of oxygen.(66)

Interestingly, another unexplained anomaly regarding the PG effect is its variability in frequency when tested in different growth media, which vary most notably in the amount and types of carbon source.(2,67,68) Given the intrinsic differences between the two species in metabolism and the variability of PG in

different media, the PG effect could be influenced by carbon source. This study tested the hypothesis that the type of carbon source influences the degree of *C. albicans* PG. Data from the 72 hour time kill analysis of *C. albicans* exposed to CASPO with different carbon sources demonstrates increased PG in fermentable sugars compared to non-fermentable carbon sources.

This study demonstrated considerable variability in endpoint growth for *C. albicans* in different carbon sources. Despite this, a clear trend was observed in all media evaluated: exponential growth of the GC, and fungicidal activity at inhibitory (2 mg/L) CASPO concentrations. Growth at 16 mg/L CASPO was fungistatic by CFU determination for all media tested; however, by OD_{600} evaluation, *C. albicans* grown in fermentable sugars grew as large cell aggregates and demonstrated a significant increase in PG compared to the un-budded yeast observed in non-fermentable sugars. The difference in growth observed between CASPO concentrations and medium carbon source further adds to the evidence for a physiological difference between PG cells and yeast grown at inhibitory CASPO concentrations.

The carbon sources chosen for these experiments represent physiologically important sugars in different host environments. Dietary sources of glucose and galactose are absorbed from the gastrointestinal tract into the bloodstream, reaching concentrations up to 0.1-0.15% (6-8 mM), and can be excreted into the urine.(69,70) Lactate is present in the gastrointestinal tract and vagina from bacterial metabolites, and in anaerobic sites from host metabolism.(71) While glycerol is not present in significant quantities in the host, it can be metabolized

via aerobic respiration in the absence of a more optimal source of carbon; it is also a significant osmolyte, protecting cells against hyper-osmotic stress.(72)

Central carbon metabolism is the fundamental process involving the breakdown of carbon sources that is required for cellular energy and provides building blocks for biosynthetic processes of proteins, lipids, nucleotides, and carbohydrates. It is not only essential for cellular function and replication, changes to *C. albicans* central carbon metabolism and growth in different carbon sources are known to affect antifungal susceptibility, morphogenesis, virulence, and interaction with host immune cells.(73-75) As such, it is not surprising that carbon source also affects the PG response to CASPO.

Adaptability in carbon source assimilation is essential to survival in different host niches and at different stages of infection, as evidenced by transcriptional changes to central carbon metabolism upon nutrient limitation and interaction with host immune cells.(12,71) *C. albicans* mutants of specific carbon metabolic pathways are unable to cause infection in some host environments while fully virulent in others.(12) Given that PG cells are unable to proliferate in non-fermentable carbon sources the potential to demonstrate this phenomenon *in vivo* is questionable, but is likely dependent on the host environment of the infection model.

Only two *in vivo* investigations into the PG effect have been conducted for *Candida* spp. in mammals: an intraperitoneal abscess murine model with *C. tropicalis*, and a murine model of systemic candidiasis with *C. albicans*.(21,22) Both studies demonstrated decreased effectiveness of CASPO with increased

dosages in one or more isolates by measuring CFU per abscess or CFU per kidney pair, respectively, but were not reproducible in subsequent experiments. The authors concluded there were insufficient evidence for an *in vivo* PG effect but could not exclude it entirely. In contrast, the PG effect was reproducibly demonstrated in a neutropenic murine model of invasive pulmonary aspergillosis, in which increased fungal burdens and inflammatory pathology was observed.(76,77) This investigation was superior as it evaluated fungal burden by real-time quantitative PCR, CFU per lung, and histological examination of lung tissue. I have demonstrated significant differences between methods of detecting PG *in vitro*, and the ability to detect PG *in vivo* will also require multiple methods, including both culture and non-culture methods, to evaluate the infection burden.

In addition to the mammalian models to evaluate PG *in vivo*, there have been two investigations using invertebrates: a toll-deficient *Drosophila melanogaster* (fruit fly) model(78), and a *Galleria mellonella* (wax moth) model(3). Both models evaluated virulence by survival rates of the host infected with *C. albicans* pre-exposed to CASPO. There was no difference in virulence of PG cells in the fly model, though this could have been due to the low infecting inoculum (10^2 cells per fly). There was a significant decrease in virulence observed in the moth model, as a significant increase in survival was observed for PG cells compared to the control. PG cells also demonstrated increased encapsulation and melanization, a process of the innate immune response of the moth to clear the infection.(79) It was suggested that this decreased virulence of

PG cells could account for the failure to reproducibly demonstrate this effect in murine models.(3) However, it is premature to extend these conclusions to animal models or clinical data, as these invertebrate models have several major deficiencies. Firstly, both invertebrate models pre-treated *C. albicans* with only one CASPO concentration. The authors in the moth study demonstrated the PG effect is reversible, as PG cells will revert to normal growth in the absence of CASPO. The moth model, therefore, does not account for changes that could result from the absence of drug over the course of the infection. This study could also not determine whether these effects are specific to PG or whether these effects are seen at all CASPO concentrations, as only one CASPO concentration was evaluated. Secondly, while these models have an innate immune system required to control fungal infections(80), they likely lack the complexity of a mammalian model that provides greater confidence in extrapolating results to humans. A more thorough investigation using invertebrate models may be useful, but the multiple cellular adaptations of PG cells suggest that particular host niches may play a role *in vivo*.

I have demonstrated an increase in PG with fermentable carbon sources, and host environments rich in these sugars are likely more amenable to PG *in vivo.* As such, infection models evaluating PG must take this into account in the design and evaluation of PG by fungal burden. Tissue, such as kidney, is frequently collected and cultured to assess infection burden in disseminated candidiasis, as was the case for the study evaluating the PG with *C. albicans* in murine models.(22) However, the nutrient availability in tissues is likely transient,

requiring the utilization of multiple carbon sources simultaneously. This is evidenced by the upregulation of the seemingly opposite pathways of glycolysis, gluconeogenesis, and the glyoxylate cycle simultaneously.(12) If PG cells cannot persist in environments with non-fermentable carbon sources, as in tissues, this effect may not be significant *in vivo*, or at least not readily detected. The evaluation of multiple sample types that represent environments rich and poor in fermentable carbon sources, such as blood and tissue, respectively, will more fully clarify the potential for PG *in vivo*.

Infection model outcomes are affected by a multitude of factors, including the growth medium of the infecting inoculum, the inoculum density, the animal species and strain, and the infection site evaluated.(81) I have demonstrated that both the inoculum density and the carbon source of the growth medium affect PG of *C. albicans in vitro*, and these factors likely influence the ability to detect PG *in vivo*. There have been insufficient investigations into the *in vivo* potential of PG to contribute to treatment failure with CASPO, as previous studies tested insufficient numbers of isolates in two similar animal models, only evaluating for the presence or absence of growth.(21,22) Further *in vivo* investigations are required, utilizing multiple infection sites in a disseminated mammalian model of candidiasis evaluating the fungal burden by multiple methods.

4.4.2. Correlation of central carbon metabolism and PG

Previous analyses on the effect of media carbon source demonstrated changes to *C. albicans* proteome, secretome, and antifungal susceptibility.(75)

Most notably, yeast grown in alternative carbon sources, such as fatty acids and non-fermentable carbon sources, are more resistant to osmotic stress and antifungals.(74) In particular, lactate grown cells demonstrated altered cell wall architecture that renders them less susceptible to recognition and killing by the host immune system compared to glucose grown cells.(82) Lactate grown cells are more resistant to cell wall stress, postulated to be due to cell wall changes associated with this carbon source, it was expected that the PG effect would be enhanced in a lactate containing medium. Likewise, since glycerol is an osmolyte that is imported into the cell to modulate the effects of osmotic or cationic stress, it was assumed that growth in glycerol would also increase PG by modulating the osmotic stress induced by CASPO. Instead, the opposite was observed in which a significant decrease in PG was observed in non-fermentable carbon sources.

The fungistatic cellular response of PG cells grown in non-fermentable carbon sources as a sole energy source is suggestive of a reduced or inhibited respiratory capacity. Non-fermentable carbon sources are metabolized through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation through the electron transport chain (ETC).(40)(Figure 4.1) In *C. albicans*, inhibition of part of the respiratory chain (ETC) can result in either a severe growth defect or complete inhibition of growth on non-fermentable carbon sources.(83) Inhibition of the ETC in *C. parapsilosis* results in increased susceptibility to CASPO(84), further demonstrating a link between respiration and cellular response to CASPO.

