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MICONAZOLE RESISTANCE IN FUNGAL BIOFILMS: A MOLECULAR POINT OF VIEW

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ALS	agglutinin-like sequence
ALT	antifungal lock therapy
ATCC	American type culture collection
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary DNA
CDR	*Candida* drug resistance
cfu	colony forming units
CoA	coenzyme A
DCFHDA	2,7-dichlorofluorescein diacetate
DEPC	diethyl pyrocarbonate
DHS-1-P	dihydrosphingosine-1-phosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPM	extracellular polymeric matrix
ESCRT	endosomal sorting complex required for transport
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
GRAS	generally regarded as safe
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
IHEM	Institute of Hygiene and Epidemiology
IMS	immunomagnetic separation
MAPK	mitogen-activated protein kinase
MDR	multi-drug resistance
MF	major facilitator
MIC	minimum inhibitory concentration
MIP2C	mannose inositol phosphate-2-ceramide

MOPS	3-(N-morpholino)propanesulfonic acid
MUCL	Mycothèque de l'Université Catholique de Louvain
n	number of replicates
NCYC	National Collection of Yeast Cultures
P	P-value, indicating the level of significance
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDTC	pyrrolidinedithiocarbamate
PHS-1-P	phytosphingosine-1-phospate
PS	physiological saline
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	standard error of the mean
spp.	species
SSGO	significantly shared gene ontology
tRNA	transfer RNA
WT	wild type
YNB	yeast nitrogen base
YPD	yeast peptone dextrose

CHAPTER 1

INTRODUCTION

YEASTS

Yeasts are unicellular microorganisms belonging to the kingdom of the fungi. They are widespread in nature and can be isolated from air, water and soil. Furthermore, plants are a preferred niche and several yeasts are commensally associated with animals and humans (1-3).

Cellular organization

Yeasts are eukaryotic cells with a diameter of 2-10 µm. The nucleus and other cell organelles are surrounded by membranes, which distinguishes yeasts from prokaryotes. The cytoplasmatic membrane and the cell wall form the barrier between the cytoplasm and the environment (Figure 1.1). The cytoplasmatic membrane consists of a phospholipid bilayer with sterols (principally ergosterol) and proteins, and forms an impermeable barrier for hydrophilic molecules. The sterols determine membrane rigidity, while the proteins are involved in signaling, anchoring of the cytoskeleton and transport of molecules across the cytoplasmatic membrane (4-6).

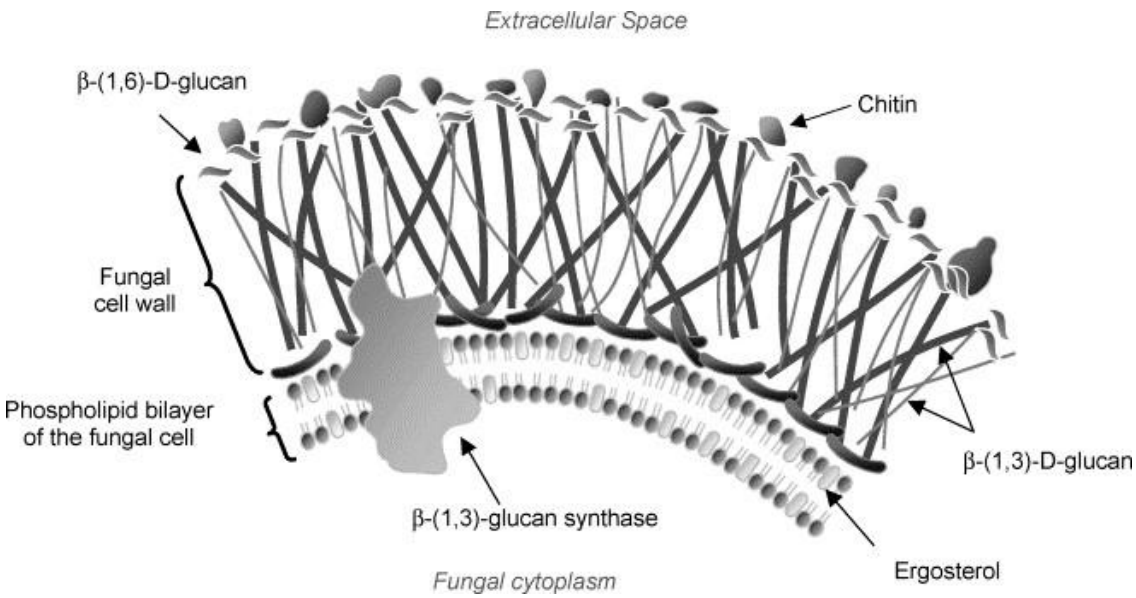

Figure 1.1. Fungal cytoplasmatic membrane and cell wall (7).

The cell wall contains mainly polysaccharides, and to a lesser extent lipids and proteins. The most abundant polysaccharide is β-glucan, which consists of glucose monomers linked by β-1,3 and β-1,6 glycosidic bonds. Chitin, another polysaccharide consisting of β-1,4-linked N-acetylglucosamine units, is found in smaller amounts and is covalently bound to glucans. Mannans, consisting of α-1,2- and α-1,3-linked mannose monomers bound to an α-1,6-linked backbone, are also found. Together with mannoproteins, mainly localized at the outer surface, the cell wall forms a complex network, involved in cell protection, maintenance of shape and cellular interactions (5-9).

Growth forms

The most common mode of vegetative reproduction in yeasts is budding. A daughter bud arises at the cell surface of the mother cell and starts growing. At the end of the cell cycle a septum is formed between both cells, which are subsequently separated. When the daughter cell remains attached to the mother cell, pseudohyphae are formed. These are chains of elongated and ellipsoid yeast cells attached to each other. Another mode of filamentous growth is the formation of true hyphae, long cells with parallel-sided walls and without constrictions between the cells (Figure 1.2). Both filamentous growth forms often show a branching pattern. The morphological switches of yeast cells are reversible and depend on the environmental conditions (10-12).

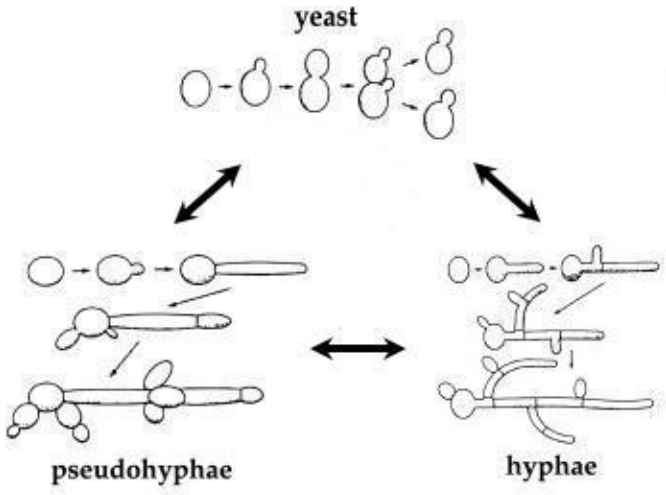

Figure 1.2. Morphology of yeast (13).

Candida albicans

Pathogenesis

Candida species are human commensals found in the mouth, gastrointestinal tract and vagina. However, they are opportunistic human pathogens and can cause infections upon disturbance of the normal mucosal flora (e.g. after broad-spectrum antibacterial therapy) and immune dysfunction (e.g. HIV infection, chemotherapy) or underlying illness (e.g. diabetes) in patients. Most *Candida* infections are superficial and include vulvovaginal or oropharyngeal candidiasis (oral thrush), with *C. albicans* being the most common cause of disease (14, 15). The use of medical devices such as prostheses and catheters may increase the risk of *Candida* related infections and is often at the origin of nosocomial infections (16). When *Candida* cells penetrate and cross the epithelial barrier, they may enter the bloodstream and cause candidemia. This bloodstream infection is frequently associated with surgical interventions or with treatment of patients at an intensive care unit (17-20). Once *Candida* spp. enter the bloodstream, they may disseminate to the organs and cause life threatening invasive candidiasis (14, 15, 20). These infections show a high (and still increasing) global incidence, leading to an increased morbidity and a prolonged hospital stay, at an enormous financial cost for the society. In addition, invasive *Candida* infections are responsible for a high mortality rate, particularly among critically ill patients (18, 21-23).

Virulence

The pathogenicity of *C. albicans* is attributed to a wide range of virulence factors. The first step in pathogenesis towards host cells is adherence. Adhesins, including the agglutinin-like sequence (ALS) proteins, Hwp1 and Eap1 are cell surface molecules involved in the adhesion to other cells and to biotic or abiotic surfaces. They all encode glycosylphosphatidylinositol cell wall proteins, covalently linked to β-1,6-glucans in the cell wall (3, 24). Among the eight members of the Als protein family, especially *ALS3* expression is important for adhesion (3, 25, 26).

Contact of yeast cells with a surface triggers the yeast-to-hyphae switch, which is another important virulence factor of *C. albicans*. Hyphal formation is a critical step in the invasion of epithelial cells and contributes to damage of the host cells. Moreover, this

morphological switch is important for biofilm formation, which drastically increases the virulence of *C. albicans*. This topic will be discussed in more detail below. The invasion of epithelial cells is enhanced by the expression of invasins (Als3 and Ssa1), which induce endocytosis. Furthermore, three classes of degradative enzymes (proteases, phospholipases and lipases) are produced to facilitate active penetration into the host cells. This active penetration is also believed to be mediated by physical forces (3, 15, 26, 27).

Additionally, its metabolic flexibility as a reaction to the available nutrients, the regulation of environmental pH and the response to extracellular stresses allows *C. albicans* to survive in a broad range of conditions (3).

Quorum sensing, communication between cells in a population regulated by signal molecules, is also associated with virulence (27, 28). In *C. albicans*, quorum sensing is reportedly regulated by the gene *CHK1* (29). Two quorum sensing molecules with an opposite effect have been identified in *C. albicans*. Farnesol inhibits the yeast-to-hyphae transition, while tyrosol promotes hyphae development. Initially, farnesol was a promising compound to treat invasive candidiasis, but as addition of endogenous farnesol increases the virulence of *C. albicans*, it needs to be considered as a virulence factor itself (27, 28).

Immunology

The first immune response to the human pathogen *C. albicans* is mediated by the innate immune system. Monocytes, macrophages and neutrophils contribute to this immune response by phagocytosis and immediate killing of the pathogen through oxidative (reactive oxygen species; ROS) and nonoxidative mechanisms (defensins, cationic peptides, iron sequestration). In a later stage, the adaptive immune system, mainly mediated by dendritic cells, is activated with the generation of T-cells, leading to a more specific immune response. These T-cells stimulate the protective immunity to *Candida* (Th1 and Th17), but may also temper or regulate the inflammatory response (Th2 and Treg, respectively). For *C. albicans*, being a human commensal, it is important to maintain a successful host-fungal interaction, which requires a balance between pro- and anti-inflammatory signals of the immune system (30-33).

Diagnosis

Fast, sensitive and reliable diagnostic tests for invasive candidiasis are important for starting an adequate therapy. The culture of blood samples is often used to detect *Candida* spp. in the bloodstream and may be performed by a fully automated system. However, this method still lacks sensitivity and culture growth takes several days. The components of the cell wall of *Candida* spp. can be used as markers for invasive candidiasis. Particularly diagnostic marker tests for (1,3)-β-D-glucan show a very good sensitivity and specificity (34, 35). Molecular genetic methods, such as PCR, are useful for rapid detection of candidemia. However, these tests are not commercially available and do not distinguish between DNA from living and dead cells (34, 36). Recently, the combination of immunomagnetic separation (IMS) of cells followed by solid-phase cytometry, proved to be fast and sensitive (37).

Other *Candida* species

Besides *C. albicans*, several other *Candida* spp. have been identified as human pathogens, with *C. glabrata*, *C. parapsilosis* and *C. tropicalis* being the most frequently isolated species. Together, these four species cover about 90% of invasive *Candida* infections (21).

C. glabrata is the second most common *Candida* species after *C. albicans* and its incidence is increasing over the last years. It has been identified in oral infections and on dentures in elderly patients, but it is also a significant cause of systemic infections. *C. glabrata* has a haploid genome, which makes it more closely related to *Saccharomyces cerevisiae* than the other medically important diploid *Candida* species. Futhermore, it does not exhibit polymorphism and has a lower virulence. *C. glabrata* is known to have a lower susceptibility to azoles. Therefore, the increase in azole use to treat fungal infections may have contributed to the increase of *C. glabrata* infections (14, 38, 39).

C. parapsilosis is able to form pseudohyphae, unlike true hyphae, explaining its lower virulence compared to *C. albicans*. Nevertheless, it particularly causes nosocomial infections due to its ability to survive in the hospital environment, which is quite unique among *Candida* species. Additionally, *C. parapsilosis* is involved in different infections ranging from superficial infections to invasive candidiasis (14, 40, 41).

C. tropicalis has genetically the highest similarity to *C. albicans* and is able to form pseudohyphae. It is rarely isolated from oropharyngeal infections, but mainly causes disseminated infections in oncology patients (14, 42, 43).

C. krusei, *C. dubliniensis*, *C. guilliermondii*, *C. lusitaniae* and *C. rugosa* are less frequently to rarely associated with human diseases. Of these species, *C. dubliniensis* is most closely related to *C. albicans* and also has the ability to grow as true hyphae. Although both species have a similar epidemiology, *C. dubliniensis* is rarely observed in invasive candidiasis (44). *C. krusei* is of particular importance due to its multidrug resistance pattern and consequently its potential to become an emerging pathogen (14, 45).

Saccharomyces cerevisiae

The yeast *S. cerevisiae* is one of the most intensively studied microorganisms. The entire genome of *S. cerevisiae* has been sequenced and genetic manipulations can be performed easily. *S. cerevisiae* is widely used in the food industry for the production of beer, wine and bread. Furthermore, it plays an important role in the production of bioethanol and has certain applications in the chemical and pharmaceutical industry for the production of secondary metabolites (e.g. glycerol, propanediol, isoprenoids, organic acids). Metabolic engineering of *S. cerevisiae* or optimization of the growth conditions is often required to enable or enhance the production of these secondary metabolites (46-49). *S. cerevisiae* is also used for its biosorption activities to detoxify wastewaters containing heavy metals (50). Because it is such a preferred organism for many fermentation-derived products, metabolic engineering projects are in continuous progress (46, 48).

Over the years, *S. cerevisiae* has become a well established eukaryotic model organism for fundamental research. Many eukaryotic gene functions have been derived from yeast experiments and can be extended to other eukaryotes. Furthermore, *S. cerevisiae* is a useful model for studying human diseases (51, 52).

Although *S. cerevisiae* is classified as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration, there are case reports describing invasive *Saccharomyces* infections. These infections remain very rare, but an increased incidence has been noticed since 1990 especially in immunocompromised patients. Invasive *Saccharomyces* infections can be nosocomially acquired, but the portal of entry is mainly

digestive. A remarkably high association has been observed with the use of probiotic products containing *S. boulardii* (actually a specific strain of *S. cerevisiae*) (53).

BIOFILMS

Biofilms are microbial communities of cells which are attached to a surface and embedded in an extracellular polymeric matrix (EPM) (54). It is widely accepted that a biofilm is a preferred and beneficial microbial life-style (55). The molecular mechanisms involved in C. albicans biofilm formation have been extensively studied, but not completely been elucidated. Biofilm formation is a complex phenomenon, regulated by several transcription factors, controlling a total network of target genes which comprise about 15% of the genome (56).

Biofilm formation in *C. albicans*

C. albicans biofilm formation proceeds in distinct developmental phases (Figure 1.3). The initial step is the adhesion of blastospores (yeast form of the cells) to a surface. Biotic surfaces, such as teeth, skin and mucosal membranes, but also abiotic surfaces such as prostheses, dentures and catheters can be colonized. Differences in chemical properties, hydrophobicity and surface roughness of these medical devices affect biofilm formation. Nevertheless, the formation of *C. albicans* biofilms has been observed on different materials, such as silicone, polyurethane and polyvinyl chloride. *In vivo*, a conditioning film on the biomaterials, created by the surrounding body fluids, masks the chemical characteristics of surfaces and may influence biofilm formation (16, 57-60). Several adhesins are involved in the cell-cell and cell-substrate adhesion, including Als1, Als3, Hwp1 and Eap1 (25, 61, 62). The latter is controlled by the transcription factor Efg1 and the first three are controlled by the transcription factors Bcr1 and Tec1. Mutant strains affected in all three transcription factors show severe defects in biofilm formation (56, 61).

Shortly after adhesion, the yeast cells start growing and microcolonies are formed. Simultaneously, hyphal formation is induced. The transcription factors, Efg1, Tec1, Ndt80 and Brg1 are involved in filamentation. Deletion of *EFG1* inhibits yeast-hyphae transition. Hyphal formation contributes to the formation of a robust biofilm, but biofilm defects are not always due to the inability to form true hyphae (61-65). During the maturation of the biofilm, the amount of EPM increases, resulting in a complex three-

dimensional structure of yeasts and filaments, covered by EPM. The EPM of biofilms is mainly composed of carbohydrates, with glucose being the most abundant monosaccharide. Some of the glucose in the matrix is present as β-1,3 glucan. Proteins and hexosamine are found in smaller amounts. Futhermore, the EPM contains extracellular DNA, which contributes to maintaining the structural integrity of mature *C. albicans* biofilms (66-69). The zinc-responsive transcription factor Zap1, is a regulator of the EPM production (61, 70). Recently, two glucan transferases (Bgl2 and Phr1) and a glucanase (Xog1) were found to be crucial for the transport of β-1,3 glucans to the matrix and for accumulation of the matrix biomass. These enzymes do not affect the glucan assembly of the cell wall and work independently of the transcription factor Zap1 (71). The transcription factor Rob1 and the genes *SUV3*, *NUP85*, *MDS3* and *KEM1* are also known to play a role in biofilm development. However, their biochemical function could not be related to specific mechanisms required for biofilm formation (56, 72).

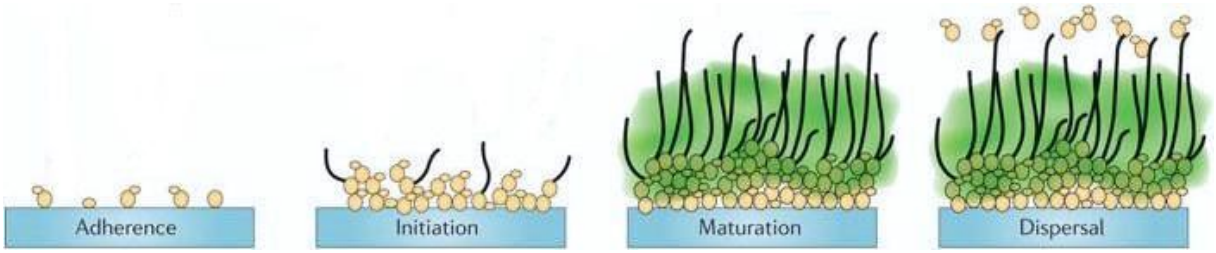

Figure 1.3. The different phases in fungal biofilm development (61).

Finally, cells can detach from the biofilm, disperse in the environment and start colonization on a different location (55, 59, 62). The genes *UME6*, *PES1* and *NRG1* have previously been described to be involved in this process. Furthermore, it is suggested that dispersal could be induced in response to quorum sensing molecules (61, 62).

Clinical relevance of *C. albicans* biofilms

Fungi often colonize biotic and abiotic surfaces to form biofilms. *C. albicans* is able to form biofilms on many medical devices, including vascular and urinary catheters, voice prostheses, endotracheal tubes, cardiac valves and pacemakers. The majority of *Candida* bloodstream infections (50 to 70%) is associated with biofilms on central venous catheters (73, 74). Dispersal of these sessile cells may lead to candidemia and systemic infections. These dispersed sessile cells show a specific phenotype with increased virulence compared to planktonic cells (75). Patients with diabetes mellitus or urinary catheterization have an increased risk for *Candida* bloodstream infections (76). Fungal biofilms are of high medical importance due to their increased resistance to antifungals (mechanisms of resistance are discussed below). Biofilm related *Candida* infections lead to a prolonged hospital stay, higher costs of antifungal therapy and a significantly higher mortality rate compared to non-biofilm related *Candida* infections (76, 77). The choice of initial antifungal therapy is crucial for the patient's outcome and total cost. Patients receiving a highly active anti-biofilm therapy (echinocandins) need a shorter hospital stay and have reduced risk of death compared to patients that have been treated with azoles (76, 78). Furthermore, the colonization of medical devices often leads to their dysfunction. As antifungal treatment rarely achieves clinical cure, replacement of the infected medical device is often the only valuable solution, increasing the medical costs (16, 57, 79).

Biofilm formation in *S. cerevisiae*

Biofilm formation by *S. cerevisiae* is distinct from that by *C. albicans*. The main difference is the absence of dimorphism in *S. cerevisiae*, although the formation of pseudohyphae has been observed in diploid strains (80, 81). *S. cerevisiae* is able to adhere to different surfaces and to complete the initial steps of biofilm formation. Therefore, *S. cerevisiae* is a valuable model for fungal biofilm formation (82, 83). Cell-cell adhesion is regulated by all members of the flocculin gene family, including *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. A first group of genes (*FLO1*, *FLO5* and *FLO9*) encodes cell wall proteins which act as lectins, while *FLO10* and *FLO11* confer adhesion, including cell-surface adhesion, by increasing cell surface hydrophobicity (84-86). Previous research showed that one *FLO* gene can compensate for the absence of another (87). Flo11 is also

known for inducing pseudohyphal growth in diploid strains (82, 83). The production of EPM, mainly containing mono- and polysaccharides, is also observed in a *S. cerevisiae* biofilm, resembling biofilm formation in pathogenic fungi (83, 88). Additionally, flocculation has important industrial applications. It is an easy and low cost method to separate yeast cells from their fermentation products (89).

Two other genes (*AGA1* and *FIG2*) encode cell wall adhesins in *S. cerevisiae*. They are not involved in biofilm formation, but are highly induced during mating (90, 91).

THERAPY

The rise in fungal infections also increases the need for effective antifungal agents. Different classes of antifungals are available for treatment of superficial and systemic infections. The majority of antifungals interacts with the biosynthesis of ergosterol. Additionally, damage to the cytoplasmatic membrane, inhibition of DNA and RNA synthesis or alteration of the cell wall composition are modes of action of antifungals (Figure 1.4).

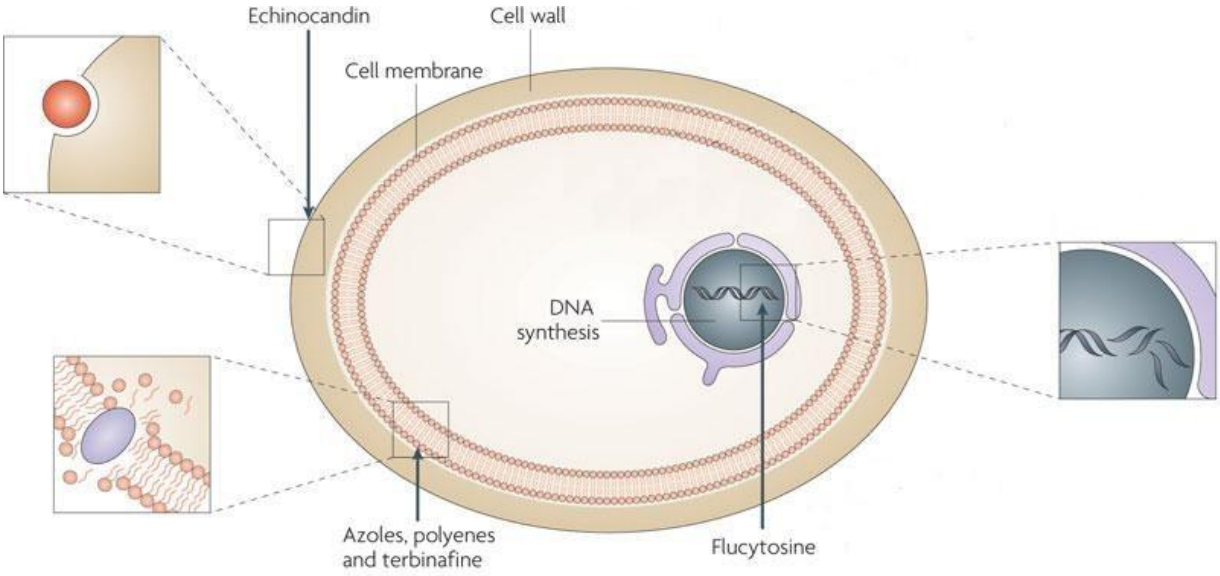

Figure 1.4. Mechanisms of action of antifungals (92).

