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Genotypic and Phenotypic Characterization of *Candida albicans* Lebanese Hospital Isolates Resistant and Sensitive to Caspofungin

By

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Genotype and Phenotype Characterization of *Candida albicans* Lebanese Hospital Isolates Resistant and Sensitive to Caspofungin

Maggie Nazih Toutounji

Abstract

Candida albicans is both a commensal and a significant opportunistic fungal human pathogen, present in the digestive tract and the mouth as a part of the normal human microflora. Overgrowth of *C. albicans* can cause oral and vaginal candidiasis as well as a number of serious mucosal, and systemic life threatening infections. The antifungal drug caspofungin of the echinocandin family is the latest generation of antifungal drugs to be developed. It functions by inhibiting glucan synthase thus weakening the fungal cell wall leading to death. Recently reports of resistance to caspofungin have been reported mainly through mutations in the *FKS* encoded subunits of glucan synthase at Hot spot 1 (amino acids 641 to 649, FSTLSLRDP) and hot spot 2 (amino acids 1357 to 1364, DWIRRYTL). Our study aimed at sequencing both hot spots from 16 *C. albicans* Lebanese hospital isolates resistant and sensitive to caspofungin to determine whether mutations in these hotspots are present, and whether such mutations also impart resistance to our isolates. In addition we wanted to determine any relationship between resistance and pathogenicity related attributes such as virulence, adhesion, filamentation, resistance to cell wall disrupting agents such as Congo red, biofilm formation, and cell wall chitin deposition. Five isolates were found to contain mutations with the mutations restricted to resistant strains.

Within hotspot 1 substitution at positions S642, T643, L644, R647, and D648 were found, while within hotspot 2 substitutions at positions L1364, T1363, and R1360, W1358 and R1361 were identified. Interestingly some of the mutations found have not been previously documented. In addition strains that are resistant to caspofungin also showed increased resistance to Congo red but decreased biofilm formation and attenuated virulence in a mouse model of infection. Caspofungin sensitive strains showed decreased resistance to Congo red yet increased virulence and biofilm formation. All filamentous strains were adhesive but only some were virluent. Chitin content analysis showed that caspofungin resistant strains had elevated levels of chitin resulting in cell wall thickening that counters the effect of caspofungin, while sensitive strains showed decreased chitin content. Our results demonstrate an inverse correlation between resistance and virulence whereby resistance is due to thickening of the cell wall preventing the cell from gaining virulence attributes, while a more susceptible cell wall increases susceptibility to drugs but allows increased virulence.

Keywords: *Candida albicans*, Caspofungin, HS1, HS2, Chitin, Resistance, Virulence

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Chapter 1

Literature Review

1.1 Overview of *Candida albicans*

Candida albicans, a diploid fungus that grows as yeast and filamentous cells, is a widespread commensal and a significant opportunistic fungal human pathogen, present in the digestive tract and the mouth as part of the normal microflora in 80% of human population without causing harmful effects. Overgrowth of *C. albicans* however can cause oral and vaginal candidiasis as well as a number of serious mucosal and systemic infections (Mayer, Wilson, & Hube, 2013). *C. albicans*-found only in warm blooded animals-has the ability to infect all major organs and tissues in the human body with no exceptions (Mora, Tittensor, Adl, Simpson, & Worm, 2011). *C. albicans* infections termed candidiasis is the fourth leading cause of hospital acquired infections with a crude death rate of 50% (Gow, van de Veerdonk, Brown, & Netea, 2011). Individuals that are susceptible to *Candida* infections are mainly immunocompromised individuals such as AIDS patients, cancer patients undergoing chemotherapy, and organ or bone marrow transplant recipients. In addition the usage of broad spectrum antibacterials favors fungal growth since the fungus no longer has to compete with bacteria for space and nourishment (M. A. Pfaller & Diekema, 2007)

C. albicans makes up 75% of total population of *Candida* species in the oral cavity (Walker et al., 2009). Usually the colonization remains benign in healthy individuals but individuals with weak immune system might suffer from recurring infections in the oral cavity resulting in oral candidiasis (OC) (Pappas et al., 2009). These infections caused by *C. albicans* have a direct effect on the esophagus and/or

oropharynx of an individual with a weak immune system (Hurley & De Louvois, 1979).

Vulvovaginal candidiasis (VVC) by *C. albicans* affects 75% of all women at least once in their lifetime (Sobel et al., 1998). Some women (5-8%) suffer reoccurrence of VVC several times per year (Fidel et al., 2004). Some factors that put women at a higher risk for VVC are the use of antibiotics, pregnancy, hormone therapy, oral contraception, and diabetes mellitus (Perlroth, Choi, & Spellberg, 2007)

Systemic candidiasis has high crude death rate even when antifungal therapy is used (Perlroth et al., 2007). Systemic candidiasis takes place when an individual has neutropenia and gastrointestinal mucosa damage resulting in the spread of *Candida* to other parts of the body (Spellberg & Lipsky, 2012). Nosocomial infection is particularly worrisome in the case of *C. albicans* as the fungus can obtain direct access to the bloodstream, through central venous catheters, trauma or gastrointestinal surgery (Berman & Sudbery, 2002).

C. albicans, a polymorphic, generally diploid organism, has a 16-Mb (haploid) genome that is arranged in 8 chromosomes (Wright & Archard, 2012). The genome of *C. albicans* has high degree of plasticity that helps it adapt and combat the stresses posed by the organism it is colonizing. This plasticity displays itself through an increase in genetic diversity and lack of chromosomal stability by various structural chromosomal rearrangements such as chromosome length polymorphisms (contraction/expansion of repeats), reciprocal translocations, chromosome deletions, loss of heterozygosity, and aneuploidies including trisomy's and monosomies of individual chromosomes (P. Sudbery, Gow, & Berman, 2004).

These genomic changes lead to phenotypic alterations allowing adaptation in a host (Butler et al., 2009). It is interesting to note that *C. albicans* does not possess a meiotic

cycle even though orthologues of mating genes are present and the organism can mate. Diploids mate generating tetraploids that shed chromosomes at random reverting to a diploid form. This parasexual cycle has many advantages over a classical sexual pathway in that it is less predictable generating more diversity allowing for survival, and avoiding the process of sporulation associated with meiosis, as spores are highly antigenic and easily recognized by the host immune system (B. Magee & Magee, 2000; R. J. Bennett & Johnson, 2005; Richard J. Bennett & Johnson, 2003; *Fungal Genomics*, 2004; Tzung et al., 2001).

1.2 *C. albicans* Mechanisms of Pathogenicity

As can be seen in Figure 1 a wide range of virulence factors and fitness attributes allow *C. albicans* to infect hosts living in different environments (Mayer et al., 2013). These virulence attributes are: morphological changes between two main forms: yeast and hyphal, the expression of adhesins and invasins on the cell surface, biofilm formation thigmotropsin, and the secretion of hydrolytic enzymes, while the fitness attributes include rapid adaptation to fluctuations in environmental pH, metabolic flexibility, powerful nutrient acquisition systems and strong stress responses (Mayer et al., 2013).

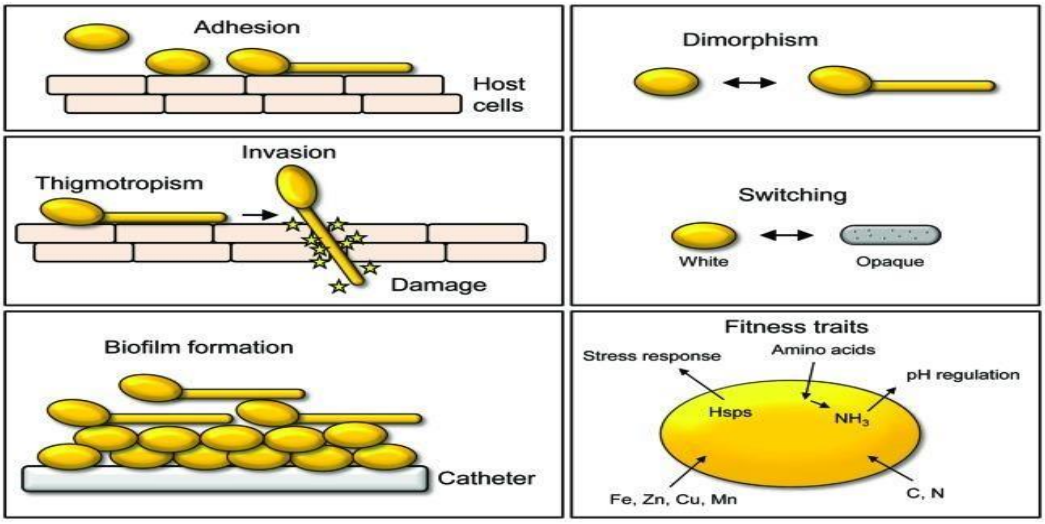


Figure 1. *C. albicans* pathogenicity mechanisms. Infection begins with the fungal attachment to cell surfaces of the host through adhesins. Transition of yeast cells to hyphae occurs only upon contact, then fungal-induced endocytosis takes place with the help of thigmotropism and invasins. Biofilms containing both a basal yeast form and an upper hyphal form start forming upon the attachment of yeast cells to abiotic surfaces. Biofilm formation requires white-opaque switching. Many fitness traits were developed by *C. albicans* allowing it to evade host cells, such traits include: pH and stress responses proteins, and nutrient and metal acquisition proteins (Mayer et al., 2013).

1.2.1 Morphological forms of *C. albicans*

C. albicans is a polymorphic fungus that grows either as budding single celled ovoid yeast, an elongated ellipsoid cells with constrictions at the septa (pseudohyphae) or as parallel-walled true hyphae that originate from unconstricted germ tubes, as seen in Figure 2. This latter form is mostly associates with systemic infection. However interplay and interchange between both the true hyphal and yeast form is important for successful infection since the yeast form allows for clonal expansion while the filamentous form is needed for virulence. Cells locked in either form are avirulent. (P. Sudbery et al., 2004). Dimorphism is the change between both growth forms (yeast and hyphal), knowing that both growth forms are important for pathogenicity (Saville, Lazzell, Monteagudo, & Lopez-Ribot, 2003). The hyphal form is more invasive than the yeast form (P. Sudbery et al., 2004).While the presence of smaller yeast forms is said to be associated with dissemination (Lo et al., 1997). Mutants that cannot form hyphae under in vitro conditions are said to be weak or attenuated in virulence (Zheng, Wang, & Wang, 2004).

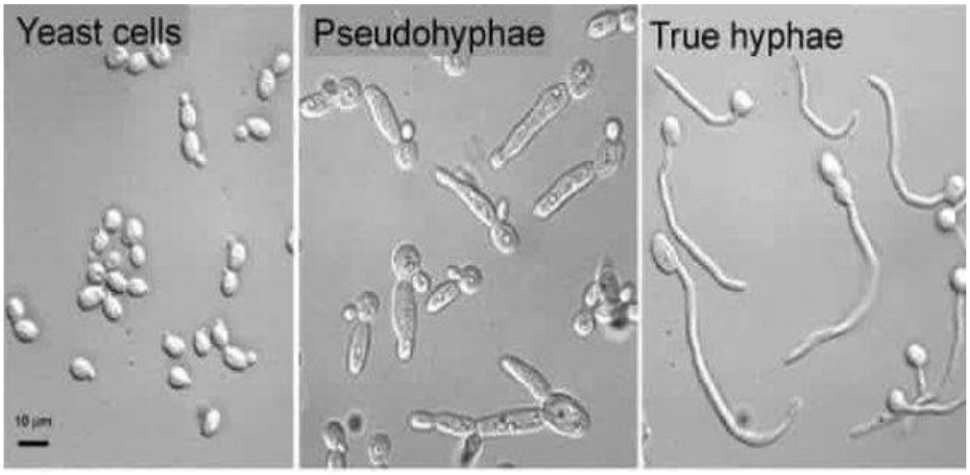


Figure 2. DIC images of the three *C. albicans* cell forms yeast, pseudohyphea, and true hyphae. Yeasts cells are round budding cells, the elongated cells are hyphal forms that are arranged in filaments, and pseudohyphea cells are present during the transition phase between both morphologies (Sudbery et al. 2004).

White and opaque cells (as seen in Figure 3) are also different morphologies that a yeast cell can take. White cells are more virulent in a systemic infection model while opaque cells have adapted to superficial infections (Soll, 2009).

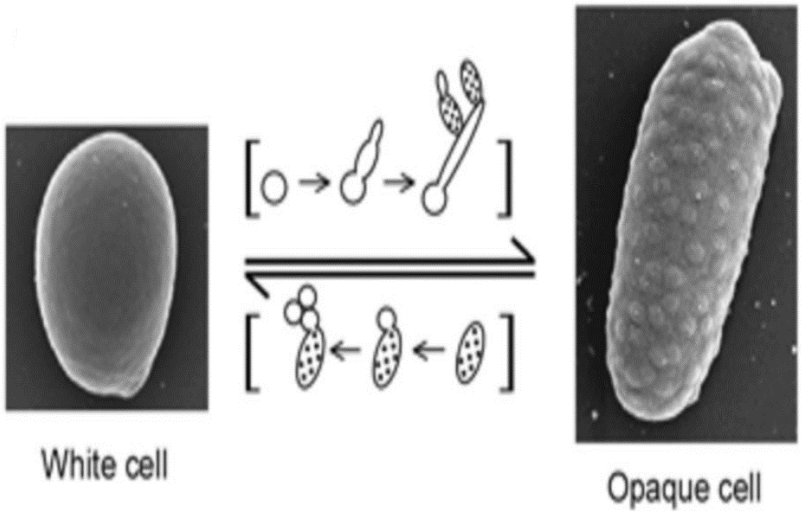


Figure 3. White and opaque cellular phenotype. The white cell has a smooth, round to ovoid surface while the opaque cell is larger in size and has cell wall pimples. A white daughter cell is formed during the switch from opaque to white, and a hypha-like transition phenotype appear during the white to opaque switch (Bergen, Voss, & Soll, 1990).

In addition, chlamydospores, which are thick-walled spore-like structures (Staib & Morschhäuser, 2007) are found on the tip of hyphal cells. They are only produced under extremely harsh conditions like hypoxia and starvation (Whiteway & Bachewich, 2007). The role of chlamydospores in vivo is unclear as they have not been observed in patient samples and they lack the ability to germinate as is the case with bacterial spores (Soll, 2009).

C. albicans morphology is affected by the environment (Mayer et al., 2013). For example, at low pH (< 6) *C. albicans* cells grow in the yeast form, while at physiological pH and higher (> 7) *C. albicans* cells grow in the hyphal form (P. E. Sudbery, 2011). The formation of hyphae is induced by starvation, the presence of serum or N-acetylglucosamine, physiological temperature, and physiological CO_2 concentrations (Albuquerque & Casadevall, 2012).

Morphogenesis, a mechanism in which microbes communicate, is controlled by quorum sensing, which is a system of stimulation and response affected by population density (Hornby et al., 2001). In *C. albicans*, the most important quorum sensing molecules are: farnesol, tyrosol, and dodecanol (Jacobsen et al., 2012). Due to quorum sensing, high cell densities (> 10^7 cells ml^{-1}) induce yeast growth, while low cell densities (< 10^7 cells ml^{-1}) induce hyphae growth (Mayer et al., 2013). The formation of hypha is associated with the expression of genes encoding virulence factors, these virulence factors are not involved in hyphal formation in itself (Mayer et al., 2013). Some of the hypha-associated proteins are: the hyphal wall protein Hwp1, the agglutinin-like sequence protein Als3, the secreted aspartic proteases Sap4, Sap5 and Sap6, and the hypha-associated proteins Ece1 and Hyr1 (Mayer et al., 2013). Deletion of *HGC1*, encoding a hypha-specific G1 cyclin-related protein, will not allow cells to produce hyphae however it will grow normally in the yeast form. The *HGC1*Δ/Δ

mutant cells express at least four hypha-associated genes (*HWP1*, *ECE1*, *HYR1* and *ALS3*) (Zheng et al., 2004).

1.2.2 Expression of adhesins and invasins

A group of proteins termed adhesins are present in *C. albicans*, these proteins allow the adherence to other *C. albicans* cells, other microorganisms, abiotic surfaces, and to host cell (Verstrepen & Klis, 2006). Adhesion is the first step in the infection process and cements the pathogen to its host. Most adhesins are cell wall associated proteins.