The ETC utilizes the byproducts of the TCA and oxygen to transfer electrons through a series of mitochondrial inner membrane proteins to produce a

proton gradient and generate adenosine triphosphate (ATP). *C. albicans* has several pathways in the ETC: the classical (primary) respiratory chain, the parallel respiratory chain, and the alternative respiratory chain.(83) Fungi vary in their capacity for respiration, but most have evolved to contain both the classical and alternative pathways.(85) Importantly, *S. cerevisiae* and *C. glabrata* do not have an alternative or parallel respiratory pathway.(86)

Reactive oxygen species (ROS) are produced during aerobic respiration and neutralized under normal circumstances by key mitochondrial enzymes. However, mitochondrial dysfunction from cellular stress can lead to ROS accumulation, causing DNA damage, lipid degradation, protein damage, and apoptosis, as demonstrated by *C. albicans* exposed to low concentrations of CASPO.(87) One of the ways *C. albicans* manages ROS accumulation is by modifying its ETC, by increasing the transfer of electrons through the alternative pathway and significantly reducing ROS production.(83) Induction of this pathway decreases the susceptibility of *C. albicans* to azoles(88), but the role of the alternative pathway has not been investigated in the response of *C. albicans* to CASPO. It is a promising pathway to investigate in its contribution to the PG effect, given the difference in metabolism of PG cells and the lack of alternative respiration in the PG negative *C. glabrata*.

The decreased PG in yeast grown in non-fermentable carbon sources may also be due to increased energy demands of coping with CASPO induced stress. PG cells are known to have cell wall alterations, with an increase in chitin and decrease in β-glucans.(Figure 1.5) *C. albicans* grown in fermentable carbon

sources derive biosynthetic components for some amino acids, nucleosides, and other carbohydrates through glycolysis, partitioning pathway intermediates for use in the cell wall as required.(40)(Figure 4.1) In non-fermentable carbon sources cell wall biosynthetic components are derived from gluconeogenesis, requiring the input of energy first to convert pyruvate to glucose and or fructose intermediates and secondly to generate cell wall components. PG cells may not be able to produce enough energy from non-fermentable carbon sources to cope with CASPO induced stress and proliferate, especially if there is indeed a reduced or inhibited respiratory capacity.

There is growing evidence for a link between metabolism, stress responses, morphogenesis, and cell wall remodeling. Studies with null mutants to evaluate specific cellular traits are demonstrating detrimental effects in unexpected and seemingly unrelated pathways, often with altered susceptibility to cell wall inhibitors. For example, null mutants of the Sorting and Assembly Machinery 37 protein (Sam37p), a subunit of the complex required for mitochondrial membrane biogenesis, are slow growing, hypo-filamentous cells that have a media dependent increased CASPO susceptibility.(89,90) Similarly, a null mutant in Growth and Oxidant Adaptation 1 (Goa1p), a protein that relocalizes to mitochondria in response to oxidative stress, is a slow growing, hypo-filamentous yeast with reduced respiratory capacity and increased sensitivity to cell wall inhibitors.(91,92) My data demonstrate PG cells are slow growing with altered carbon metabolism and morphology. The similarities between mitochondrial mutants defective in cell wall regulation and PG cells

suggests the PG effect may be a model to further investigate the common thread between metabolism, morphology, and cell wall integrity.

4.4.3 Differences in PG morphology in fermentable and non-fermentable carbon sources.

C. albicans is a pleomorphic organism, transitioning between yeast, hyphae, and pseudohyphae. Yeast appear as unicellular ovoid cells, hyphae are cylindrical filaments that grow from the tips, and pseudohyphae are filaments of unseparated buds distinguishable from true hyphae by their constrictions (septa) at the growth point from the bud. The ability to switch between morphologies is believed to be important for its evolution as a pathogen: yeast forms are essential for colonization and dissemination; hyphae are important for tissue invasion; and mutants defective in morphogenesis are associated with decreased virulence.(93,94) Morphogenesis is essential to *C. albicans* survival under diverse environments, and evaluating morphology in response to CASPO will elucidate its physiological state.

PG cells previously characterized by electron microscopy demonstrate morphological alterations with cell clumping, abnormal septa, enlarged cells, and absence of hyphae.(6) Similarly, I observed large yeast-like aggregates with incomplete bud separation with PG in fermentable carbon sources. However, I observed the enlargement of yeast with cessation of budding with PG in non-fermentable carbon sources. This difference in growth and morphology between

PG in fermentable and non-fermentable carbon sources has never been identified, and may be indicative of the mechanisms for the PG effect in *C. albicans.*

A number of factors affect the morphogenesis of *C. albicans*, including temperature, pH, bacterial metabolites, inoculum density, and carbon source.(95,96) Of particular interest is the difference in morphology of *C. albicans* grown in different carbon sources. Depending on the concentration of fermentable sugars, *C. albicans* appears as yeast, pseudohyphae, and hyphae to varying degrees; when grown on non-fermentable carbon sources, *C. albicans* typically grows only in yeast form.(96,97) This correlates with the difference in metabolism between the two forms, as yeast predominantly utilize aerobic respiration to generate energy, while hyphae have a tendency towards fermentation.(98,99) This difference in metabolism between the two forms is also demonstrated when *C. albicans* is grown anaerobically where cells can only utilize fermentation, and only hyphae are observed.(100) While suppression of respiration is not essential for the transition of yeast to hyphae, there is substantial evidence for a correlation with carbon metabolism and respiration between yeast and hyphae.(99)

The distinct morphology of PG cells has been characterized as "incomplete separation" of budded yeast with the absence of filamentation, attributed to the changes in cell wall composition.(3,6) However, this morphology is too similar to pseudohyphae to assume that these cell aggregates are yeast that remain attached after budding. While the distinction of morphology may seem insignificant, there are physiological distinctions between yeast and hyphae

demonstrated by transcriptional and metabolomic analyses.(101,102) These differences are believed to contribute to survival in different environments and virulence in the host. Considering these differences and their significance, it would be prudent to fully evaluate the morphology of PG cells.

In relation to their morphology of unseparated elongated buds, pseudohyphae are characterized by the capability of invading agar and an extended period of polarized growth compared to yeast, resulting in a delay in G2 of the cell cycle.(103) By evaluating the delayed growth of PG cells by cell cycle analysis, I demonstrated that PG cells have a noticeable delay in the G2/M phase not observed with low concentrations of CASPO. The G2/M delay and the PG cell morphology is suggestive of pseudohyphae growth.

Cell proliferation is the fundamental goal after cell survival, requiring the accurate duplication and partitioning of cellular components regulated by the cell cycle. The cell cycle is sensitive to changes in energy metabolism and ROS accumulation, and oxidative stress can lead to cell cycle arrest, often in G2/M.(104) In *C. albicans*, G2/M arrests tend towards polarized growth(103), as seen with genetic mutations in some cell cycle transcription factors and proteins required for septum formation that demonstrate a G2 delay and constitutive pseudohyphae morphology.(105,106) Integration of cell wall components for septum formation and cytokinesis, especially the linkage of chitin to β-1,3-glucans, is essential for the control of bud growth and cell cycle progression.(107) *C. albicans* resistant to CASPO demonstrate increased chitin synthesis and

alternative septum formation(108,109), and a similar mechanism may contribute to the PG effect.

CASPO induces the HOG, calcineurin, and CWI pathways in *C. albicans*.(110)(Figure 1.6) These pathways contribute to resistance caused by GS "hot spot" mutations and the PG effect(30,31), likely due to the upregulation of chitin synthesis by these pathways.(111) Increased cell wall chitin is associated with increased CASPO MICs and can be associated with abnormal cellular morphology.(109) The increase in chitin after exposure to CASPO is conserved in *Candida* spp., but is notably absent in *C. glabrata*.(112) Given that I have confirmed PG does not occur in *C. glabrata* by broth micro-dilution testing, this could explain the difference in PG between species. However, Rueda et al demonstrated that increased cell wall chitin "is necessary but not sufficient for CASPO adaptation" at PG concentrations, as a significant increase in cell wall chitin was observed in yeast grown in CASPO concentrations as low as the MIC yet growth was inhibited at the MIC.(3) Also, other conditions result in elevated cell wall chitin content. For example, pseudohyphae of S. cerevisiae demonstrate increased cell wall chitin that accumulates over the entire cell surface(113), similar to that observed in PG cells. Therefore, while the stimulation of stress pathways at PG CASPO concentrations coordinates the activation of multiple cellular processes that includes increased chitin synthesis, other unknown cellular factors contribute to the adaptation and morphology of PG cells.