Azoles

Azoles interfere with the ergosterol biosynthesis by inhibiting the enzyme lanosterol 14α-demethylase. This leads to a decrease in the production of ergosterol, an important constituent of the cytoplasmatic membrane, and an increase of other sterols to toxic levels, resulting in an altered membrane permeability. Azoles have a fungistatic effect (92-94).

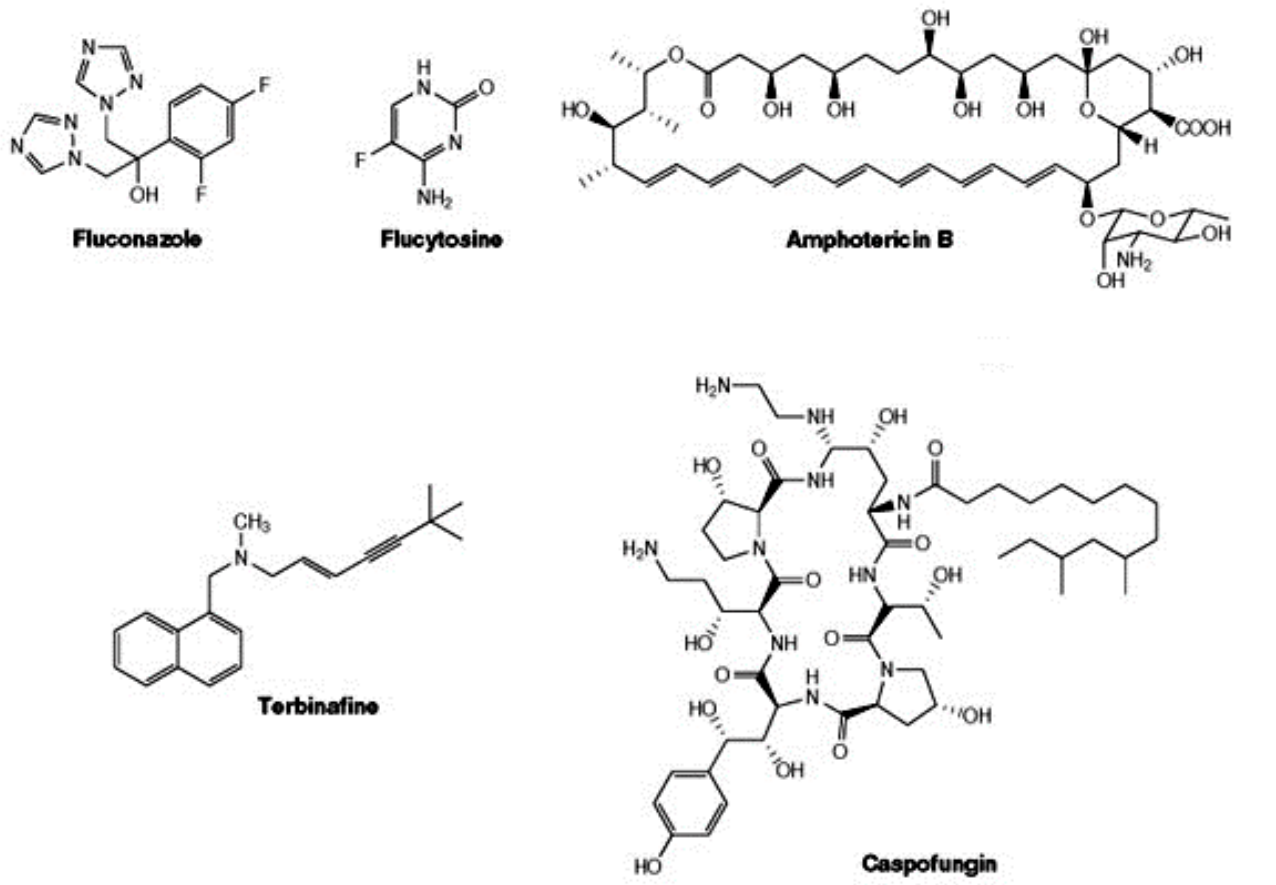

Figure 1.5. Chemical structures of main representatives of each class of antifungal drugs (95).

Azole antifungals can be categorized into two groups: imidazoles and triazoles, having two and three nitrogen atoms in the heterocyclic ring, respectively. Miconazole, ketoconazole and clotrimazole are imidazoles and fluconazole (Figure 1.5), itraconazole, voriconazole and posaconazole are triazoles. Fluconazole is one of the most widely used azoles for the treatment of mucosal and systemic candidiasis and it has a high oral bioavailability. Toxic side effects are rare and the high aqueous solubility of this compound makes it an excellent drug for intravenous delivery. The MIC range of fluconazole for *C. albicans* strains varies between 0.25 and 8 μg/ml. The increased MIC values for *C. glabrata* strains (8 to 64 μg/ml) indicate its lower susceptibility to fluconazole, while *C. krusei* has intrinsic resistance to this antifungal (93). Voriconazole

is a chemical derivative of fluconazole, with enhanced potency. Its MIC range for *Candida* spp. (0.02 to 2 μg/ml) is lower compared to that of fluconazole. Additionally, voriconazole shows enhanced activity against *C. glabrata* and *C. krusei* (93). It has a good oral bioavailability and a cyclodextrin formulation increases the aqueous solubility of this compound, enabling intravenous administration. Itraconazole and posaconazole are only orally administered and exhibit a higher potency than fluconazole (96, 97). The MIC range of itraconazole against *C. albicans* strains is between 0.03 and 0.5 μg/ml and that of posaconazole is even lower with MIC values between 0.02 and 0.12 μg/ml. Similar to fluconazole, both antifungals show increased MICs for *C. glabrata* and *C. krusei*, ranging from 1 to 4 μg/ml and from 0.5 to 2 μg/ml, respectively (93).

Initially, ketoconazole and miconazole were systemically administered, but toxicity limited their use. Miconazole and clotrimazole are available in topical formulations for treatment of mucosal candidiasis (93).

Miconazole

In contrast to the other azoles, miconazole (Figure 1.6) has fungicidal activity against *Candida* spp. cells in suspension but also in young and mature biofilms (98, 99). The MIC of *Candida* spp. for miconazole varies between 0.03 and 1 μg/ml, and is slightly higher for *C. krusei* (4 μg/ml) (99, 100). Previous experiments indicate that miconazole may disturb the cytoplasmatic membrane, causing leakage of essential compounds (101, 102). Furthermore, an increase of the intracellular levels of ROS in *Candida* spp. has been observed upon miconazole treatment, contributing to its fungicidal effect (99, 103, 104). It is likely that the ROS increase is preceded by changes in the actin cytoskeleton (105). Disruption of membrane rafts, enriched domains of sphingolipids and ergosterol in the cytoplasmatic membrane, leads to a decreased miconazole activity and also influences the miconazole-actin cytoskeleton stabilization and ROS accumulation. Probably, membrane rafts play a primary role in the action of miconazole by intracellular accumulation of the latter (106).

Miconazole was initially administered intravenously to treat systemic fungal infections. Due to many adverse effects, partially related to the carrier solution, its use is nowadays limited to topical administration for treatment of mucosal infections (107-109).

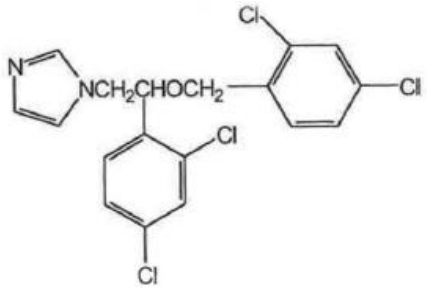

Figure 1.6. Chemical structure of miconazole (110).

Polyenes

Polyenes were the first class of antifungals to be widely used. They are produced by *Streptomyces* spp. and have fungicidal activity (93). Amphotericin B is the main compound within this class (Figure 1.5). It binds to ergosterol in the cytoplasmatic membrane, reducing the stability of the latter. Furthermore, pores are formed, leading to efflux of intracellular material and ultimately cell death (111). Recent data indicate that the ergosterol binding is the primary cause of its fungicidal activity (112). Additionally, treatment with amphotericin B is associated with the induction of oxidative stress (111, 113). The MIC range of amphotericin B for *Candida* spp. varies between 0.04 and 5 µg/ml. Due to its poor oral bioavailability, amphotericin B is only administered intravenously. Taken together, amphotericin B is a very potent and broad-spectrum antifungal for treatment of invasive fungal infections (93, 111). However, its use has been limited due to nephrotoxicity. Lipid formulations of amphotericin B drastically reduce the toxic effects and these formulations can be used in higher doses (92, 114). No statistically significant differences were observed in the MIC between amphotericin B and its lipid formulations (115). Despite the widespread use of the lipid formulations of amphotericin B for treatment of candidemia and invasive candidiasis, resistance remains low (93).

Nystatin, another polyene drug, is not gastro-intestinally absorbed and shows systemic toxicity. Therefore, its use has been restricted to topical applications. Similar to amphotericin B, liposomal formulations of nystatin increase the efficacy and reduce toxicity, allowing its use for treatment of systemic fungal infections (115).

Echinocandins

Echinocandins are the most recent class of antifungal agents targeting a specific component of the fungal cells and not mammalian cells (92). By inhibiting the synthesis of β-1,3 glucan, which is an important constituent of the cell wall, they have a fungicidal activity against *Candida* species. Recent research showed that the fungicidal activity of caspofungin was caused by both apoptosis and necrosis. Cellular apoptosis was observed at subinhibitory concentrations (116). Caspofungin (Figure 1.5), micafungin and anidulafungin are only available for intravenous administration. The spectrum of these three drugs is very similar, showing MICs for *Candida* spp. between 0.03 and 1 μg/ml. A remarkably lower susceptibility has been observed for *C. parapsilosis* with a MIC range between 2 and 4 μg/ml. The echinocandins exhibit a very low toxicity and are generally well tolerated. Their efficacy in the treatment of systemic *Candida* infections is similar to that of amphotericin B, but with fewer adverse effects (7, 93, 94, 117).

Flucytosine

Flucytosine is a fluorinated pyrimidine (Figure 1.5), originally synthesized as an anticancer drug, but also having antifungal properties. After fungal uptake of flucytosine, it is converted into 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate. The former metabolite inhibits the DNA synthesis and the latter is incorporated into RNA, inhibiting protein synthesis. It is used in second- or third line therapy and is often administered in combination with amphotericin B to prevent a rapid emergence of resistance. The MICs of flucytosine for *Candida* spp. are between 0.06 and 1 μg/ml, but the MIC for *C. krusei* is higher (32 μg/ml) (93, 94, 118, 119).

Allylamines

Like azoles, allylamines interfere with the ergosterol biosynthesis but already at an early stage. They inhibit the epoxidation of squalene, leading to accumulation of this intermediate, which damages the fungal membrane. Simultaneously, there is a decrease of ergosterol. The allylamine terbinafine (Figure 1.5) has a fungistatic effect against *C. albicans* and is used in topical preparations (94, 110, 120, 121).

Antifungal treatment of biofilms

The outcome of antifungal treatment of planktonic and sessile cells is generally different, as the latter show higher antifungal resistance (discussed below). While young biofilms are susceptible to fluconazole and miconazole (using a concentration of 256 µg/ml), with miconazole showing the highest antifungal activity, mature biofilms show resistance to both azoles (98, 122). However, in very high concentrations (2000 µg/ml, exceeding 1000-fold the MIC for planktonic cells), miconazole is able to exert fungicidal activity against *Candida* spp. (99). Newer azole antifungals, such as voriconazole (256 µg/ml) and posaconazole (64 µg/ml) have no activity against *Candida* biofilms (123).

Amphotericin B shows a concentration-dependent fungicidal activity against *C. albicans* biofilms, with more than 95% of the biofilm cells killed at concentrations of 8 to 64 µg/ml. Unfortunately, these concentrations are well above the therapeutic ones (0.125 to 1 µg/ml). (122, 124) Lipid formulations of amphotericin B may overcome this problem as they exhibit inhibitory activities against biofilms with similar MICs as observed for planktonic cells (0.25 to 1 µg/ml) (125). The lipid formulations of amphotericin B have been proven to be useful for antifungal lock therapy, leading to a complete eradication of catheter-associated *C. albicans* biofilms (126).

The concentration-dependent fungicidal activity of caspofungin against *C. albicans* biofilms is within its therapeutic concentration range (122, 124). *C. albicans* biofilm MICs range from 0.03 to 0.06 µg/ml (123). At a concentration of 2 µg/ml, caspofungin shows fungicidal activity against *Candida* spp. biofilms, independent of the stage of their development (127). As also other echinocandins (including anidulafungin) show similar activities against *C. albicans* biofilms at concentrations within the therapeutic range (0.03 to 0.5 µg/ml), this strongly suggests a class dependent phenomenon (123, 128, 129).

Combination therapy is not recommended in clinical practice and is restricted to persistent candidemia originating from a biofilm-associated infection. Combinations of fluconazole or amphotericin B with caspofungin did not show a synergistic effect against *C. albicans* biofilms. In contrast, a synergistic effect was observed for the combination amphotericin B and posaconazole (130, 131).

RESISTANCE

Resistance has been observed to antifungal drugs of all classes. A good understanding of the mechanisms is important as a basis to overcome fungal resistance. Planktonic and sessile cells have similar mechanisms of resistance, but biofilms exhibit some additional mechanisms to be discussed separately. Overall, resistance is a multifactorial, complex phenomenon.

General mechanisms of resistance

Mutations in the pathways where antifungals interfere is a mechanism described for azoles, polyenes, echinocandins and flucytosine. Furthermore, upregulation of the target gene and of efflux pumps has been observed for azole resistance. Figure 1.7 gives an overview of these mechanisms of resistance, which are discussed below in more detail.

Efflux pumps

Two types of efflux pumps are involved in azole resistance in *Candida*: the ATP binding cassette (ABC) transporters, including Cdr1 and Cdr2 (*Candida* drug resistance), and the major facilitator (MF) transporters including Mdr1 (multi drug resistance). These transporters actively remove azoles from the cytoplasm preventing inhibition of the ergosterol biosynthesis. The drug efflux by ABC transporters is ATP dependent, while MF transporters utilize the proton gradient across the cytoplasmatic membrane for drug efflux. Upregulation of the genes encoding these efflux pumps has been observed in fluconazole resistant clinical isolates. Further evidence of the involvement of efflux pumps in resistance was obtained from studies with mutants. Strains with disruptions in the genes encoding efflux pumps were hypersusceptible, whereas overexpression of these genes led to increased resistance. While ABC transporters are associated with resistance to all azoles, MF transporters appear to be selective for fluconazole (132-136).

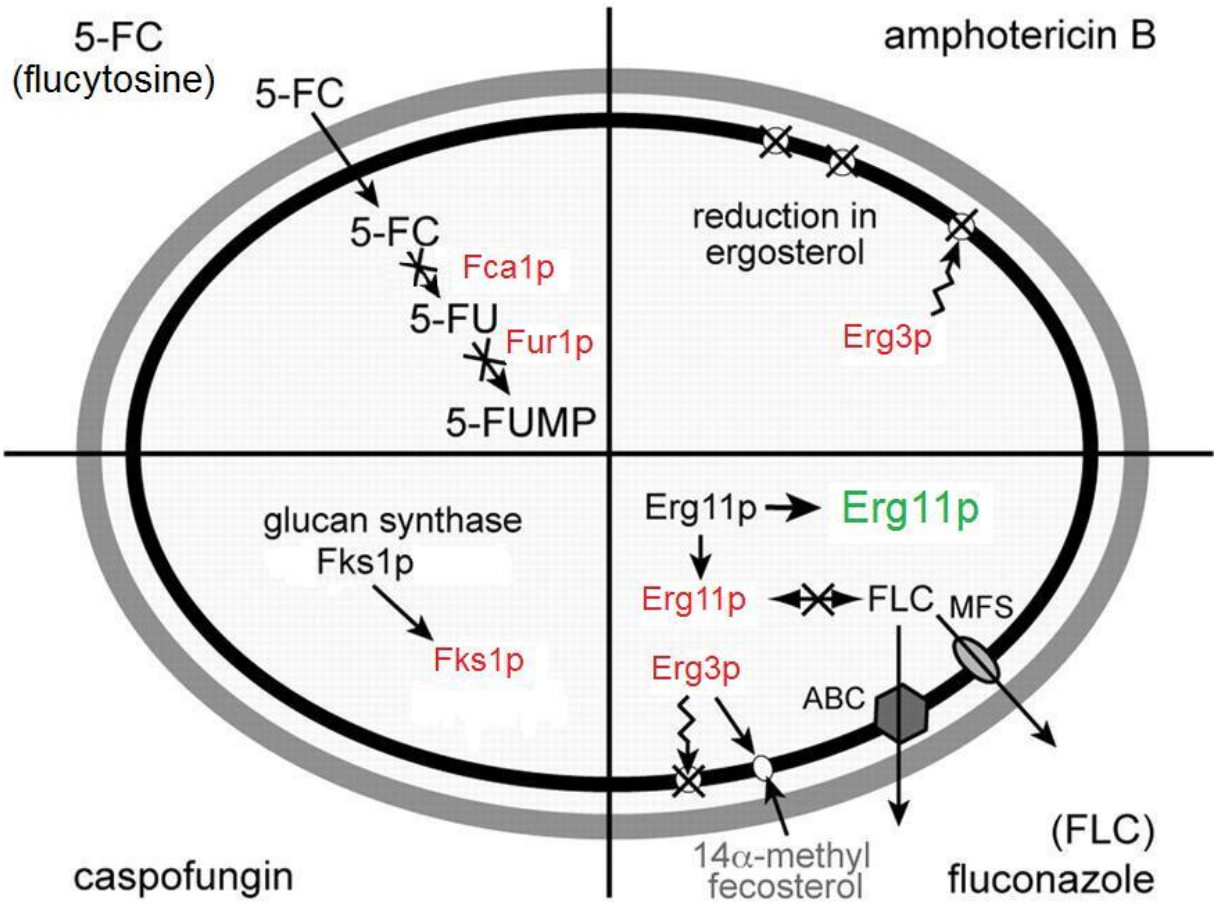

Figure 1.7. Mechanisms of resistance to antifungal drugs in *C. albicans*. Proteins in red indicate mutations and proteins in green indicate overexpression (137).

Overexpression of target gene

The upregulation of *ERG11* has been associated with azole resistance. In this way a complete saturation of lanosterol 14α-demethylase with azoles is avoided and the production of ergosterol can be maintained. Simultaneously with the upregulation of *ERG11*, genes located upstream (*ERG1*, *ERG7*, *ERG9* and *ERG10*) or downstream (*ERG2*, *ERG3* and *ERG25*) of *ERG11* are also upregulated upon azole treatment. This indicates a global upregulation of the ergosterol biosynthetic pathway (138-140).

Mutations

Resistance to polyenes is rather uncommon. However, a mutation in *ERG3* or *ERG6* in the ergosterol pathway has been associated with resistance to polyenes. These mutations lead to a decreased content of ergosterol in the cytoplasmatic membrane, and thus a reduced presence of the target of polyenes. The rigidity of the cytoplasmatic membrane is then maintained by other sterols (141, 142).

Mutations in *FUR1*, encoding uracil phosphoribosyltransferase, or in *FCA1*, encoding cytosine deaminase, lead to a decrease of flucytosine metabolites which interfere with RNA and DNA synthesis. Consequently, the action of flucytosine is counteracted (119, 138).

Resistance to echinocandins was obtained by the mutation of the gene *FKS1*, involved in the biosynthesis of β-1,3 glucan. Probably, this mutation leads to an altered drug binding, inhibiting the effect of echinocandins (138, 143).

Erg11 is the target of azoles. Mutations in *ERG11* result in reduced affinity for azoles, leading to resistance. Also, mutations in *ERG3* contribute to resistance, as these will lead to accumulation of 14α-methylfecosterol, which can compensate for the depletion of ergosterol and the consequent growth inhibition (144, 145).

Stress responses

Contact with antifungals or immune response factors and changes in the environment may induce general stress responses also contributing to resistance. The mitogen-activated protein kinase (MAPK) pathway is activated in response to different stresses and occupies a central position in the regulation of appropriate stress responses, thereby activating the high-osmolarity glycerol (HOG1) pathway, the protein kinase C cell wall integrity (MKC1) pathway and the *C. albicans* ERK-like kinase (CEK1) pathway. The first one is mainly activated upon osmotic or heavy metal stress, while the second one is particularly induced by cell wall stress. Additionally, both pathways are induced by oxidative stress and increase the level of antioxidants (137, 146, 147). The CEK1 pathway is involved in cell wall construction.

Besides this complex regulatory network, the protein calcineurin is activated upon several stresses, especially membrane stress. Calcineurin affects the transcription of other stress regulators, which leads to an increased tolerance to osmotic stress, to pH

changes and to certain antifungals. Calcineurin is stabilized by the heat-shock protein 90 (Hsp90), which is also induced by the MAPK pathway (137).

Biofilm specific mechanisms of resistance

The general mechanisms of resistance discussed before are also observed in fungal biofilms. However, biofilms show an increased resistance to antifungals compared to planktonic cells. The architecture of biofilms and their cell density provide additional mechanisms of resistance that do not, or only to a lesser extent, occur in planktonic cells (Figure 1.8).

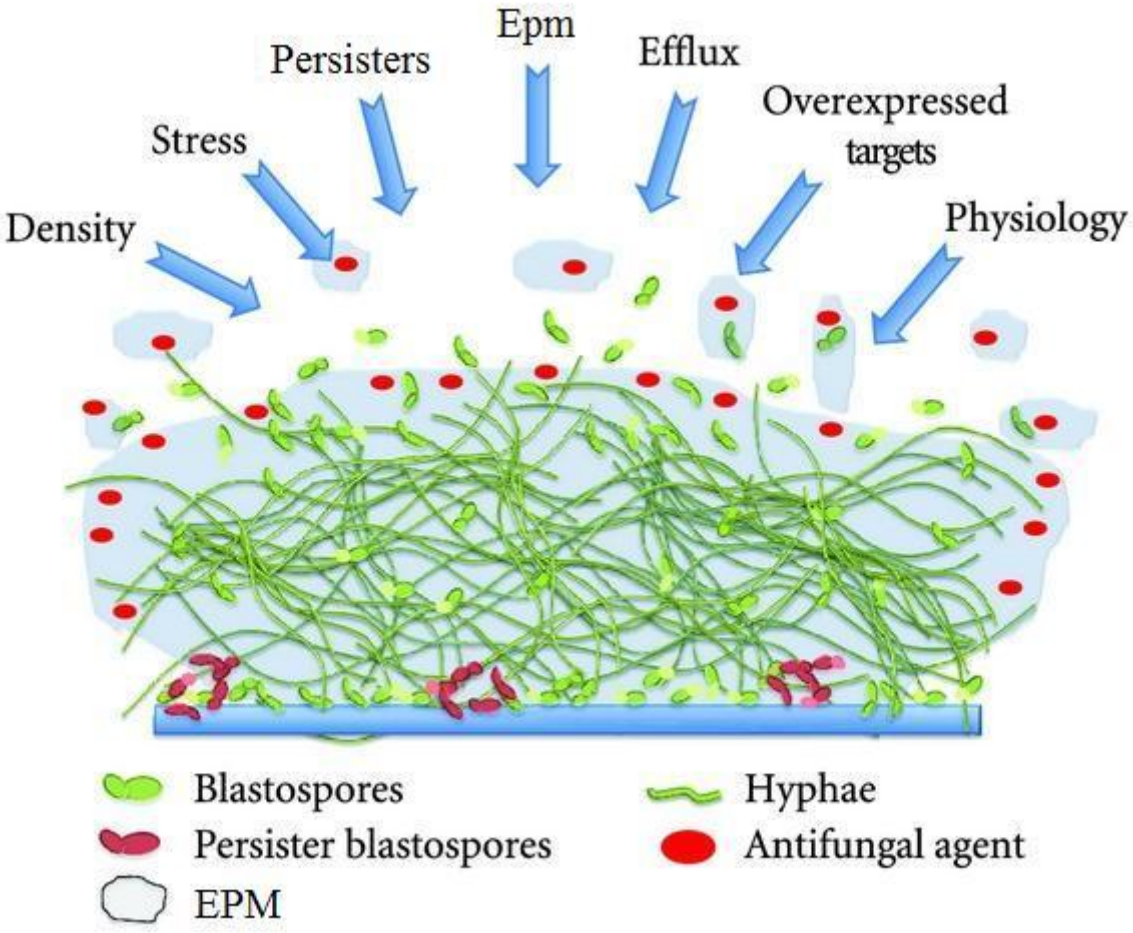

Figure 1.8. Schematic overview of fungal biofilm resistance mechanisms (148).