In *C. albicans* the major family of adhesins are the agglutinin-like sequence (ALS) proteins a family made up of eight members (Als1–7 and Als9) (Mayer et al., 2013). The *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoprotein (Mayer et al., 2013). From the eight Als proteins, Als3, which is the hypha-associated adhesin, is the main adhesin (Murciano et al., 2012). The expression of *ALS3* gene is upregulated during: oral epithelial cells infection in vitro and vaginal infection in vivo (Murciano et al., 2012;(Nobile et al., 2008).

Another adhesion protein is Hwp1, which is a hypha-associated GPI-linked protein (Cheng et al., 2005). Hwp1 might link *C. albicans* hyphae to host cells since it acts as a substrate for mammalian transglutaminases. Hwp1 and Als3 are also associated with biofilm formation (Nobile et al., 2008).

Adhesion can also be achieved with the help of morphology-independent protein. The GPI-linked proteins (Eap1, Iff4 and Ecm33), non-covalent wall-associated proteins (Mp65, a putative β-glucanase, and Phr1, a β-1,3 glucanosyl transferase), cell-surface associated proteases (Sap9 and Sap10), and the integrin-like surface protein (Int1), all are morphology independent proteins that are needed for adhesion (Naglik, Moyes, Wächtler, & Hube, 2011).

To invade host cells, *C. albicans* uses two different mechanisms: induced endocytosis and active penetration (Naglik et al., 2011; Zhu & Filler, 2010).

Induced endocytosis is characterized by the binding to host ligand that is induced by invasins, specialized proteins that are expressed on the cell surface, such as the binding of E-cadherin on epithelial cells by a clathrin-dependent mechanism (Phan et al., 2007), and N-cadherin on endothelial cells (Phan et al., 2007), thus the phagocytosis of the fungal cell into the host cell takes place. Two invasins have been identified Als3 and Ssa1 (member of heat shock protein 70 family)(Murciano et al., 2012; Phan et al., 2007). Mutations in these 2 genes might cause reduction in: epithelial adherence, invasion reduction, and virulence of oropharyngeal candidiasis (Naglik et al., 2011).

Active penetration requires viable *C. albicans* hyphae depending on fungal adhesion and physical forces(Wächtler, Wilson, Haedicke, Dalle, & Hube, 2011). Secreted aspartic proteases (Saps) are said to be a part of active penetration while Lipases and phospholipases are not (Sun et al., 2010; Wächtler et al., 2011).

1.2.3 Biofilm formation

The cell surface of *C. albicans* that allows for biofilm formation is a complex, well studied structure. It consists of an innermost chitin thick polysaccharide layer necessary for rigidity followed by a layer of β-1,3 glucan, and an outermost layer of β-1,6 glucan that is involved in anchorage of most cell surface glycoproteins.

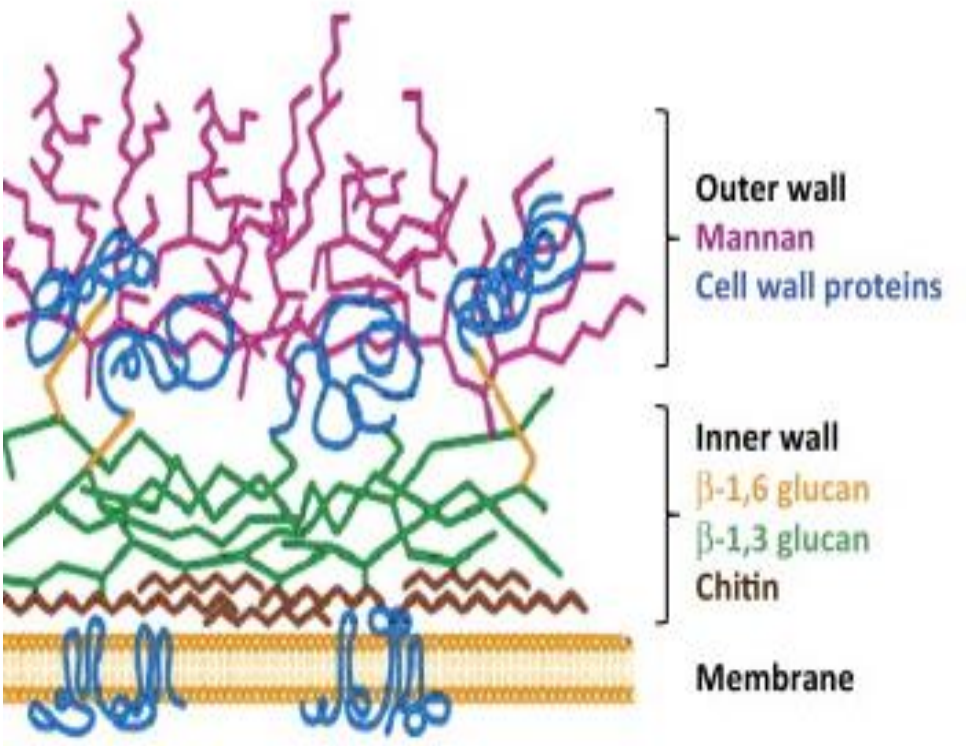


Figure 4. Schematic representation of the cell wall components organization of *C. albicans*. Mannan sugars and proteins on the outer wall attach to the inner wall which is made up of β-1,6-glucans, β-1,3-glucans, and chitin (Brown, D. Brown, Netea, & Gow, 2014).

The formation of biofilms on abiotic or biotic surfaces represents a virulence factor of *C. albicans.* The most common substrates are catheters, dentures (abiotic) and mucosal cell surfaces (biotic) (Fanning & Mitchell, 2012).

As seen in Figure 5, the process of biofilms formation starts with the attachment of yeast cells to the substrate, multiplication of these yeast cells at the base while cells on the upper part undergo morphological switching, elongate and produce hyphal cells. Biofilm cells secrete extracellular matrix material which is assembled into a mesh like network of cells, (Finkel & Mitchell, 2011).

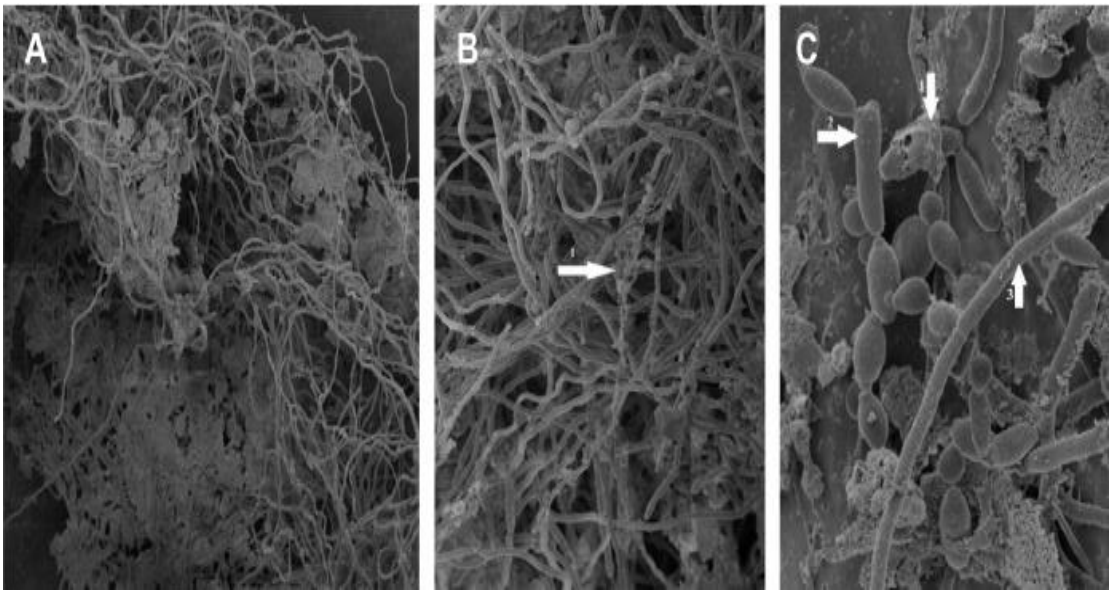


Figure 5. A scanning electron microscope of the different morphological forms of *C. albicans* biofilm. The *C. albicans* biofilm is formed in the inner layer of a central venous catheter and shown in different magnifications. (A) X450, (B) X1100, and (C) 4500. The arrow in (B) refers to the extracellular matrix. In (C) the first arrow refers to pseudohyphal form, the second refers to the yeast form, and the third refers to the true hyphal form (S.M.L et al., 2013).

The complex architecture of biofilms, the matrix of the biofilm, the expression of drug efflux pumps in an increased manner, and metabolic plasticity are factors that result in an increase resistance of *C. albicans* to antifungal agents (Fanning & Mitchell, 2012).

Biofilm regulation is a complex process with several transcription factors been known to control its formation such as Bcr1, Tec1, and Efg1 (Fanning & Mitchell, 2012). In addition there are reports of novel factors that are also involved in biofilm production such as Ndt80, Rob1 and Brg1. Deletion of any of these regulators results in defective biofilm formation (Nobile et al., 2012). In addition many factors control the production of extracellular matrix. The major component of biofilm matrix is β-1,3 glucan that is negatively regulated by the zinc-responsive transcription Zap1 (Nobile et al., 2009). While β-1,3 glucan production is positively regulated by Glucoamylases

(Gca1 and Gca2), glucan transferases (Bgl2 and Phr1) and the exo-glucanase, Xog1f (Murciano et al., 2012; Phan et al., 2007).

Biofilms are more susceptible to the antifungal agent, fluconazole, in both vivo and vitro when Bgl2, Phr1, and Xog1 are not present (Taff et al., 2012). *C. albicans* biofilms are resistant to killing by neutrophils and do not trigger production of reactive oxygen species (ROS) therefore β-glucans in the extracellular matrix protect *C. albicans* from these attacks (Xie et al., 2012).

1.2.4 Secretion of hydrolase

C. albicans hyphae secrete hydrolases, which are needed for the active penetration of fungus into host cells (Wächtler et al., 2012). Extracellular nutrient acquisition is enhanced by hydrolases (Naglik, Challacombe, & Hube, 2003). The three different classes of secreted hydrolases by *C. albicans* are: proteases, phospholipases and lipases (Naglik et al., 2003)

The family of secreted aspartic proteases (Saps) is the most studied of the hydrolases and consists of a family of ten members, Sap1–10. Sap1–8 are secreted and released to the surrounding medium, while Sap9 and Sap10 remain bound to the cell-surface (Naglik et al., 2003).

Sap1–3 has been shown to be required to destroy the reconstituted human epithelium (RHE) in vitro ((Schaller, Schäfer, Korting, & Hube, 1998). The invasion into RHE does not require the presence of Saps and that Sap1–6 are not essential for virulence in the case of disseminated candidiasis (Correia et al., 2010).

The family of phospholipases is made up of four different classes (A, B, C and D) (Niewerth & Korting, n.d.). Class B is made up of five members (PLB1–5) that are extracellular and might be associated with pathogenicity through interference with the

host membrane (Mavor, Thewes, & Hube, 2005). The family of lipases is made up of 10 members (LIP1–10) (Naglik et al., 2008).

1.2.5 Contact sensing and direct growth via thigmotropism

Contact sensing is an important environmental factor that initiates hypha and biofilms formation in *C. albicans*. Due to contact with a surface, yeast cells switch to hyphal growth and biofilms formation takes place (Kumamoto, 2008). Hyphae can invade into the substratum when substrates such as agar or mucosal surfaces are present (Kumamoto, 2008). When surfaces have different topologies (such as the presence of ridges) directional hyphal growth (thigmotropism) may occur (Brand et al., 2007).

The extracellular calcium uptake through the calcium channels regulates the thigmotropism of *C. albicans* hyphae (Brand et al., 2007). Other mechanisms involve the polarisome Rsr1/Bud1-GTPase module (Brand & Gow, 2009). Correct sensing and response to both abiotic (biofilm formation) and biotic (invasion) surfaces is important for pathogenicity (Brand & Gow, 2009).

1.2.6 pH-sensing and regulation

C. albicans must be able to adapt to pH changes because environmental pH changes regularly ranging from slightly alkaline to acidic (Davis, 2009). The human blood and tissues pH is slightly alkaline (pH 7.4), while the digestive tract pH ranges from very acidic (pH 2) to more alkaline (pH 8), and the vaginas pH is around pH 4 (Davis, 2009).

pH-sensitive proteins and nutrient are affected negatively when *C. albicans* is under stress, this stress is caused when *C. albicans* is present in a neutral to alkaline pH (Davis, 2009).

The proteins that are important for the adaptation of *C. albicans* to pH changes are the two cell wall β-glycosidases Phr1 (expressed in neutral-alkaline pH thus

required for systemic infections) and Phr2 (expressed in acidic pH thus required for vaginal infections) (Mühlschlegel & Fonzi, 1997).

pH-sensing takes place through the Rim101 signal transduction pathway where the environmental pH is estimated by the Dfg16 and Rim21 (plasma membrane receptors) (Davis, 2009). Once these receptors are activated a signaling cascade is induced leading to the activation of transcription factor Rim101 that will enter the nucleus and cause pH-dependent response (Davis, 2009).

The virulence of *C. albicans* is affected by the Rim101 and the signal transduction pathway. *C. albicans* can modulate extracellular pH and induce hypha formation when the pH is alkaline and when there is nutrient starvation, knowing that hypha formation is a virulence factor (Mayer et al., 2013)

1.2.7 Metabolic flexibility and metal acquisition

Survival and growth of all microorganisms depends on nutrition. Metabolic adaptability to various nutritional sources and environmental alterations is important for pathogenic organisms during infections(Brock, 2009; Otzen, Bardl, Jacobsen, Nett, & Brock, 2014). *C. albicans* is normally found in the gastrointestinal tract where the concentration of nutrient is relatively high but the growth of *C. albicans* is kept in check by the presence of other microbes in the GIT as part of the normal flora (Brock, 2009).

When dissemination of *C. albicans* into the bloodstream takes place, the fungus has access to glucose, a preferred source of energy for most organisms. However phagocytic cells in the blood (macrophages and neutrophils) phagocytose *C. albicans* changing drastically the nutrient source available (Brock, 2009). The environment switches to a nutrient starvation one and forcing the fungus to switch from glycolysis to gluconeogenesis with the conversion of amino acids and simple two carbon lipid compounds within macrophages into glucose allowing for energy generation, hyphal

production and lysis of the macrophage (Lorenz, Bender, & Fink, 2004). Therefore metabolic plasticity is essential for *C. albicans* to adapt to such an environment. (Lorenz et al., 2004).

Metals such as iron, zinc, manganese, and copper are important for the survival of all living organisms because they act as cofactors that regulate the function of many proteins and enzymes (Mayer et al., 2013). Most metals available from the host such as iron are usually hidden from the fungus as they are part of a ferritin complex that is not easily accessible. *Candida* has evolved an iron uptake mechanism utilizing the adhesion Als3 that in this case can bind iron from ferritin and render it accessible to the fungus (Hood & Skaar, 2012).

1.2.8 Response to environmental stress

As a pathogen in warm blooded organisms *C. albicans* is in a constant battle with the host and with the host immune system. This environmental host derived stress takes on many forms: oxidative and nitrosative stress produced mainly by phagocytic cells of the immune system (Mayer et al., 2013), heat shock stress in response to temperature changes, and osmotic stress in response to varying concentration of solutes. *C. albicans* has evolved an arsenal of proteins and pathways to deal with such stresses. Mutants lacking genes encoding stress response regulators are usually attenuated in virulence (Brown et al., 2012).

Oxidative stress response is promoted by reactive oxygen species (ROS), such as peroxides, superoxide anions, and hydroxyl radicals (Brown et al., 2012) generated by macrophages. Detoxification of ROS in *C. albicans* is done by Catalase Cta1 and superoxide dismutases, Sod1 and Sod5 (Martchenko, Alarco, Harcus, & Whiteway, 2004). On the other hand nitrosative stress response in phagocytosed *C. albicans* cells is promoted by reactive nitrogen species (RNS) that are produced by neutrophils, this

response promotes flavohemoglobin-related protein Yhb1 a protein involved in detoxification of RNS (Hromatka, Noble, & Johnson, 2005).