Pseudohyphae and hyphae are often collectively referred to as the filamentous or invasive forms of *C. albicans*, as both can invade solid media *in*

vitro and are believed to have developed to forage for nutrients. However, these forms represent distinct cellular states, as they are induced under different conditions and demonstrate critical differences in their cell cycles.(114) Likewise, there are physiological differences between pseudohyphae and yeast. In addition to differences in cell cycle, pseudohyphae express low levels of some hyphae-specific proteins.(115) The pseudohyphae morphology is assumed to be an intermediate between yeast and hyphae, regulated by a shared transcriptional regulation in a dose dependent manner with hyphae.(116) Despite being considered a distinct yet intermediate state between yeast and hyphae as pseudohyphae demonstrate similarities of both, pseudohyphae generally do not develop true hyphae and vice versa.(106) PG cells do not resemble hyphae, but the distinction between unseparated yeast and pseudohyphae may have implications for infection models, as pseudohyphae are an invasive morphology.

Increased fermentative metabolism is associated with hyphae, but the metabolic status of pseudohyphae is unknown. The ability to invade tissues *in vivo* and solid agar media *in vitro* likely requires cells to cope with micro-aerophilic or anaerobic environments, suggesting similar metabolic preferences of hyphae and pseudohyphae. In *S. cerevisiae* there are three defined pathways regulating pseudohyphal growth that are conserved in *C. albicans*: the MAPK pathway, the protein kinase A pathway, and the sucrose non-fermentable pathway. These pathways are also involved in regulating the cell response to nutrient availability and either directly target mitochondrial function or are influenced by mitochondrial function.(117,118) Although these pathways are

conserved between *S. cerevisiae* and *C. albicans*, the response to respiratory dysfunction is not. This further supports future investigations of mitochondrial function and respiratory capacity in the mechanism of the PG effect.

Investigations into the effects of specific cellular factors has revealed that no one factor dictates morphology, as combinatorial factors in complex environments affect morphogenesis differently than manipulations of any one single factor. As stated by Brown and Ene of general compensatory mechanisms to antifungals(12), are these changes to morphology and metabolism a requirement for the cell to adapt to the antifungal or is this response an indirect by-product of exposure to the antifungal agent? Further investigation into the PG effect is required to answer this, along with the many questions regarding the clinical potential and significance in antifungal therapy.

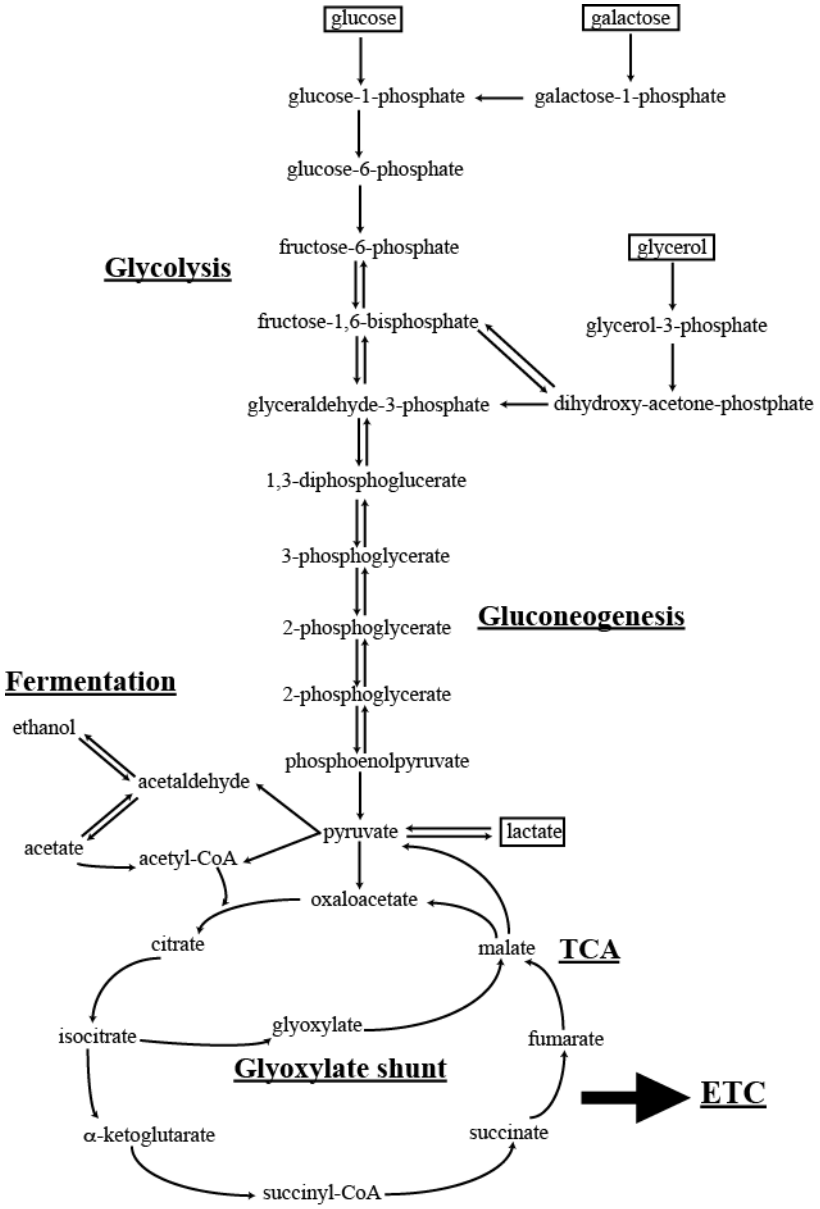

Figure 4.1. *C. albicans* central carbon metabolism. The major pathways for carbon metabolism were obtained from the CGD.(40) Carbon sources used in this study are indicated in boxes. Glycerol and lactate are non-fermentable carbon sources and are utilized strictly through the TCA and ETC; glucose and galactose are preferentially utilized through the TCA and ETC, but can also be fermented. Unlike *S. cerevisiae* and *C. glabrata*, *C. albicans* is capable of utilizing multiple carbon sources simultaneously, which appears important for colonization, dissemination, and survival in the host.

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Chapter 5: Conclusions

5.1. Future directions

This project began when I noticed a trend of unexplained anomalies in echinocandin research, in particular the noticeable growth above the MIC and the unexplained intrinsic resistance of some species despite highly similar GS sequences. The assumption regarding the mechanism of action of echinocandins is that they inhibit glucan synthesis, resulting in cell wall fragility and eventual lysis due to overwhelming osmotic forces. However, the anomalies surrounding the response of fungi to echinocandins are suggestive of additional cellular effects not necessarily associated with the cell wall. To me, these anomalies are an opportunity to evaluate the cellular effects of echinocandins in the hope of maximizing their therapeutic potential. Perhaps it is as the late Isaac Asimov said: "The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' but 'That's funny...' "

My research has contributed to the overall understanding of what is happening in PG cells, but has not determined why it is happening or why it is only happening at elevated concentrations. Based on the significant morphological and physiological differences in PG cells, future investigations into this effect to answer these questions will likely provide significant insight into the connections between cellular response to cell wall stress, cellular metabolism, and antimicrobial resistance. Identifying the mechanism of PG will contribute to our understanding of mechanisms of intrinsic echinocandin resistance, may provide a means of increasing the efficacy profile to include more fungal species, and determine the potential for this effect to contribute in treatment failure.

5.1.1. Clinical significance of the PG effect

C. albicans is an important organism. It is not only a significant contributor to human disease, it is a model organism to study morphogenesis, virulence, and pathogenesis of fungi. It is a fast growing organism, and in my opinion this yeast is easier than, for example, filamentous fungi to manipulate in the laboratory. *S. cerevisiae* is the most widely studied fungus but is not an ideal model as it is rarely pathogenic. Approximately 80% of *C. albicans'* genes are homologous to *S. cerevisiae'*s, allowing a direct comparison of conserved cell pathways and functions between the two organisms; however, at least 13% of genes are unique to *C. albicans*, and highlight the importance of investigating species specific cell functions, especially those pertaining to pathogenesis or antifungal response.(1) In addition to being an important human pathogen, *C. albicans* is an excellent organism to investigate due to its practicality in ease of use and extensive genetic information available.

C. albicans is one of the most prevalent fungi in humans, asymptomatically colonizing the skin, genital-, and intestinal-mucosa of up to 70% of healthy individuals.(2) *Candida* spp. are responsible for over 70% of all IFIs in North America, with *C. albicans* accounting for almost 50%.(3) The annual incidence of invasive disease caused by *C. albicans* in the Calgary Health Region is 1.5 per 100,000.(4) This is similar to other regions, which are also demonstrating increasing rates of candidemia.(5) The burden of these infections is significant in terms of cost to both the patient and the health care system, and

warrants continuing efforts to decrease the risk and progression of these infections.