Cell density

It has been observed that increased biofilm resistance is a phase-dependent phenomenon, indicating that the high cell density of mature biofilms may play a role in resistance (149). At equal cell densities, planktonic cells show a similar resistance to antifungals as sessile cells. Additionally, dissociated biofilm cells at a low density (similar to that of planktonic cells), show a higher susceptibility to antifungals. These changes have been observed for different classes of antifungals (azoles, polyenes and echinocandins). It is possible that quorum sensing molecules are involved in the increased resistance of high density populations, although the effect of farnesol on the regulatory system of Chk1 does not seem to be a factor (150). In contrast, resuspended biofilm cells still show a resistant phenotype for fluconazole, but are less resistant than intact biofilms (151).

Growth rate

A change in growth rate of *C. albicans* biofilms did not influence the effect of amphotericin B treatment, indicating a minor role for growth rate in polyene resistance (68). Furthermore, it has been found that the metabolic activity increases during biofilm development, indicating that a lower metabolic activity is not associated with the increased resistance of a developing biofilm (152). However, as biofilms are heterogeneous structures, it is possible that they contain resistant subpopulations with a lower growth rate (153).

Efflux pumps

The role of efflux pumps in general resistance to antifungals has been discussed above. However, they also play a specific role in fungal biofilm resistance where they show a phase dependent involvement. Although *CDR1*, *CDR2* and *MDR1* are expressed during all phases of biofilm development, increased transcription levels of these genes were observed during the early phase. Young biofilms formed from strains with single, double or triple mutations in the *CDR* and *MDR1* genes were hypersusceptible to azoles. This confirms that efflux pumps play a role in azole resistance at this biofilm stage. However, mature biofilms of these mutants showed a resistant azole phenotype,

indicating that efflux pumps do not contribute to azole resistance at later stages of biofilm development (149, 151).

Extracellular polymeric matrix

The production of EPM is a typical characteristic of biofilms. The amount of EPM produced depends on the growth conditions: biofilms grown under a dynamic flow produce more EPM than statically grown biofilms (66, 67, 154). However, the extent of matrix formation did not affect the susceptibility of biofilm cells to amphotericin B, flucytosine or fluconazole (154). The EPM does not inhibit the penetration of fluconazole, amphotericin B or flucytosine, indicating that this is not a major mechanism of resistance (155). However, another study showed that increased EPM production also increased resistance against amphotericin B. The contrast with previous findings is probably due to differences in the growth conditions (67).

The most recent studies focus on the composition of the EPM and demonstrate a biofilm specific role in resistance for extracellular DNA and β-1,3 glucans in the EPM. Extracellular DNA contributes to the maintenance and stability of mature *C. albicans* biofilms. Treatment of these biofilms with DNase decreased the biomass and increased the susceptibility to amphotericin B (and to a lower extent to echinocandins), but not to fluconazole (67, 69, 156). The β-1,3 glucan synthesis by Fks1, is probably of high importance for the resistance of biofilms (157). Additionally, the glucan transferases Bgl2 and Phr1 and the glucanase Xog1 are crucial for β-1,3 glucan transport to the matrix and for the accumulation of the matrix biomass, which suggests a role in drug resistance (71). During biofilm development, an increase in β-1,3 glucan has been observed in the cell wall and the surrounding matrix, enhancing its ability to sequester antifungals (azoles, polyenes, echinocandins and flucytosine) and preventing them from reaching their target (73, 157-159). The presence of β-1,3 glucans also seems to hinder the penetration of the larger components of the immune system (160, 161).

Persisters

Persisters, defined as a subpopulation of cells that have entered into a dormant state are highly tolerant to antifungals. This dormant state is characterized by a transcriptional downregulation of genes involved in energy production, leading to slow or no growth. Persister cells probably play an important role in recalcitrant and

recurrent biofilm-related infections. They survive antifungal treatment and are protected by the EPM as this forms a barrier for the larger compounds of the immune system. After antifungal therapy, the surviving cells start growing again, causing a new infection (160, 162). Persister cells have been demonstrated in *C. albicans* biofilms upon amphotericin B treatment, exhibiting a biphasic killing pattern. Isolation and reinoculation of these persister cells results in the formation of a biofilm with a new subpopulation of persister cells, indicating that these *C. albicans* persisters are not mutants, but phenotypic variants of the wild type. *C. albicans* persister cells exclusively occur in biofilms. Their formation is not induced by drug treatment, but is triggered by adherence to a surface (163).

The level of persister cells varies between *Candida* spp. and between *C. albicans* strains (164, 165). Persisters were not observed in *C. glabrata* and *C. tropicalis* biofilms upon amphotericin B treatment and were also absent in the widely used *C. albicans* laboratory strain SC5314 (164). However, miconazole treatment of the latter resulted in a persistent fraction (166). The levels of persister cells in *Candida* strains isolated from patients with long term oral *Candida* carriage were significantly higher than in *Candida* strains isolated from patients with transient carriage. However, these high-persister strains did not show an altered MIC compared to low-persister strains. Furthermore, the level of persistence within each strain was maintained when the persister cells were isolated and reinoculated. These data confirm that persisters are phenotypic variants, but suggest that an underlying genetic change determines the level of persistence (165).

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CHAPTER 2

AIMS

AIMS

Candida species are human commensals, but also opportunistic pathogens, causing infections in immunocompromised patients. They have the ability to colonize biotic and abiotic surfaces and form biofilms, which are highly resistant to commonly used antifungals. As fungal biofilm formation and resistance are an increasing medical problem, there is also a growing need for new and potent antifungals.

Azoles are known for their fungistatic activity against *Candida* species. Miconazole is reportedly an exception within this class, as it was previously shown to have fungicidal activity (1-5). A first aim of this doctoral research was to investigate whether miconazole also has fungicidal activity against mature biofilms of *Candida* species and whether these biofilms showed resistance against this antifungal.

The extensive research on fundamental aspects of fungal biofilm formation and resistance have led to new insights and has broadened our knowledge. However, due to the complexity of these processes, the mechanisms involved are not completely understood yet. If we want to discover new therapies for fungal biofilms and fight resistance against antifungals, fundamental research is important. For this reason, the second aim of this doctoral research was to unravel the molecular mechanisms involved in fungal biofilm formation and in the resistance to miconazole. Based on the results of the screening of a *S. cerevisiae* deletion mutant bank, genes identified to play a role in *S. cerevisiae* biofilm formation or resistance to miconazole were further examined in *C. albicans*.

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CHAPTER 3

FUNGICIDAL ACTIVITY OF MICONAZOLE AGAINST *CANDIDA* SPP. BIOFILMS

Based on:

Vandenbosch D, Braeckmans K, Nelis HJ, Coenye T. 2010. Fungicidal activity of miconazole against *Candida* spp. biofilms. J Antimicrob Chemother **65**:694-700.

ABSTRACT

Although azole antifungals are considered to be fungistatic, miconazole has fungicidal activity against planktonic *C albicans* cells, presumably associated with the induction of ROS production. Only few data are available concerning the effect of miconazole against sessile *C. albicans* cells. In the present study, the fungicidal activity of miconazole against *in vitro*-grown mature *Candida* biofilms, and its relationship with the induction of ROS and ROS-dependent apoptosis were examined.

The effect of miconazole on mature biofilms formed by 10 *C. albicans* strains and 5 strains from other *Candida* species was evaluated by plate counting and measuring the level of ROS induction. MIC tests were performed in the absence and presence of ascorbic acid, a quencher of ROS. The apoptotic population in *C. albicans* cells was determined using annexin-Cy3.

Miconazole showed a significant fungicidal effect against all mature *Candida* biofilms tested and caused elevated ROS levels, both in planktonic and sessile cells. Addition of ascorbic acid drastically reduced these levels. While ROS quenching decreased the susceptibility to miconazole of planktonic cells of most *Candida* strains, no reduced fungicidal activity of miconazole against biofilms was observed. Miconazole did not cause a significant increase in apoptosis.

ROS levels increased in all *Candida* biofilms upon addition of miconazole. However, ROS induction was not the only factor that underlies its fungicidal activity, as quenching of ROS did not lead to an enhanced survival of biofilm cells. ROS-induced apoptosis was not observed in *C. albicans* cells after miconazole treatment.

INTRODUCTION

Candida species are frequently associated with nosocomial infections in immunocompromised hosts (1). Device-related infections caused by this organism often involve biofilm formation, a process in which planktonic yeast cells adhere to a biotic or abiotic surface, ultimately resulting in the formation of a complex three-dimensional structure of yeast cells, filaments and extracellular polymeric matrix (2, 3). There are profound differences between planktonic and sessile cells, including an increased tolerance of the latter towards antifungal agents (4).

Azole antifungals are widely used to treat infections with *Candida* spp. These compounds inhibit the 14α-demethylation of lanosterol by interacting with cytochrome P450, a crucial enzyme in the ergosterol biosynthetic pathway. The resulting decrease in ergosterol levels and the accumulation of toxic sterol intermediates in the cytoplasmatic membrane lead to growth inhibition (5). Miconazole (an imidazole) has reportedly a higher *in vitro* activity against planktonic *C. albicans* cells than the more recently developed and presumably more active fluconazole (a triazole) (6-8). However, few data are available about the effect of miconazole against *Candida* biofilms. A fungicidal activity was observed for miconazole against *C. albicans* biofilms, but only against young (2–6 h) biofilms (9). Recent research has shown that the fungicidal activity of miconazole against planktonic *C. albicans* cells is related to the induction of ROS. Although the exact mechanism of this enhanced ROS accumulation is not completely understood, combined inhibition of catalase and peroxidase, as well as changes in the actin cytoskeleton, appear to be involved (7, 8). It is well known that antioxidants can act as a reductant for ROS. Antioxidative compounds are important for the prevention of peroxidation and free radical accumulation (10). Furthermore, ROS are inducers of apoptosis (11). Programmed cell death was observed in *Saccharomyces cerevisiae* exposed to different types of oxidative stress (12, 13). Hyperactivation of the RAS signalling pathway by stabilization of the actin cytoskeleton leads to an increase in cAMP, followed by the loss of the mitochondrial membrane potential and the accumulation of ROS, ultimately leading to apoptosis (14). Apoptosis is also induced in *C. albicans* upon treatment with low doses of H_2O_2, acetic acid or amphotericin B (15).

The aim of the present study was to investigate the activity of miconazole against *Candida* biofilms. To this end, the effect of miconazole and fluconazole against mature biofilms of 10 *C. albicans* strains and 5 strains belonging to other *Candida* species was compared. ROS levels were determined in miconazole-treated and untreated mature *Candida* biofilms to verify whether there is a correlation with the activity of miconazole. Finally, we investigated whether increased apoptosis contributes to the antimicrobial effect of miconazole.

MATERIALS AND METHODS

Strains

The following strains were used: *C. albicans* SC5314 (ATCC MYA-2876) (American Type Culture Collection, Teddington, UK); *C. albicans* ATCC 10231; *C. albicans* IHEM 10284 (Institute of Hygiene and Epidemiology—Mycology Section, Brussels, Belgium); *C. albicans* IHEM 9559; *C. albicans* NCYC 1467 (National Collection of Yeast Cultures, Norwich, UK); *C. albicans* MUCL 29800 (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium); *C. albicans* MUCL 29903; *C. albicans* MUCL 29981; *C. albicans* MUCL 29919; *C. albicans* MUCL 30112; *C. dubliniensis* IHEM 14280; *C. glabrata* MUCL 15664; *C. krusei* IHEM 1796; *C. parapsilosis* IHEM 3270; and *C. tropicalis* IHEM 4225. A stock culture of all these strains was kept in Microbank Tubes (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at −80°C. Cells were routinely transferred to Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, UK) plates or Sabouraud dextrose broth (SDB) (Oxoid) and incubated at 37°C for 24 h.

Biofilm formation on silicone discs

Candida biofilms were grown on sterile silicone discs (4 mm in thickness and 13 mm in diameter) in a 24-well microtitre plate (TPP, Trasadingen, Switzerland). Silicone sheets were prepared from a medical grade silicone rubber kit (Q7-4735; Dow Corning Corp., Midland, MN, USA), according to the manufacturer's instructions. The discs were punched from the sheets, washed in 2% RBS 35 detergent (Sigma–Aldrich, St Louis, MO, USA) and in MilliQ water (Millipore, Billerica, MA, USA), and autoclaved. Inoculum suspensions were prepared by incubating the cells in SDB for 16 h at 37°C. After removing the supernatant, the cells were washed three times with and finally resuspended in 1 mL of physiological saline (0.9% NaCl; Novolab, Geraardsbergen, Belgium) (PS). This inoculum was further diluted with yeast nitrogen base 0.1× (BD, Franklin Lakes, USA) (YNB) supplemented with 5 mM glucose (Sigma–Aldrich) to yield an optical density of 0.07 at a wavelength of 600 nm. Then, 1 mL of a 1:100 dilution of the inoculum in YNB 0.1× was added to each well containing a silicone disc and the 24-well microtitre plates were incubated for 1 h at 37°C. Subsequently, the silicone discs were rinsed three times with 1 mL of PS to remove non-

adherent cells and aseptically transferred to a new well. Following the addition of 1 mL of diluted YNB (0.004× with a final glucose concentration of 0.2 mM) to each well, the plates were further incubated for 24 h at 37°C.

Biofilm formation in 96-well microtitre plates

Candida biofilms were grown in round-bottomed 96-well microtitre plates (TPP), as described previously (16), with an adhesion phase of 1 h followed by a growth phase of 24 h (both at 37°C in SDB).

Treatment of biofilms with antifungal agents

The silicone discs containing the mature biofilms were transferred to a new 24-well microtitre plate. Then, 1 mL of a solution of fluconazole (Diflucan, Pfizer, Brussels, Belgium) or miconazole nitrate (Certa, Braine–l'Alleud, Belgium) (final concentration 5 mM, corresponding to 1,531 mg/L for fluconazole and 2081 mg/L for miconazole) in phosphate buffered saline (PBS) with 2% dimethyl sulfoxide (Sigma–Aldrich) (DMSO) was added to the biofilms. Appropriate controls were also included. The plates were incubated at 37°C for 24 h. The silicone discs were subsequently washed three times with 1 mL PS. The number of cfu on each silicone disc was determined by pour plating. To this end, the silicone discs with biofilms were transferred to 10 mL of SDB and biofilm cells were removed from the silicone by three cycles of 30 s of sonication and 30 s of vortex mixing. With this procedure, all sessile cells were removed from the silicone discs and clumps of cells were broken apart (17). Serial 10-fold dilutions of the resulting cell suspension were made, and 1 mL of each dilution was plated and SDA was added, resulting in a lower limit of detection of 10 cfu per disc. Plates were incubated for 24 h at 37°C, after which the number of cfu per disc was calculated by counting colonies on the plates. For each strain and treatment, biofilms formed on at least three silicone discs in at least three independent experiments ($n \geq 9$) were included.

Detection of ROS

Mature biofilms formed in round-bottomed 96-well microtitre plates were rinsed with 100 µL of PS and treated for 24 h at 37°C with 100 µL of a miconazole suspension (5 mM in PBS with 2% DMSO). Appropriate controls were included and incubated under identical conditions. ROS accumulation was measured in a fluorometric assay using 2,7-dichlorofluorescein diacetate (DCFHDA) (Invitrogen, Carlsbad, CA, USA) (6). To this end, biofilms were incubated with 10 µM DCFHDA, simultaneously with the antifungal treatment. Fluorescence was measured after 24 h of incubation using a Wallac Victor Multilabel Counter (Perkin Elmer, Wellesley, MA, USA) (λ_{ex} = 485 nm; λ_{em} = 535 nm). The values obtained were corrected for background fluorescence (measured in the absence of cells) and compared with those obtained with untreated biofilms. ROS levels were quantified in duplicate on at least three biofilms ($n \geq 6$) for each strain. To determine whether fluorescence is generated in the extracellular environment or intracellularly, the entire content of the well (supernatant and biofilm) was removed and cells were separated from the supernatant by centrifugation. The fluorescence of the supernatant and of the resuspended sessile cells in PBS was measured separately, as described above.

Influence of antioxidants on miconazole-treated biofilms

A set of antioxidative compounds was used to investigate their possible protective effect against miconazole activity, including cysteine (0.025% w/v and 0.25% w/v) (Sigma–Aldrich), mannitol (10 and 100 mM) (Merck, Darmstadt, Germany), glutathione (1.5 and 15 mM) (Sigma–Aldrich), ascorbic acid (10 and 100 mM) (Merck) and pyrrolidinedithiocarbamate (PDTC) (10 µM and 1 mM) (Sigma–Aldrich). The level of ROS was determined in miconazole-treated biofilms using DCFHDA (10 µM) in the presence and absence of the antioxidants. The number of cfu on silicone discs of *C. albicans* SC5314 treated with miconazole in combination with the selected antioxidative compounds was determined by plating in at least three independent experiments on at least three silicone discs for each condition ($n \geq 9$).

Antifungal susceptibility assay

The MIC of miconazole in the presence and absence of ascorbic acid (10 mM) was determined according to the protocol of the European Committee on Antimicrobial Susceptibility Testing (18). The medium used for these experiments was RPMI-1640 2× with l-glutamine and without sodium bicarbonate (Sigma–Aldrich), supplemented with 2% w/v glucose (Sigma–Aldrich) and buffered to pH 7.0 with MOPS (Sigma–Aldrich). Flat-bottomed 96-well microtitre plates were inoculated with *Candida* to obtain 5×10^5 cells/mL in each well. After 24 h of incubation at 35°C, the absorbance was measured at a wavelength of 590 nm using a Wallac Victor microtitre plate reader (Perkin Elmer). DCFHDA (10 μM) was also added to each well and the absorbance measurement was immediately followed by a measurement of fluorescence (λ_{ex} = 485 nm; λ_{em} = 535 nm).

Detection of apoptosis

To investigate a possible apoptosis-inducing effect of miconazole on *C. albicans* SC5314 biofilms, the Apoptosis Detection Kit Annexin V-CY3 (Sigma–Aldrich) was used. This test allows differentiation between living cells (green fluorescence), necrotic cells (red fluorescence) and apoptotic cells (green and red fluorescence). Biofilms were grown in 96-well microtitre plates and treated with miconazole, as described above. Sessile cells were removed and diluted 1:10 in PBS, after which 50 μL was spotted on a microscope slide and left at room temperature, allowing the cells to be adsorbed to the slide. The adsorbed cells were carefully washed three times with binding buffer (supplied with the apoptosis kit), and treated with a mixture of 6-carboxyfluorescein diacetate (500 μM) and annexin V-Cy3 conjugate (1 mg/L) for 10 min. Excess labelling agent was removed by washing the cells three times with binding buffer. *C. albicans* biofilms treated with acetic acid (60 and 300 mM) and hydrogen peroxide (5 and 25 mM) were included as positive controls in this assay. Apoptosis in planktonic *C. albicans* SC5314 cells was also tested. Therefore, overnight cultures were treated with miconazole (10× MIC) for 24 h and compared with untreated planktonic cultures. The staining procedure was performed as described above for sessile cells. Results were observed using a fluorescence microscope (Olympus BX40, Olympus, Tokyo, Japan). For each condition, between 241 and 1174 cells were photographed, and co-localization of the fluorescein and Cy3 fluorescence signal was quantified on a cell-per-cell

basis with an in-house-developed image-processing program based on Matlab to differentiate between living, necrotic and apoptotic cells.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. The non-parametric Mann–Whitney test was used to compare the results.

RESULTS

Effect of azoles on *Candida* biofilms

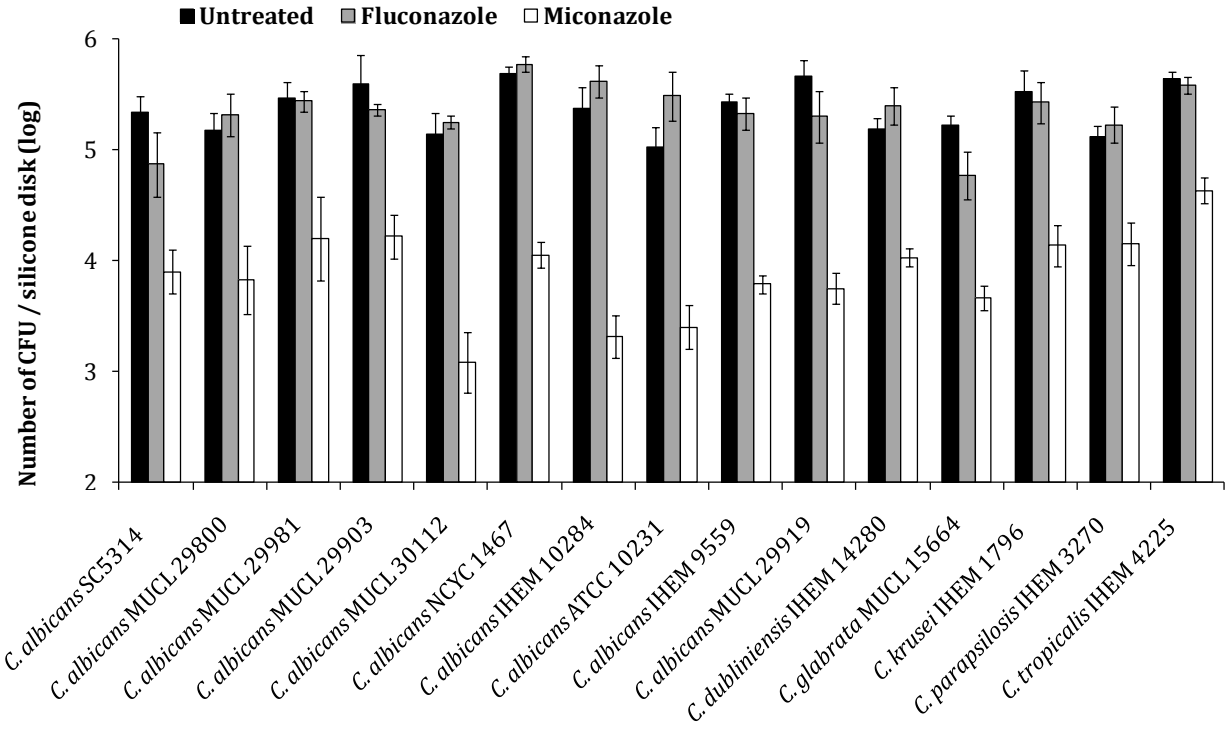

Figure 3.1. Number of cfu (logarithmic) per silicone disc of *Candida* biofilms in the presence and absence of antifungals. Black bars, untreated mature biofilms; grey bars, mature biofilms treated with 5 mM fluconazole; white bars, mature biofilms treated with 5 mM miconazole. Data presented are the mean and SEM of at least three independent experiments on at least three biofilms ($n \geq 9$). Statistical analysis with the Mann–Whitney test indicated significant differences in biofilm biomass between miconazole-treated and untreated biofilms for all strains tested ($P < 0.05$), but not between fluconazole-treated and untreated biofilms.

The effect of fluconazole and miconazole on *Candida* biofilms formed on silicone discs was determined for 15 strains (Figure 3.1). Untreated biofilms contained 10^5–10^6 cfu/disc, depending on the strain tested. Treatment with fluconazole did not result in a

significant reduction in cfu. In contrast, treatment with miconazole resulted in a substantial reduction (ranging from 89.3% to 99.1%; $P < 0.05$) in the number of cfu recovered from the discs for all strains investigated. The lowest reductions were observed for *C. parapsilosis* IHEM 3270 (89.3%) and *C. tropicalis* IHEM 4225 (90.3%), and the highest reductions were observed for *C. albicans* MUCL 30112 (99.1%) and *C. albicans* IHEM 10284 (99.1%).

Our results showed only a fungistatic effect for fluconazole, whereas miconazole showed fungicidal activity against *Candida* biofilms.