Heat shock proteins are activated when a sudden increase in temperature is sensed by an organism. These proteins act as molecular chaperones preventing protein unfolding and aggregation (Mayer et al., 2013). The sugar trehalose can also act as a chemical chaperone that stabilizes proteins that might unfold, it accumulates in *C. albicans* when thermal stress occur (Brown et al., 2012). It is noteworthy to mention that as a pathogen of warm blooded hosts *C. albicans* does not face major changes in temperature. Accordingly many of the Hsp have been transcriptionally rewired and are involved in virulence attributes instead. Six major Hsps have been identified in *C. albicans*: Hsp104, Hsp90, Hsp78, two Hsp70 proteins (Ssa1 and Ssa2), and Hsp60. Hsp needed for proper biofilm formation is encoded by *HSP104* (Fiori et al., 2012). Hsp90 is a major Hsp in *C. albicans* and controls drug resistance, morphogenesis, biofilm formation and virulence. An uncharacterized Hsp that is transcriptionally upregulated in response to phagocytosis by macrophages is encoded by *HSP78* (Shapiro et al., 2009; Singh et al., 2009)

On the cell surface, the two *C. albicans* Hsp70 family members, Ssa1 and Ssa2 (stress-70 subfamily A), are expressed and function as receptors for antimicrobial peptides (e.g., Ssa2 binds histatin 5) (Li, Sun, Okamoto-Shibayama, & Edgerton, 2006; Sun et al., 2010). Ssa1 also acts an invasin (Sun et al., 2012). Lastly, a putative mitochondrial Hsp of unknown function is encoded by *HSP60* (Leach, Stead, Argo, & Brown, 2011).

The transcription factor heat shock factor 1(Hsf1) controls the expression of Hsps (Sorger & Pelham, 1988). Transcription of Hsp-encoding genes via binding to heat shock elements (HSEs) in their promoters is induced by Hsf1 that is

phosphorylated in response to heat stress. *C. albicans* Hsf1 is vital for viability and a mutant that is uncapable of activating Hsf1, displays attenuated virulence in systemic candidiasis (Mayer et al., 2013).

The osmotic stress response in *C. albicans* is regulated by the transcriptional activator Hog1. Hog1 is a MAPK protein that is activated by Ssk2 and Pbs2 resulting in intracellular accumulation of glycerol. The accumulation of glycerol decreases water concentration inside the cell and hence prevents water loss through osmosis. Interestingly Hog1 is also thought to play a role in oxidative stress response (Brown el at., 2012).

1.3 Candidiasis Management

There are many risk factors that are associated with a high risk of fungal infection such as the use of broad-spectrum antibacterial agents, use of central venous catheters in hospitals resulting in nosocomial infections, neutropenia, use of implantable prosthetic devices, and use of immunosuppressive agents (Pappas et al., 2009; Tortorano et al., 2004). *Candida* infections are on the rise mainly due to the increase in use of antifungal agents as prophylactics and the limited number of antifungal agents available for treatment. As a eukaryote fungi share a relatively high rate of gene orthology and a high rate of conserved pathways with humans that limits the number of structures and mechanisms that can be targeted in the fungus specifically without harming the human host. One structure that differs significantly between fungi and higher eukaryotes is the cell surface. Fungal cell walls rely on ergosterol as the main sterol for fluidity and rigidity while humans have cholesterol. In addition fungi have a cell wall which is lacking in humans (Rex et al., 2003).

As can be seen in Figure 6 there are four major classes of systemic antifungal agents currently used for the treatment of Candidiasis. They are: the Amphotericin polyenes (AmB-d, L-AmB, AmB lipid complex [ABLC], and AmB colloidal dispersion [ABCD]), the triazoles (fluconazole, itraconazole, voriconazole, and posaconazole), the echinocandins (caspofungin, anidulafungin, and micafungin), and flucytosine (Pappas et al., 2009).

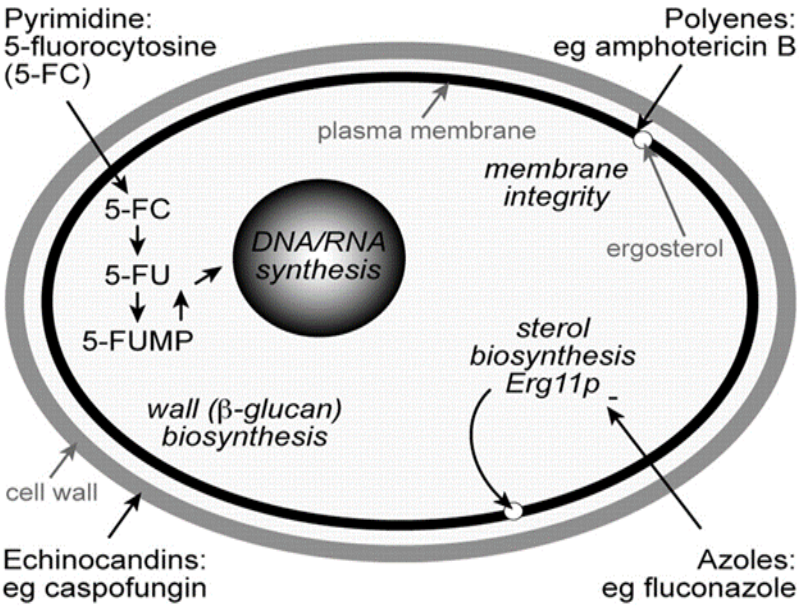


Figure 6. Antifungal drugs and their *C. albicans* targets. Echinocandins target the cell wall, azole targets sterol biosynthesis, polyenes target the membrane integrity, and pyrimidines target DNA/RNA synthesis (Cannon et al., 2007).

1.3.1 Amphotericin B

The two different types of AmBs are: AmB with the deoxycholate preparation (AmB-d) and lipid formulation of amphotericin (LFAmB) that can be divided into three types: amphotericin lipid complex (ABLC), amphotericin B colloidal dispersion (ABCD), and liposomal amphotericin B (L-AmB) (Pappas et al., 2009). LFAmBs have the same spectrum of activity as AmB-d (Pappas et al., 2009).

Amphotericin B acts by binding to ergosterol and creating pores in the fungal membrane. These pores impair membrane function and disturb the delicate osmotic balance resulting in cell death (Andreoli, 1974).

AmB-d and LFAmBs are used for the treatment of invasive candidiasis however AmB-d has adverse effects such as nephrotoxicity resulting in acute renal failure since it disrupt the permeability of the cell membrane causing an alteration in the tubular and vascular smooth muscle cell function thus defects in the tubular transports and vasoconstriction (Deray, 2002).

LFAmBs are more expensive than AmB-d and have less nephrotoxic effects with AmB-L providing the greatest renal protection and used in urinary tract candidaisis (Walsh et al., 1998; Wingard, 2002) and in CNS infections (Groll et al., 2000).

1.3.2 Triazoles

Fluconazole, itraconazole, voriconazole, and posaconazole act in a similar fashion against most *Candida* species (Espinel-Ingroff et al., 2005; M. A. Pfaller, Diekema, & Sheehan, 2006). They function through the inhibition of cytochrome P450 enzyme 14 alpha-demethylase encoded by the *ERG11* gene preventing the formation of ergosterol from lanosterol (Pappas et al., 2009)

Fluconazole is more efficient than AmB-d for the treatment of candidemia (Rex et al., 2003) and it is the therapy of choice for oropharyngeal, esophageal, and vaginal candidiasis (Goldman et al., 2005; Sobel et al., 2001). It is used for CNS treatment because of its great ability to penetrate the CSF and vitreous body (Arndt et al., 1988). Itraconazole is taken by patients with mucosal candidiasis after treatment failure when fluconazole is used (Pappas et al., 2009). Voriconazole is used for mucosal, CNS, and invasive candidiasis (Pappas et al., 2009). Patients with renal

dysfunction will have cyclodextrin accumulation because it is linearly related to creatinine clearance (Pappas et al., 2009). Patients with a creatinine clearance > 50 mL/min cannot be given cyclodextrin, knowing that the intravenous form voriconazole is cyclodextrin since voriconazole has limited aqueous solubility (von Mach, Burhenne, & Weilemann, 2006). Posaconazole is used mainly for the treatment of oropharyngeal candidiasis (Pappas et al., 2009).

1.3.3 Echinocandins

Caspofungin, anidulafungin, and micafungin are the latest generation of drugs on the market. They are mainly used for the treatment of esophageal candidiasis (Krause et al., 2004; Villanueva et al., 2001) and invasive candidiasis ((Mora-Duarte et al., 2002; Pappas et al., 2007).

Echinocandins show few adverse effects. Dose adjustment is not required for renal insufficiency or dialysis (Pappas et al., 2009). Caspofungin and micafungin have the least hepatic metabolism and they are not a major substrate for cytochrome P450 (Pappas et al., 2009). The only echinocandin that requires dose adjustment is caspofungin when used with patients suffering from moderate to severe hepatic dysfunction (Pappas et al., 2009). Echinocandins inhibit the 1,3 beta glucan synthase enzyme needed to synthesize 1,3 beta glucans, a primary constituent of fungal cell walls. Lack of synthesis of glucans results in cell death (Sucher, Chahine, & Balcer, 2009).

1.3.4 Flucytosine

Flucytosine is one of the oldest antifungal drugs on the market. Flucytosine has an antifungal activity against most *Candida* species with the exception of *C. krusei.* It is primarily used for treatment of urinary tract candidiasis. Dose adjustment should be taken into consideration for patients with renal dysfunction (Stamm et al., 1987).

Flucytosine is usually given with AmB for patients with invasive diseases, such as *Candida* endocarditis or meningitis (Pappas et al., 2009). Flucytosine is converted intrafungally to 5-fluorouridinetriphosphate (FUDP) that inhibits protein synthesis leading to death. An alternative pathway converts flucytosine into 5-fluorodeoxyuridinemonophosphate (FdUP) that inhibits DNA synthesis (Chandra, Mohammad, & Ghannoum, 2009).

1.4 Antifungal Drug Resistance

Resistance to flucytosine is due to a mutation in the enzyme uracil phosphoribosyltransferase (*FUR1*) this will inhibit the conversion of 5-fluorouracil to 5-fluorouridine monophosphate, needed in the biosynthesis of FUDP and FdUP. Resistance to polyenes is rare and is the result of mutations in *ERG3* and *ERG* 11 resulting in a decrease in the levels of ergosterol that Amphotericin B can bind to. (Akins, 2005).

C. albicans resistance to azoles is due to many factors. Overexpression of *ERG11,* or the presence of point mutations causes reduction of azole binding (Akins, 2005; Sanglard, 2003; White, Marr, & Bowden, 1998) and thus decreases susceptibility to azoles. In addition high resistance of *C. albicans* to azoles is associated with the overexpression of MFS drug pump Mdr1p, or the ATP-binding cassette (ABC) pumps Cdr1p or Cdr2p in plasma membrane by overexpression of *TAC1*, the transcriptional activator of the *CDR* genes (Perea et al., 2001; White et al., 1998). These pumps pump out the drug and prevent its interference in ergosterol biosynthesis. LOH (loss of heterozygosity) is another factor that decreases susceptibility to the drug. LOH involves the loss of a wild type copy of *TAC1* and duplication of a mutated copy that overexpresses the gene (Coste et al., 2006).

Limited and rare resistance of *C. albicans* to echinocandins has been recently reported. These echinocandins-resistant *C.albicans* have point mutations in β-(1,3)-D-glucan synthase subunit Gsc1p (Baixench et al., 2007). Mechanisms of resistance to echinocandins is detailed in the below chapter. See Figure 7 for a brief schematic of drug resistance.

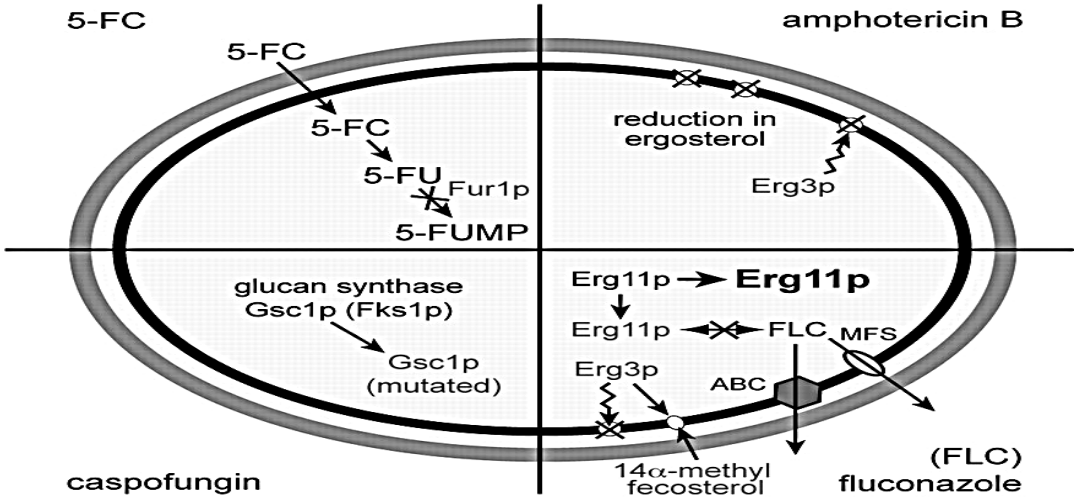


Figure 7. Mechanisms of resistance to antifungal drugs in *C. albicans.* Different mutations cause resistance to different categories of antifungal drugs. Echinocandin resistance is caused by a mutation in the FKS1 gene, while azole resistance is caused overexpression or a mutation in the Erg11p. Flucyotsines resistance is due to a mutation the *FUR1*, and polyene resistance is due to a mutation in *ERG3* (Cannon et al., 2007).

1.5 *C. albicans* Resistance to Echinocandins

The inhibition of β-(1,3)-D-glucan synthase (GS) is caused by echinocandins, that target the GS *FKS* subunits. These subunit are encoded by 3 genes, *FKS1*, *FKS2*, and *FKS3* (Michael A. Pfaller, Messer, Woosley, Jones, & Castanheira, 2013; Michael A. Pfaller, Moet, Messer, Jones, & Castanheira, 2011).

The resistance to echinocandins treatment is associated with a single residue amino acid substitutions in *FKS1* (*C. albicans, C. tropicalis, C. krusei, C. glabrata*) and *FKS2* (*C. glabrata*) (Michael A. Pfaller et al., 2011) that increases the MIC and

cause complications in controlling and managing the infection (Michael A. Pfaller et al., 2013). Currently, the incidence of echinocandins resistance in *C. albicans* is <1% (Lackner et al., 2014).

In *C. albicans* that are resistant to echinocandin only amino acid substitutions within *FKS1* encoding the β-(1,3)-D-glucan synthase were isolated (Katiyar et al., 2012, p. 1).

The most common amino acid changes are at positions F641, L642, T643, L644, S645, R647, D648, P649, W1358, and R1361 (Park et al., 2005) within hot spot 1 (HS1) (amino acid positions 641 to 649, FLTLSLRDP) (Katiyar et al., 2012) and hot spot 2 (HS2) (amino acid positions 1357 to 1364, DWIRRYTL) (Lackner et al., 2014).

Silent mutations were also determined one within hotspot 1 T643 and five outside the hotspot regions P547, T551, I554, I1375, and A1410. In addition, two missense mutations were identified within hotspot 1 R647G and P649L, respectively (Lackner et al., 2014).

C. albicans is a diploid fungus thus it carries pair of each chromosome and at least two alleles of each gene locus (Butler et al., 2009). A substitution of an amino acid in one allele (heterozygous) will affect half the total amount of cellular glucan synthase, while substitutions both alleles (homozygous) will affect the whole amount of cellular glucan synthase (Lackner et al., 2014). Differences in caspofungin MIC values between homozygous with heterozygous *fks1* mutants were determined. The homozygous f*ks1/fks1* mutant *C.albicans* had the highest MIC value as compared with that of the wild-type (Ben-Ami et al., 2011). The fitness and virulence attributes of heterozygous and homozygous *fks1* mutant strains were studied. Differences were seen when both strains were compared with the wild-type's growth rate, filamention, cell wall chitin content, and virulence. The homozygous strains exhibited impaired growth

rates, defected filamentation abilities, increased chitin content, and decreased virulence (Ben-Ami et al., 2011)

β-1,3-D-glucan synthase that is important for cell wall integrity targets Rho, a GTP-binding protein that regulates the glucan synthase biosynthetic capacity and Fks, a catalytic subunit that is encoded by two genes of *C. albicans FKS1*, and *FKS2* (Mazur & Baginsky, 1996). During growth the structure of the fungal cell changes, therefore requiring remodeling of β-1,3- and β-1,6-glucans (Perlin, 2015). Fungal cell survival depends mainly on the cell wall, drugs like echinocandins causes cellular stress affecting the cell wall integrity. Fungal cells protect themselves against such stresses through adaptive response mechanisms (Perlin, 2007; Walker, Gow, & Munro, 2010).