C. albicans becomes an opportunistic pathogen when host defense barriers become compromised, such as a break in the mucosal barrier or disruption of the host immune system. Superficial infections affect a significant proportion of the population; oral candidiasis is prevalent among HIV and cancer patients receiving chemotherapy or radiation, and many women suffer from recurrent vulvovaginal candidiasis.(6) While generally not life threatening, these infections are often persistent and cause discomfort and distress to patients. Systemic infections occur after yeast gain access to the bloodstream or tissues, often secondary to invasive medical procedures, the use of broad-spectrum antibiotics, and immune suppressing therapies, diseases, and infections.(7) While the incidence of candidemia is increasing in parallel with increasing populations at risk, the mortality rate has remained the same.(5) Still, the mortality rate remains high, as candidemia is associated with a 14.5% and 10% increase in mortality in adult and pediatric patients, respectively.(8)

The severe impact to morbidity and mortality associated with *Candida* spp. infections highlights the imminent need for appropriate diagnostics, prevention, and treatment.

The current Canadian guidelines for first line therapy of candidemia caused by *C. albicans* is fluconazole or echinocandins, and due to the increasing resistance to fluconazole, echinocandins are preferred first line therapy if there is a previous exposure to azoles.(1,9) Fluconazole is an effective first line therapy

with minimal side effects, but resistance is increasing with widespread use, and prior exposure significantly increases the risk of recovering *Candida* spp. isolates with decreased fluconazole susceptibility.(2,10) Although there are increasing reports of clinical isolates with FKS mutations conferring resistance to echinocandins, the overall prevalence of resistance remains low with only 2-3% of *C. albicans* and related species.(3,4,11,12)

The high safety and efficacy of echinocandins in treating *C. albicans* is due to their limited drug interactions and fungal specific target. Because of this, the use of echinocandins is increasing, with more than 60% of candidemia patients receiving an echinocandin(5,12), and their use in high dose strategies is an attractive option to increase efficacy. With the current dosing regimen of a 70 mg CASPO loading dose, peak plasma concentrations reach 11—12 mg/L.(6,13) My data demonstrate this concentration range is too low to stimulate PG *in vitro* in most isolates, as only 13 of the 24 *C. albicans* isolates I evaluated demonstrated PG within this concentration range (8—32 mg/L), and only with an elevated starting inoculum. If the PG effect can occur *in vivo*, it is not likely to occur with the current dosing regimen. Dose escalation to 150 and 200 mg CASPO can achieve peak plasma concentrations of 29.4 and 33.5 mg/L, respectively(7,14), concentrations at which PG occurs *in vitro*. However, plasma concentrations are continuously fluctuating and it is unknown how this could affect PG.

Echinocandins are highly protein bound and are sequestered in tissues even after plasma concentrations begin to decline.(5,13,15) Addition of serum to MIC testing medium results in increased MIC values 2- to 4-fold and eliminates

the PG effect with concentrations up to 64 mg/L.(8,16,17) However, due to a several fold increase in MIC, it is likely that the concentrations were not high enough to properly evaluate the effects of serum on PG, as it is likely the PG concentrations also increase by this factor. Whether PG is achievable in the presence of serum is dependent on if these concentrations (>64 mg/L) could be sustained *in vivo*, even with dose escalation. Peak and trough plasma concentrations, the effects of protein binding, and the high sequestration of echinocandins in tissues are all complex factors that must be addressed to fully understand appropriate dosing for treatment management as well as the potential for PG *in vivo*.

Dose escalation could increase efficacy for isolates with increased MICs, providing a means to treat yeast isolates with FKS mutations. However, in a murine model of invasive candidiasis, CASPO dose escalation was not consistently effective against *C. albicans* isolates with FKS1 mutations.(18) The authors only evaluated 3 isolates and did not determine pharmacokinetics, but the lack of treatment success with increasing concentrations is a cause for concern. A similar study also determined that dose escalation was not effective against *C. albicans* with hetero- or homozygous FKS1 mutations; moreover, this study determined that while the EUCAST clinical breakpoints could predict *in vivo* responses with standard dosing, they could not predict responses with elevated doses.(19) The maximum tolerated dose of CASPO is unknown, but high doses are well tolerated in patients with invasive candidiasis and aspergillosis up to 210 mg.(20) The use of dose escalation in empirical therapy is appealing, but it is not

known whether these treatment failures correlate with *in vivo* PG or if increased exposure with dose escalation could further contribute to acquired echinocandin resistance.

One potential infection model that is of particular interest in regards to the PG effect is antifungal (or catheter) lock therapy. Long-term intravascular devices, such as central venous and hemodialysis catheters, are prone to biofilm formation.(21) Biofilms are organized communities of cells embedded within an extracellular matrix that can adhere to abiotic surfaces, such as a catheter lumen, and are more resistant to host defenses and antifungal therapy.(22,23) Current guidelines for catheter associated blood stream infections by *Candida* spp. recommend removal of the device and reinsertion at an alternate site.(24) However, this process can contribute to morbidity and mortality, and may not be practical in patients in which another site is not accessible. Antifungal lock therapy involves administering highly concentrated drug solution to sterilize the device; while this is generally not applied clinically yet, the use of amphotericin B, ethanol, and echinocandins are potential antifungal lock therapy agents based on animal studies and a collection of case reports.(21) There have been no consensus on dosage for echinocandin use in antifungal lock therapy as some demonstrated success with as little as 2 mg/L CASPO(25), therefore it is premature to draw conclusions or extrapolate the potential for PG from *in vitro* data. However, given that antifungal lock therapy utilizes highly concentrated drug, this application has the potential to demonstrate PG if echinocandin concentrations used were within the PG range.

Despite increasing use since the introduction of CASPO in 2001, echinocandin resistance remains rare, and reports of breakthrough infections are limited to case reports.(26) With little evidence of clinical failure, the significance of the PG effect is poorly understood. PG cells are slow growing, and many reports evaluated the PG effect over a 48—120 hour period to demonstrate significant growth.(16,27) The requirement of an extended growth period also questions the clinical significance of PG *in vivo*, as current doses are administered daily (24 hours). My data demonstrate significant PG is achievable at 24 hours, and highlights the potential for this effect to occur during treatment if it is achievable to maintain elevated concentrations *in vivo*.

In addition to the possibility of PG, it is unknown what effect, if any, dose escalation would have on the incidence of echinocandin resistance. Some authors have justified dose escalation by claiming resistance is less likely to occur as the drug exposure level would be high enough to eradicate the infecting organism, preventing any colonizers or persisting yeast to acquire resistance.(12) In my opinion there is too little information on the effects of dose escalation in the treatment of candidiasis, and any conclusions based on the assumption, such as that made by DS Perlin, of a "mutant prevention concentration window" with elevated doses is premature. Exposure to echinocandins is a risk factor for developing FKS mutations(28), and it is possible that higher doses would lead to a longer exposure period given that echinocandins persist in tissues long after plasma levels begin to decline. Further *in vivo* investigation of echinocandin dose

escalation utilizing a recovery mammalian model is warranted to monitor clinical efficacy for signs of emerging resistance and the PG effect.

Echinocandin resistance and the PG effect are distinguished by MICs: resistance refers to an elevated MIC based on clinical breakpoints that predict clinical failure, while the PG effect does not correlate with MIC. This lack of correlation with MIC, and presumably clinical therapy, is the reason the PG effect has generally been discounted. Resistance mechanisms of *C. albicans* are generally attributable for rendering an isolate untreatable with an echinocandin, such as a mutation in FKS1; the PG effect has been considered an *in vitro* phenomenon of echinocandin tolerance as a result of an adaptive physiological mechanism in response to cell wall damage.(27,29) There are marked similarities in resistant FKS mutants and PG cells, most notably significant increases in cell wall chitin as a result of upregulated calcineurin and CWI pathways.(30) The PG effect is not associated with FKS1 mutations(16,27), but the similarity is response pathways may be indicative of shared compensatory mechanisms for the PG effect and resistance. There are no reports of clinical failure due to *C. albicans* without an FKS1 mutation, and only one report of a laboratory mutant with reduced susceptibility to CASPO as a result of mutations in two chitinase genes (but no FKS1 mutations).(31)

The possibility of persistent growth, either due to a mechanism for resistance or PG, at elevated echinocandin concentrations poses a significant drawback to a drug class that is otherwise proven safe and effective for candidiasis. Further study of the PG phenomenon will help determine its

significance to treatment failure, identify cellular response mechanisms contributing to resistance, identify ways to fight it, and improve options for antifungal therapy.

5.2. Major findings

The major findings of this thesis that contribute to the field of echinocandin research can be summarized as follows:

1. All *C. albicans* demonstrate the PG effect when exposed to elevated concentrations of CASPO.

2. Demonstration of the PG effect in *C. albicans* with *in vitro* broth microdilution is dependent on the starting inoculum.