ROS-accumulation in miconazole-treated biofilms

Accumulation of ROS following treatment with miconazole was measured using DCFHDA. As the conversion of this dye depends on the number of metabolically active cells in the biofilm, results were normalized to the number of cfu/disc. Treatment with miconazole resulted in a significant increase in ROS accumulation for all strains investigated ($P < 0.005$) (Figure 3.2). No ROS accumulation could be observed for untreated *C. dubliniensis* IHEM 14280, *C. glabrata* MUCL 15664 and *C. parapsilosis* IHEM 3270 biofilms. In contrast, all untreated *C. albicans* biofilms tested showed a basal ROS accumulation. A remarkably high fluorescence could be observed for *C. albicans* MUCL 30112. The highest increases (> 100-fold) in ROS accumulation after miconazole treatment could be observed for *C. albicans* MUCL 29919, *C. albicans* IHEM 9559 and *C. albicans* MUCL 29800. The lowest impact of miconazole treatment was detected for *C. tropicalis* IHEM 4225 (2-fold increase) and *C. albicans* NCYC 1467 (7-fold increase). Measurements were also carried out separately in sessile *C. albicans* SC5314 cells and in the supernatant. The ROS-induced increase in fluorescence was only observed for the cells and not for the supernatant (data not shown), indicating an intracellular origin.

In conclusion, miconazole caused a significant intracellular increase in ROS accumulation in all *Candida* strains investigated.

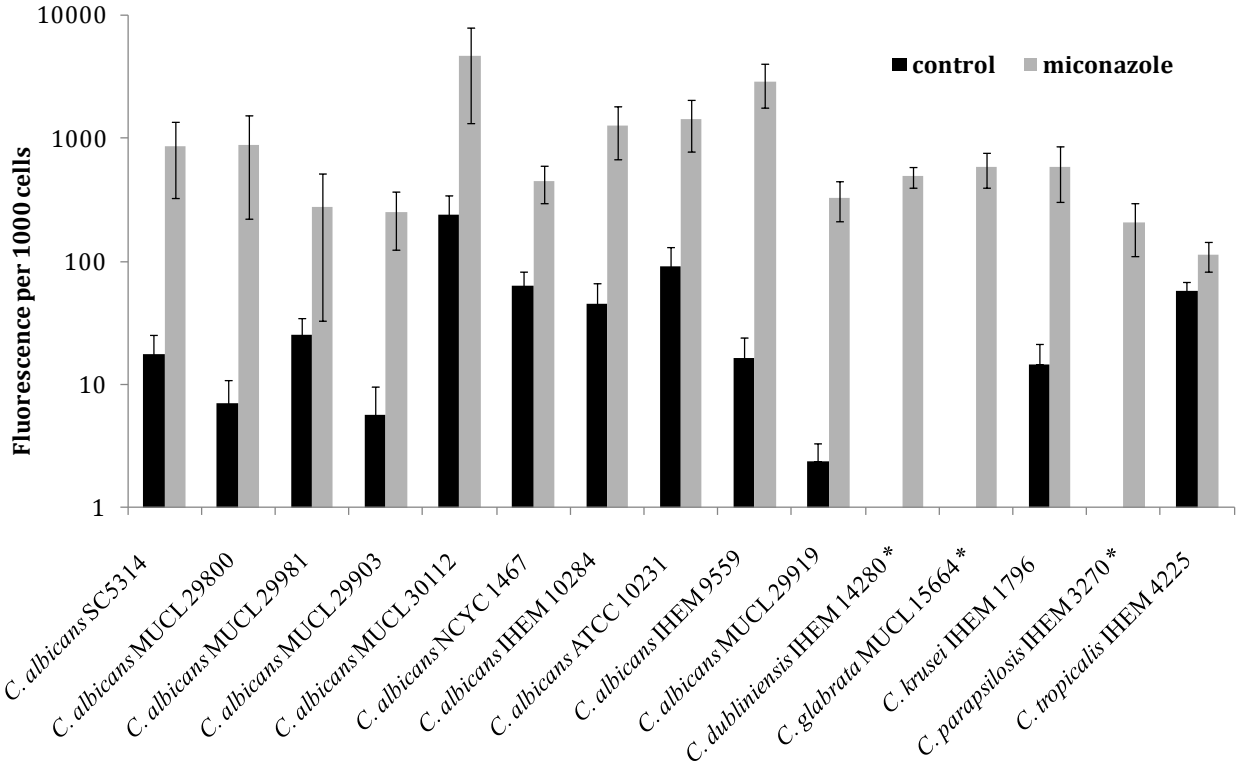

Figure 3.2. Accumulation of ROS expressed as fluorescence per 1000 cells in mature *Candida* biofilms in the presence and absence of miconazole. Black bars, untreated mature biofilms; grey bars, mature biofilms treated with 5 mM miconazole. Data presented are the mean and SEM of two independent experiments on at least three biofilms ($n \geq 6$). Statistical analysis with the Mann–Whitney test indicated a significant difference ($P < 0.05$) in fluorescence between miconazole-treated and untreated biofilms. *Fluorescence below background level.

Effect of antioxidants on miconazole-treated biofilms

The effect of the addition of five compounds with antioxidative properties on miconazole-treated *C. albicans* SC5314 biofilms was investigated using DCFHDA ($n \geq 9$ for each treatment). Only cysteine (0.25% w/v), glutathione (15 mM), PDTC (10 μM) and ascorbic acid (10 and 100 mM) significantly reduced ($P < 0.05$) miconazole-induced ROS accumulation (Figure 3.3). However, the addition of antioxidants to miconazole-treated biofilms did not result in a statistically significant increase in survival (Figure 3.3). Ascorbic acid (10 mM) was selected to further examine the effect of ROS quenchers on other

miconazole-treated *Candida* biofilms. The addition of ascorbic acid did not significantly increase the number of cfu on the silicone discs, but resulted in a reduction of ROS accumulation following treatment with miconazole for all strains tested (Table 3.1). This reduction was statistically significant ($P < 0.05$) when compared with biofilms treated with miconazole alone ($n = 6$ for each strain), except for *C. albicans* MUCL 29919 and *C. glabrata* MUCL 15664.

Ascorbic acid reduced the miconazole-induced ROS accumulation in sessile cells, but did not cause an enhanced survival.

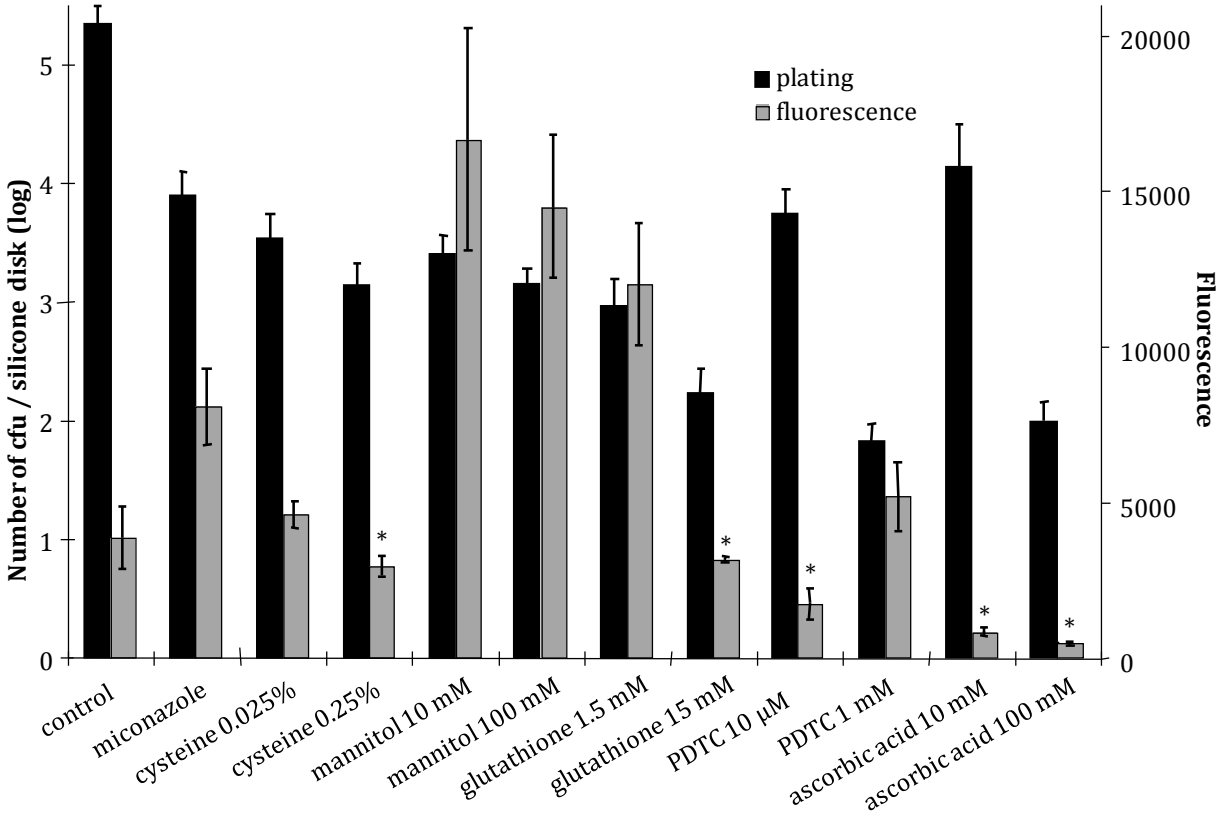

Figure 3.3. Number of cfu of *C. albicans* SC5314 biofilms (left-hand *y*-axis) and ROS accumulation (right-hand *y*-axis) following treatment with miconazole in the absence or presence of antioxidants. Black bars, log cfu per silicone disc; grey bars, fluorescence. Data presented are the mean and SEM of at least three independent experiments on at least three biofilms ($n \geq 9$). All treated biofilms showed a significant decrease ($P < 0.05$) in cfu compared with the control. Significant reductions ($P < 0.05$) in ROS accumulation compared with miconazole-treated biofilms are marked with an asterisk.

Table 3.1. Number of cfu of *Candida* biofilms treated with miconazole alone or in combination with ascorbic acid (10mM) and ROS accumulation after miconazole treatment and the addition of ascorbic acid (10 mM). Plate counts correspond to the mean ± SEM of at least three independent experiments on at least three silicone disks ($n \geq 9$). There was no significant increase in cfu after the addition of ascorbic acid compared with miconazole-treated biofilms without ascorbic acid. Fluorescence results correspond to the mean ± SEM of two independent experiments on three biofilms ($n = 6$). Statistically significant reductions ($P < 0.05$) in ROS accumulation are marked with an asterisk.

	PLATING MICONAZOLE (log cfu / silicone disk)	PLATING MICONAZOLE + ASCORBIC ACID (log cfu / silicone disk)	FLUORESCENCE (% compared to miconazole treatment without ascorbic acid)
***C. albicans* SC5314**	3.89 ± 0.20	4.13 ± 0.41	10.4 ± 2.3*
***C. albicans* MUCL 29800**	3.82 ± 0.31	2.00 ± 0.02	32.2 ± 7.7*
***C. albicans* MUCL 29981**	4.20 ± 0.38	2.99 ± 0.20	30.7 ± 4.2*
***C. albicans* MUCL 29903**	4.22 ± 0.20	3.47 ± 0.04	60.3 ± 14.1*
***C. albicans* MUCL 30112**	3.08 ± 0.27	2.53 ± 0.31	17.9 ± 5.6*
***C. albicans* NCYC 1467**	4.05 ± 0.12	4.16 ± 0.21	25.0 ± 5.9*
***C. albicans* IHEM 10284**	3.32 ± 0.19	3.19 ± 0.27	47.6 ± 5.5*
***C. albicans* ATCC 10231**	3.40 ± 0.19	2.89 ± 0.32	33.4 ± 2.5*
***C. albicans* IHEM 9559**	3.78 ± 0.08	3.28 ± 0.15	11.5 ± 3.9*
***C. albicans* MUCL 29919**	3.75 ± 0.14	3.80 ± 0.19	81.7 ± 16.9
***C. dubliniensis* IHEM 14280**	4.03 ± 0.08	3.90 ± 0.21	23.3 ± 2.3*
***C. glabrata* MUCL 15664**	3.66 ± 0.11	3.21 ± 0.19	64.4 ± 13.3
***C. krusei* IHEM 1796**	4.14 ± 0.19	2.73 ± 0.16	20.3 ± 4.5*
***C. parapsilosis* IHEM 3270**	4.15 ± 0.19	3.36 ± 0.22	53.2 ± 8.5*
***C. tropicalis* IHEM 4225**	4.63 ± 0.11	4.05 ± 0.22	26.5 ± 3.8*

Effect of ascorbic acid on the susceptibility of planktonic cells

Miconazole also induced ROS accumulation in planktonic *Candida* cultures. Addition of ascorbic acid to planktonic cultures incubated with miconazole reduced ROS production (47.8% to 89.9% reduction) for all strains tested (data not shown). The MIC of miconazole increased 2- to 64-fold for most strains following the addition of ascorbic acid. This increase was not observed for two strains with an intermediate MIC (0.125 mg/L) (*C. albicans* IHEM 10284 and MUCL 29919) and for most strains with a high MIC (1.0 – 4.0 mg/L) (*C. glabrata* MUCL 15664, *C. krusei* IHEM 1796 and *C. parapsilosis* IHEM 3270) (Table 3.2).

For planktonic cells of most strains, the addition of ascorbic acid reduced the ROS accumulation and the susceptibility to miconazole.

Table 3.2. MIC of miconazole in the absence and presence of ascorbic acid.

	MIC miconazole (mg/L)	MIC miconazole (mg/L) in the presence of 10 mM ascorbic acid	Fold change in MIC
***C. albicans* SC5314**	0.063	0.250	4
***C. albicans* MUCL 29800**	0.031	0.125	4
***C. albicans* MUCL 29981**	0.031	2.000	64
***C. albicans* MUCL 29903**	0.063	0.250	4
***C. albicans* MUCL 30112**	0.063	0.500	8
***C. albicans* NCYC 1467**	0.063	0.500	8
***C. albicans* IHEM 10284**	0.125	0.125	1
***C. albicans* ATCC 10231**	0.125	0.250	2
***C. albicans* IHEM 9559**	0.125	0.250	2
***C. albicans* MUCL 29919**	0.125	0.125	1
***C. dubliniensis* IHEM 14280**	0.063	0.125	2
***C. glabrata* MUCL 15664**	1.000	1.000	1
***C. krusei* IHEM 1796**	4.000	4.000	1
***C. parapsilosis* IHEM 3270**	1.000	1.000	1
***C. tropicalis* IHEM 4225**	1.000	4.000	4

Apoptosis in miconazole-treated planktonic and sessile *C. albicans* cells

To determine whether the effect of miconazole was due to increased apoptosis, we quantified the number of apoptotic cells in treated and untreated biofilms and planktonic cultures (Figure 3.4A and Figure 3.4B). Untreated planktonic *C. albicans* SC5314 cultures contained 14.0% ± 7.0% apoptotic cells, which was not significantly different (P = 0.83) from miconazole-treated cells (14.6% ± 4.5%). The number of necrotic cells in a planktonic culture treated with miconazole (13.2% ± 3.6%) was not significantly different (P = 0.41) from untreated planktonic cells (7.5% ± 2.3%). Untreated *C. albicans* SC5314 biofilms contained 9.7% ± 4.0% apoptotic cells. The number of apoptotic cells upon treatment of these biofilms with miconazole alone (14.4% ± 11.5%) or in combination with ascorbic acid (18.5% ± 6.5%) did not significantly increased (P = 0.40 and P = 0.19, respectively). In contrast, the fraction of necrotic cells increased significantly (P < 0.05) compared with the untreated biofilms (8.9% ± 3.7%) following treatment with miconazole alone (25.1% ± 1.9%) or in combination with ascorbic acid (32.7% ± 8.6%). Low concentrations of acetic acid (60 mM) and hydrogen peroxide (5 mM) resulted in a significant increase (P < 0.05) in the fraction of apoptotic cells (34.6% ± 5.1% and 20.6% ± 5.2%, respectively). The hydrogen peroxide-treated biofilms also showed a significant increase in the amount of necrotic cells (26.1% ± 3.5%).

Miconazole did not result in increased apoptosis in planktonic or in sessile *Candida* cells.

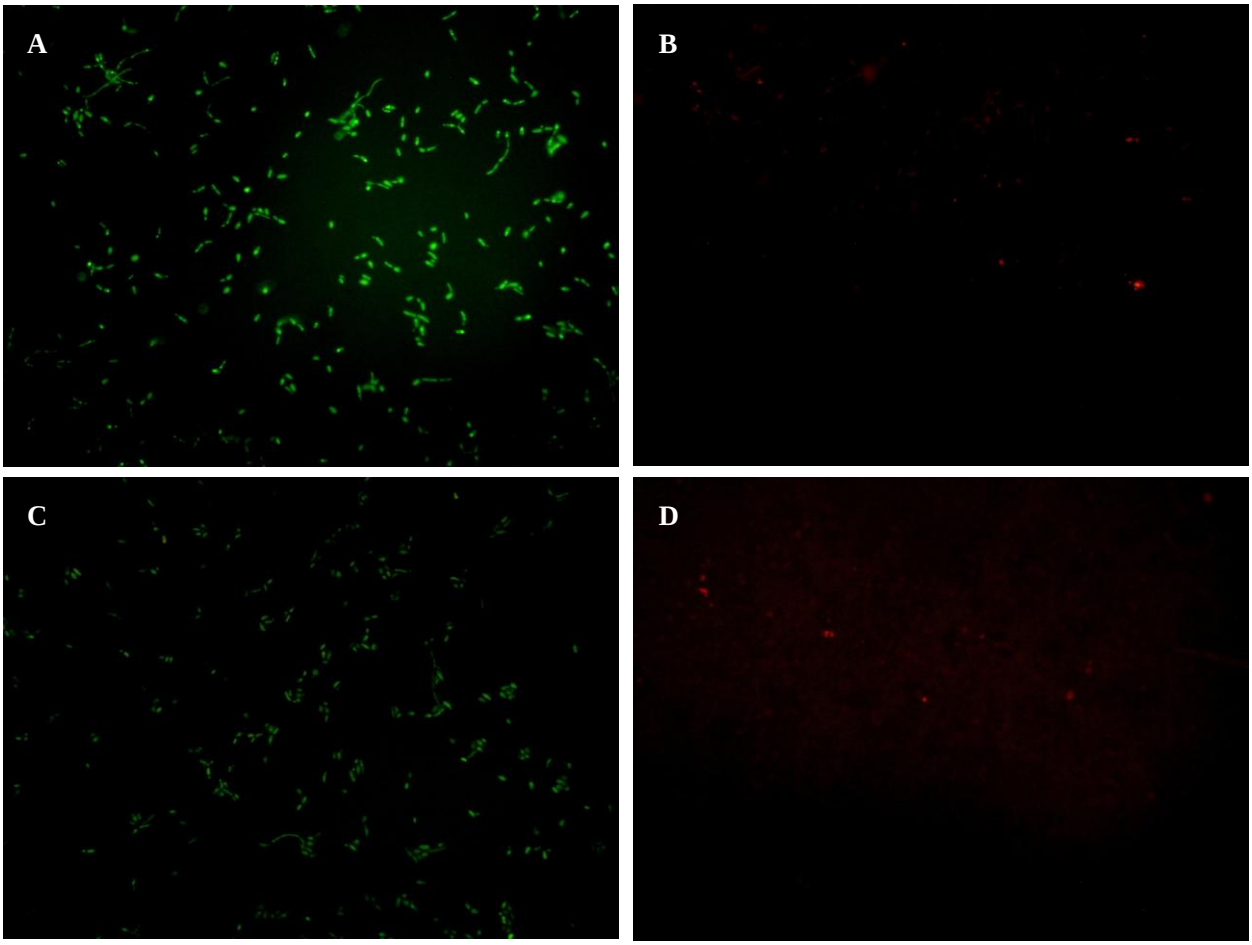

Figure 3.4A. *C. albicans* sessile cells stained with the Apoptosis Detection Kit Annexin V-CY3. Picture A, untreated sessile *C. albicans* cells (green fluorescence). Picture B, untreated sessile *C. albicans* cells (red fluorescence). Picture C, miconazole-treated sessile *C. albicans* cells (green fluorescence). Picture D, miconazole-treated sessile *C. albicans* cells (red fluorescence).

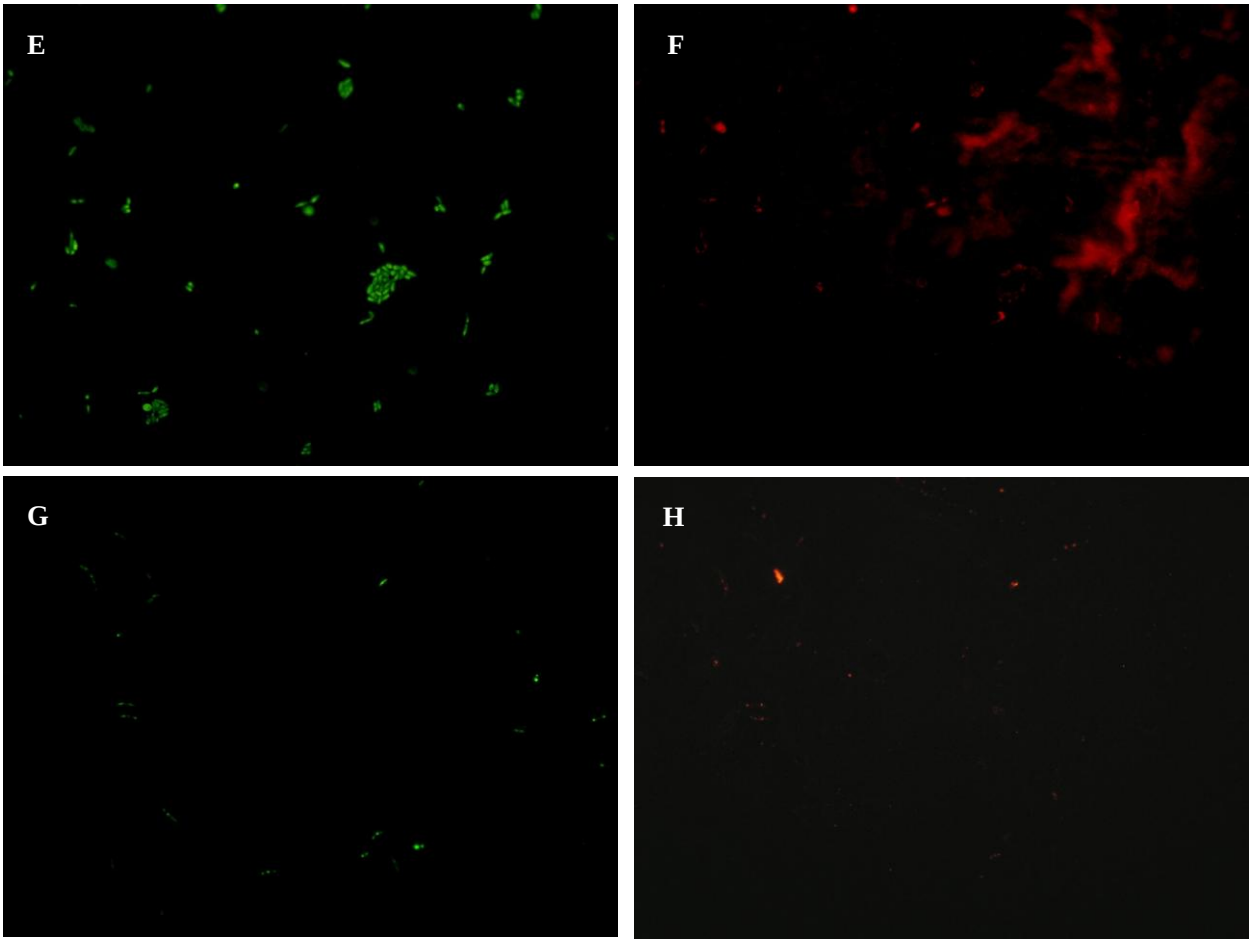

Figure 3.4B. *C. albicans* sessile cells stained with the Apoptosis Detection Kit Annexin V-CY3. Picture E, miconazole- and ascorbic acid-treated sessile *C. albicans* cells (green fluorescence). Picture F, miconazole- and ascorbic acid-treated sessile *C. albicans* cells (red fluorescence). Picture G, hydrogen peroxide-treated sessile *C. albicans* cells (green fluorescence). Picture H, hydrogen peroxide-treated sessile *C. albicans* cells (red fluorescence).

DISCUSSION

In the present study, the antifungal activity of miconazole against *Candida* biofilms was investigated.