Genes that are required for cell wall integrity, cell wall architecture, and genes that are also required for protein kinase C (PKC) cell integrity–signaling pathway are induced by echinocandins (Reinoso-Martín, Schüller, Schuetzer-Muehlbauer, & Kuchler, 2003). A group of effectors are activated once stress signals are transmitted to Rho1 GTPase (Perlin, 2015). Once the cell wall integrity signaling pathways are triggered, cell wall architecture and carbohydrate polymers of the cell wall change (Levin, 2011).

Echinocandins inhibition of glucan biosynthesis will induce PKC, Ca^{2+}/calcineurin/Crz1, and HOG (high osmolarity glycerol) pathways(Munro et al., 2007). It also induce the activation of Hsp90 causing echinocandin drugs tolerance through calcineurin and Mkc1 proteins and co-chaperone Sgt1 (Singh et al., 2009). In addition resistance can be due to the increase in chitin synthesis in response to echinocandin exposure. This is a compensatory mechanism in response to decreased glucan synthesis. Chitin replaces the β-1,3-glucan to maintain the cell wall structural

integrity causing a decreased sensitivity to the drug (Gow et al., 2007; Munro et al., 2007; Walker et al., 2008).

PKC, HOG, and calcineurin signaling pathways are responsible for coordinating the compensatory increase in chitin (Perlin, 2015). Chs2 and Chs8 activation allows most *Candida* species to survive even in the presence of high levels of echinocandins (Walker, Gow, & Munro, 2013). Moreover, the increase in cell wall chitin of *C. albicans* reduces fitness and virulence. The thickened, chitin rich cell wall will impair the cells ability to remodel and switch knowing that filamentation in *C. albicans* is critical for phagocytic resistance (Ben-Ami et al., 2011).

1.6 Antifungal Resistance of *C. albicans* in Lebanon

Our lab has previously addressed the issue of antifungal drug resistance in Lebanese hospital isolates to azoles, AmB, caspofungin anidulafungin (Basma, 2007; Bitar, Khalaf, Harastani, & Tokajian, 2014; Khalaf, Basma, Barada, & Ojaimi, 2009). An initial study on hospital isolates found resistance to all drug categories with the exception of echinocandin (caspofungin). *C. albicans* isolates showed 12% for IT, followed by 7.7% for VO, 6% for KE, 5% for FL, 1.7% for AP and 0% for CS. Three isolates (2.6%) showed resistance to all azoles used, with an isolate that was resistant to all drugs used except CS (0%) (Khalaf et al., 2009). A later study looked at resistance to micafungin, a novel echinocandin and attempted to correlate resistance with biofilm formation-a virulence attribute. The study demonstrated the presence of resistance to anidulafungin (11.9%). The study also found out that there is a strong correlation between *C. albicans* ability to form biofilm with drug resistance (Bitar et al., 2014).

1.7 Aim of the Study

This study aimed at sequencing HS1 and HS2 of the *FKS1* gene from previously isolated *C. albicans* from Lebanese hospital to determine whether mutations are present in Lebanese isolates, and if mutations are present, to determine possible novel mutations specific to Lebanese isolates within these HS regions. If mutations do exist, we would like to determine whether such mutations contribute to resistance to caspofungin or whether these mutations are distributes randomly amongst resistant or non-resistant isolates.

In addition, we also wanted to determine any possible relationship between drug resistance and pathogenicity related attributes such as virulence, adhesion, filamentation, biofilm formation, cell wall chitin deposition, and resistance to cell wall disrupting agents such as Congo red. Since as mentioned above, fitness and virulence factors of resistant *C. albicans* to echinocandins might include production of thickened chitin rich cell wall, abnormal filamentation, (Ben-Ami & Kontoyiannis, 2012). The logic behind that is that if a mutation results in increased chitin deposition then the cell might not be able to filament well, and as such cannot infect properly resulting in attenuated virulence. In addition it will not be able to form a thick mesh like biofilm as the upper biofilm layer consists of filamentous cells. Such cells however will be resistant to cell wall disruptive agents due to the thickened chitin that prevents such agents from entering the cell. Such a phenotype of cell wall thickening has previously been observed in our lab when a deletion of *PIR32* a cell wall protein resulted in increased chitin deposition and resistance to SDS and hydrogen peroxide (Bahnan et al., 2012).

Chapter 2

Materials and Methods

2.1 Clinical Isolates

A total of 16 clinical *Candida* isolates were previously provided by 2 major hospitals in Beirut: Saint Georges Hospital, and AUBMC. The samples were recovered from sputum, stool, urine, abscess, bronchial lavage, and Deep Tracheal Aspirate (DTA) (Basma, 2007; Bitar, Khalaf, Harastani, & Tokajian, 2014). Samples were obtained from cryobanks stored at -80 °C and grown on potato dextrose agar at 37°C.

2.2 DNA Extraction

For DNA extraction, fresh colonies were collected upon culturing samples on PDA for 48 hours at 30 °C. Extraction was performed using the ZR Fungal/Bacterial DNA MiniPrep™ kit Zymo Research USA according to the manufacturer's instructions. The extracted DNA was then stored at -20 °C until needed.

2.3 Typing of HS1 and HS2 of the *FKS1* gene

2.3.1 Pre-sequencing PCR

Amplification of the hotspots region HS1 and HS2 was accomplished by adding 0.4 µl (20 pmol/ µl) of the forward primer HS1 (5'- AAT GGG CTG GTG CTC AAC AT-3') HS2 (5'- AAG ATT GGT GCT GGT ATG GG-3') 0.4 µl (20 pmol/ µl) of the reverse primer HS1 (5'- CCT TCA ATT TCA GAT GGA ACT TGA TG-3') HS2 (5'- TAA TGG TGC TTG CCA ATG AG-3'), 9.7µl deionized water, and 12.5µl (250 U) of the AmpliTaq Gold PCR Master Mix (Applied Biosystems). The PCR thermal cycling

conditions consisted of the initialization step (9 min at 95 °C), 30 cycles of denaturation (30 s at 95 °C), annealing (30s at 54 °C), elongation (1:40 min at 72 °C), and a single final elongation (10 min at 72 °C). The samples were then stored at 4 °C. Ethidium bromide (6 µl) was then used to stain the amplified DNA fragments to visualize it using 1% agarose gel and 1X TAE buffer at 80 V.

Table 1. Primers used for the amplification of the HS1 and HS2 of the *FKS1* gene showing the DNA sequence of each.

Primer Name	Sequence 5'-3'
Cand-F1HS1-F	AAT GGG CTG GTG CTC AAC AT
Cand-F1HS1-R	CCT TCA ATT TCA GAT GGA ACT TGA TG
Cand-F1HS2-F	AAG ATT GGT GCT GGT ATG GG
Cand-F1HS2-R	TAA TGG TGC TTG CCA ATG AG

2.3.2 PCR cleanup

0.4 µl of Exonuclease I (Thermo Scientific) and 1 µl of Fast Alkaline Phosphatase (Thermo Scientific) were added to 6 µl of the pre-sequencing PCR products in order to purify it. The thermal conditions for this step are 37 °C for 15 min followed by 80 °C for 15 min. The purified products were stored at 4 °C until further use.

2.3.3 Sequencing PCR

BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) was used to sequence the purified PCR products. The sequencing reaction was performed by adding 4 µl of 5X-diluted Bigdye® premix, 3 µl of 1.2 µM sequencing forward/reverse primers, and 3 µl of the purified PCR product in a total volume of 10 µl, PCR was performed consisting of an initial denaturation step at 96 ˚C

for 1min, 26 cycles of denaturation (96 ˚C for 10 s), annealing (50 ˚C for 5 s) and extension (60 ˚C for 4 min). The final product was stored at 4 °C until needed.

2.3.4 BigDye X-Terminator Purification

BigDye X-Terminator Purification Kit (Applied BioSystems) was used to purify the sequencing products according to the manufacture instructions. The plate was then loaded for sequencing electrophoresis on an ABI 3500 Avant Genetic Analyzer (Applied Biosystems). For sequence analysis, the software: CLC Main Workbench 5 (CLC Bio) was used to analyze and align the protein sequences.

2.4 Antifungal Susceptibility Testing

The Minimum Inhibitory Concentrations (MICs) to caspofungin for all 16 samples was determined using the Etest strips (biomerieux, France). RPMI 1640 (with L-glutamine and no bicarbonate) with MOPS and glucose (AB Biodisks) was prepared according to the manufacturer's instructions. Fungal suspension at 0.5 McFarland turbidity (or 10^5 CFU/ml) was streaked on the media. Antifungal strips were applied on the inoculated plate and incubated at 37 ˚C for 48 hours. The CLSI quality control reference strain *C. albicans* ATCC 90028 was also utilized in this study and its E test MIC was provided by the E test manufacturer. MICs of the controls strain fell within the acceptable range (0.25µg/ml) (Wanjare, Gupta, & Mehta, 2016).

Table 2. MIC values for Caspofungin against *C. albicans* ATCC90028 (as per CLSI document M27-S4, 2012).

	MIC values (µg/ml)		
Candida albicans	Susceptible (S)	Intermediate (I)	Resistant (R)
	≤0.25	0.5	≥1

2.5 Murine Study of Disseminated Candidiasis

Forty eight 4 to 6 week-old BALB/c mice (20-30 g) were injected with 10^7 cells of *C. albicans* through the tail (3 mice per strain). Survival of the infected mice was monitored on a daily basis for 21 days. Water and food was given to the mice ad libitum. Moribund mice were euthanized. (Dib, Hayek, Sadek, Beyrouthy, & Khalaf, 2008).

2.6 Cell Wall Disrupting Agents

Strains were grown until exponential phase and serially diluted tenfold. 5µl of the serial dilutions (10^5 – 10^2 cfu/ml) were spotted on PDA plates pretreated with 10, 30, and 50µg/ml Congo red (Sigma-Aldrich, Germany). A control plate lacking cell surface disrupting agents was spotted with the exact same volume of the three strains. The plates were incubated at 30 ºC for 3-4 days (Plaine et al., 2008).

2.7 Chitin Quantification

Cell wall chitin content was measured for all strains in addition to the control strain SC5314 according to a modified protocol described previously (Bahnan et al., 2012) . Briefly, 6N HCl was used to hydrolyze 50mg wet weight purified cell walls at 100ºC overnight. After centrifugation the pellet was reconstituted in 1 ml of distilled water. A 0.1 ml aliquot of this sample was added to 0.1 ml of solution A (1.5 N Na_2CO_3 in 4% acetylacetone). The mixture was incubated at 100ºC for 20 minutes and after cooling to room temperature, 0.7 ml of 96% ethanol was added to the mixture followed by addition of a 0.1 ml of solution B (1.6 g of *p*-dimethyl-aminobenzaldehyde in 30 ml of concentrated HCl and 30 ml of 96% ethanol). The mixture was incubated for 1 hour at room temperature and absorbance of the samples was measured

spectrophotometrically at 520 nm. The results were plotted against a standard curve generated by the use of known glucosamine standards taken through the same procedure as our samples. Chitin concentrations were expressed as a percentage of control strain strain SC5314 (Younes & Khalaf, 2013).

2.8 Biofilm Formation

Biofilm formation was assessed using a modified protocol as previously described (Dimassi, Khalaf, Younes, & Bahnan, 2011; Hashash et al., 2011). Briefly, an overnight culture of *C. albicans* in PDB was diluted to approximately 10^7 cfu/ml. 24 well polystyrene microtiter plates pre-treated by overnight incubation with 5% FBS at 4 ºC were inoculated with five hundred microliters of *C. albicans* and incubated for 3 h in a shaking incubator (75 rpm) at 37 ºC to allow adhesion. After incubation, wells were washed twice with PBS, and 1 ml of PDB was added to the adherent *C. albicans* cells. The plates were left to incubate at 37 ºC for 48 h. Afterwards, wells were washed with PBS to remove non-adherent cells. Five hundred microliters of 99% methanol was added to the wells for 15 min to fix the biofilm to the wells. After removing the methanol and air-drying the wells for 20 min, 500 µl of a 0.2% crystal violet solution was added to the plates and left to react with the biofilm cells for 20 min. The crystal violet was then washed 5 times with distilled water and released by adding 750 µl of 33% acetic acid. Absorbance was measured at 590 nm and expressed as percentage of control strain SC5314 (ATCC MYA-2876).

2.9 Filamentation and Adhesion

Strains were spotted on PDA plates and incubated for 14 days at 37 ^{0}C after which cells were washed vigorously with tap water. Strains that adhered to agar and

underwent invasive filamentous growth resisting washing were photographed and compared with pre washing conditions (Younes & Khalaf, 2013).

2.10 Statistical Analysis

Cell wall chitin assay was repeated twice in duplicates, biofilm formation assay was repeated twice in triplicates, and murine study of disseminated candidiasis was performed on 3 mice per strains.

Chapter 3

Results

3.1 Molecular Analysis of Hotspots within the *FKS1* gene

As can be seen in Table 3, the amino acid sequences of HS1 and HS2 region of the *FKS1* gene encoding 1,3-β-glucan synthase for all 16 *C. albicans* isolates were generated. Only 5 isolates conferred amino acid substitutions within the hotspots, whereas no amino acid substitution was found in the remaining 11 *C. albicans* isolates or within the sequence adjacent to the hotspots. Amino acid substitutions within the hotspots were at positions 642, 643, 644, 647, 648, 1358, 1360, 1361, 1363, and 1364. Mutations of amino acids within HS1 were L642V, T643I, L644V, L644G, R647K, D648S, and within HS2 were W1358S, R1360L, R1361L, T1363G, and L1364C.

Table 3. Amino acid sequences of HS1 and HS2 of the *FKS1* gene. The HS1 and HS2 region was sequenced and analyzed but only the region harboring the amino acid substitutions is shown, with the amino acid changes in red.

Isolates	HS1 region (amino acid positions 641-649) FLTLSLRDP	HS2 region (amino acid positions (1357-1364) DWIRRYTLS	Interpretation
IB006	FLTLSLRDP	DWIRRYTLS	Wild type
IB014	FLT**V**SL**K**S**S**P	DWI**L**RYTLS	**Mutant**
14	FLTLSLRDP	DWIRRYTLS	Wild type
IB017	FLTLSLRDP	DWIRRYTLS	Wild type
IB018	FLTLSLRDP	DWIRRYTLS	Wild type
IB020	FLTLSLRDP	DWIRRYTLS	Wild type
IB032	FLTLSLR**S**P	DWIRRYT**C**S	**Mutant**
52	FLTLSLRDP	DWIRRYTLS	Wild type
55	FLTLSLRDP	DWIRRYTLS	Wild type
IB059	F**V**T**V**SLR**S**P	DWIRRY**G**LS	**Mutant**
IB070	FLTLSLRDP	DWIRRYTLS	Wild type
IB075	FL**I**LSLRDP	DWI**L**LYTLS	**Mutant**
IB078	FLTLSLRDP	DWIRRYTLS	Wild type
IB084	FLTLSLRDP	DWIRRYTLS	Wild type
IB108	FL**IG**SLR**S**P	D**S**IR**L**YTLS	**Mutant**
IB115	FLTLSLRDP	DWIRRYTLS	Wild type

3.2 Antifungal Susceptibility Testing

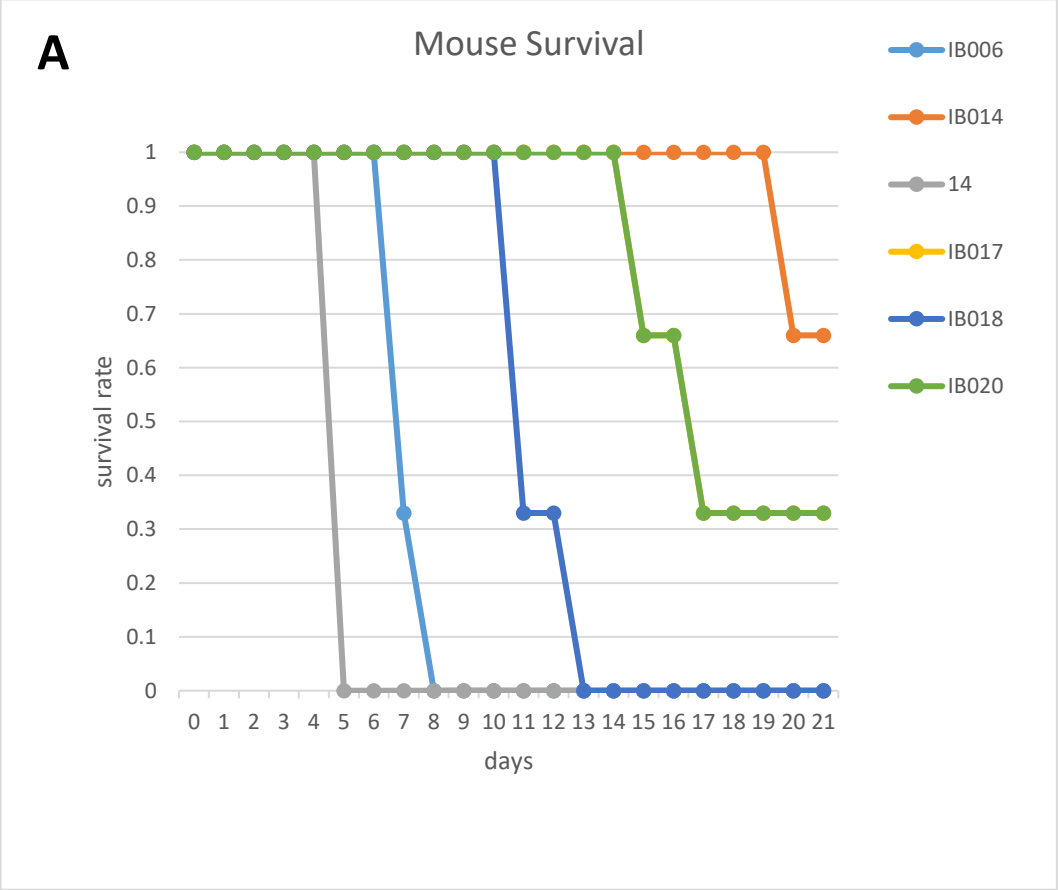
Caspofungin antifungal susceptibility testing was performed on all 16 isolates using the E-test method. As can be seen in Table 4 IB014, IB059, IB075, and IB108 showed resistance against caspofungin, while IB017, IB020, IB032, and 52 showed intermediate resistance against caspofungin. All strains that were resistant displayed a mutation in the hotspot region. The results are summarized in Table 4.