3. Confirmation that *C. glabrata* does not demonstrate the PG effect under the same test conditions as *C. albicans*.

4. GS sequence alone does not account for or predict the cellular response of different fungal species to echinocandins.

5. The PG effect is modulated by carbon source.

6. PG cells demonstrate a marked cell cycle delay in G2/M not observed with inhibitory concentrations of CASPO.

5.3 Conclusions

Due to the lack of correlation between MIC or treatment failure and the PG effect, it has been assumed to be a purely *in vitro* phenomenon and irrelevant in the management of infections. While the *in vivo* potential of this effect has yet to be fully evaluated for candidiasis, my research demonstrates how growth conditions influence the degree of PG and may influence the testing and evaluation of PG *in vivo*. Inoculum density influences the PG effect, and this research demonstrates that all *C. albicans* but no *C. glabrata* exhibit PG with *in vitro* broth microdilution testing. This has two major implications: first, the clinical potential of this effect has been previously underestimated by assuming only a subset of strains is capable of PG; and secondly, the differences between these organisms can be utilized to investigate the mechanism for PG. I also demonstrated that carbon source modulates the PG effect, where this effect is significantly more prominent in fermentable carbon sources. While murine models of candidiasis have been inconclusive in the demonstration of a reproducible PG effect, this research provides evidence for investigating PG in different infection models to investigate whether different host niches are more amenable to the PG effect due to differing nutrient availabilities.

My research findings contribute to the growing body of evidence that suggests the action of echinocandins is not entirely concentration dependent. Most

studies only evaluate sub-inhibitory or PG concentrations of echinocandins. This research highlights the significant physiological differences between yeast grown at PG and inhibitory CASPO concentrations, including changes to metabolism, morphology, and cell cycle inhibition, demonstrating the need to evaluate subinhibitory, inhibitory and PG concentrations to fully understand the cellular effects of echinocandins.

The echinocandins have variable efficacy among different fungal species, limiting their use in management of IFIs. My research provided a fully resolved gene phylogeny to accurately infer the evolution of GS and highlights the lack of correlation between susceptibility and amino acid sequence of the putative GS echinocandin binding sites. My findings further our understanding of GS and provide the foundation for future research to investigate the sub-cellular localization and post-transcriptional regulation of GS that contributes to decreased susceptibility in some fungi.

5.4. References

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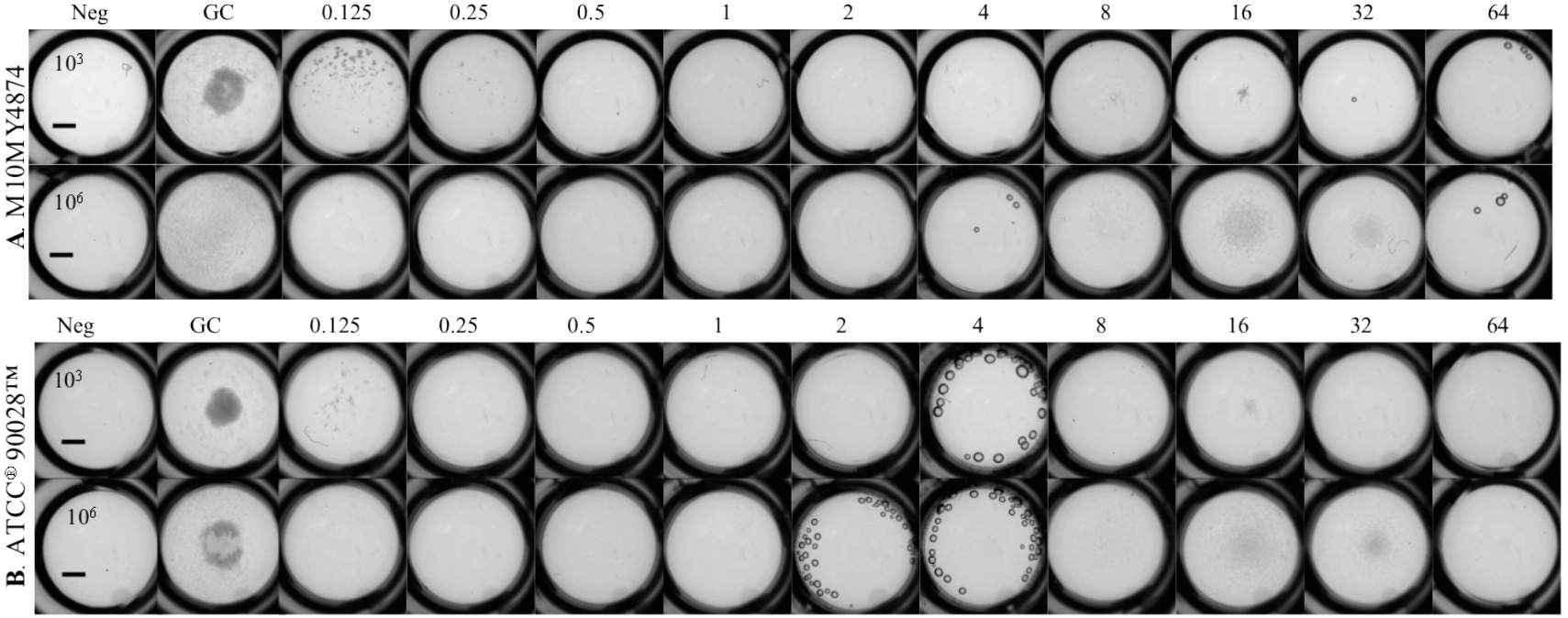
Appendices

Appendix 1. Stereomicroscopy of *C. albicans* microdilution growth patterns. Individual wells of 96-well microdilution plates containing *C. albicans* exposed to CASPO for 24 hours were visualized and photographed under a stereomicroscope at 1.2X magnification. Each organism was tested using the standard 10^3 (upper image) and increased 10^6 inoculum (lower image). Numbers above images indicate CASPO concentrations (mg/L), the growth control (GC), and negative control (Neg).

Scale bars represent 1 mm.



Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
C. ATCC® 24433™
D. M10MY2168
E. M10MY8596

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
F. M10MY7506
G. M10MY7837
H. M10MY4875

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
I. M 10M Y5059
J. M 10M Y2166
K. M 10M Y1585

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
L. M10MY2938
M. M10MY3029
N. M10MY3028

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
O. M10MY2978
P. M10MY4529
Q. M10MY5068

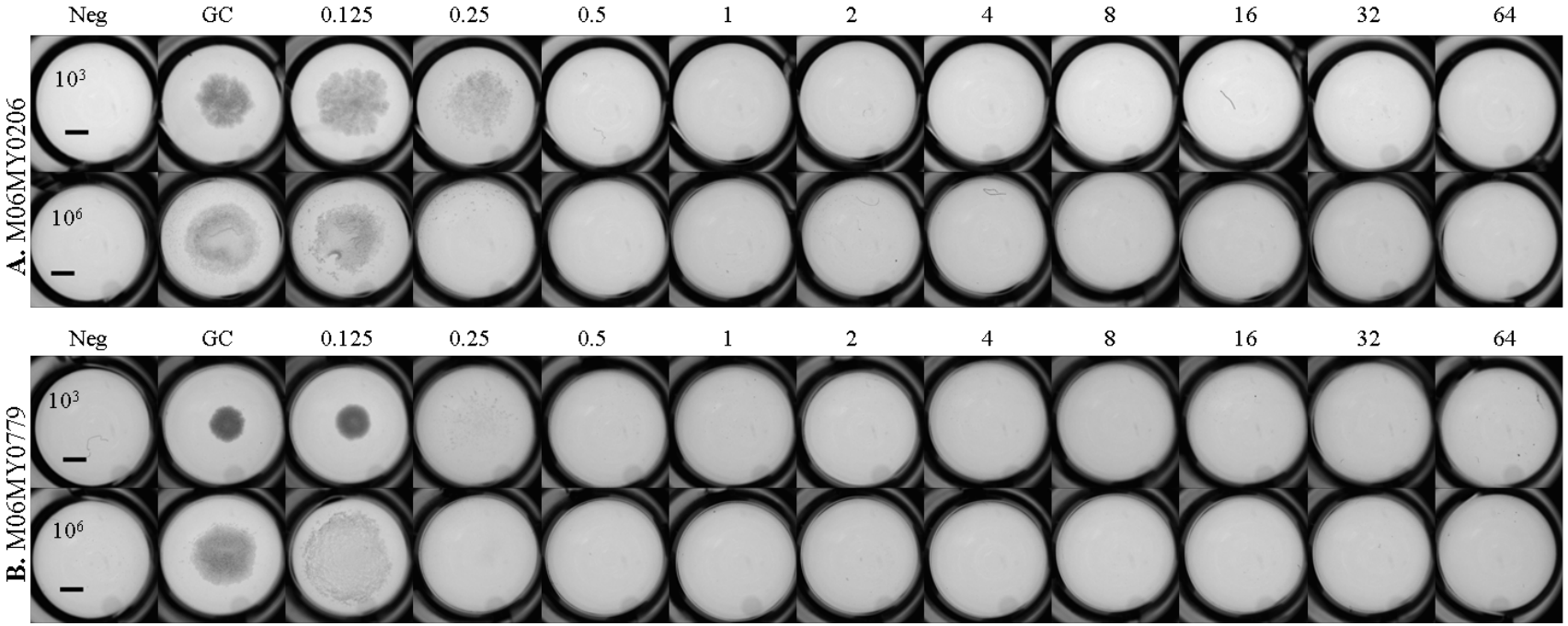
Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
R. M10MY2333
S. M10MY2222
T. M10MY2678

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
U. M10MY2273
V. M10MY2883
W. M10MY2797

X. M10MY2163
Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6

Appendix 2. Stereomicroscopy of *C. glabrata* microdilution growth patterns. Individual wells of 96-well microdilution plates containing *C. glabrata* exposed to CASPO for 24 hours were visualized and photographed under a stereomicroscope at 1.2X magnification. Each organism was tested using the standard 10^3 (upper image) and increased 10^6 inoculum (lower image). Numbers above images indicate CASPO concentrations (mg/L), the growth control (GC), and negative control (Neg).