Results from our study showed that miconazole, unlike fluconazole, has a pronounced antibiofilm effect against *C. albicans* and other *Candida* spp. It should be noted that the antifungal concentration used in our *in vitro* experiments (5 mM) was higher than the common therapeutic *in vivo* concentrations. However, this high concentration is achievable during antifungal lock therapy (19, 20) and allowed us to investigate the mechanism of action of miconazole.

We observed that the accumulation of ROS was strongly increased in sessile *Candida* cells treated with miconazole, indicating that ROS may be responsible for the fungicidal effect. ROS are generally described as important inducers of apoptosis in yeasts (10), but ROS induced by treatment with miconazole did not cause an increase in programmed cell death in sessile *C. albicans* cells. The majority of cells killed by miconazole were necrotic.

The addition of ascorbic acid to miconazole-treated *Candida* biofilms considerably reduced ROS accumulation for all strains. Surprisingly, this did not lead to a reduction of the fungicidal activity of miconazole. Sessile *Candida* cells are reportedly more tolerant to oxidative stress than their planktonic counterparts (21). This inherent tolerance may explain why several antioxidative compounds did not result in an additional protection. Furthermore, previous studies have shown that prior to the induction of ROS miconazole affects the organization of the actin cytoskeleton in yeasts (8). The coupling of mitochondria to the actin cytoskeleton might lead to an association of actin with channels in the mitochondrial membranes. The opening of these channels is followed by reduction of the membrane potential and, finally, the release of ROS into the cytoplasm (22). The targeting of the actin cytoskeleton by miconazole may have other effects, which are not counteracted by ascorbic acid. Alternatively, a yet unknown mechanism may contribute to the fungicidal activity of miconazole against sessile *Candida* cells.

An increase in ROS accumulation caused by miconazole treatment was also observed in all planktonic *Candida* cultures tested, confirming previous observations in *C. albicans* (6, 7). The addition of ascorbic acid to miconazole-treated planktonic *Candida* cells reduced ROS induction for all strains. Furthermore, a simultaneous decrease in

susceptibility to miconazole was observed for most strains, which confirmed previous findings concerning the protective effect of antioxidants during miconazole treatment (6). However, we demonstrated that the protective effect of ascorbic acid was limited in the case of strains with intermediate to high MICs of miconazole.

Our data suggest that miconazole may be useful for the treatment of biofilm-related *Candida* infections. We have also shown that ROS induction is probably not directly responsible for the reduction in the number of cfu. So far, the basis for the fungicidal activity of miconazole remains unclear and further investigations are needed.

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CHAPTER 4

GENOME WIDE SCREENING FOR GENES INVOLVED IN BIOFILM FORMATION AND MICONAZOLE SUSCEPTIBILITY IN *SACCHAROMYCES CEREVISIAE*

Based on:

Vandenbosch D, De Canck E, Dhondt I, Rigole P, Nelis HJ, Coenye T. 2013. Genome wide screening for genes involved in biofilm formation and miconazole susceptibility in *Saccharomyces cerevisiae*. Submitted.

ABSTRACT

Infections related to fungal biofilms are difficult to treat due to the reduced susceptibility of sessile cells to most antifungal agents. Previous research has shown that 1% - 10% of sessile *Candida* cells survive treatment with high doses of miconazole (a fungicidal imidazole) (Chapter 3). The aim of this study was to identify genes involved in fungal biofilm formation and to unravel the mechanisms of resistance of these biofilms to miconazole. To this end, a screening of a *S. cerevisiae* deletion mutant bank was carried out. Our results reveal that genes involved in peroxisomal transport and the biogenesis of the respiratory chain complex IV play an essential role in biofilm formation. On the other hand, genes involved in transcription and peroxisomal and mitochondrial organization seem to highly influence the susceptibility to miconazole of yeast biofilms. Additionally, our data confirm previous findings on genes involved in biofilm formation and in general stress responses. Our data for the first suggest the involvement of peroxisomes in biofilm formation and miconazole resistance in fungal biofilms.

INTRODUCTION

Biofilms are microbial communities of cells attached to a surface and embedded in an EPM (1). Biofilms can be formed both on host tissues and artificial surfaces, including medical devices such as catheters and prostheses (2-4). Fungal biofilm related infections have increased in frequency and result in increased morbidity and mortality in immunocompromised patients (5, 6). One of the major fungal human pathogens is *C. albicans*. This opportunistic human commensal causes infections ranging from superficial mucous membrane infections to life-threatening systemic diseases. The increased resistance to antifungals of sessile compared to planktonic cells often prevents a successful therapy (7). Production of extracellular matrix, increased cell density, upregulation of efflux pumps, decreased growth rate, overexpression of drug targets and presence of persister cells are known to play a role in the resistance of sessile cells (7-16).

Azoles are widely used to treat fungal infections. These antifungal compounds decrease the production of ergosterol by interacting with cytochrome P450 and inhibiting the 14α-demethylation of lanosterol. As ergosterol is an important constituent of the cytoplasmatic membrane, treatment with azole antifungals leads to growth inhibition (17). Besides this fungistatic mechanism of action, recent data indicate a fungicidal effect for miconazole (an imidazole) against *Candida* spp. cells in suspension and in young and mature biofilms (18, 19) (Chapter3). Accumulation of ROS appears to be involved in this process, although it is likely that other mechanisms also account for the fungicidal activity (20-22). Despite the observed fungicidal effect of miconazole on biofilms, 1% - 10% of the sessile *C. albicans* cells survive exposure to high levels of this antifungal agent (19) (Chapter 3).

The aim of the present study is to identify genes involved in fungal biofilm formation and to unravel the mechanisms of resistance of these biofilms to miconazole. To this end, we screened a deletion mutant bank of *S. cerevisiae* for biofilm formation and miconazole susceptibility. Previous work indicated that *S. cerevisiae* forms biofilms and could be used as a model for fungal biofilm formation (23). Furthermore, the staining procedure used in this study has been optimized previously, and showed to be a reliable method to measure biofilm formation (24, 25).

MATERIALS AND METHODS

Strains

The strains used in this study are *S. cerevisiae* BY4741, the BY4741-derived haploid set of deletion mutants in non-essential genes from the EUROSCARF collection (*n* = 4,961), *C. albicans* SC5314, *C. albicans* Δ*pex4*/Δ*pex4* (26) and *C. albicans* Δ*pex8*/Δ*pex8* (26). Stock cultures of these strains were kept at -80°C. Strains were cultured on SDA (Oxoid) at 37°C for at least 48 h.

Screening

Suspensions of *S. cerevisiae* BY4741 and BY4741-derived deletion mutants, containing approximately 10^7 cells/ml, were prepared in Yeast-Peptone-Dextrose (YPD) medium (BD). 100 µl of these cell suspensions were added to the wells (12 replicates per strain) of a U-bottomed 96-well microtitre plate (SPL Lifesciences, Pocheon, Korea) to initiate biofilm formation. After 1 h incubation at 37°C, the supernatant was removed and the wells were rinsed with 100 µl of PS to remove unattached cells. The microtitre plates were further incubated for 24 h at 37°C after addition of 100 µl YPD medium to each well. Subsequently, the supernatant was removed and the mature biofilms were rinsed with 100 µl PS before treatment with miconazole (1,000 µg/ml) (Certa). To this end, 100 µl of a miconazole suspension in PBS containing 2% DMSO (Sigma-Aldrich) was added to six biofilms of each strain and 100 µl of PBS containing 2% DMSO to the other six biofilms (control). After 24 h incubation at 37°C, the supernatant was removed and 120 µl of a diluted resazurin solution (CellTiter-Blue 1:6 in PS) (Promega, Leiden, The Netherlands) was added to each well. Fluorescence was measured after 2 h incubation in the dark at 37°C using an Envision microtitre plate reader (Perkin Elmer) (λ_{ex} = 535 nm; λ_{em} = 590 nm). As six mutants (*HTL1*, *CDC26*, *ACB1*, *YDJ1*, *CDC40* and *SAM37*) were not able to grow at 37°C, the incubation temperature was lowered to 25°C for these strains. Two mutants (*ADH1* and *SDS23*) failed to grow under the conditions of our study and were consequently not included.

Mutants showing a significant difference in either biofilm formation or susceptibility to miconazole in the first screening, were retested at least once and the average result of all tests was calculated.

Calculations and statistical analysis

For each deletion mutant, relative values for biofilm formation and susceptibility to miconazole were calculated. Biofilm formation, expressed as the average fluorescence of untreated biofilms (corrected for the blank) was compared between the wild type (WT) and each mutant. Values lower than 1 indicate decreased biofilm formation and values higher than 1 indicate increased biofilm formation compared to the WT. The susceptibility to miconazole, expressed as the ratio of the average fluorescence of miconazole-treated biofilms to untreated biofilms (both corrected for the blank) was compared between the WT and each mutant. Values lower than 1 indicate an increased susceptibility to miconazole and values higher than 1 indicate a decreased susceptibility to miconazole compared to the WT. Statistical analysis was performed using the non-parametric Mann-Whitney U Test (SPSS Statistics 17.0 software). Results were considered significantly different when $P < 0.01$.

Data processing

For a general overview of the data, mutants with significant differences in biofilm formation or susceptibility to miconazole were grouped using the Gene Ontology Slim Mapper (27) according to the biological processes in which the deleted genes are involved.

For a more in-depth analysis, genes knocked out in mutants that showed a significant difference in at least one phenotype were categorized using the Gene Ontology Term Finder (27), searching for significantly shared gene ontology (SSGO) terms ($P < 0.01$). The frequency of SSGO terms in our dataset was compared to that in the total genome of *S. cerevisiae* S288C.

Detection of ROS

Biofilms of *S. cerevisiae* and *C. albicans* were grown as described above. ROS accumulation was measured in a fluorometric assay using 2',7'-dichlorofluorescein diacetate (DCFHDA) (Invitrogen) (20). To this end, biofilms were incubated with 10 µM DCFHDA, simultaneously with the antifungal treatment. Fluorescence was measured after 0 h and 6 h incubation using an Envision microtitre plate reader (Perkin Elmer) (λ_{ex} = 485 nm; λ_{em} = 535 nm). Values obtained were corrected for background

fluorescence (measured in the absence of cells) and compared to those obtained with untreated WT biofilms. ROS levels were quantified in triplicate on six biofilms (n = 18) for each strain.

Gene expression

C. albicans biofilms were grown and treated as described above. Sessile cells were collected and cell disruption, RNA purification and DNase treatment were performed according to the manufacturers' instructions (RiboPure-Yeast kit, Applied Biosystems, Carlsbad, CA). The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used for the reverse transcriptase reaction. To this end, 1 µl reverse transcriptase and 4 µl reaction mix were added to each tube (5 min at 25°C, 30 min at 42°C and 5 min at 85°C). A forward (FW) and a reverse (RV) primer were developed for the genes *PEX4 (*FW: TTGTTAGACCAACCCGAGCCAGAC ; RV: TTTGCTGCATCGATGTTCAACGGC), *PEX8 (*FW: AGCTTGGGTCCTCAAGGTAGAGC ; RV: ATTTGGGGTGCCCAGCAAGG), *LSC2 (*FW: CGTCAACATCTTTGGTGGTATTGT ; RV: TTGGTGGCAGCAATTAAACCT) and *RPP2B (*FW: TGCTTACTTATTGTTAGTTCAAGGTGGTA ; RV: CAACACCAACGGATTCCAATAAA) and their efficiency was calculated based on a standard curve and should be within the range of 90% to 110%. The specificity was tested by the determination of the melt curve of the amplified product and should show a unique dissociation peak. Real-time PCR (CFX96 Real Time System, Bio-Rad) was performed using the Sso Advanced SYBR Green Supermix (Bio-Rad). The expression levels of the genes of interest were normalized using two stably expressed reference genes (*LSC2* and *RPP2B*). Experiments were performed in triplicate and analyzed with the Bio-Rad CFX Manager software (Bio-Rad).

RESULTS AND DISCUSSION

General overview

A total of 4,961 haploid *S. cerevisiae* deletion mutants were screened for biofilm formation and susceptibility to miconazole (Figure 4.1). 341 mutants (6.9%) showed significant differences ($P < 0.01$) in biofilm formation compared to the WT. The majority (242 of 341 mutants) formed less biofilm as opposed to 99 mutants showing significantly increased biofilm formation. 387 mutants (7.8%) exhibited significantly different ($P < 0.01$) susceptibility to miconazole compared to the WT, 136 mutants being more susceptible and 251 mutants more resistant compared to the WT. 84 mutants showed both decreased biofilm formation and decreased susceptibility, 37 mutants showed decreased biofilm formation and increased susceptibility, 7 mutants showed increased biofilm formation and decreased susceptibility, and 18 mutants showed increased biofilm formation and increased susceptibility (Figure 4.1).

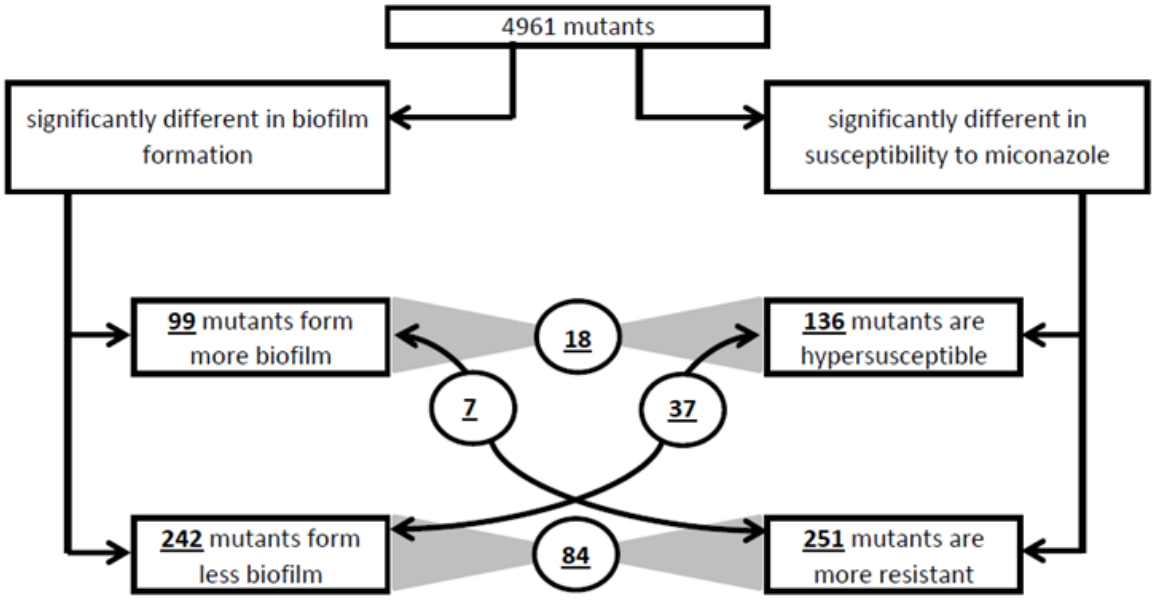

Figure 4.1. Overview of screening of *S. cerevisiae* deletion mutant bank for biofilm formation and susceptibility to miconazole.

A decrease in biofilm formation was observed in the mutants of which the deleted genes are involved in mitochondrial organization, protein complex biogenesis and protein targeting. An increase in biofilm formation was observed mainly in the mutants of which the deleted genes are involved in transcription, cell wall organization and mitotic cell cycle (Table 4.1). Deletion of several genes involved in protein targeting, transcription and response to stress increased the susceptibility to miconazole, while deletion of several genes involved in mitochondrial organization and protein complex biogenesis led to increased resistance to miconazole. The deletion of several genes involved in lipid metabolism also affected miconazole susceptibility, with about half of the mutants showing increased susceptibility and about half showing decreased susceptibility (Table 4.1).

Table 4.1. Frequency of genes, categorized according their biological processes involved. The corresponding mutants showed a significantly different phenotype in biofilm formation or miconazole susceptibility compared to the WT.

	Biofilm formation		Miconazole susceptibility	
GO Term	decreased	increased	increased	decreased
Total	242	99	136	251
biological process unknown	45	26	20	68
mitochondrial organization	39	7	5	28
protein complex biogenesis	24	0	11	18
protein targeting	23	4	18	6
mitochondrial translation	23	5	1	10
transmembrane transport	18	3	8	10
carbohydrate metabolic process	15	3	8	8
generation of precursor metabolites and energy	15	2	5	12
transcription from RNA polymerase II promoter	14	10	18	14
response to DNA damage stimulus	13	6	14	8
proteolysis involved in cellular protein catabolic process	13	1	12	5
lipid metabolic process	12	6	13	16
cofactor metabolic process	12	0	5	9
ion transport	12	3	7	13
response to chemical stimulus	11	7	17	9
cellular respiration	11	1	3	6
peroxisome organization	11	0	9	2
protein phosphorylation	10	4	6	6
signaling	10	5	8	11
regulation of translation	10	1	2	10
regulation of organelle organization	9	1	4	6
cellular amino acid metabolic process	9	2	1	12
cytoplasmatic translation	9	2	5	9
regulation of cell cycle	8	5	5	9
DNA repair	7	4	10	4
response to starvation	7	1	2	5
cell wall organization or biogenesis	7	9	8	6
mitotic cell cycle	7	8	8	8
nucleobase-containing small molecule metabolic process	7	1	1	9
mRNA processing	6	2	6	2
RNA splicing	6	2	3	4
nuclear transport	6	3	4	0
cytokinesis	6	4	4	8
vacuole organization	6	0	4	3
nucleobase-containing compound transport	6	2	5	1
cytoskeleton organization	5	2	6	6
RNA modification	5	3	3	2
endosomal transport	5	2	5	2
DNA recombination	5	3	1	0
cellular ion homeostasis	5	4	6	12
sporulation	5	6	3	2
rRNA processing	5	1	2	7
protein modification by small protein conjugation or removal	5	3	8	3
organelle fusion	5	0	2	3
membrane fusion	5	0	1	4
ribosomal small subunit biogenesis	4	1	1	5
chromatin organization	4	6	12	6
RNA catabolic process	4	0	2	6
organelle inheritance	4	0	2	2

	Biofilm formation		Miconazole susceptibility	
GO Term	decreased	increased	increased	decreased
meiotic cell cycle	4	2	3	3
response to osmotic stress	4	0	4	5
vesicle organization	4	0	2	2
tRNA processing	4	3	3	1
regulation of transport	4	2	4	3
Golgi vesicle transport	4	2	3	5
endocytosis	4	1	3	5
amino acid transport	4	1	1	4
histone modification	3	2	5	4
peptidyl-amino acid modification	3	2	3	3
protein folding	3	0	1	5
transcription from RNA polymerase III promoter	3	0	0	0
pseudohyphal growth	3	4	3	5
protein maturation	3	0	1	2
telomere organization	3	1	1	2
nucleus organization	2	0	2	2
protein glycosylation	2	1	2	0
membrane invagination	2	0	3	4
organelle assembly	2	1	1	2
cell budding	2	3	1	5
transcription from RNA polymerase I promoter	2	0	2	0
ribosome assembly	2	1	0	1
oligosaccharide metabolic process	2	1	2	0
DNA-dependent transcription, elongation	2	2	3	0
protein acylation	2	1	2	2
invasive growth in response to glucose limitation	2	1	3	3
ribosomal large subunit biogenesis	2	0	0	2
translational initiation	1	1	2	3
DNA-dependent transcription, initiation	1	1	0	1
DNA-dependent transcription, termination	1	0	0	1
conjugation	1	3	2	2
regulation of protein modification process	1	0	2	1
DNA replication	1	1	3	2
response to oxidative stress	1	1	3	1
protein alkylation	1	0	3	0
regulation of DNA metabolic process	1	2	4	3
translational elongation	1	0	0	4
vitamin metabolic process	1	1	0	3
response to heat	1	0	4	1
organelle fission	1	0	5	1
tRNA aminoacylation for protein translation	1	1	0	0
cell morphogenesis	0	1	0	1
exocytosis	0	1	0	2
protein dephosphorylation	0	3	0	2
lipid transport	0	1	2	1
chromosome segregation	0	1	3	1
protein lipidation	0	0	3	0
carbohydrate transport	0	0	1	1
other	5	4	3	14
not yet annotated	1	0	0	0

Validation of screening

A total of 917 mutants (18.5 %) was retested following the first screening because of a significant difference ($P < 0.01$) in biofilm formation or miconazole susceptibility compared to the WT. The results for 819 of these 917 retested mutants (89.3%) confirmed the previously observed significant differences (including 237 mutants that showed a significantly different phenotype at a lower level of significance; $P < 0.05$). Overall, these data indicate a good repeatability of our method and confirm the validity of our screening.

As a second measure of the validity of our screening, we compared our results with results previously obtained with selected *C. albicans* mutants. Deletion of *SUN4*, encoding a cell wall protein related to glucanases, led to decreased biofilm formation in *S. cerevisiae*. It was previously demonstrated that its ortholog in *C. albicans*, *SUN41*, is required for biofilm formation (28, 29). Similarly, our data show that *SUV3* is required for biofilm development in *S. cerevisiae*, as was previously also reported for *C. albicans* (30).

Deletion of *LCB4*, a gene involved in sphingolipid biosynthesis, increased susceptibility to miconazole. We have previously shown that a heterozygous *LCB4/lcb4 C. albicans* deletion mutant was also hypersusceptible to miconazole (25) (Chapter 5).

In addition, our experiments identified several genes involved in general and azole specific resistance in *C. albicans* biofilms. Deletion of ergosterol biosynthesis genes (*ERG2*, *ERG4*, *ERG24* and *ERG28*) increased the susceptibility to miconazole, probably due to a decreased level of ergosterol, an important constituent of the cytoplasmatic membrane. The failure of adapting the sterol composition of the cytoplasmatic membrane as a mechanism to inhibit the penetration of miconazole in these mutants may also contribute to the observed phenotype. Induction of ROS production has been observed in planktonic and sessile *Candida* cells after miconazole treatment (19, 22) (Chapter 3). This increased ROS production was preceded by changes in the actin cytoskeleton (21) and may contribute to the fungicidal action of miconazole. We identified three genes (*SIT4*, *VPS1* and *END3*) that were involved in the organization of the actin cytoskeleton and which appear to play a role in the resistance to miconazole. Furthermore, superoxide dismutases are important for ROS detoxification and their protective effect against miconazole-induced cell death in *C. albicans* biofilms was observed previously (31). Also, in the present study a deletion of *SOD1*, encoding a zinc-

copper superoxide dismutase, led to increased susceptibility to miconazole. Deletion of *MXR1* resulted in a similar phenotype. The latter gene encodes methionine sulfoxide reductase, and is known for its antioxidative capacities leading to increased survival of cells (32, 33). Trehalose, a disaccharide, is also known for its protective effect against ROS. Deletion of *TPS2*, encoding trehalose-6-phosphate phosphatase, leads to a decrease of trehalose levels and the accumulation of trehalose-6-phosphate to toxic levels (34, 35). Data from the present study suggest that *TPS2* is also involved in resistance of yeast biofilms to miconazole.

Mutants showing decreased biofilm formation

The SSGO terms of the mutants with decreased biofilm formation could be divided in 10 categories (Figure 4.2). The growth rate of several mutants within these categories was determined (Table 4.2). As only a minority of mutants showed a significant increase in doubling time (2 of 15), we may conclude that there is no major link between growth rate and biofilm formation, and that an affected metabolic activity in the mutants is not directly involved in decreased biofilm formation.

Table 4.2. Doubling times of mutants with lower biofilm formation. Significantly ($P < 0.05$) increased doubling times compared to the WT are marked with an asterisk.