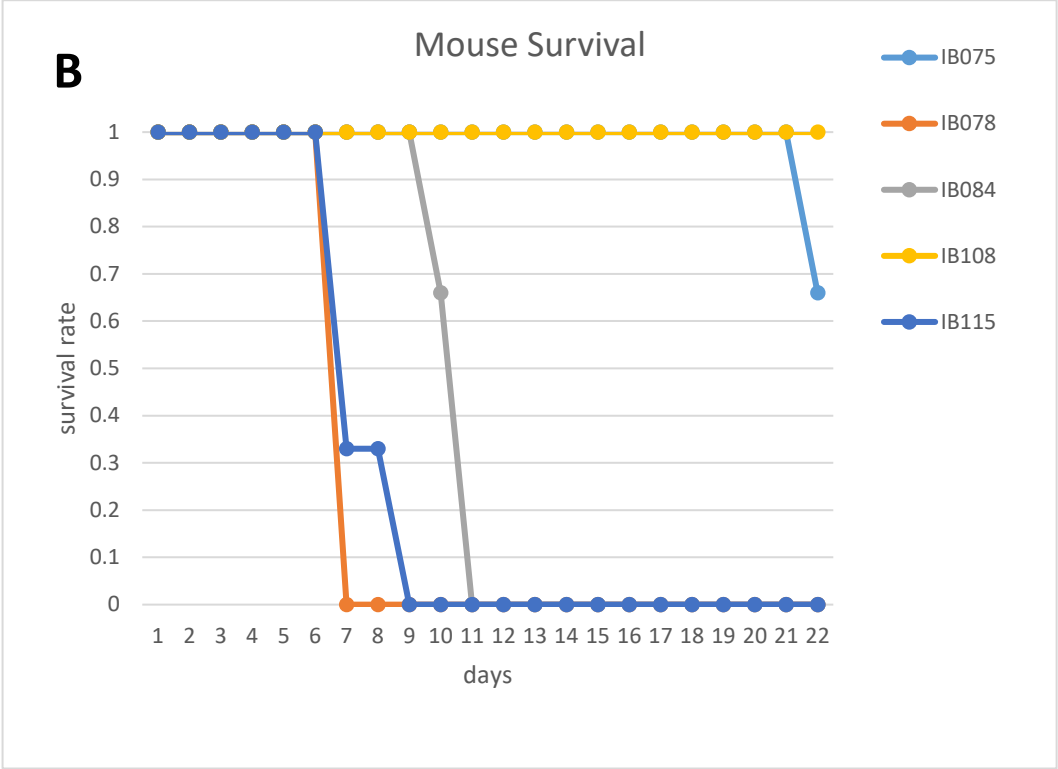
Table 4. Caspofungin MICs of all 16 *C. albicans*. 4 isolates were resistant, 4 displayed intermediate resistance, and the remaining 8 isolates were sensitive.

***C. albicans* Isolates**	**MIC values(µg/ml)**
IB006	0.023 (S)
IB014	1.5 (R)
14	0.023 (S)
IB017	0.38 (I)
IB018	0.042 (S)
IB020	0.38 (I)
IB032	0.5 (I)
52	0.38 (I)
55	0.25 (S)
IB059	1 (R)
IB070	0.042 (S)
IB075	1 (R)
IB078	0.032 (S)
IB084	0.064 (S)
IB108	1.5 (R)
IB115	0.025 (S)

3.3 Murine Study of Disseminated Candidiasis

3 BALB/c mice per strain were injected with 10^7 cells of *C. albicans* in the tail vein causing disseminated candidiasis. Mice survival was monitored for 21 days. As can be seen in Figure 8 virulence was mainly observed in isolates that showed no mutations in the hotspots and that were sensitive to caspofungin where 0-33% of mice injected with sensitive isolates survived. This was seen in isolates IB006, 14, IB078, and IB115. On the other hand 67-100% of all mice injected with resistant isolates survived. This was seen in isolates IB014, IB032, IB059, IB075, and IB108.





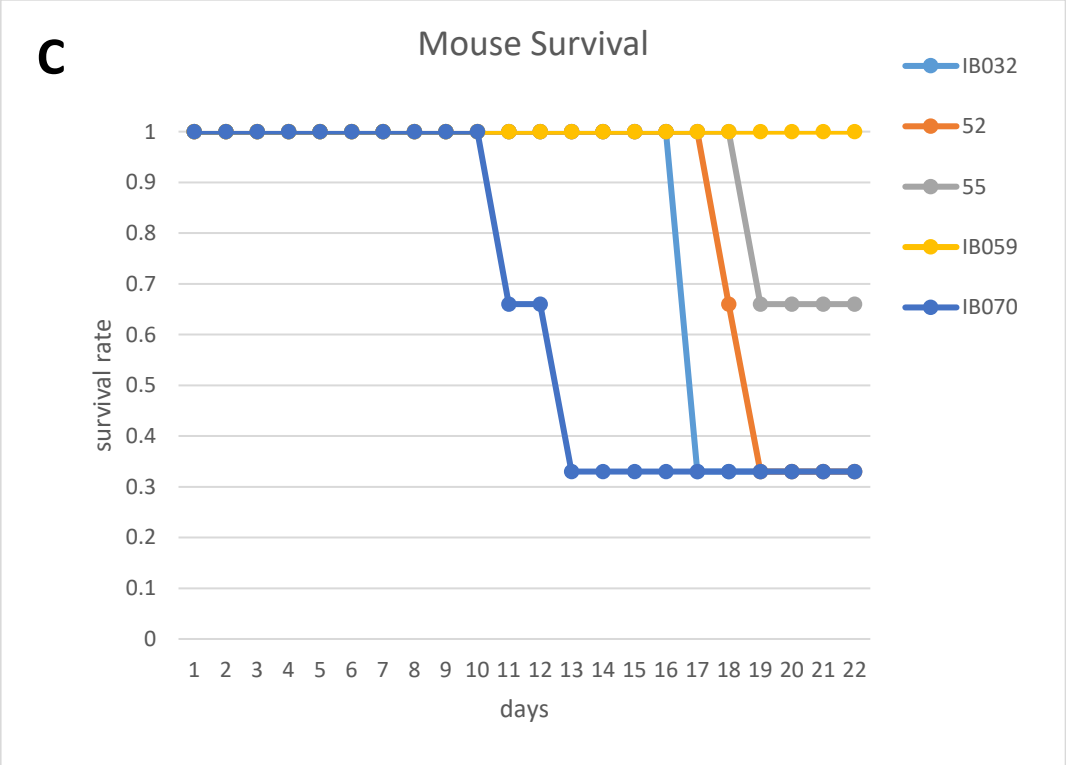


Figure 8. BALB/c mice injected with 10^7 cells of sensitive and resistant *C. albicans* isolates and monitored for survival for 21 days. Mice injected with sensitive strains survival rate was low when compared with the survival rate of mice injected with resistant strains, implying that sensitive strains are more virulent than resistant ones.

3.4 Cell Wall Disrupting Agents

As can be seen in Figure 5 the sixteen *C. albicans* isolates were spotted on PDA plates containing 30µg/ml Congo red, a cell wall disrupting agent. Strains conferring mutations within the hotspots were resistant to Congo red such as IB014, IB032, IB059, IB075, and IB0108 while strains IB006, 14, IB078, and IB115 that showed no mutations within the hotspots were sensitive. As a control, all strains grew on PDA media without Congo red as seen in Figure 10.

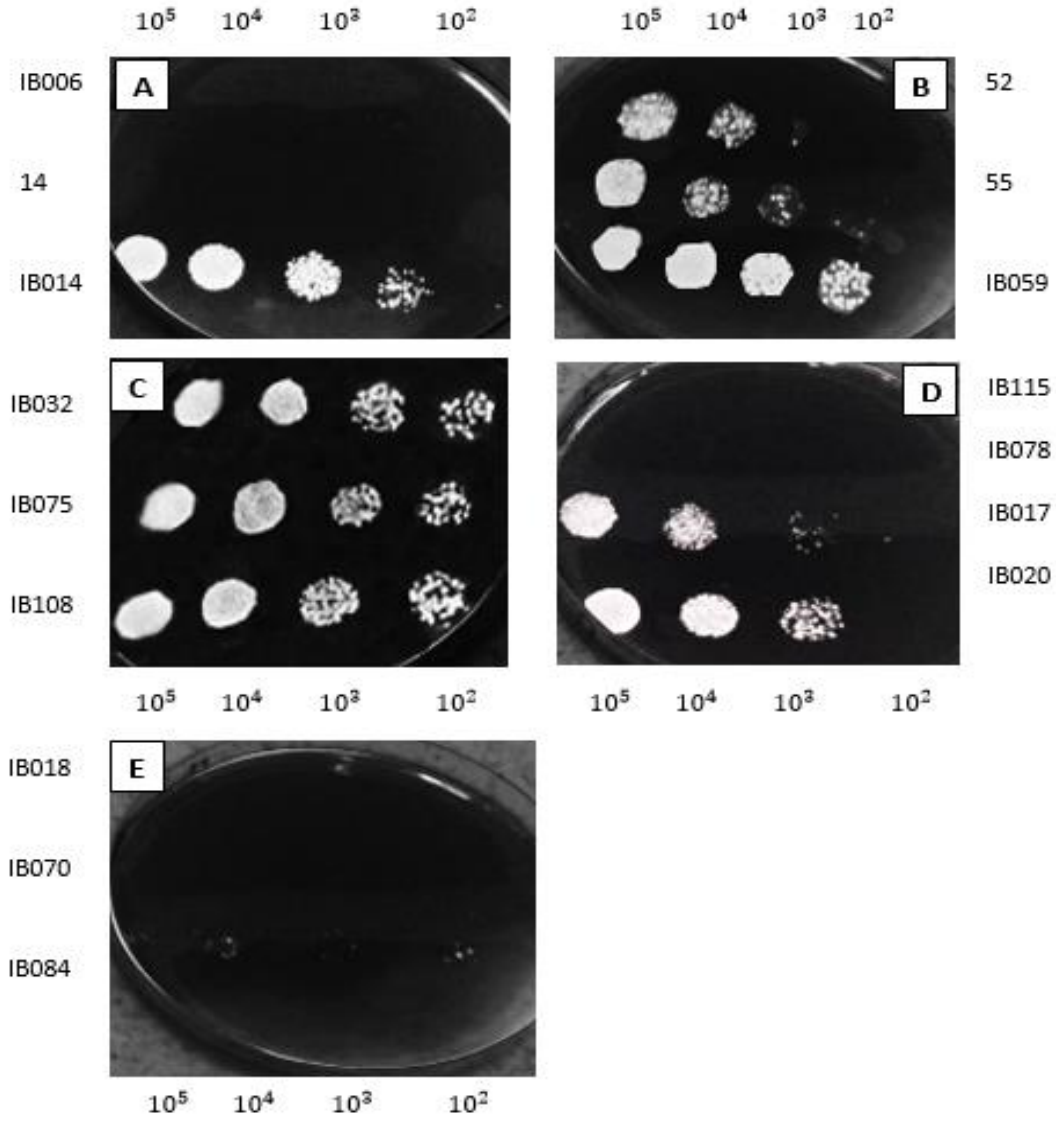


Figure 5. Sixteen *C. albicans* strains were spotted on PDA plates treated with Congo red. Figures A, B, C, D, and E show the growth of sensitive and resistant strains with the presence of Congo red. No growth was seen in sensitive strains.

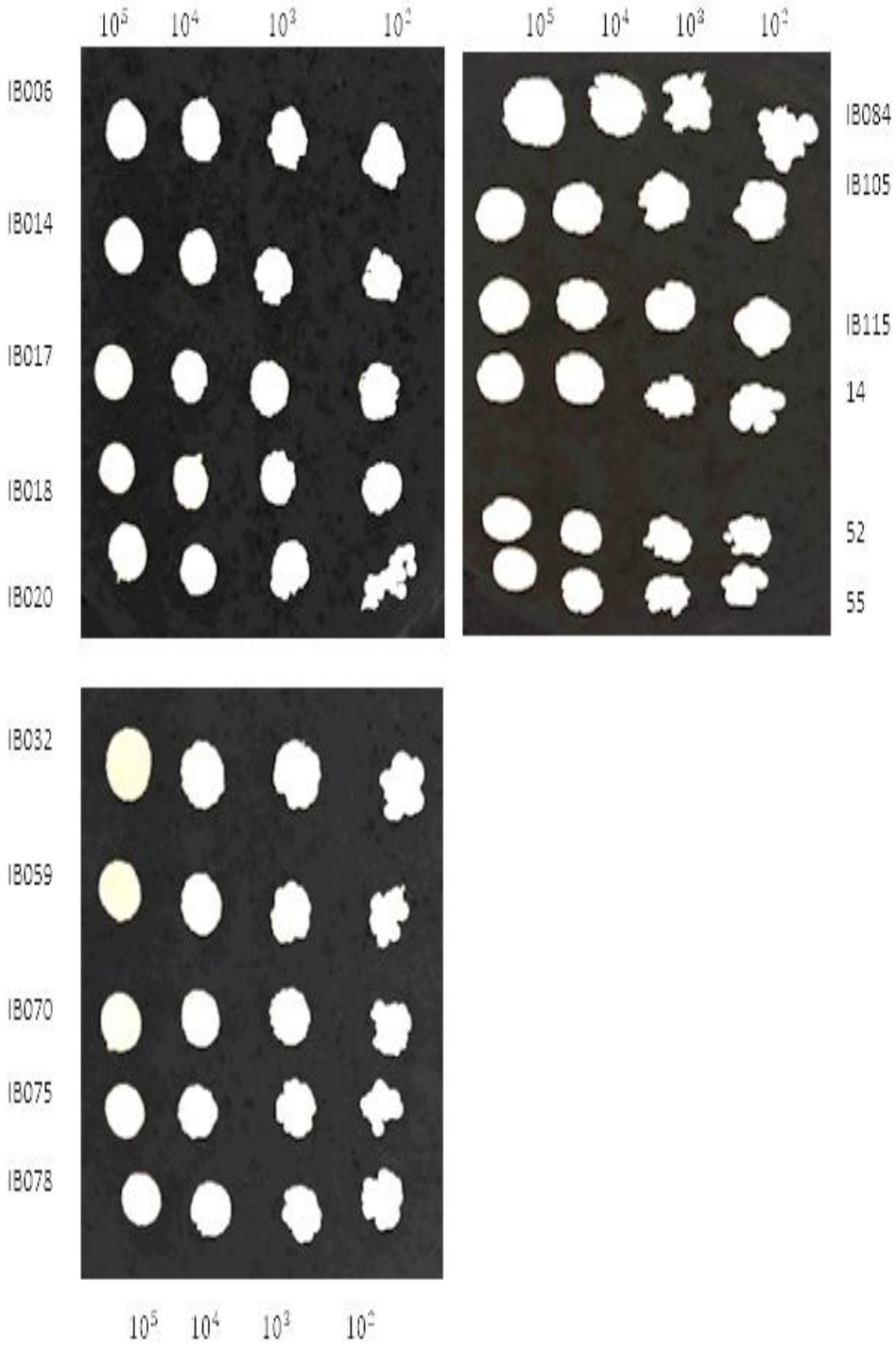


Figure 10. Control plate lacking Congo red. All sixteen *C. albicans* strains grew fully on PDA plates lacking Congo red.