Scale bars represent 1 mm.



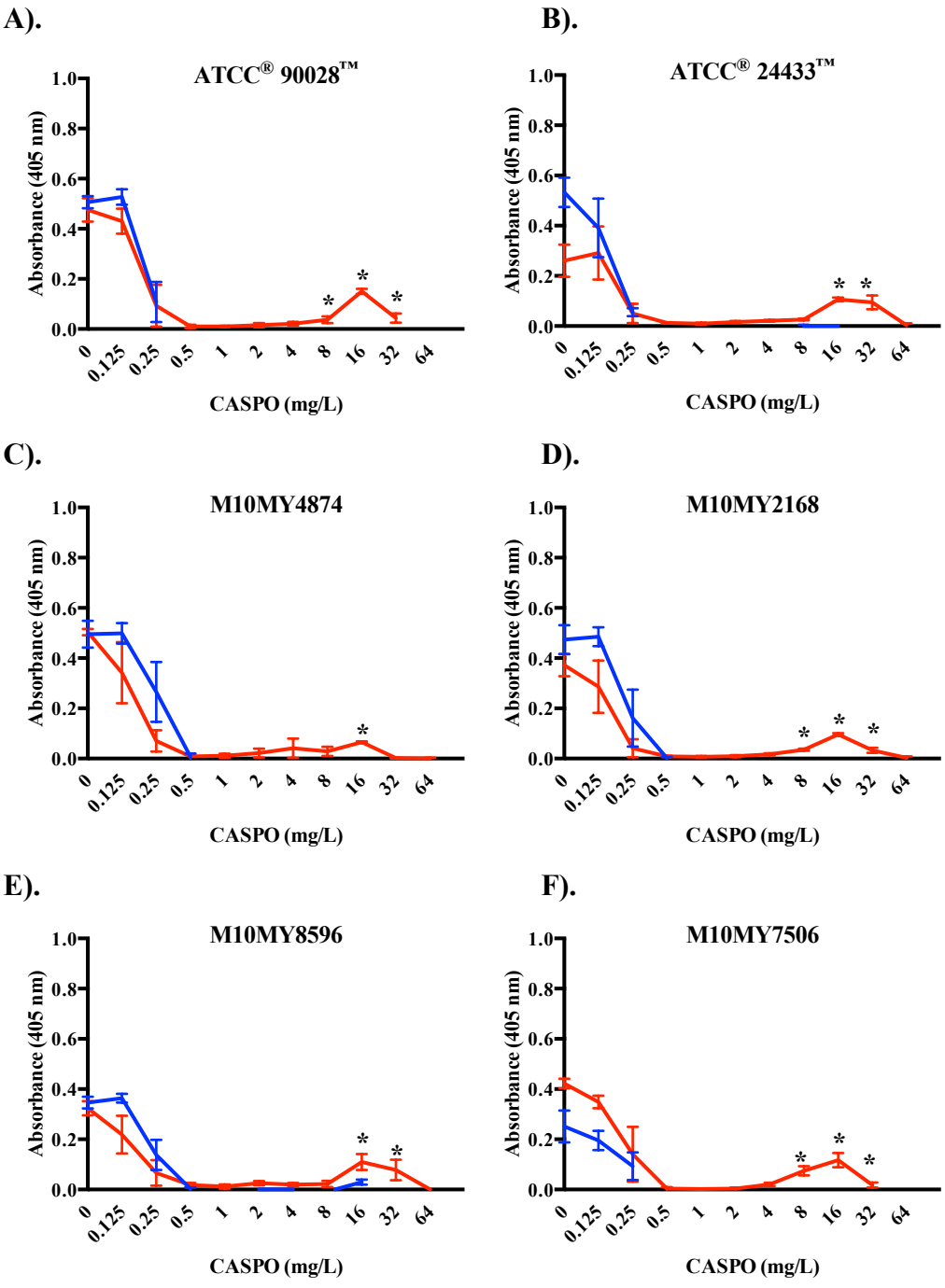
Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
C. M06MY1064
D. M06MY2192
E. M06MY2346

Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10^3
10^6
F. M06MY2882
G. M06MY3156
H. M06MY3590

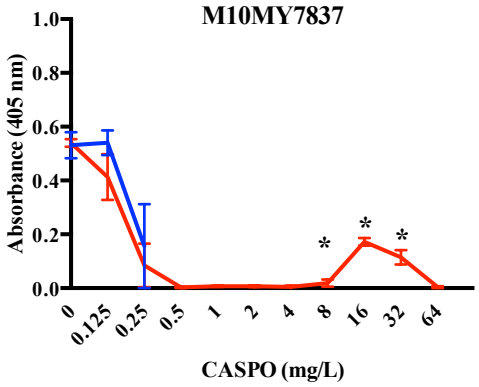
I. M06MY3593 **J.** M06MY4294 **K.** M10MY1407

L. ATCC® 15126™
Neg
GC
0.125
0.25
0.5
1
2
4
8
16
32
64
10³
10⁶

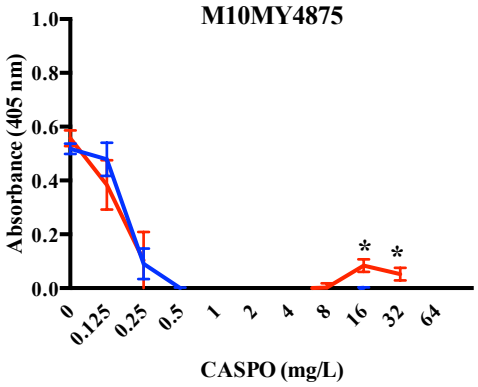
Appendix 3. *C. albicans* PG microdilution absorbance profiles: . Absorbance readings of 24 *C. albicans* **(A—X)** exposed to CASPO at 10^3 (blue) and 10^6 (red) starting inocula assessed by broth microdilution at 24 hours. Plotted absorbance readings (405nm) represent the average of three independent replicates after removal of baseline (media control). Error bars represent standard error of measure. *Statistically significant growth above the MIC ($p<0.05$)



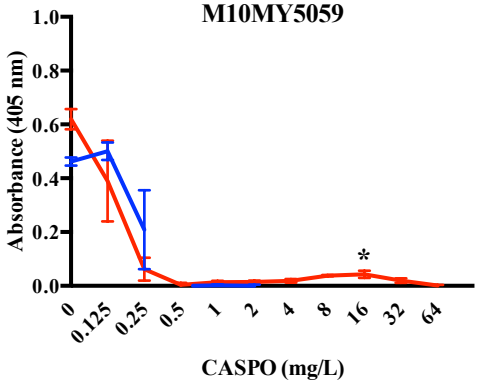
G).


M10MY7837
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*
*
*

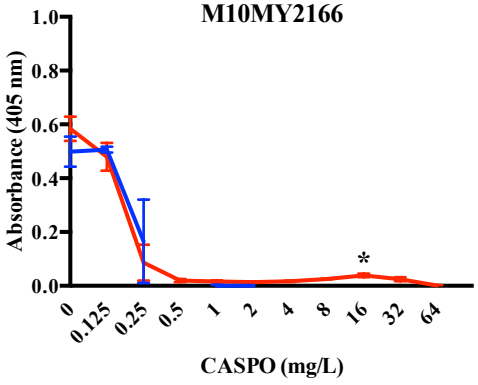
H).


M10MY4875
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*
*

I).


M10MY5059
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

J).