	Doubling time (minutes)	STDEV (minutes)	*P*-value
WT	137	22	
Δ*atg11*	125	26	0.481
Δ*coq1*	154	7	0.056
Δ*coq4*	142	12	0.421
Δ*coq5*	163 *	9	0.027
Δ*csg2*	110	4	0.035
Δ*fis1*	131	17	0.687
Δ*pex1*	181	72	0.205
Δ*pex10*	170	48	0.057
Δ*pex12*	159	27	0.101
Δ*pex13*	201 *	70	0.009
Δ*pex5*	157	43	0.257
Δ*pex6*	164	51	0.301
Δ*slt2*	203	113	0.365
Δ*vps15*	134	16	0.850
Δ*ydc1*	113	4	0.035

Compared with the overall prevalence in the genome, genes involved in peroxisomal transport were overrepresented among the mutants showing decreased biofilm formation (10-fold more compared to the total genome). Pex1, Pex5, Pex6, Pex10, Pex12 and Pex13 are involved in the peroxisomal matrix protein import, and deletion of the corresponding genes led to decreased biofilm formation. Pex5 is the receptor which binds to the peroxisomal proteins in the cytosol. A set of peroxins, including the ones mentioned before, are anchored in the peroxisomal membrane and are involved in the binding of the receptor-protein complex, its dissociation, the uptake of the peroxisomal protein and the release of the Pex5 receptor into the cytosol (36). Furthermore, Pex3, of which the deficiency also led to decreased biofilm formation in *S. cerevisiae*, is required for the proper localization of peroxisomal membrane proteins (37). So far, a link between biofilm formation and the activity of peroxisomes has not been described. Peroxisomes are organelles with a single membrane in which β-oxidation of fatty acids takes place (38, 39). They also contain antioxidative systems to neutralize ROS produced during metabolism (40). As β-oxidation in yeast cells exclusively takes place in peroxisomes (38, 39), it is not surprising that mutations in peroxins may lead to a disordered balance in lipid composition and may therefore change the composition of cellular membranes. Differences in the distribution of lipids between planktonic and sessile cells have been described previously. Furthermore, lipids are important for adhesion and play a critical role in the formation of biofilms (41, 42).

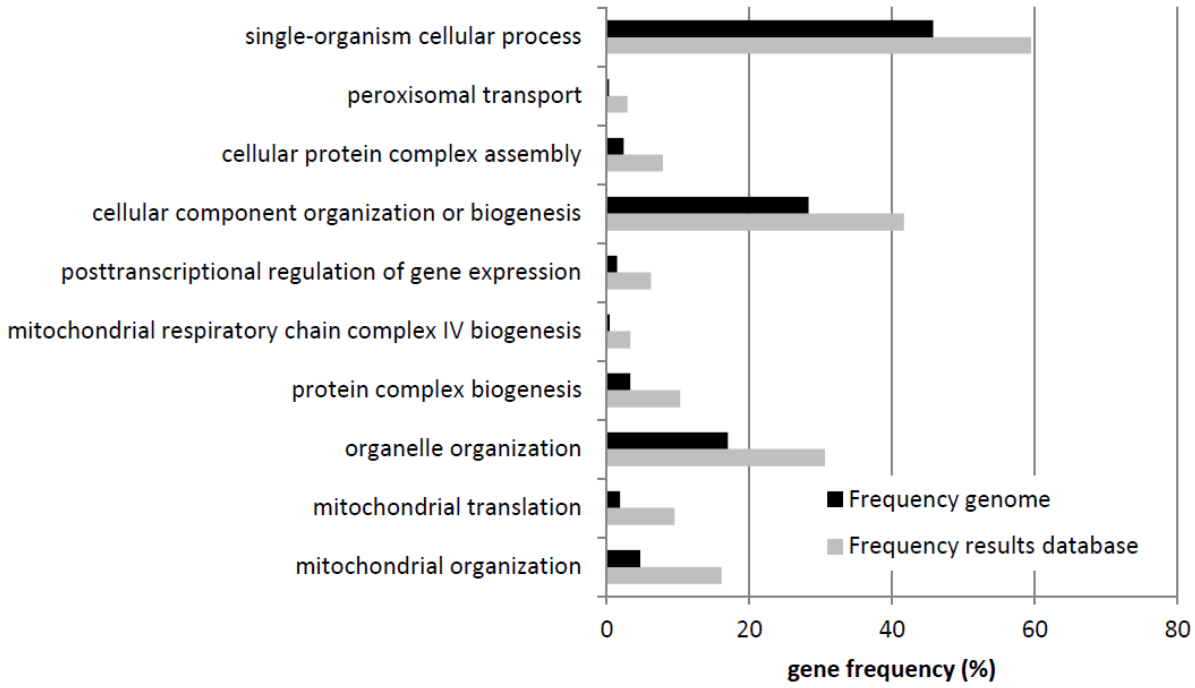

Figure 4.2. Frequency of SSGO terms of the mutants with decreased biofilm formation in the results database compared to the overall frequency in the *S. cerevisiae* genome.

Many mutants in which genes involved in mitochondrial organization are deleted, showed significantly decreased biofilm formation. Particularly genes involved in the biogenesis of the respiratory chain complex IV (*PET54*, *PET100*, *PET122*, *COX12*, *COX14*, *COX16*, *COX20* and *MSS51*) were overrepresented in this group (8-fold more prevalent than in the total genome). Complex IV, or cytochrome c oxidase, is the final enzyme in the electron transport chain, creating the proton gradient necessary for ATP production. Mutants in which *PET54*, *PET100* or *PET122* are deleted are known to form so called 'petite' colonies. Petite mutants often arise spontaneously during growth. The partial or complete loss of mitochondrial DNA leads to a lower growth rate and the formation of small colonies (43). It is likely that the reduced growth rate of these mutants is responsible for the decreased biofilm formation. *COX12* plays a role in the assembly of complex IV, but does not seem to be required for its function (44). For this reason, the mechanism by which *COX12* influences the biofilm formation remains unclear. The deletion of *COX14, COX16* and *MSS51* leads to a defect in respiratory growth; the function of *COX16* is unknown (45). The decreased ATP production in these mutants is likely to

result in a lack of energy for the formation of a dense biofilm, although other mechanisms may decrease biofilm formation.

In *S. cerevisiae* the flocculin gene family, including *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*, encodes cell wall proteins which are important for cell-cell adhesion. The latter gene is also involved in cell-surface adhesion. A first group of Flo proteins (encoded by *FLO1*, *FLO5* and *FLO9*) acts as lectins, while Flo10 and Flo11 confer adhesion by increased cell surface hydrophobicity. (46-48). *S. cerevisiae* strain BY4741 used in the present study, a derivative of S288C, expresses low levels of *FLO11*, and therefore has reduced biofilm forming capacity (49). Nevertheless, confocal laser scanning microscopy images of the biofilms in our study showed a biofilm-like morphology and washing with PS did not affect this structure (Figure 4.3). The flocculin mutants did not show a change in biofilm formation compared to the WT under the conditions of our study, supporting the idea that one *FLO* gene can compensate for the absence of another (50).

The transcriptional network controlling biofilm formation has been extensively studied in *C. albicans* and consists of six regulators: Tec1, Efg1, Ndt80, Rob1, Brg1 and Bcr1 (51). However, there are differences in the controlled target genes (Tec1 and Efg1) or differences in the function (Ndt80) between the orthologs in *C. albicans* and *S. cerevisiae*. For Rob1 and Brg1, the regulatory function is only detectable in species closely related to *C. albicans*. Finally, Bcr1 orthologs have not been found in *S. cerevisiae* (51). None of the *S. cerevisiae* orthologs of these *C. albicans* genes seems to be involved in biofilm formation.

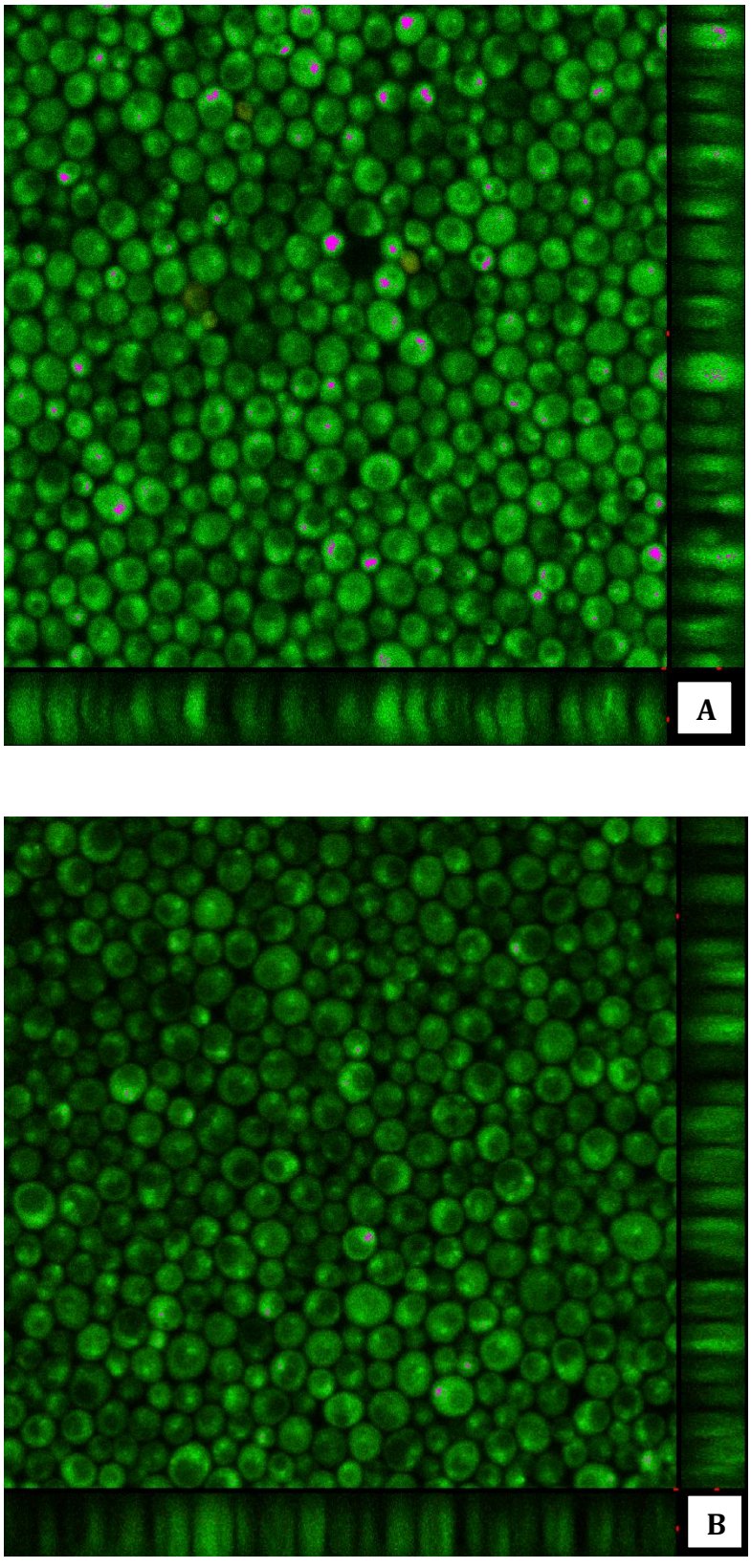

Figure 4.3. Confocal laser scanning microscopy images of *S. cerevisiae* BY4741 WT (A) and *S. cerevisiae* BY4741 Δ*FLO11* (B).

Mutants showing increased biofilm formation

Only 99 mutants showed a significantly increased biofilm formation and no SSGO terms could be detected within this category, indicating the involvement of a broad variation of biological processes. Furthermore, 25% of the deleted genes were associated with unknown biological processes. We hypothesise that the deletion of genes leading to increased biofilm formation disturbs the global metabolic balance and that other pathways compensate as reaction. This idea is also supported by the presence of a relatively high number of genes involved in transcription within this group of 99 mutants. Their deletion influences a variety of other cellular metabolic pathways and, probably, a combination of factors is responsible for the observed phenotype.

Mutants showing increased susceptibility to miconazole

The SSGO terms of the mutants with increased susceptibility to miconazole could be divided in 12 categories (Figure 4.4). Compared with the overall prevalence in the genome, we observed that genes involved in regulation of transcription by glucose were overrepresented among the mutants showing increased susceptibility to miconazole (18-fold more compared to the total genome). Although only five genes are clustered in this group (*NRG2*, *TUP1*, *VPS36*, *SNF8* and *GCR1*), they seem to be highly involved in the resistance to miconazole. As all of these genes regulate the expression of several other genes involved in various pathways, it is likely that a combination of factors account for the increased susceptibility to miconazole. Tup1, a general repressor forming a complex with Ssn6, regulates the expression of over 300 genes involved in metabolic processes, transport, meiosis, cell wall organization, stress responses and transcription (52-54). Gcr1 is a transcriptional activator that coordinates the expression of several glycolytic enzymes (55, 56). Nrg2 is known to repress a large number of stress responsive genes. However, Nrg1 (a paralog of Nrg2) is more important for this regulation. Furthermore, genes involved in mitochondrial organization, carbon and nitrogen signaling, cell wall organization, mating and transcription are also known to be Nrg-repressed (57). Finally, *SNF8* and *VPS36* derepress the transcription of *SUC2*, encoding a sucrose hydrolyzing enzyme (58). Vps36 and Snf8 are both components of the ESCRT-II (endosomal sorting complex required for transport) complex, which is involved in protein sorting and the biogenesis of multivesicular bodies. This complex also regulates the formation of ESCRT-

III (59-61). Interestingly, defects in the ESCRT machinery lead to the accumulation of receptors and transporters in the cytoplasmatic membrane (62), which possibly results in an enhanced uptake of miconazole and a consequent increase in susceptibility.

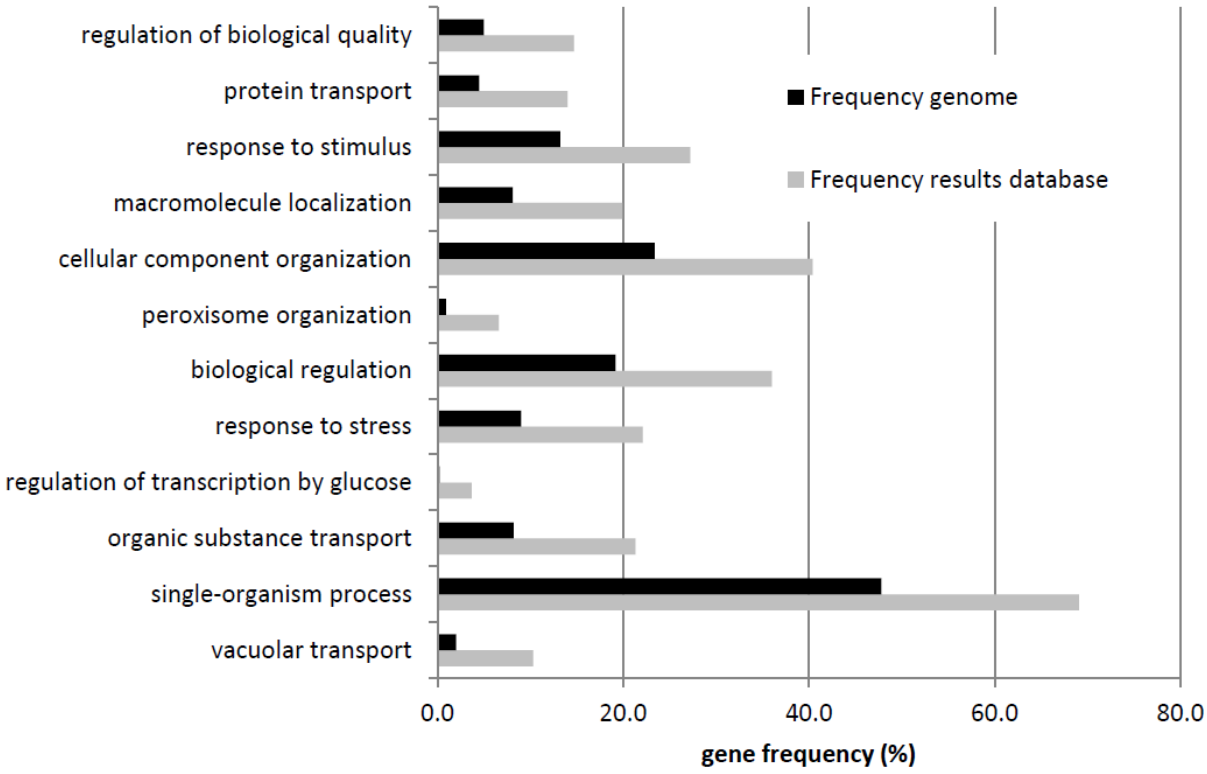

Figure 4.4. Frequency of SSGO terms of the mutants with increased susceptibility to miconazole in the results database compared to the overall frequency in the *S. cerevisiae* genome.

A second cluster of genes overrepresented among hypersusceptible mutants (seven fold more prevalent than in the total genome) was involved in peroxisomal organization: *PEX2*, *PEX4*, *PEX8*, *PEX34*, *VPS1*, *VPS15*, *SLT2*, *FIS1* and *ATG11*. Three of these genes (*PEX2*, *PEX4* and *PEX8*) have a function in the peroxisomal matrix protein import machinery (36), which was also found to be involved in biofilm formation. *VPS15*, *SLT2* and *ATG11* are involved in the degradation of peroxisomes. Their corresponding mutants are deficient in pexophagy (63, 64). In contrast, *VPS1*, *FIS1* and *PEX34* are involved in determining the number of peroxisomes per cell (65, 66). It seems contradictory that genes involved on one hand in the degradation of peroxisomes and on

the other hand in the development of peroxisomes play a role in the resistance to miconazole. However, this probably indicates that a balanced level of peroxisomes is crucial for sessile cells. A low number of peroxisomes may disturb the lipid housekeeping and the antioxidative capacities of the cells. A decrease in the degradation of peroxisomes is known to induce intracellular protein aggregation and this affects the catalase activity and consequently increases ROS levels (67, 68). As the induction of ROS has previously been linked to the fungicidal activity of miconazole (19, 22), this may contribute to the hypersusceptibility of these peroxisomal mutants. Furthermore, it is possible that strains affected in peroxisome functioning show an altered sphingolipid composition which consequently influences the susceptibility to miconazole (Chapter 5).

Additional experiments showed a possible effect of altered antioxidative capacities for strains affected in their peroxisome functioning, as all *S. cerevisiae* mutants within this category showed increased ROS-levels upon miconazole treatment compared to the WT, ranging from a 1.5 to 6.5 fold increase (Figure 4.5). Similar to *S. cerevisiae*, mutant biofilms of *C. albicans* Δ*pex4*/Δ*pex4* and Δ*pex8*/Δ*pex8* were hypersusceptible to miconazole compared to the WT (relative values of 0.65 and 0.48, respectively). In contrast, no decrease in the MIC for miconazole was observed for both strains compared to the WT, suggesting that the hypersusceptibility of both strains is biofilm specific. Biofilms of both mutants also showed an increased ROS-level upon miconazole treatment, which was more pronounced for *C. albicans* Δ*pex8*/Δ*pex8* (three-fold increase compared to WT) than for *C. albicans* Δ*pex4*/Δ*pex4* (two-fold increase compared to WT). Miconazole treatment of *C. albicans* WT biofilms led to a two-fold overexpression of the genes *PEX4* and *PEX8* compared to untreated biofilms. The susceptibility to fluconazole (included as negative control) of the biofilms of both *C. albicans* mutants was similar to that of the *C. albicans* WT (relative values of 1.02 for *C. albicans* Δ*pex4*/Δ*pex4* and 1.15 for *C. albicans* Δ*pex8*/Δ*pex8*), and fluconazole treatment did not induce ROS. Taken together, these additional experiments confirm the biofilm specific importance of peroxisomes in miconazole resistance of *S. cerevisiae* and *C. albicans* biofilms.

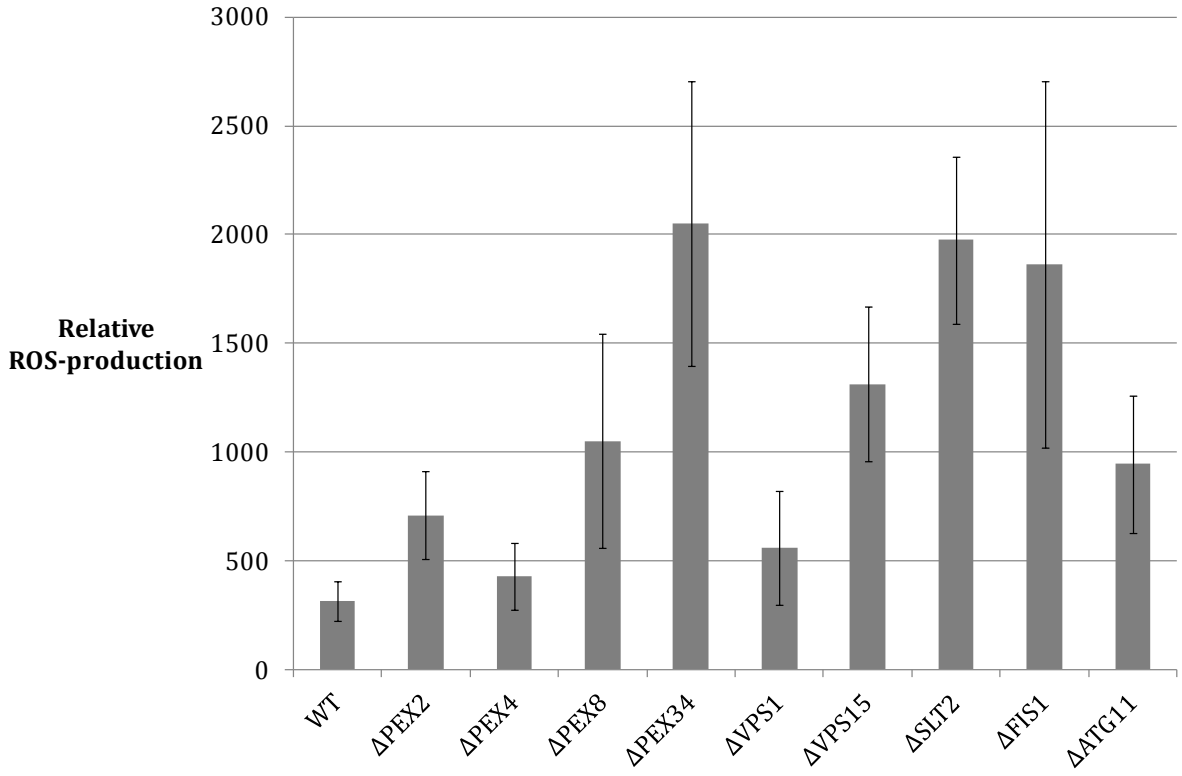

Figure 4.5. ROS-production in *S. cerevisiae* biofilms after miconazole treatment. The results were compared to untreated WT biofilms. Experiments were performed in triplicate on six biofilms. Error bars represent the standard error of the mean.

Mutants showing decreased susceptibility to miconazole

Within the large group of mutants (251) with decreased susceptibility to miconazole, only two categories with SSGO terms could be distinguished; i.e. genes involved in mitochondrial respiratory chain complex assembly and in posttranscriptional regulation of gene expression, respectively (Figure 4.6). This indicates that the majority of the genes in this group play a role in a broad range of different biological processes. The genes involved in the assembly of the mitochondrial respiratory chain complex (*CBP6*, *MZM1*, *EMI1*, *COX12*, *COX14*, *COX16*, *COX19*, *COA6* and *CBP3*) were seven times more prevalent among the mutants showing decreased miconazole susceptibility than in the total *S. cerevisiae* genome. Genes belonging to this category play a role in the production of ATP under aerobic conditions. Their deletion may lead to decreased ATP levels, and as a result a dormant state of the cells. Probably this mechanism is important for making these mutants more resistant to miconazole, as

observed in previous research (12, 69-71). However, it is possible that also other mechanisms are responsible for the observed phenotype. The second category of genes, of which deletion led to decreased susceptibility to miconazole, is involved in posttranscriptional regulation and therefore affecting the expression of many other genes. Two main groups may be distinguished: genes related to mitochondrial organization (*CBP6*, *CBS2*, *ICP55*, *COX14* and *ATP25*) and genes related to ribosomal organization (*RPS9B*, *RPL31A* and *ASC1*). As they have a global regulatory effect, it is not clear which mechanisms exactly contribute to the observed decrease in susceptibility to miconazole.