3.5 Chitin Quantification

Chitin content was quantified for all 16 strains through acid hydrolysis. The amount of chitin was normalized to reference strain SC 5314 and expressed as a percentage of the strain. As can be seen in Figure 6 below, resistant strains IB014, IB032, IB059, IB075, and IB108 showed a significant increase in total chitin amount compared to sensitive strains with more than a two fold increase with respect to the reference strain.

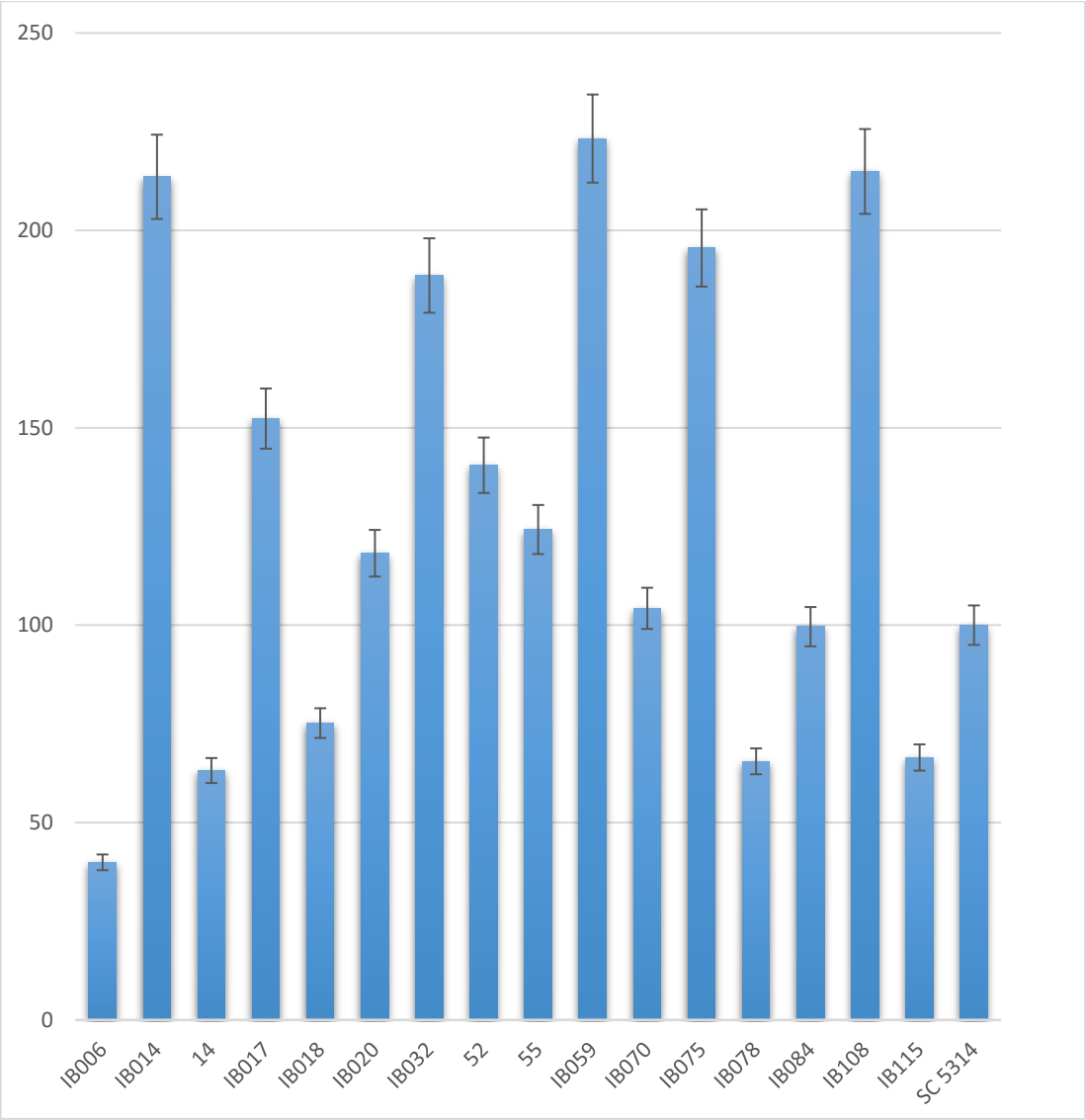


Figure 11. Cell wall chitin content of all 16 *C. albicans* isolates. Chitin content was determined through acid hydrolysis. Chitin content of sensitive and resistant isolates was normalized to that of SC 5314. Resistant strains contained a much higher amount of chitin than sensitive strains.

3.6 Biofilm Formation

The biofilm forming capacity for all 16 strains was determined by crystal violet staining. OD values were normalized to SC 5314 and expressed as a percentage of the reference strain. Differences in biofilm formation between sensitive and resistant strains is clear with resistant strains IB014, IB032, IB059, IB075, and IB108 showing decreased biofilm formation compared to the sensitive strains.

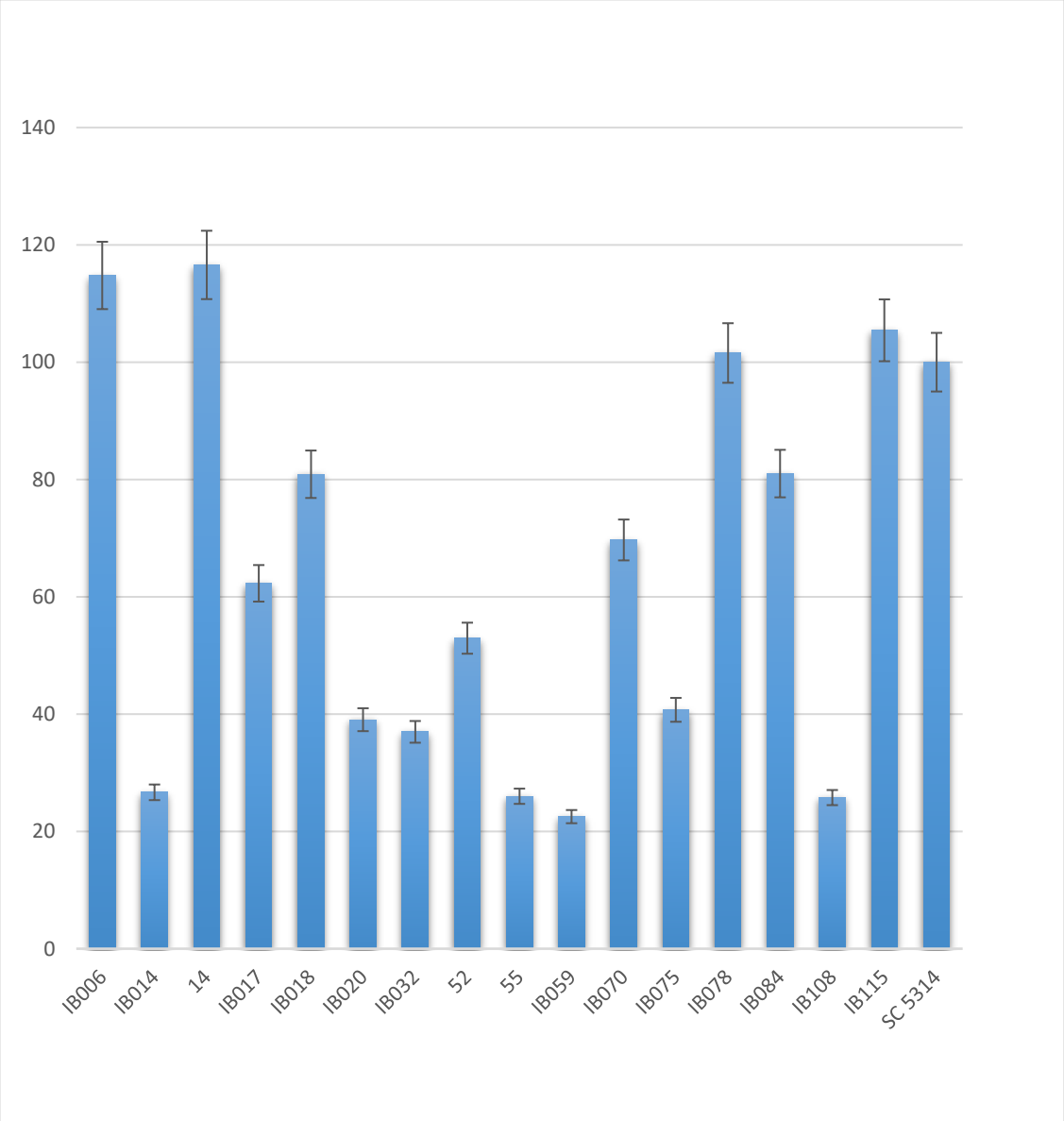
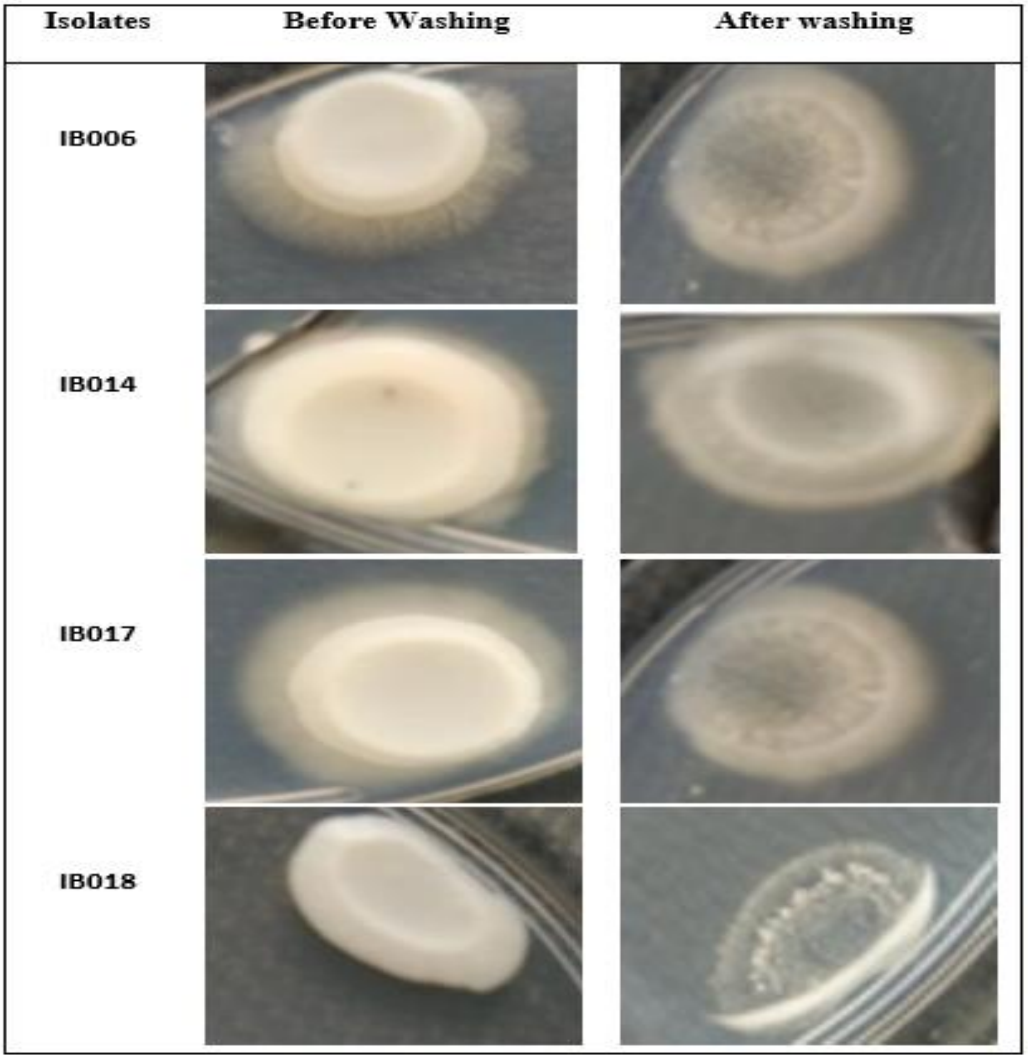


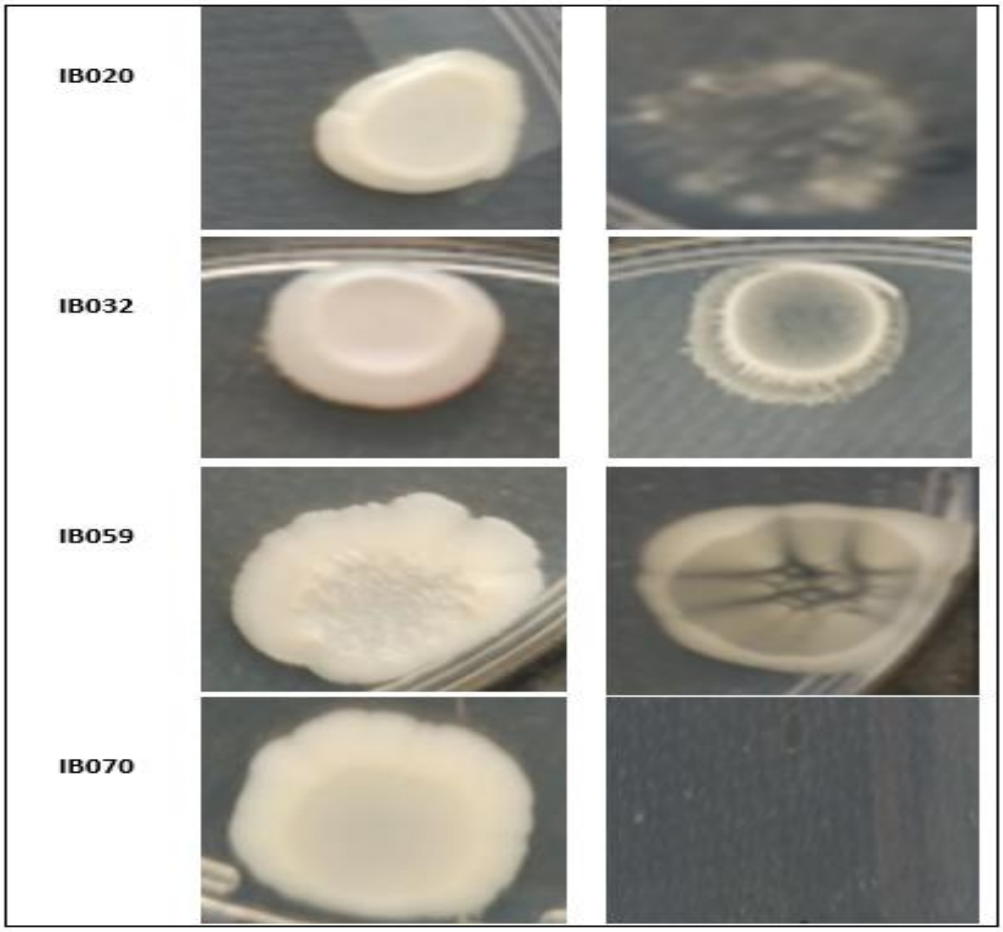
Figure 12. Biofilm forming capabilities of all 16 isolates. Resistant *C. albicans* isolates showing a 60% to 74% decrease in biofilm formation as compared to the reference strain SC 5314 whereas sensitive strains were able to form biofilm in the same rate of the reference strain.