M10MY2166
Absorbance (405 nm)
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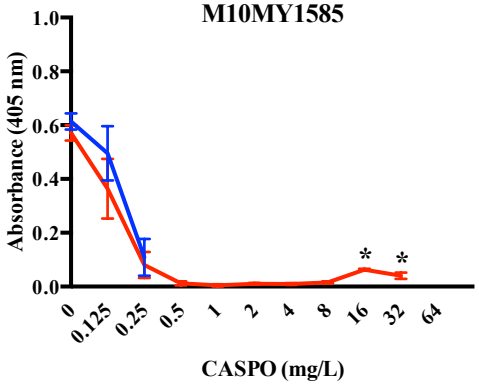
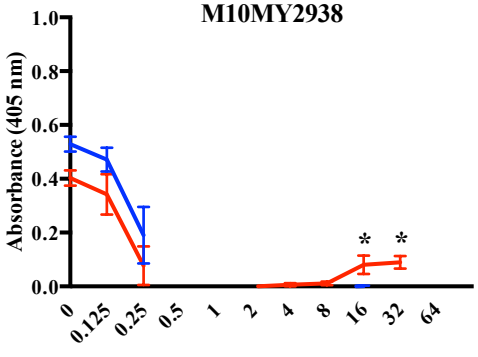
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

K).


M10MY1585
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*
*

L).


M10MY2938
Absorbance (405 nm)
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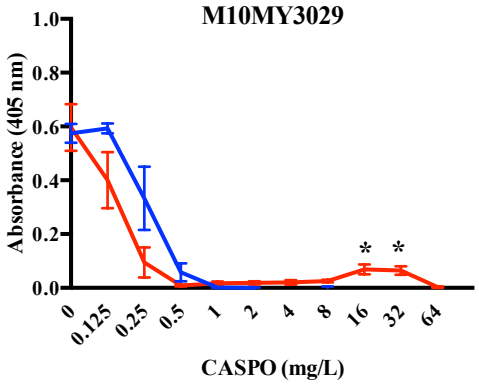
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
*
*

M).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Absorbance (405 nm)
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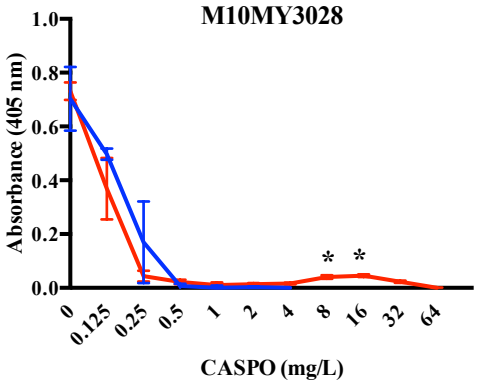
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
* *

N).


M10MY3028
Absorbance (405 nm)
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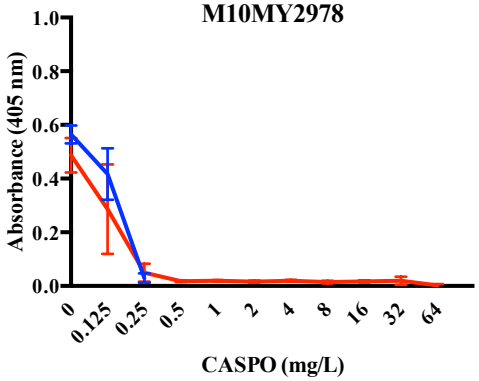
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
* *

O).


M10MY2978
Absorbance (405 nm)
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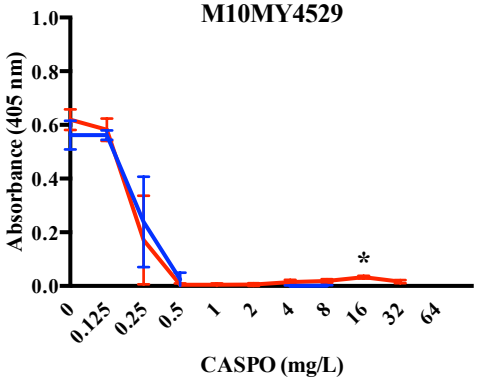
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

P).


M10MY4529
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
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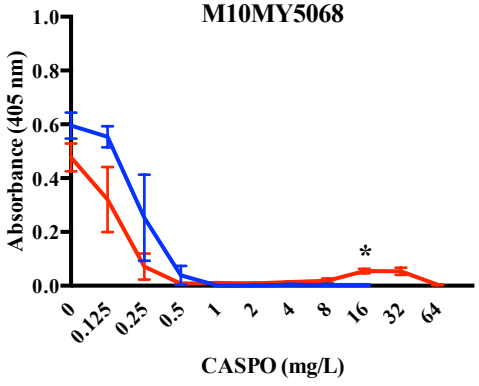
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

Q).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Absorbance (405 nm)
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0.8
0.6
0.4
0.2
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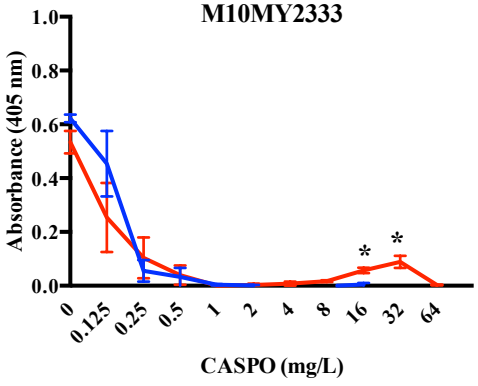
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

R).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Absorbance (405 nm)
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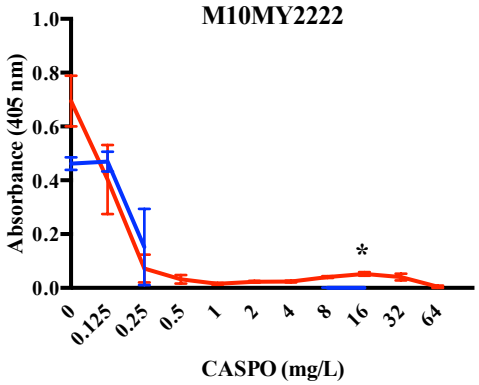
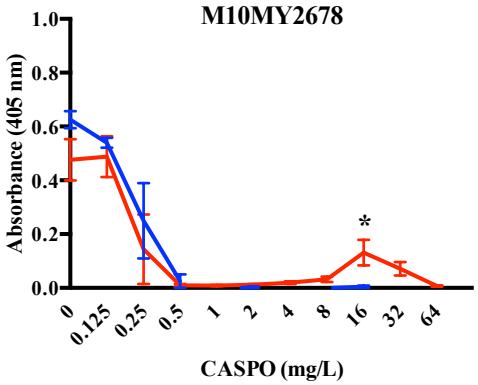
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
* *

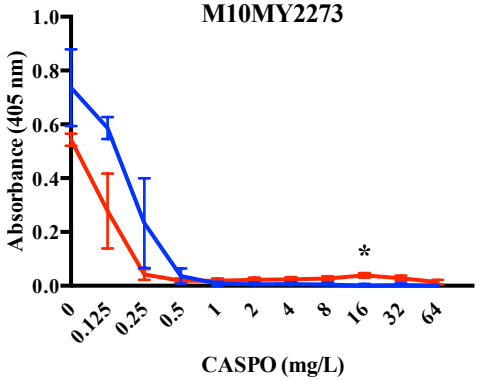
S).


M10MY2222
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

T).


M10MY2678
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

U).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Absorbance (405 nm)
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0.6
0.4
0.2
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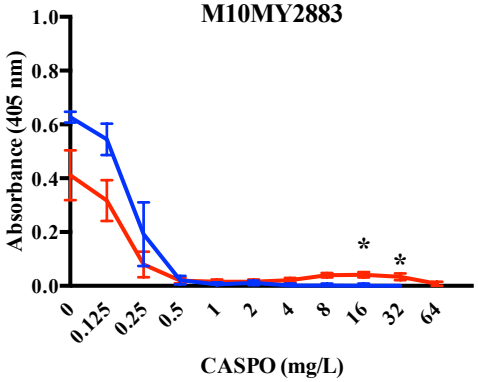
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

V).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Absorbance (405 nm)
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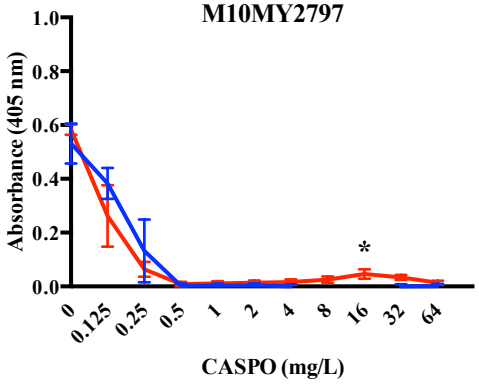
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*
*

W).