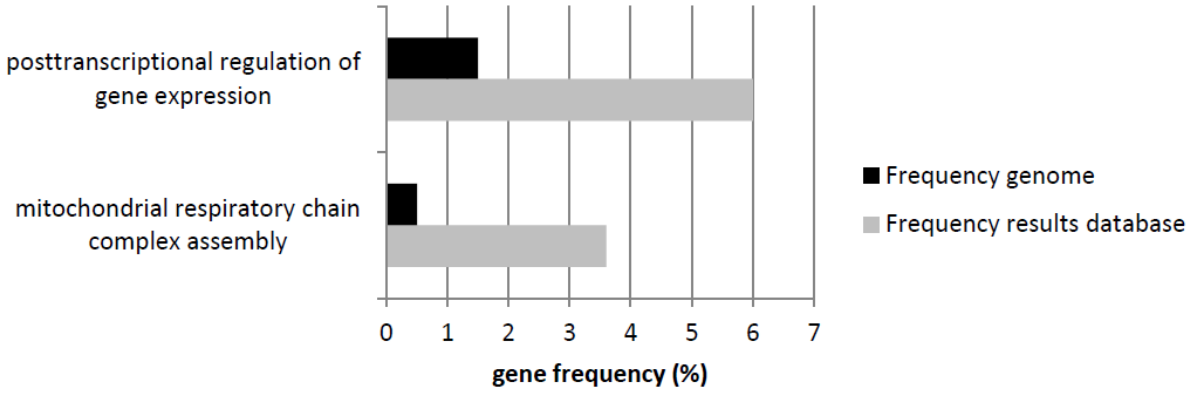

Figure 4.6. Frequency of SSGO terms of the mutants with decreased susceptibility to miconazole in the results database compared to the overall frequency in the *S. cerevisiae* genome.

Mutants affected in biofilm formation and miconazole susceptibility

146 mutants were affected both in biofilm formation and miconazole susceptibility. SSGO terms could not be defined for all mutants within each combination (Figure 4.7). Most mutants affected in both phenotypes were found to exhibit decreased biofilm formation and increased resistance (84 mutants). This is not completely unexpected, as genes involved in mitochondrial organization in this group were highly represented (4-fold more prevalent than in the total genome) and it is likely that a lower

ATP level both decreases biofilm formation and increases resistance by inducing dormancy. A second significantly overrepresented group of mutants with decreased biofilm formation and increased resistance to miconazole have defects in posttranscriptional regulation of gene expression. Our results also reveal that genes involved in peroxisomal organization play an important role both in the formation of biofilms and the resistance to miconazole, although they do not have significantly shared genes for this biological process: biofilm formation is regulated mainly by genes related to peroxisomal transport, while the resistance of sessile cells to miconazole is particularly induced by genes involved in the general peroxisomal organization.

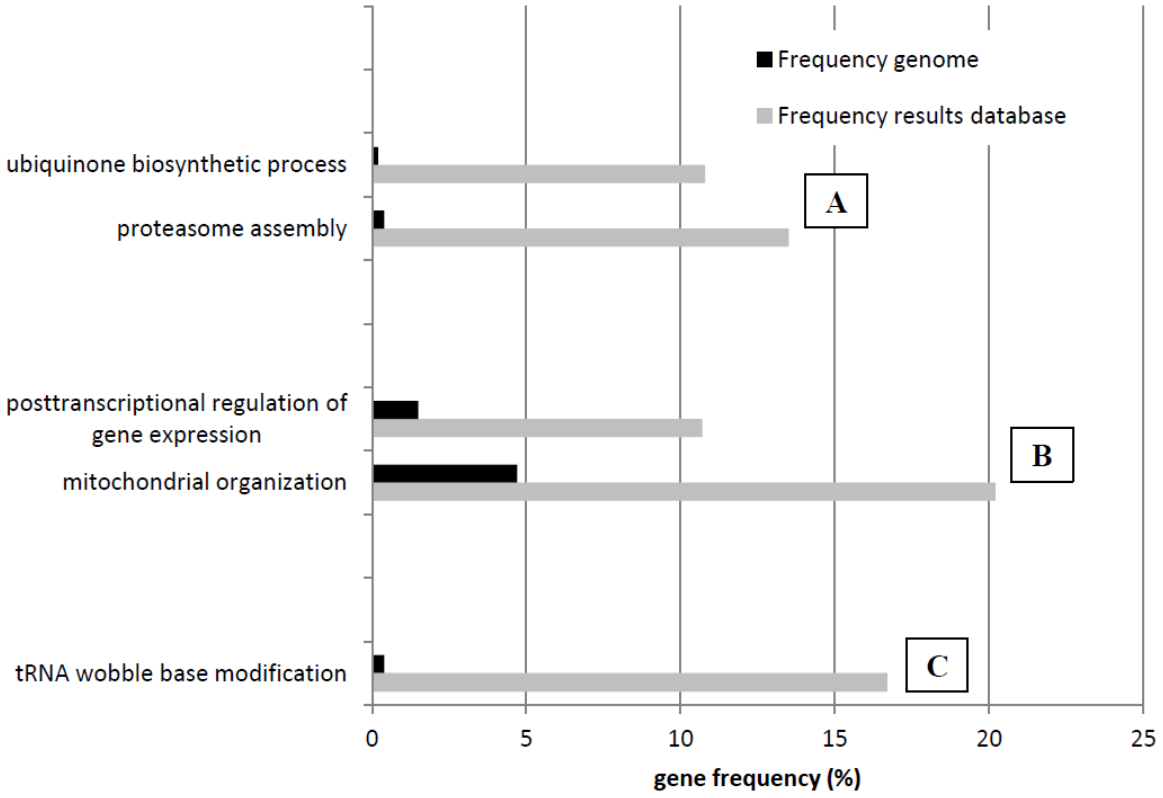

Figure 4.7. Frequency of SSGO terms in the results database compared to the overall frequency in the *S. cerevisiae* genome for mutants with decreased biofilm formation and increased susceptibility to miconazole (A), for mutants with decreased biofilm formation and decreased susceptibility to miconazole (B) and for mutants with increased biofilm formation and increased susceptibility to miconazole (C).

Within the group of 18 mutants with increased biofilm formation and increased susceptibility to miconazole, transfer RNA (tRNA) wobble uridine modification is a SSGO term represented by *SIT4*, *IKI3* and *TRM9*. These modifications play an important role in the folding and stability of tRNA. Furthermore, they are necessary for an accurate and efficient translation and they have recently been linked to the control of gene expression in response to stress (72, 73).

Finally, we found two SSGO terms for mutants with decreased biofilm formation and increased susceptibility, i.e. the biosynthesis of ubiquinone, a component of the electron transport chain, important for ATP production by respiration, and the assembly of proteasomes, protein complexes responsible for the degradation of unneeded or damaged proteins. As many other genes involved in mitochondrial organization showed decreased biofilm formation, probably due to a dormant state induced by lower ATP production, it is very likely that a decrease in ubiquinone may also contribute to this phenomenon. In contrast to our previous hypothesis that this dormant state leads to increased resistance, deletion of four genes (*COQ1*, *COQ4*, *COQ5* and *COQ8*) in the ubiquinone biosynthetic pathway increases the susceptibility to miconazole. The anti-oxidative property of the reduced form of ubiquinone (74-76) may be important for the latter phenomenon as a decrease in ubiquinone may diminish the protection against miconazole-induced ROS. Also, proteasomes are involved in the response to oxidative stress (77). Deletion of the genes involved in proteasome assembly (*UMP1*, *PRE9*, *NAS2*, *IRC25* and *POC4*) may impair the proteasome activity in the mutants and may consequently contribute to the hypersusceptibility to miconazole. Inactivation of proteasomes has previously been shown to decrease the growth rate of *C. albicans* and to inhibit its biofilm formation (78).

CONCLUSION

A large number of genes was found to be involved in biofilm formation and drug resistance in *S. cerevisiae*, indicating the complexity of both processes. The validity of the screening was confirmed by the identification of genes previously observed to be involved in biofilm formation and drug resistance in *C. albicans*. Peroxisomal transport and mitochondrial organization appear to be important for yeast biofilm formation. Additionally, genes involved in transcription, peroxisomal and mitochondrial organization influence the susceptibility to miconazole. Peroxisomes were also found to be important for miconazole resistance in the human pathogen *C. albicans* and this may offer perspectives for the treatment of fungal biofilm-related infections. However, a considerable number of genes identified in the present study is associated with unknown biological processes, requiring further research.

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CHAPTER 5

PHYTOSPHINGOSINE-1-PHOSPHATE IS A SIGNALING MOLECULE INVOLVED IN MICONAZOLE RESISTANCE IN SESSILE *CANDIDA ALBICANS* CELLS

Based on:

Vandenbosch D, Bink A., Govaert G, Cammue BP, Nelis HJ, Thevissen K, Coenye T. 2012. Phytosphingosine-1-phosphate is a signaling molecule involved in miconazole resistance in sessile *Candida albicans* cells. Antimicrob Agents Chemother **56**:2290-4.

ABSTRACT

Previous research has shown that 1% to 10% of sessile *Candida albicans* cells survive treatment with high doses of miconazole (a fungicidal imidazole) (Chapter 3). In the present study, we investigated the involvement of sphingolipid biosynthetic intermediates in this survival. We observed that the *LCB4* gene, coding for the enzyme that catalyzes the phosphorylation of dihydrosphingosine and phytosphingosine, is important in governing the miconazole resistance of sessile *S. cerevisiae* and *C. albicans* cells. The addition of 10 nM phytosphingosine-1-phosphate (PHS-1-P) drastically reduced the intracellular miconazole concentration and significantly increased the miconazole resistance of a hypersusceptible *C. albicans* heterozygous *LCB4/lcb4* mutant, indicating a protective effect of PHS-1-P against miconazole-induced cell death in sessile cells. At this concentration of PHS-1-P, we did not observe any effect on the fluidity of the cytoplasmatic membrane. The protective effect of PHS-1-P was not observed when the efflux pumps were inhibited or when tested in a mutant without functional efflux systems. Also, the addition of PHS-1-P during miconazole treatment increased the expression levels of genes coding for efflux pumps, leading to the hypothesis that PHS-1-P acts as a signaling molecule and enhances the efflux of miconazole in sessile *C. albicans* cells.

INTRODUCTION

Candida albicans is a fungal pathogen that frequently causes nosocomial infections in immunocompromised hosts. The adhesion of the cells to biotic or abiotic surfaces results in the formation of a complex three-dimensional biofilm consisting of yeast cells and filaments embedded in a self-produced exopolymeric matrix. These sessile cells show increased antifungal resistance compared to planktonic cells, resulting in recurrent infections that are very difficult to treat. Therefore, there is an urgent need to unravel the molecular mechanisms of resistance of biofilms to find new and effective therapies (1-4).

Azoles are widely used to treat *Candida* infections. These antifungal compounds decrease the production of ergosterol by interacting with cytochrome P450 and inhibiting the 14α-demethylation of lanosterol. As ergosterol is an important constituent of the cytoplasmatic membrane, treatment with azole antifungals leads to growth inhibition (5). Besides this fungistatic mechanism of action, recent data indicate a fungicidal effect of miconazole (an imidazole) against *Candida* species cells in suspension and in young and mature biofilms (6, 7) (Chapter 3). The accumulation of ROS appears to be involved in this process, although it is likely that other mechanisms are also involved in the fungicidal activity (8-10). Despite the observed fungicidal activity of miconazole also against biofilms, 1% to 10% of sessile *C. albicans* cells survive exposure to high levels of this antifungal agent (7) (Chapter 3).

Previous research has shown that membrane rafts, which are tightly packed domains of sphingolipids and sterols, and both the sphingolipid and ergosterol contents of the membrane are critical factors in the mode of action of miconazole against yeast cells (11). However, possible mechanisms of miconazole resistance related to membrane rafts and, more specifically, sphingolipids have not been investigated with *C. albicans* biofilm cells. Sphingolipids are characterized by their typical long-chain amino-alcohol backbone, which can be phosphorylated or form more complex structures after acylation with fatty acids (ceramides) and the further addition of sugar residues (12). Sphingolipids are incorporated into different cellular membranes, including the cytoplasmatic membrane. Because of their physicochemical properties, sphingolipids are involved in a broad range of biological functions, including intracellular transport, cell-cell interactions, and molecular

sorting (13). Membrane rafts are involved in cell signaling, the sorting of membrane-bound proteins, and the maintenance of polarity during mating (14-16).

The aim of the present study was to investigate the role of sphingolipids in the resistance of sessile *C. albicans* cells to miconazole.

MATERIALS AND METHODS

Strains

The strains used in this study are listed in Table 5.1. A stock culture of all these strains was kept at −80°C. Cells were transferred onto SDA (Oxoid) plates and incubated at 37°C for 48 h.

Table 5.1. List of strains used in this study.

Species	Strain	Description	Source or reference
C. albicans	SC5314 (ATCC MYA-2876)	Wild Type	American Type Culture Collection
C. albicans		LCB4/Δlcb4::his3	(17)
C. albicans	DSY1050	Δcdr1::hisG/ Δcdr1::hisG; Δcdr2::hisG/ Δcdr2::hisG; Δmdr1::hisG-URA3-hisG/ Δmdr1::hisG	D. Sanglard
S. cerevisiae	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF
S. cerevisiae	YOR171c	BY4741, Δlcb4::KanMX4	EUROSCARF
S. cerevisiae	YLR260w	BY4741, Δlcb5::KanMX4	EUROSCARF
S. cerevisiae	YDR294c	BY4741, Δdpl1::KanMX4	EUROSCARF
S. cerevisiae	YKR053c	BY4741, Δysr3::KanMX4	EUROSCARF
S. cerevisiae	YJL134w	BY4741, Δlcb3::KanMX4	EUROSCARF
S. cerevisiae	YDR297w	BY4741, Δsur2::KanMX4	EUROSCARF
S. cerevisiae	YHL003c	BY4741, Δlag1::KanMX4	EUROSCARF
S. cerevisiae	YKL008c	BY4741, Δlac1::KanMX4	EUROSCARF
S. cerevisiae	YPL087w	BY4741, Δydc1::KanMX4	EUROSCARF
S. cerevisiae	YBR183w	BY4741, Δypc1::KanMX4	EUROSCARF
S. cerevisiae	YMR272c	BY4741, Δscs7::KanMX4	EUROSCARF
S. cerevisiae	YPL057c	BY4741, Δsur1::KanMX4	EUROSCARF
S. cerevisiae	YBR036c	BY4741, Δcsg2::KanMX4	EUROSCARF
S. cerevisiae	YBR161w	BY4741, Δcsh1::KanMX4	EUROSCARF
S. cerevisiae	YDR072c	BY4741, Δipt1::KanMX4	EUROSCARF

Determination of miconazole susceptibility of *S. cerevisiae* deletion mutants

Suspensions of *S. cerevisiae* BY4741 and BY4741-derived deletion mutants, containing approximately 10^7 cells/ml, were prepared in YPD medium (BD). Biofilms were grown in round-bottomed 96-well microtitre plates (SPL Lifesciences) containing 100 µl cell suspension per well (12 replicates per strain). After 1 h of incubation at 37°C, the supernatant was removed, and the wells were rinsed with 100 µl of PS to remove unattached cells. The microtitre plates were further incubated for 24 h at 37°C after the addition of 100 µl YPD medium to each well. Subsequently, the mature biofilms were rinsed with 100 µl PS before treatment with miconazole (1,000 µg/ml) (Certa). To this end, 100 µl of a miconazole suspension in PBS containing 2% DMSO (Sigma-Aldrich) was added to six biofilms of each strain, and 100 µl of PBS containing 2% DMSO was added to the other six biofilms (control). After 24 h of incubation at 37°C, the supernatant was removed, and 120 µl of a diluted resazurin solution (CellTiter-Blue at a 1:6 dilution in PS) (Promega) was added to each well. Fluorescence was measured (λ_{ex} = 535 nm; λ_{em} = 590 nm) after 2 h of incubation at 37°C using an Envision microtitre plate reader (Perkin-Elmer). For each deletion mutant, a relative value for susceptibility to miconazole was calculated. To this end, the ratios of the average fluorescence of miconazole-treated biofilms to that of untreated biofilms (both corrected for the blank) were compared between the WT and each mutant.

Analysis of gene expression in sessile *C. albicans* cells

C. albicans biofilms were grown on silicone disks in 24-well microtitre plates and treated with miconazole and phytosphingosine-1-phosphate (PHS-1-P) or miconazole alone, as described previously (7). Biofilm cells were collected and washed with physiological saline. Untreated sessile *C. albicans* cells were used as a control. Cell disruption, RNA purification, and DNase treatment were performed according to the manufacturer's instructions (RiboPure-Yeast kit; Applied Biosystems, Carlsbad, CA). The isolated RNA was concentrated with an Amicon Ultra filter (Millipore) and subsequently diluted with diethyl pyrocarbonate (DEPC)-treated water until a final volume of 15 µl was

reached. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used for the reverse transcriptase (RT) reaction. To this end, 1 µl reverse transcriptase and 4 µl reaction mix were added to each tube (5 min at 25°C, 30 min at 42°C, and 5 min at 85°C). A forward primer and a reverse primer were developed for the *LCB1*, *LCB2*, *LCB3*, *LCB4*, *KSR1*, *SUR2*, *LAG1*, *YDC1*, *HSX11*, *DPL1*, *SCS7*, *AUR1*, *IPT1*, *MIT1*, *CDR1*, *CDR2*, *MDR1*, *RIP*, and *LSC2* genes (Table 5.2) and their efficiency was calculated based on a standard curve and should be within the range of 90% to 110%. The specificity was tested by the determination of the melt curve of the amplified product and should show a unique dissociation peak. Real-time PCR (CFX96 Real Time system; Bio-Rad) was performed by using iQ SYBR green Supermix (Bio-Rad). The expression levels of the genes of interest were normalized using two stably expressed reference genes (*RIP* and *LSC2*). Experiments were performed as five independent biological repeats, each consisting of six technical repeats, and analyzed with Bio-Rad CFX Manager software (Bio-Rad).

Table 5.2. Sequences and concentrations of the primers used for expression analysis of genes involved in sphingolipid biosynthesis in *C. albicans* SC5314.

GENE	FUNCTION		PRIMER SEQUENCE (5' => 3')	CONCENTRATION
LCB1	serine C-palmitoyltransferase	forward	ACAAGACGGCAGTGAACCTTGCT	300 nM
		reverse	CACCCACACCGGCCGATCTAA	300 nM
LCB2	serine C-palmitoyltransferase	forward	TGACGAGTGCACGTGCCAGA	600 nM
		reverse	ACCAGGAACTTTACATCCACCAGCA	600 nM
LCB3	sphingosine-1-phosphate phosphatase	forward	CCACGATCGCCACCTTTGCAT	600 nM
		reverse	TGGCCAGGTGAGACGAGGGAA	600 nM
LCB4	sphingosine kinase	forward	GTGGCCCGGAAATACCCCCTT	600 nM
		reverse	CGCCATGCGGGTTTATCAATACCAA	600 nM
KSR1	3-ketosphinganine reductase	forward	GGCTACTCGCAATATGCCCCCAT	50 nM
		reverse	CCCAGGGAAAACACATGTGACACGA	50 nM
SUR2	ceramide hydroxylase	forward	CGAGGGTACTTTCACAGCCCACT	100 nM
		reverse	CAACAACCGGCACAATCAAGGCA	100 nM
LAG1	sphingosine N-acyltransferase	forward	CATTTCACTTGGATGGGATTGGCTGTT	100 nM
		reverse	AATGGACCGGTAATGGGAGATTCCAAA	100 nM
YDC1	dihydroceramidase	forward	GGAAGGACACGGATGGTGGCA	300 nM
		reverse	AACAACAGGCAACCCCCATATCCA	300 nM
HSX11	ceramide glucosyltransferase	forward	GCAAAATACCCAACAGTTGATGCCCAA	100 nM
		reverse	GGCATGAACGAACCCCTTTGCT	100 nM
DPL1	sphinganine-1-phosphate aldolase	forward	CGGTTCAGTCTGTTTAGCAAGAGATA	100 nM
		reverse	ATTTGAGATGACACCGTAGAACTAACA	100 nM
SCS7	fatty acid alpha-hydroxylase	forward	TCAGAGCCCCATTACTGCGTTGA	600 nM
		reverse	CCGTGCAAAAGAAAGTGGAGAGCA	600 nM
AUR1	inositol phosphoceramide synthase	forward	CCATGGAGGAGCAGCTGGGAA	600 nM
		reverse	TTGGGCCACCGACGGCATTA	600 nM
IPT1	inositol phosphoryl transferase	forward	CCCCAATGCTGCACCTTGGTTT	600 nM
		reverse	TCCACTCGAATTAATCCAGCGGCATA	600 nM
MIT1	inositolphosphoceramide mannose transferase	forward	TCAACGTAATTGGTTGGTGCCGTACAT	600 nM
		reverse	CCGCACTTTCCCAGCTTCAGGA	600 nM
CDR1	multidrug transporter of ATP-binding cassette superfamily	forward	CAGCAACCATGGGTCAATTATG	300 nM
		reverse	GTAGCCAAATTGGCAGCATTATC	300 nM
CDR2	multidrug transporter of ATP-binding cassette superfamily	forward	GATTCAAGCCATTCTTTCTACTGGAT	300 nM
		reverse	AGTAACCAATTCTCTAGGTGCACAAG	300 nM
MDR1	multidrug transporter of major facilitator superfamily	forward	TTCCGTGTTGGGTTTCATCA	300 nM
		reverse	TGGTCCGTTCAAGTAAAACAAAACT	300 nM
RIP	ubiquinol cytochrome c-reductase	forward	TGTCACGGTTCCCATTATGATATTT	300 nM
		reverse	TGGAATTTCCAAGTTCAATGGA	300 nM
LSC2	succinate-CoA ligase	forward	CGTCAACATCTTTGGTGGTATTGT	600 nM
		reverse	TTGGTGGCAGCAATTAAACCT	600 nM

Determination of susceptibility of sessile *C. albicans* cells to miconazole

Biofilms of *C. albicans* WT strain SC5314, the *LCB4/lcb4* mutant, and DSY1050 (in which *CDR1*, *CDR2*, and *MDR1* are inactivated) (18) were grown in 96-well microtitre plates and treated with miconazole as described above. The biofilm susceptibility to miconazole was also determined in the presence of the sphingolipid biosynthetic intermediate dihydrosphingosine-1-phosphate (DHS-1-P) (10 nM and 100 nM) (Sigma-Aldrich) or PHS-1-P (10 nM and 100 nM) (Avanti Polar Lipids, Alabaster, AL), the oxidative phosphorylation inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (5 μM) (Sigma-Aldrich), and/or the serine palmitoyltransferase (*LCB1*) inhibitor myriocin (10 μM) (Sigma-Aldrich). Susceptibility to miconazole was evaluated with the resazurin-based cell viability assay as described above.

Pyrene lateral diffusion assay

Lateral diffusion was measured by the excimerization of pyrene. This small molecule can bind to the long-chain fatty acids of the cytoplasmatic membrane. Excited pyrene monomers are able to form a complex with unexcited pyrene molecules, leading to the formation of pyrene dimers, which emit fluorescence at a higher wavelength (19, 20). Biofilms of *C. albicans* WT strain SC5314 and the *LCB4/lcb4* mutant were grown in a 96-well microtitre plate and treated as described above, with and without the addition of sphingolipid biosynthetic intermediates (DHS-1-P or PHS-1-P). For each experimental condition, the cells of six biofilms were collected and were resuspended in 10 ml PBS with 0.25% formaldehyde. The cell suspensions were centrifuged (4 min at 5,000 rpm), washed twice with PBS containing 0.25% formaldehyde, and further diluted until an absorption of 0.25 at 450 nm was reached. One milliliter of these suspensions was incubated with pyrene (final concentration, 10 μM; Sigma-Aldrich) for 20 min at 37°C. The cells were washed to remove excess pyrene and resuspended in 1 ml PBS. Aliquots of 200 μl were added to the wells of a black 96-well microtitre plate (Perkin-Elmer), and the fluorescence was measured (λ_{ex} = 340 nm; λ_{em} = 380 nm and 480 nm). The ratio of pyrene dimers to pyrene

monomers (fluorescence at 480 nm divided by fluorescence at 380 nm) was calculated for each condition and is directly proportional to the membrane fluidity.

Determination of intracellular miconazole concentrations

Biofilms of *C. albicans* WT strain SC5314 and the *LCB4/lcb4* mutant were grown in a 96-well microtitre plate and treated as described above, with and without the addition of PHS-1-P. For each experimental condition, the cells of six biofilms were collected, rinsed three times with physiological saline to remove extracellular miconazole, and finally resuspended in a mixture of 70% acetonitrile–30% PBS. Glass beads were added to the cell suspensions, and the cells were subsequently lysed by shaking with a Precellys24 instrument (Bertin Technologies, Montigny-le-Bretonneux, France) for 20 s at 6,000 rpm. The cell lysate was transferred into a new tube to adjust the concentration of acetonitrile to 30%, and trifluoroacetic acid was added to 0.1%. The tubes were centrifuged for 30 min at 13,000 rpm, after which the intracellular miconazole concentration was determined by using high-performance liquid chromatography (HPLC), as described previously (11).