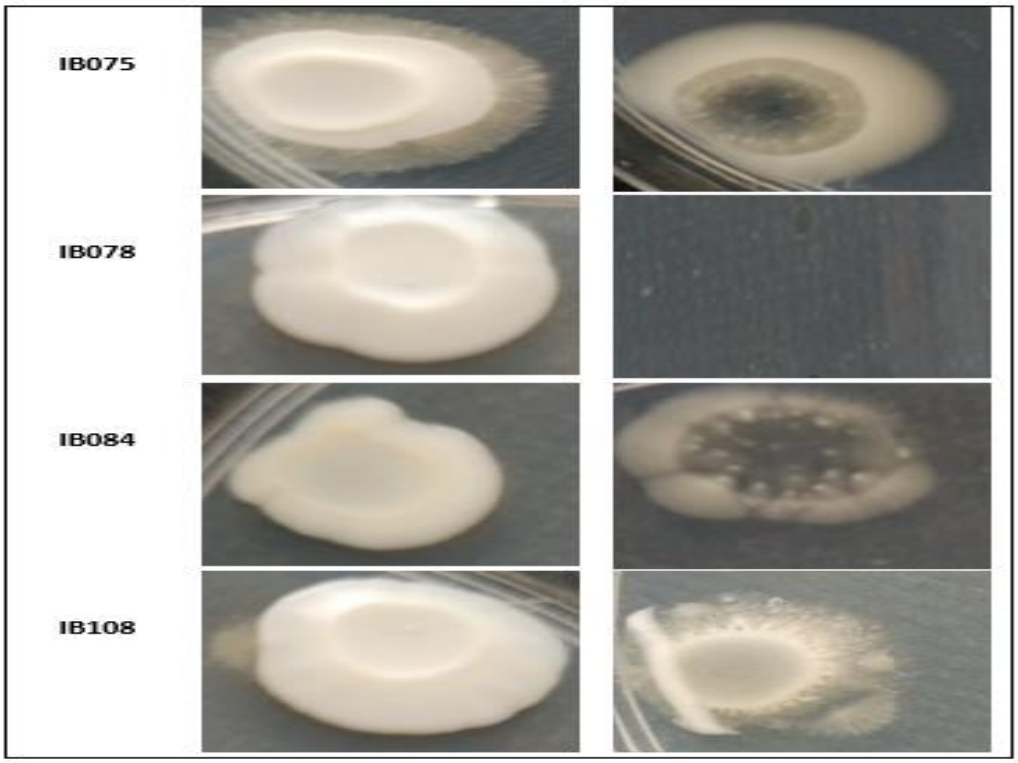
3.7 Adhesion and Filamentation

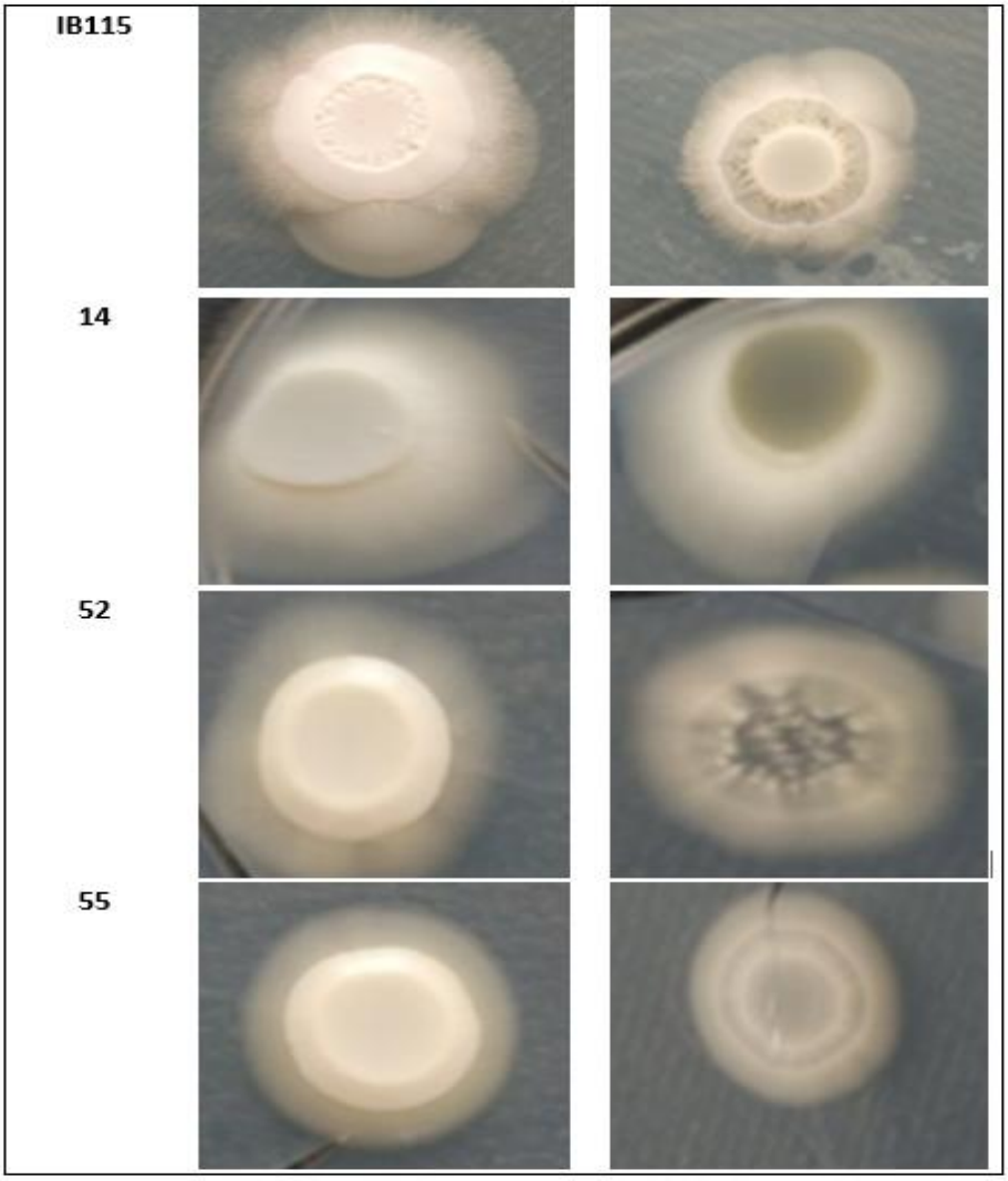
C. albicans isolates were grown on PDA for 14 days at 37ºC to allow for adhesion and invasive filamentous growth. Adhesive filamentous strains were more resistant to washing than non-filamentous strains. Strains that were filamentous were able to adhere to PDA after washing more than those that were not filamentous. No clear cut differences were observed between resistant and sensitive strains as far as adhesion. However a correlation between adhesion and filamentation was found whereby adhesive strains resisted washing by forming invasive filamentous growth.

Table 5. Adhesion and filamentation assay. All Sixteen *C. albicans* isolates were assayed for adhesion and filamentation by growth on PDA media for 14 days at 37 ºC followed by washing. Adhesive and filamentous isolates were able to remain adherent to PDA, while non adhesive and non filamentous isolates were washed away after rinsing with water.




IB020
IB032
IB059
IB070


IB075
IB078
IB084
IB108



3.8 Summary of the Results Obtained

Table 6 below summarizes the results obtained for all 16 isolates. IB014, IB032, IB059, IB075, and IB108 are resistant to caspofungin and Congo red, mutated at the *FKS1* gene, avirulent, have a high chitin content, but low biofilm forming capabilities. On the other hand IB006, 14, IB078, and IB115, are sensitive to caspofungin and Congo red have the low MIC values, and harbor a wild type *FKS1* gene. They are also virulent, with low cell wall chitin content, and a higher biofilm potential.

Table 6. Summary of results. Note that resistant strains harbor *FKS1* mutations, are attenuated in virulence, with a thicker chitin cell wall, decreased biofilm formation ability, and increased resistance to cell wall disrupting agents. Phenotypes are denoted by a cross with one cross denoting reduced levels, two crosses denoting intermediary levels, and three crosses denoting increased levels.

Isolate	Mutation	MIC	Virulence	Congo red	Chitin	Biofilm	Adhesion	Filamentation
IB006	NO	0.023 (S)	+++	+	+	+++	++	++
IB014	YES	1.5 (R)	+	+++	+++	+	++	+
14	NO	0.023 (S)	+++	+	+	+++	+++	+++
IB017	NO	0.38 (S)	++	++	++	++	++	+++
IB018	NO	0.042 (S)	+++	+	+	++	+	+
IB020	NO	0.38 (S)	++	++	++	++	+	+
IB032	YES	0.5 (I)	++	+++	+++	+	++	++
52	NO	0.38 (S)	++	++	++	++	++	+++
55	NO	0.25 (S)	+	++	++	+	+++	++
IB059	YES	1 (R)	_	+++	+++	+	++	+
IB070	NO	0.042 (S)	++	+	++	++	_	+
IB075	YES	1 (R)	+	+++	+++	+	+++	+++
IB078	NO	0.032 (S)	+++	+	+	+++	_	+
IB084	NO	0.064 (S)	+++	_	+	++	++	++
IB108	YES	1.5 (R)	_	+++	+++	+	++	++
IB115	NO	0.025 (S)	+++	_	+	+++	+++	+++

Chapter 4

Discussion

This thesis aimed at analyzing the genotypes and phenotypes of *C. albicans* strains with varying levels of sensitivities to the echinocandin, caspofungin. At the molecular level, *C. albicans* strains that are resistant to caspofungin have mutations in the *FKS* encoded subunits of glucan synthase at hot spot 1 (amino acids 641 to 649, FLTLSLRDP) and hot spot 2 (amino acids 1357 to 1364, DWIRRYTL) (Ben-Ami & Kontoyiannis, 2012; Park et al., 2005). Phenotypically, *C. albicans* strains that are resistant to caspofungin have been shown to possess impaired pathogenicity attributes (Da Silva et al., 2009; McKenzie et al., 2010; Walker et al., 2008).

Caspofungin is a relatively novel drug used in the treatment of candidiasis and accordingly levels of resistance have been found to be lower than for other traditional drugs such as azoles. This was also the case in Lebanese hospital isolates whereby a previous study found significant resistance to traditional drugs but non to caspofungin, while a later study determined the presence of low levels of resistance to anidulafungin, another member of the echinocandin family (Bitar et al 2014). Both hotspots from 16 *C. albicans* Lebanese hospital isolates resistant and sensitive to caspofungin were sequenced. Sensitive isolates were obtained from a previous study (Basma, 2007), while resistant isolates were isolates that were found to be resistant to other echinocandins such as anidulafungin (Bitar et al 2014) and tested here for caspofungin resistance. Four isolates were found to be resistant to caspofungin while another four showed intermediary resistance with eight isolates sensitive to the drug. Interestingly all 4 resistant isolates and one intermediary resistant isolate were found to contain mutations within the hotspots. These mutations included amino acid substitutions

within HS1 at positions L642V, T643I, L644V, L644G, R647K, D648S, and within HS2 at positions W1358S, R1360L, R1361L, T1363G, and L1364C. Some of the isolated mutations were not previously reported.

In addition, we wanted to determine whether any relationship between resistance and pathogenicity related attributes such as virulence, adhesion, filamentation, resistance to cell wall disrupting agents such as Congo red, biofilm formation, and cell wall chitin deposition is present in our isolates.

Strains that are resistant to caspofungin also showed increased resistance to Congo red but decreased biofilm formation and attenuated virulence in a mouse model of infection. Caspofungin sensitive strains showed decreased resistance to Congo red yet increased virulence and biofilm formation. All filamentous strains were adhesive but only those that are sensitive to caspofungin and showed no mutation were virluent. Chitin content analysis showed that caspofungin resistant strains have elevated levels of chitin resulting in cell wall thickening that counters the effect of caspofungin, while sensitive strains showed decreased chitin content. Our results demonstrate an inverse correlation between resistance and virulence whereby resistance is due to thickening of the cell wall preventing the cell from gaining virulence attributes, while a thinner cell wall increases susceptibility to drugs but allows increased virulence.

4.1 Resistance to Caspofungin

Studies have shown that echinocandins targets the fungal enzyme glucan synthase. β-1,3-glucan, a major cell wall component is synthesized through this enzyme. Once glucan synthase is inhibited by echinocandins, the fungal cell wall becomes weak causing the induction of different adaptive protective mechanisms involving stress response pathways yielding in a group of compensatory genetics responses (Perlin, 2011). These adaptive mechanisms will cause the cell to become

echinocandins tolerant, involving a decrease in enzyme sensitivity to the drug through amino acid changes in the hot spots of the FKS-encoded subunit of glucan synthase resulting in high MIC values.(Perlin, 2007, 2015)

The β-1,3-d-glucan synthase enzyme is responsible for the catalysis of sugar moieties from a specific activated donor molecule to a specific receptor molecule resulting in the formation of glycosidic bonds the UDP-glucose + ([1,3]-β-d-glucosyl) (N) → UDP + ([1,3]- β-d-glucosyl) (N + 1) reaction (Orlean, 1982). The genetics of glucan synthase have been understood through biochemical analysis and analysis of FKS-resistant mutants (Garcia-Effron, Lee, Park, Cleary, & Perlin, 2009; Garcia-Effron, Park, & Perlin, 2009; Inoue et al., 1996; López-Romero & Ruiz-Herrera, 1977).

Fks and Rho are two subunits of the enzyme complex (Kondoh, Tachibana, Ohya, Arisawa, & Watanabe, 1997). Fks, the target of echinocandin drugs, is encoded by the three related genes *FKS1*, *FKS2* and *FKS3,* and most importantly it is the catalytic subunit of the enzyme (Mio et al., 1997). *FKS1, FKS2* and *FKS3* are calcineurin dependent and the immunosuppressive drug FK506 is responsible for their downregulation (Mazur et al., 1995). The regulation of glucan synthase activity is accomplished through the GTP-binding protein in the Rho/Rac subfamily, Rho (Mazur & Baginsky, 1996).

Kinetic studies of echinocandins transport into the fungal cell, have shown that these cells have both high and low affinity transport systems to move the drug in an energy dependent manner into the cell (Paderu, Park, & Perlin, 2004). It is not known if the action of echinocadins requires its transportation into the cell. However, it has been speculated that the drugs tail may insert itself in the cells bilayer resulting in the inhibition of the glucan synthase (Perlin, 2011). This interaction shows that the drug might be acting through the extracellular part of the cell membrane on the enzyme,

implying that the entrance of the drug into the cell might not be required (Perlin, 2011). The inhibition caused by such an interaction is called non-competitive inhibition since the binding site for the substrate is different from the binding site for the drug (Garcia-Effron, Park, et al., 2009).

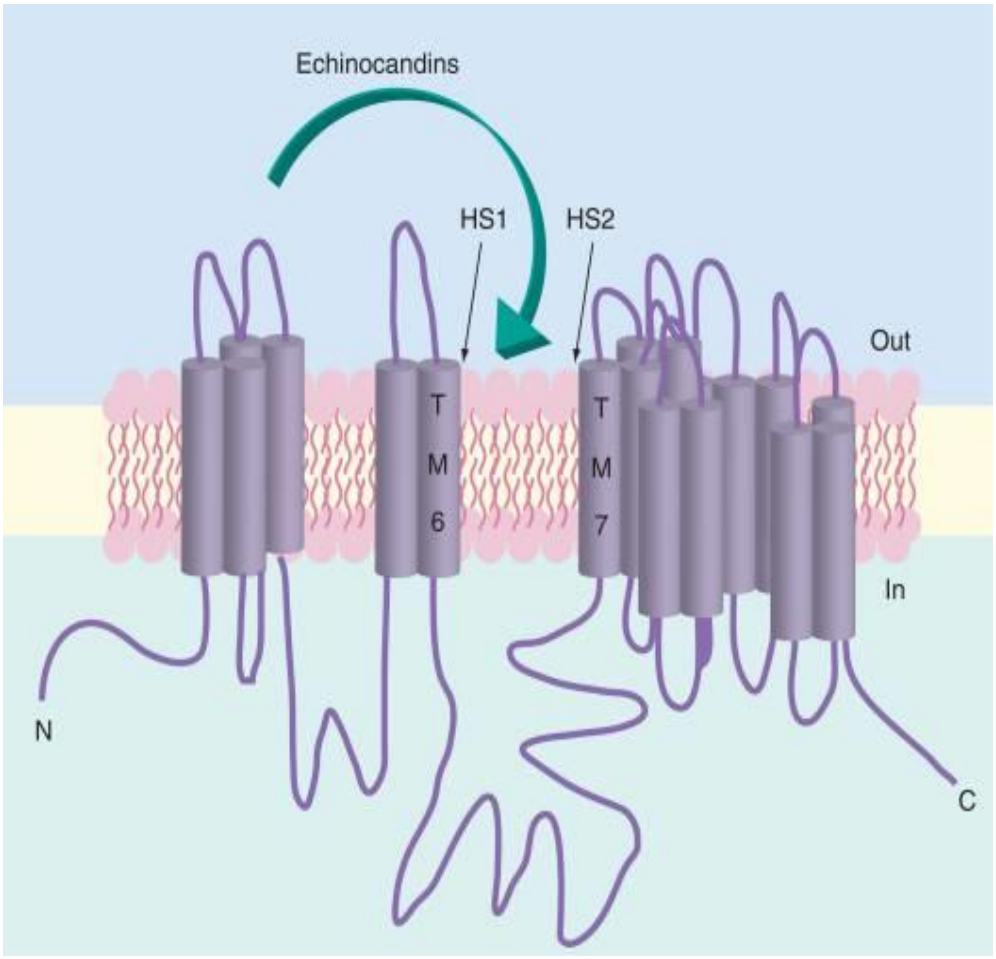


Figure 13. A membrane topology model of glucan synthase. The model shows the location of the hotspots where mutations take place (Douglas, 2001; Inoue et al., 1995)

Resistance caused by echinocandins is associated with two conserved hotspot regions of *FKS1* (amino acids 641 to 649, FLTLSLRDP) and/or *FKS2* (amino acids 1357 to 1364, DWIRRYTL) at which mutation occurs. *FKS1* is the most expressed *FKS* gene and most of the mutations occur there (Castanheira et al., 2010(Pappas et al., 2009).