M10MY2797
Absorbance (405 nm)
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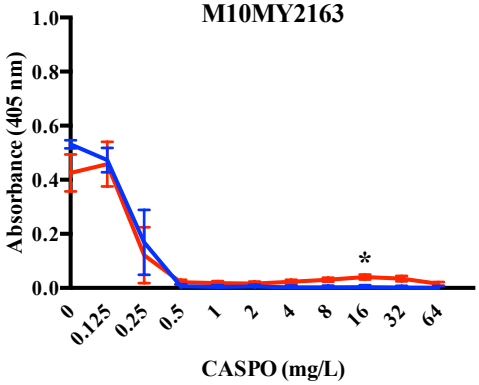
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

X).


M10MY2163
Absorbance (405 nm)
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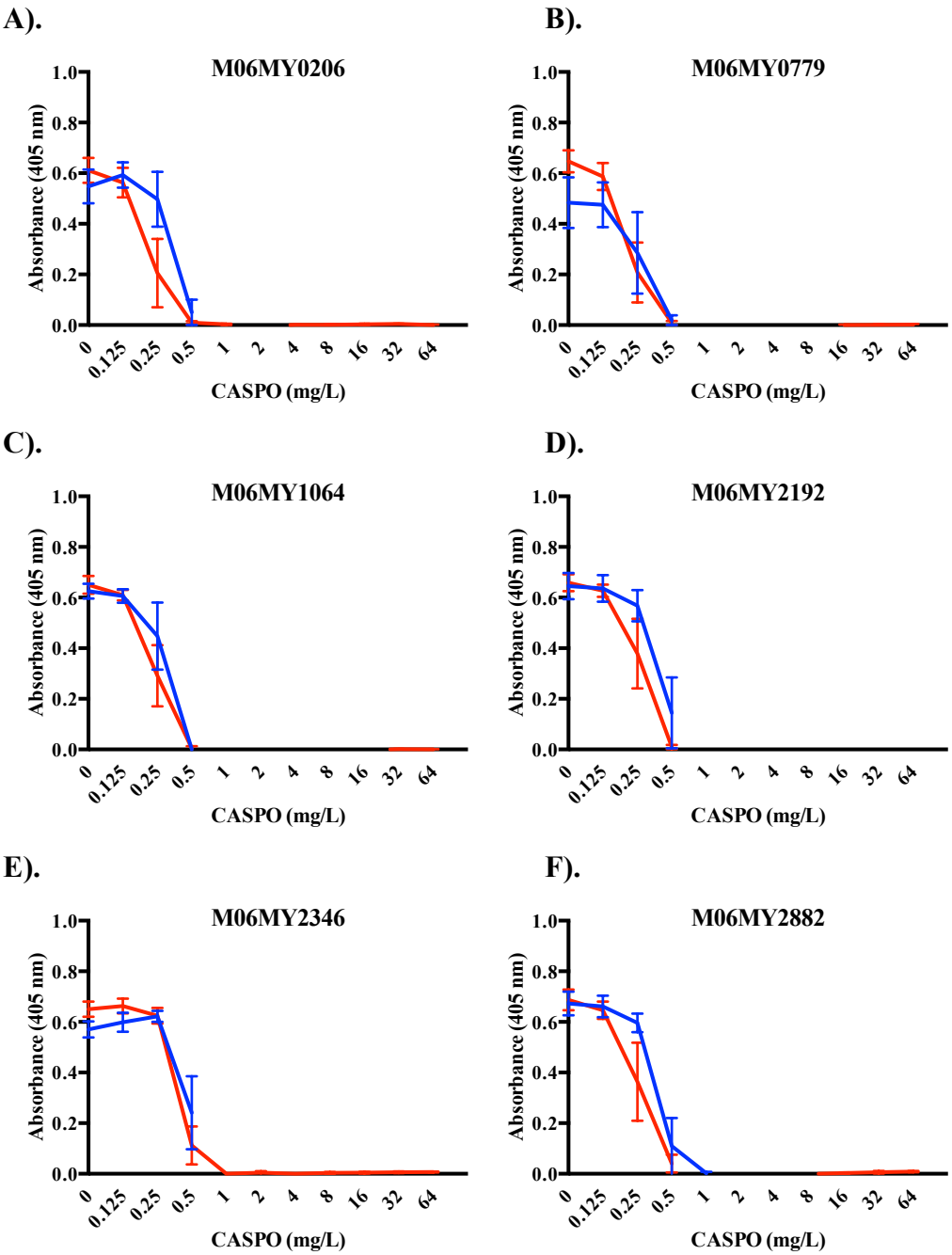
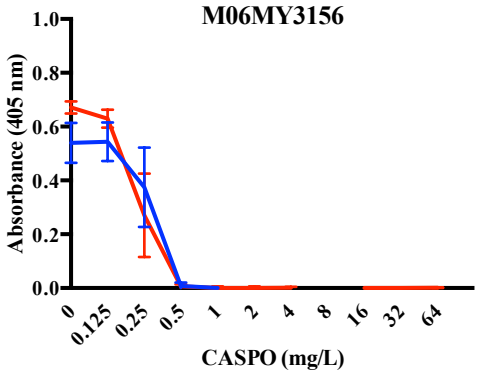
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)
*

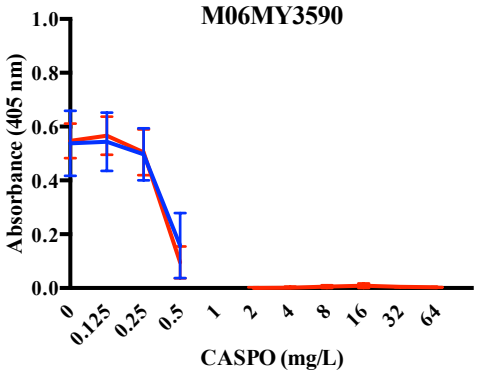
Appendix 4. *C. glabrata* PG microdilution absorbance profiles: Absorbance readings of 12 *C. glabrata* **(A—L)** exposed to CASPO at 10^3 (blue) and 10^6 (red) starting inocula assessed by broth microdilution at 24 hours. Plotted absorbance readings (405nm) represent the average of three independent replicates after removal of baseline (media control). Error bars represent standard error of measure. *Statistically significant growth above the MIC ($p<0.05$)



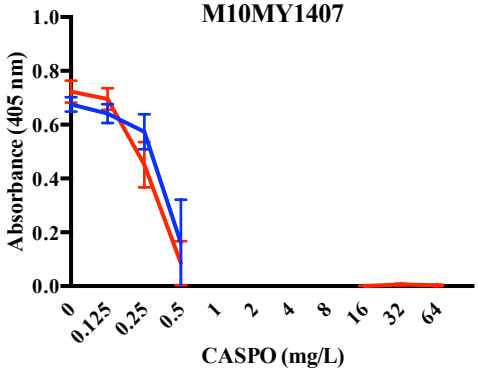
G).


M06MY3156
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

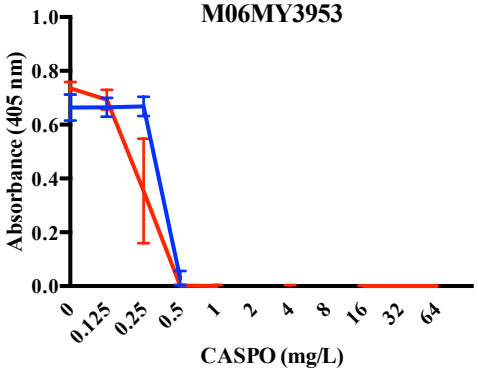
H).


M06MY3590
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

I).


M10MY1407
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

J).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Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
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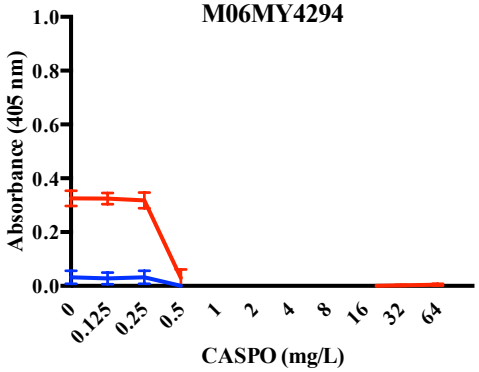
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

K).


M06MY4294
Absorbance (405 nm)
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0.8
0.6
0.4
0.2
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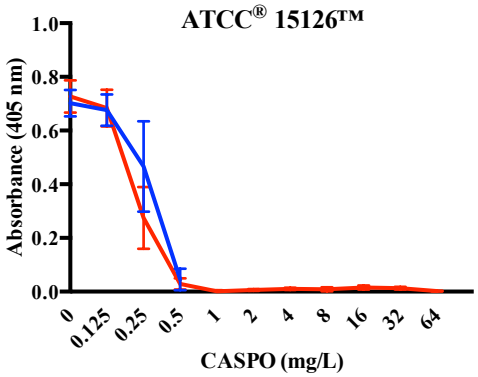
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)

L).


ATCC® 15126™
Absorbance (405 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
0.125
0.25
0.5
1
2
4
8
16
32
64
CASPO (mg/L)