It is not known exactly where caspofungin bind to its target enzyme. Regardless however, these mutations will hinder the full interaction of caspofungin with its binding site on the enzyme. In addition, these mutations occur near the transmembrane domain of the enzyme critical for function, hence drastically decreasing the catalytic efficiency (seen as a decrease in Vmax) of the enzyme by about 50% resulting in a decreased glucan deposition on the cell surface. The cell attempts to compensate this by increasing transcription levels of the enzyme, however since the enzyme is losing activity this mechanism is not successful (Perlin et al., 2015). Other mechanisms such as chitin deposition are activated and are more successful in compensating for glucan loss. (Kurtz et al., 1996). In summary resistance takes place by the drug not being able to bind the altered 3D enzyme structure, in addition to the fact that the mutation decreases capacity of the enzyme to synthesize glucans, leading to a compensatory mechanism.

Four *C. albicans* strains (IB014, IB059, IB075, and IB108) were found to be resistant to caspofungin with MIC values ≥ 1 μg/ml, 4 *C. albicans* isolates (IB017, IB020, 52, and IB032) show intermediate resistance to caspofungin with MIC values between 0.38 μg/ml and 0.5 μg/ml, and the remaining 8 *C. albicans* isolates (IB006, 14, IB018, 55, IB070, IB078, IB084, and IB115) are sensitive to caspofungin with MIC values ≤ 0.25 μg/ml (as per CLSI document M27-S4, 2012).

Isolates that are resistant to caspofungin showed amino acid substitution within the 2 hotspots and no mutations outside these regions were found in any of the isolates.

For IB014, HS1 amino acid substitutions are L644V, R647K, and D648S, and HS2 amino acid substitution is R1360L. L644V and R647K amino acid substitutions are conserved while D648S and R1360L amino acid substitutions are not conserved

since amino acid D is hydrophilic with negative polarity and S is non-polar, amino acid R is hydrophilic and negatively charged while L is hydrophobic and non-polar.

For IB059, HS1 amino acid substitutions are L642V, L644V, and D648S, and HS2 amino acid substitution is T1363G. L642 and L644V amino acid substitutions are conserved while D648S and T1363G are not since D is acidic while S is not. Also T is hydrophilic non-charged while G is hydrophobic and non-polar.

In the case of IB075, HS1 amino acid substitution is T643I, and HS2 amino acid substitutions are R1360L and R1361L. As discussed earlier a mutation at T643 is said to be silent. However this substitution is not conserved since T is hydrophilic and uncharged while I is hydrophobic and non-polar. R1361L and R1360L amino acid substitutions are not conserved since R is hydrophilic and positively charged while L is hydrophobic and non-polar.

In IB108, HS1 amino acid substitutions are T643I, L644G, and D648S and HS2 amino acid substitutions are W1358S and R1361L. The only conserved amino acid substitution is L644G, while T643I, D648S, W1358S, and R1361L are not. T is hydrophilic and uncharged while I is hydrophobic and non-polar, W is hydrophobic and non-polar while S is hydrophilic and uncharged, and R is hydrophilic and positively charged while L is hydrophobic and non-polar.

Isolates showing intermediate resistance to caspofungin with MIC values between 0.38 μg/ml and less than 0.5 μg/ml had no mutations in the hotspots with the exception of only one isolate IB032 with intermediate resistance to caspofungin with an MIC value 0.5 μg/ml that showed mutations in both hotspots: a mutation in HS1, a substitution of D648S and HS2, a substitution of L1364C. L1364C is a conserved amino acid substitution while D648S is not conserved.

IB014, IB032, IB059 and IB108 share a common mutation at the position 648 of the HS1 region, were serine (S) is substituted by aspartic acid (D). IB014 and IB075 share a common mutation is at position 1360 of the HS2 were arginine (R) is substituted by leucine (L), IB108 and IB075 share two common mutations at position 1361 of the HS2 and 643 of the HS1 were arginine (R) is substituted by leucine (L), and threonine (T) is substituted by isoleucine (I) respectively. In addition IB059 and IB014 share a common mutation is at position 644 of the HS1 were leucine (L) is substituted by valine (V).

As discussed previously some of these amino acid mutations are conserved and might not change the structure and function of the protein. However, non-conserved amino acid mutations cause a change in the 3D structure of the protein therefore changing its function.

Table 7. Non-conserved amino acid substitutions. Note the strong correlation between non-conserved mutations and high MIC values.

Isolates	Non-conserved amino acid substitution within HS1	Non-conserved amino acid substitutions within HS2	Total number of mutation	MIC
IB014	D648S	R1360L	4	1.5(R)
IB032	D648S	0	2	0.5(I)
IB059	D648S	T1363G	4	1(R)
IB075	T643I	R1361L, R1360L	3	1(R)
IB108	T643I, D648S	W1358S, R1361L	5	1.5(R)

As can be seen in Table 7 there is a strong correlation between amino acid substitution and elevated MIC values. IB032 with intermediary resistant to caspofungin, exhibits only one non-conserved amino acid substitution, whereas IB108 with the highest MIC value shows four non-conserved amino acid substitutions.

Accordingly not all *FKS* mutations have the same level of resistance, the position and number of mutations, and whether the amino acid substitution is conserved or not dramatically alter the tertiary structure of the enzyme, are responsible for increased resistance.

The most common amino acid substitutions are at positions F641, L642, T643, L644, S645, R647, D648, P649, W1358, and R1361 (Park et al., 2005) within hot spot 1 (HS1) (amino acid positions 641 to 649, FLTLSLRDP) (Katiyar et al., 2012) and hot spot 2 (HS2) (amino acid positions 1357 to 1364, DWIRRYTL) (Lackner et al., 2014). In this study we detected novel amino acid changes that were not previously characterized. These amino acid changes are within HS2 at amino acids R1360, T1363, and L1364.

4.2 Cell Wall of Caspofungin Resistant *C. albicans*

Cell wall salvage pathways are triggered once *C. albicans* is exposed to echinocandins, these salvage pathways includes high-osmolarity glycerol mitogen-activated protein kinase, protein kinase C pathways, and Ca^{+2}/calcineurin (Munro et al., 2007). Upregulation of the chitin synthase gene expression, high activity of the chitin synthase, and high chitin content in the cell wall are the results of these activated pathways (Munro et al., 2007).

C. albicans are protected from echinocandins through the high levels of chitin content in the cell wall (Walker et al., 2008). Caspofungin-resistant *FKS1* gene mutant *C. albicans* strains have high cellular chitin thus a thickened cell wall and as such strains were able to grow in high echinocandins concentrations (Stevens, Ichinomiya, Koshi, & Horiuchi, 2006; Walker et al., 2008).

Only 5 out of the 16 strains that we studied have high cellular chitin content. Interestingly all these 5 cell wall thickened strains were strains that had the highest MIC concentrations, 4 being resistant and one exhibiting intermediary resistance (IB014, IB032, IB059, IB075, and IB108). None of the sensitive strains had a significant increase in cell wall chitin. This absolute correlation strengthens the notion that the cell overcompensates for exposure to echinocandin by increasing chitin concentrations. Increased cell wall thickness has been previously reported for many cell wall proteins. In a deletion analysis of 40 cell wall proteins performed by (Plaine et al., 2008) increased cell wall thickness in response to cell wall mutations was observed for many mutants resulting in resistance to cell wall disrupting agents such as calcofluour white and caspofungin. We have also observed a 2 fold increase in cell wall chitin deposition in a *PIR 32* mutant strain that we generated. Pir 32 is a cell wall protein that is necessary for rigidity. A deletion would be expected to render the cell surface more susceptible to cell wall disrupting agents however we found the mutant to be more resistant oxidative stress and SDS this was due to an increase in chitin deposition.

In our study *FKS1* mutant strains that lacked susceptibility to caspofungin mirrored this resistance by a lack of sensitivity to Congo red, the cell wall disrupting agent. Congo red acts in a somewhat similar fashion to caspofungin by interfereing with glucan microfibril assembly in addition to chitin assembly (Bruno et al., 2006). Accordingly it makes sense that resistance to caspofungin is reflected in a resistance to Congo red. Strains that were sensitive to caspofungin were also sensitive to Congo red.

4.3 Virulence

Mechanisms of *C. albicans* virulence and host defense were investigated through the use of murine model of disseminated candidiasis. Survival rates after

intravenous *C. albicans* infection were monitored daily over a period of 3 weeks. (Lo et al., 1997).

As discussed previously, *C. albicans* strains that are less susceptible to caspofungin bearing a mutation in the *FKS1* gene, have a thickened cell wall therefore high levels of chitin. These findings are associated with reduced virulence. There are many explanations as to why increased chitin results in reduced virulence. One explanation is that increased chitin might increase rigidity and hinder the cell wall from elongating and taking on filamentous forms that are known to be associated with invasive candidiasis and virulence (Ben-Ami et al., 2011; Berman, 2006; Rueda, Cuenca-Estrella, & Zaragoza, 2014).

Another explanation is that *C. albicans* interacts with host immune cells through its cell wall, activating a Dectin-1–mediated inflammatory response. Strains with high chitin content were only capable of activating a weak host inflammatory response this might be reflecting that chitin is acting as an anti-inflammatory signal, bearing in mind that long chitin fragments have immune-regulatory functions (Da Silva et al., 2009; de Jonge & Thomma, 2009).

Thirdly increased chitin thickness might hinder deposition or secretion of proteins with virulent attributes. The lack of these proteins on the cell wall might result in attenuated virulence. As cell wall proteins are highly antigenic and involved in virulence related attributes coding for lipases, adhesins and super oxide dismutases, a restructuring of the cell surface proteome might affect virulence. We have determined recently (El Khoury, Awad, Wex, & Khalaf, 2018) that a deletion of a single cell wall protein can dramatically alter the cell surface architecture and affect multiple phenotypes including virulence.

In this study, caspofungin-resistant *FKS1* gene mutant *C. albicans* strains IB014, IB032, IB059, IB075, and IB108 with high levels of chitin, are avirulent. Surprisingly, strain 55 with a caspofungin-sensitive MIC value of 0.25 μg/ml and no amino acid substitutions within the hotspots, was also avirulent. Strain 55 however exhibited a relative increase in chitin concentration in its cell wall when compared with the remaining sensitive strains, even though chitin levels are still below values conferring resistance.

4.4 Biofilm Formation

Biofilms of mature *C. albicans* are usually found in a complex three-dimensional architecture enclosed within exopolymeric material and made up of an intricate network of hyphae, pseudohyphae, and yeasts within channels of water (Ramage, Vandewalle, Wickes, & López-Ribot, 2001). The growth of *C. albicans* biofilm depending on farnesol, a quorum sensing molecule, and on Ras/cAMP/PKA signaling pathway that in addition to biofilm, plays an important role in regulating filamentation and virulence attributes such as adhesion and dimorphism. (Uppuluri, Pierce, & López-Ribot, 2009).

The ability of our 16 *C. albicans* strains to form biofilm was tested through crystal violet absorption. Our biofilm data results were inversely correlated with our cell wall chitin data. Strains that were virulent and contained thin chitin cell walls had the ability to form a thick biofilm layer. On the other hand strains that had increased chitin cell wall deposition were attenuated in virulence and formed reduced levels of biofilm. This is to be expected since biofilm formation is a virulence attribute and reduced virulence should be reflected by reduced biofilm formation.

β-1,3-glucan, synthesized by glucan synthase is a major component of biofilms. Strains that have a mutation in the glucan synthase gene will not be able to synthesize β-1,3-glucan needed to form a thick biofilm meshwork. (Desai et al., 2013; Nett, Crawford, Marchillo, & Andes, 2010) providing an explanation as to why our *FKS1* mutants had decreased biofilm capabilities. Another possible explanation is that, as mentioned above, the increase in chitin thickness might hinder deposition of biofilm related proteins, or hinder secretion of matrix polysaccharides.

4.5 Adhesion and Filamentation

C. albicans has the ability to attach to different types of tissues and inanimate surfaces through adhesion. As a pathogen, adhesion is the first step necessary for virulence allowing *C. albicans* to lock onto its host. After adhesion is accomplished the fungus can through its arsenal of cell surface hydrolytic enzymes such as phopholipases, lipases and proteases degrade the host tissue and disseminate (Cotter & Kavanagh, 2000).

In this study the 16 *C. albicans* isolates were assayed for their ability to adhere and filament on PDA agar. Adhesion was determined by the ability of cells to withstand vigorous washing. Strains 14, 55, IB075, and IB115 showed high levels of adhesion and filamentation on PDA after washing. While strain IB018, IB020, IB70, and IB78 were neither adhesive nor filamentous, with the remaining colonies exhibiting varying levels of adhesion and filamentation.

The correlation between adhesion and filamentation was high, strains that were adhesive were filamentous. This is due to the fact that filamentation allows for invasive growth and invasive growth under the agar shields the colony from being washed off. However no strong correlation was observed between adhesion and virulence or with

caspofungin resistance. Some strains were virulent and adhesive such as strain 14 and IB115, however other strains such as strain 55 and IB075 for example were attenuated in virulence but able to adhere to agar and resist washing. On the other hand strains IB018 and IB078 were virulent but did not exhibit strong adhesive properties. Since adhesion is a necessary precursor for virulence this might come as a surprise. However it should be noted that the adhesion assay performed was performed on agar and not on human cells or under in vivo conditions, different environmental pressures might result in expression of adhesion genes not expressed under the conditions of our experiment.

Chapter 5
Conclusion and Insights

This study is the first study of its kind in Lebanon to report caspogfungin resistance in Lebanese hospital isolates. This study is also the first to genotype *C albicans* isolates from Lebanon by sequencing a fragment of the *FKS1* gene in an attempt to determine the molecular basis of resistance. The study then attempts to link drug resistance to a host of virulence related attributes and phenotypes.

We have shown that Lebanese isolates share many of the mutations previously observed in 2 hot spot regions of the *FKS1* gene. Interestingly however we have shown that novel mutations not previously characterized are present in the Lebanese populations. We have shown that these mutations confer resistance to caspofungin, with only resistant isolates exhibiting point mutations while sensitive isolates have the wild type form of the *FKS1* allele. We have also determined that drug resistant isolates have an increased cell wall chitin deposition that renders them resistant to cell wall disrupting agents such as Congo but show a decreased ability to form biofilm and are attenuated in virulence. On the other hand drug sensitive strains have low chitin content, are virulent with the ability to form extensive biofilm networks.

Such a study is one of only a handful to look into the current state of drug resistance in Lebanon and analyze in depth the mechanisms of resistance, and attempt to correlate genotype to phenotype. This study should improve our current understanding of *C. albicans* infections in Lebanese hospitals.

Future work should include increasing the sample size, carrying out an adhesion essay on human cell lines, and performing similar genotyping on *C. albicans* isolates resistant to other classes of drugs. Furthermore, the long-term goal would be to eventually perform whole genome sequencing of *C. albicans* resistant and sensitive to

caspofungin in an effort to determine novel hotspots and SNPs that correlate with resistance, and pathogenicity related phenotypic attributes such as adhesion, biofilm formation, and virulence.

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