Statistical analysis

Statistical analysis was performed by using the nonparametric Mann-Whitney U test (SPSS Statistics 17.0 software).

RESULTS

Determination of the miconazole susceptibility of *S. cerevisiae* deletion mutants

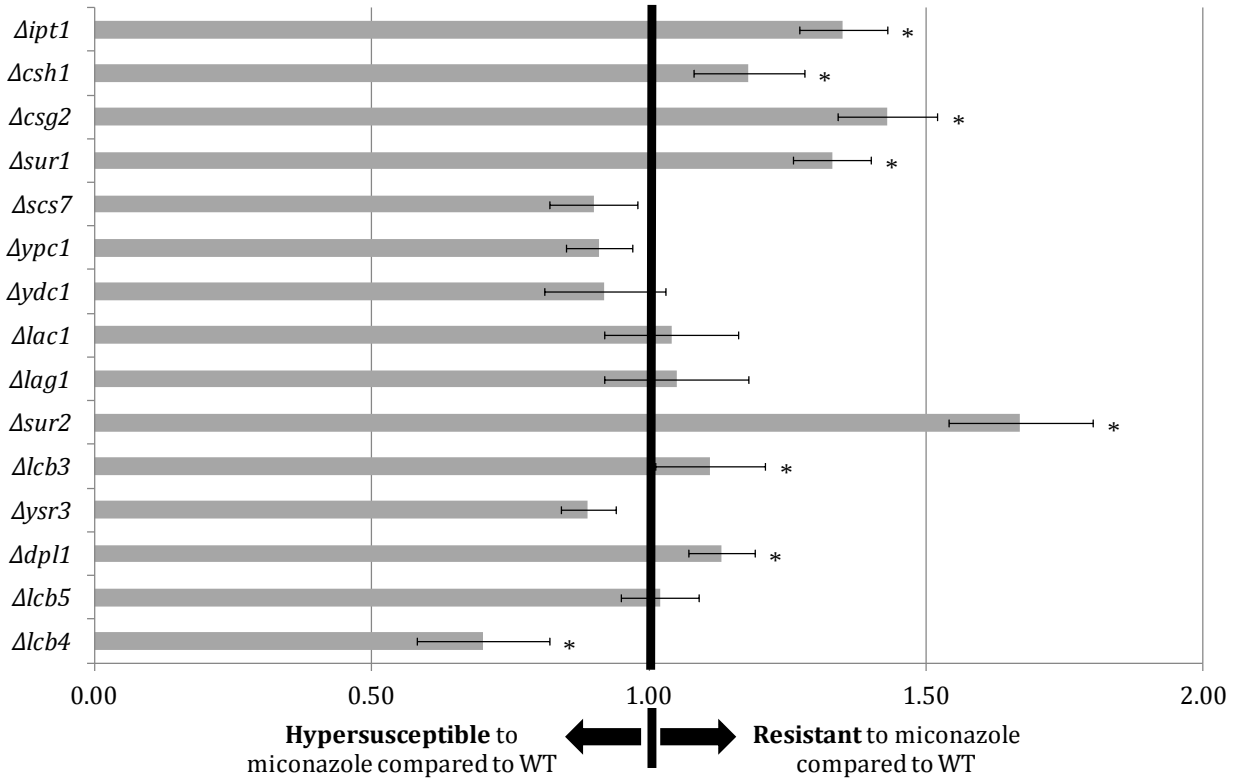

Figure 5.1. Relative susceptibilities of *S. cerevisiae* BY4741 deletion mutants to miconazole compared to the susceptibility of the WT. The values are the means of data from six replicates. Error bars represent standard errors of the means. Significant differences ($P < 0.05$) are marked with asterisks.

The susceptibility of biofilms of *S. cerevisiae* mutants affected in genes involved in sphingolipid biosynthesis to miconazole was investigated and was compared to that of the WT (Figure 5.1). Seven mutants (Δ*lcb5*, Δ*ysr3*, Δ*lag1*, Δ*lac1*, Δ*ydc1*, Δ*ypc1*, and Δ*scs7*) did not show an altered susceptibility. Sessile Δ*lcb4* cells, deficient in the phosphorylation of dihydrosphingosine and phytosphingosine, were hypersusceptible to miconazole, while

sessile Δ*lcb3* cells, deficient in the dephosphorylation of DHS-1-P and PHS-1-P, and sessile Δ*dpl1* cells, deficient in the breakdown of DHS-1-P and PHS-1-P, were more resistant to miconazole than the WT. The deletion of *SUR1*, *CSG2*, or *CSH1*, involved in the mannosylation of inositol-phosphorylceramide; the deletion of *IPT1*, encoding inositolphosphotransferase; and the deletion of *SUR2*, encoding dihydrosphingosine hydroxylase, resulted in an increased resistance of sessile cells of the corresponding mutants to miconazole.

Expression of genes involved in sphingolipid biosynthesis in sessile *C. albicans* cells

The expression levels of all genes involved in sphingolipid biosynthesis in sessile *C. albicans* cells after miconazole treatment were determined and compared with those in untreated sessile *C. albicans* cells (Figure 5.2 and Table 5.3). Four genes, *KSR1*, *YDC1*, *LCB4*, and *DPL1*, were significantly ($P < 0.05$) upregulated after miconazole treatment. The highest upregulation was observed for *LCB4* (3.1-fold). *DPL1* (2.2-fold upregulated) is involved in the breakdown of DHS-1-P and PHS-1-P. *KSR1* (2.3-fold upregulated) and *YDC1* (2.1-fold upregulated) are involved in the formation of dihydrosphingosine and phytosphingosine, respectively.

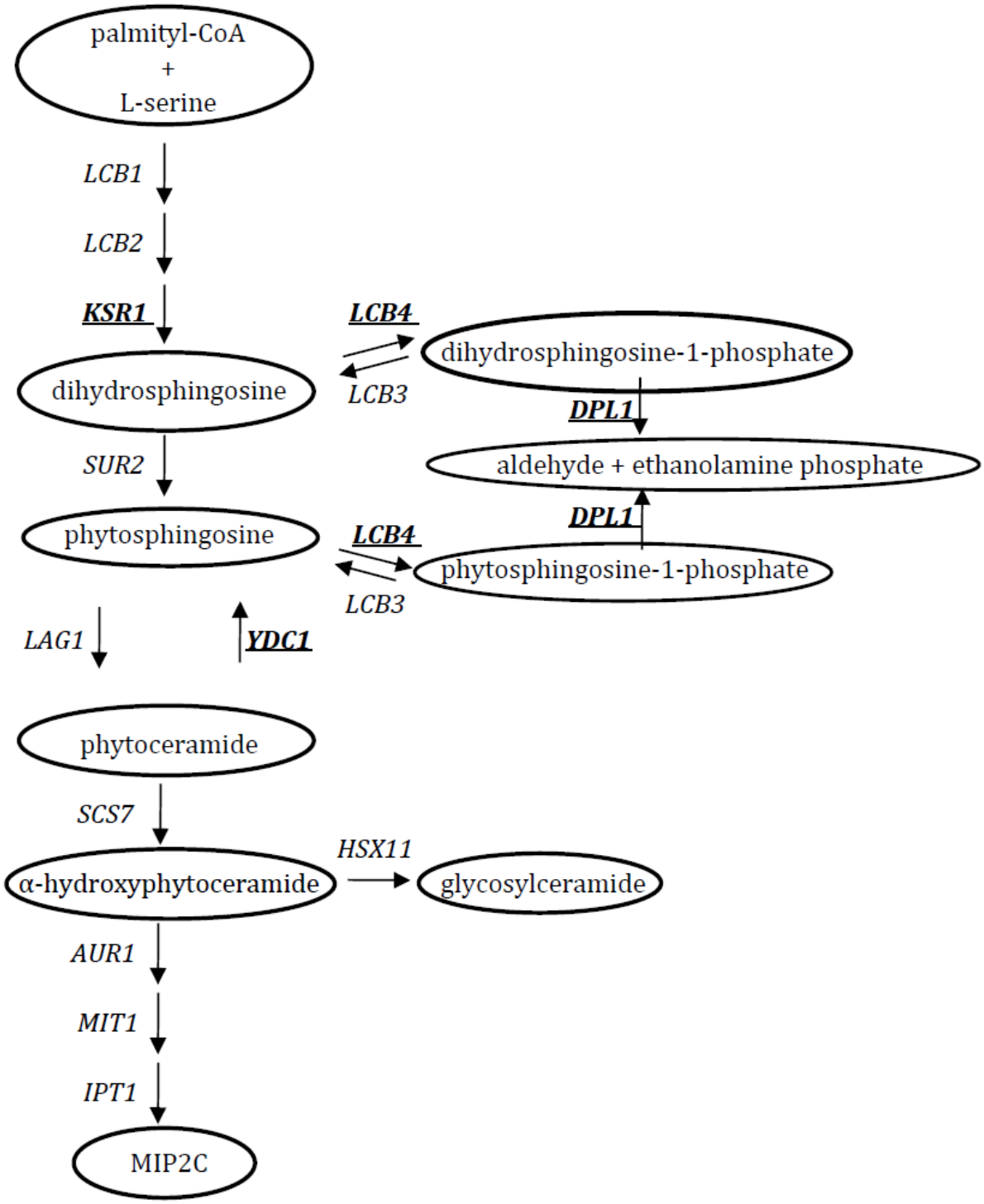

Figure 5.2. Sphingolipid biosynthesis pathway in *C. albicans*. Genes with significantly increased expression levels ($P < 0.05$) after miconazole treatment of sessile cells compared to untreated sessile cells are underlined. CoA, coenzyme A. MIP2C, mannosyl-diinositol-phosphorylceramide.

Table 5.3. Fold changes in expression levels of genes involved in sphingolipid biosynthesis in sessile *C. albicans* cells after miconazole treatment compared to untreated cells. Data presented are the means of data from five independent experiments. Genes with significantly different expression levels ($P < 0.05$) are marked with asterisks.

Gene	Average fold change	SEM
AUR1	0.6	0.1
DPL1	2.2*	0.7
HSX11	1.1	0.2
IPT1	1.4	0.2
KSR1	2.3*	0.3
LAG1	1.1	0.2
LCB1	1.3	0.2
LCB2	0.8	0.2
LCB3	1.8	0.3
LCB4	3.1*	0.8
MIT1	0.6	0.1
SCS7	0.8	0.2
SUR2	0.5	0.1
YDC1	2.1*	0.3

Determination of the susceptibility of sessile *C. albicans* cells to miconazole

In line with the observed miconazole hypersusceptibility of sessile *S. cerevisiae* Δ*lcb4* cells, sessile cells of the heterozygous *C. albicans LCB4/lcb4* mutant were also hypersusceptible to miconazole (Figure 5.3). The addition of DHS-1-P (final concentrations, 10 nM and 100 nM) during biofilm growth and miconazole treatment did not significantly alter the susceptibilities of the WT and the *LCB4/lcb4* mutant. In contrast, the *LCB4/lcb4* mutant showed significantly increased resistance ($P < 0.05$) to miconazole after the addition of PHS-1-P (final concentrations, 10 nM and 100 nM) during biofilm growth and miconazole treatment. This effect was not dose dependent, and an optimal effect was obtained at 10 nM. A similar but less explicit change in susceptibility to miconazole was obtained for the WT after the supplementation of the medium with PHS-1-P. As a control, we tested the effect of PHS-1-P in combination with the *LCB1* inhibitor myriocin, which

blocks the first step of sphingolipid biosynthesis (21). The significantly increased susceptibility of WT strain SC5314 to miconazole after the addition of 10 µM myriocin decreased again in combination with PHS-1-P (10 nM).

When CCCP (5 µM) was added simultaneously with PHS-1-P (10 nM), no changes in the susceptibility to miconazole were observed for the WT and the *LCB4/lcb4* mutant, compared to the conditions without PHS-1-P. The application of only CCCP did not influence the susceptibilities of the WT and the *LCB4/lcb4* mutant to miconazole. The addition of PHS-1-P (10 nM) during biofilm growth and miconazole treatment of the triple mutant strain DSY1050 also did not alter the miconazole resistance.

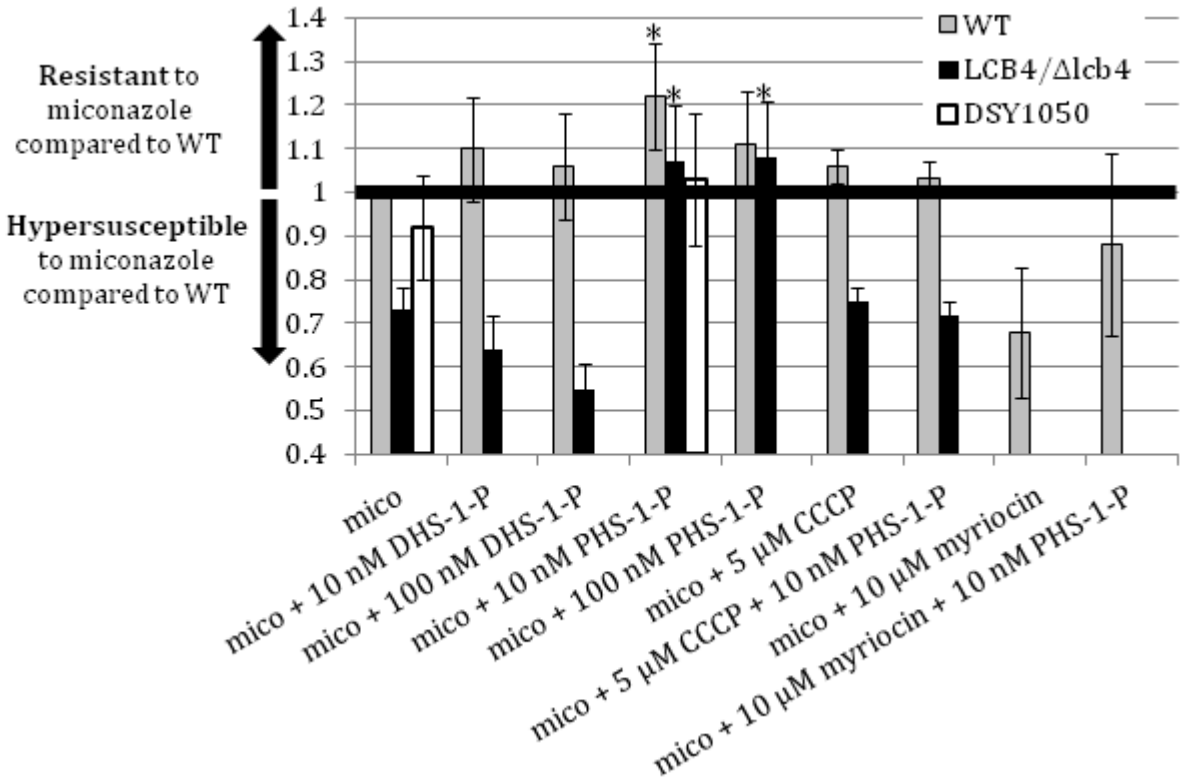

Figure 5.3. Relative miconazole susceptibilities of WT strain SC5314, the *LCB4/lcb4* mutant, and DSY1050 after the addition of dihydrosphingosine-1-phospate (DHS-1-P), phytosphingosine-1-phosphate (PHS-1-P), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and myriocin (or combinations thereof). Values marked with asterisk are significantly different from the values of the corresponding strain without the addition of the sphingolipid intermediate. Data presented are the means and standard errors of the means from three independent experiments with six biofilms.

Pyrene lateral diffusion assay

The relative fluidities of the cytoplasmatic membrane were similar in untreated sessile *C. albicans* cells of the WT and the *LCB4/lcb4* mutant (1.39 ± 0.11 and 1.58 ± 0.19, respectively). Treatment with miconazole resulted in a significant decrease ($P < 0.05$) in membrane fluidity for both strains (1.04 ± 0.06 for the WT and 0.88 ± 0.13 for the *LCB4/lcb4* mutant). This decrease was more pronounced for the *LCB4/lcb4* mutant than for the WT. The addition of PHS-1-P during biofilm growth and miconazole treatment significantly ($P < 0.05$) increased the fluidity of the cytoplasmatic membrane of WT cells (1.22 ± 0.05) but did not affect the membrane fluidity of *LCB4/lcb4* mutant cells (0.86 ± 0.11).

Intracellular miconazole concentrations

The intracellular miconazole concentration after 24 h of treatment was significantly higher ($P < 0.05$) in sessile *LCB4/lcb4* mutant cells than in sessile WT cells (2.1 µg/1,000 cells and 1.5 µg/1,000 cells, respectively) (Table 5.4). The addition of PHS-1-P (to a final concentration of 10 nM) significantly decreased ($P < 0.05$) the intracellular miconazole concentration to 0.6 µg/1,000 cells in sessile *LCB4/lcb4* mutant cells, while only a slight decrease was observed for WT sessile cells (1.3 µg/1,000 cells, $P = 0.12$). The addition of CCCP during miconazole treatment (in the presence of 10 nM PHS-1-P) significantly increased ($P < 0.05$) the intracellular miconazole concentrations in sessile cells of the WT and the *LCB4/lcb4* mutant (1.8 µg/1,000 cells for both strains).

Table 5.4. Intracellular miconazole concentrations in cells of *C. albicans* WT strain SC5314 and the *LCB4/lcb4* mutant with the addition of 10 nM PHS-1-P and/or 5 µM CCCP. The results are the means and standard deviations of data from at least four replicates.

Condition	Mean intracellular miconazole concentration (µg/1000 cells) + SD	
	Without CCCP	With CCCP
WT SC5314 cells	1.5 ± 0.2	1.6 ± 0.1
WT SC5314 cells + PHS-1-P	1.3 ± 0.1	1.8 ± 0.1
LCB4/Δ*LCB4* cells	2.1 ± 0.5(1)	2.1 ± 0.4
LCB4/Δ*LCB4* cells + PHS-1-P	0.6 ± 0.1(2)	1.8 ± 0.1(3)

(1)Significantly different compared to WT.

(2)Significantly different compared to the *LCB4*/Δ*LCB4* mutant without PHS-1-P.

3)Significantly different compared to the *LCB4*/Δ*LCB4* mutant with PHS-1-P and without CCCP.

Expression of genes coding for efflux pumps in sessile C. *albicans* cells

The expression levels of *CDR1*, *CDR2*, and *MDR1* in sessile *C. albicans* cells after treatment with miconazole and PHS-1-P were determined and compared with those in sessile *C. albicans* cells treated with miconazole alone. All three genes were highly upregulated (132-fold for *CDR1*, 29-fold for *CDR2*, and 22-fold for *MDR1*) due to the addition of PHS-1-P during miconazole treatment. The expression levels of *CDR1* and *CDR2* in untreated sessile *LCB4/lcb4* mutant cells were not significantly different compared with those in untreated sessile WT cells (2.3-fold with $P = 0.101$ and 1.2-fold with $P = 0.297$, respectively). The expression level of *MDR1* in untreated sessile *LCB4/lcb4* mutant cells was significantly higher compared with those in untreated sessile WT cells (2.9-fold, $P = 0.025$).

DISCUSSION

We previously observed that 1% to 10% of cells in a *C. albicans* biofilm survived treatment with high doses of miconazole (7) (Chapter 3). Other studies have suggested a role for sphingolipids in governing miconazole resistance in planktonic yeast cultures (11, 22), and in the present study, we focused on the role of sphingolipid biosynthesis in the molecular mechanisms underlying the resistance of *C. albicans* biofilms to miconazole.

The screening of *S. cerevisiae* deletion mutants affected in sphingolipid biosynthesis revealed the involvement of *LCB4* in governing resistance to miconazole, as biofilms of the corresponding deletion mutant showed an increased susceptibility to miconazole. *LCB4* encodes a sphingosine kinase which is involved in the phosphorylation of dihydrosphingosine and phytosphingosine (23). The increased transcription of *LCB4* in miconazole-treated sessile *C. albicans* cells confirmed its involvement in miconazole resistance. Furthermore, a significant upregulation was observed for the *KSR1* and *YDC1* genes, which encode enzymes involved in the formation of dihydrosphingosine and phytosphingosine, respectively. Increased levels of DHS-1-P and PHS-1-P are known to play a role in mediating resistance to heat stress (24), probably due to the induction of *TPS2* transcription and trehalose accumulation (25). In contrast, a rapid intracellular accumulation of DHS-1-P and PHS-1-P results in a reduced growth rate and may even be lethal for cells (26, 27). The observed overexpression of *DPL1*, involved in the breakdown of DHS-1-P and PHS-1-P to a long-chain aldehyde and ethanolamine phosphate, is probably necessary to maintain balanced levels of DHS-1-P and PHS-1-P.

DHS-1-P and PHS-1-P seem to be important for resistance to miconazole. To elucidate their role, experiments were performed using a heterozygous *C. albicans LCB4/lcb4* mutant. Sessile cells of this *LCB4/lcb4* mutant were hypersusceptible to miconazole compared to the WT. We also observed a higher intracellular miconazole concentration in treated sessile cells of the *LCB4/lcb4* mutant than in sessile WT cells. The addition of PHS-1-P (10 nM) during biofilm growth and miconazole treatment significantly increased the resistance of the *LCB4/lcb4* mutant to miconazole (similar to that of the WT without the addition of PHS-1-P) and drastically reduced the intracellular miconazole levels, clearly pointing to a protective effect of PHS-1-P against the action of miconazole. The intracellular miconazole concentration in sessile WT cells was not altered by the

addition of PHS-1-P. However, a slightly increased resistance to miconazole was observed in the presence of PHS-1-P. When we inhibited *LCB1* by adding myriocin, a similar increase in resistance was noted upon the addition of PHS-1-P, confirming the protective effect of the latter against miconazole. In contrast, DHS-1-P has no protective effect against miconazole treatment in sessile *C. albicans* cells, as the susceptibility to miconazole was unchanged when this sphingolipid intermediate was added to cells of the WT and the *LCB4/lcb4* mutant.

To investigate whether PHS-1-P has a direct or indirect effect on the structure of the cytoplasmatic membrane of sessile *C. albicans* cells, resulting in altered miconazole uptake, the fluidity of the cytoplasmatic membrane was measured by using a pyrene lateral diffusion assay. The membrane fluidities of untreated sessile cells of the WT and the *LCB4/lcb4* mutant did not differ. A more rigid structure of the cytoplasmatic membranes of both strains after treatment with miconazole was noticed. Changes in the composition of the cytoplasmatic membrane due to stress situations were described previously (28). The addition of PHS-1-P to sessile cells of the *LCB4/lcb4* mutant had neither a direct nor an indirect effect on the fluidity of the cytoplasmatic membrane, indicating that changes in membrane fluidity are not responsible for this protective effect. Previous research has shown that several cellular processes are regulated by sphingolipid long-chain bases. In *S. cerevisiae*, sphingolipid long-chain bases are thought to activate the protein kinase Pkh1 and Pkh2, leading to an activation of downstream pathways playing a role in actin cytoskeleton dynamics, the regulation of transcription and translation, stress resistance, and cell growth (29). Still, more research is needed to understand the exact regulatory mechanisms and the connections to other metabolic pathways (30). Knowledge of the role of sphingolipids in fungal pathogens like *C. albicans* is even more limited. Pasrija et al. (22) found previously that the disruption of certain sphingolipid biosynthetic genes in *C. albicans*, with the exception of Δ*ipt1*, leads to a mislocalization of efflux pumps and an increased susceptibility to drugs. The role of PHS-1-P in efflux pumps was investigated by chemically inhibiting these pumps (using CCCP) and by studying a triple mutant in which all these efflux pumps were inactivated. The addition of the oxidative phosphorylation inhibitor CCCP (31, 32) significantly increased the intracellular miconazole levels for both the WT and the *LCB4/lcb4* mutant and counteracted the protective effect of PHS-1-P. Similarly, the addition of PHS-1-P did not increase the resistance to miconazole in the triple

efflux pump mutant. Furthermore, the expression of the genes coding for efflux pumps in *C. albicans* SC5314 was highly upregulated when PHS-1-P was added during miconazole treatment. These data indicate that PHS-1-P directly or indirectly acts on efflux pumps in miconazole-treated sessile *C. albicans* cells.

In conclusion, a protective effect of PHS-1-P against miconazole-treated *C. albicans* biofilms was observed. As the structure of the cytoplasmatic membrane remained unaffected, it is likely that PHS-1-P acts as a signaling molecule and that it enhances the export of miconazole. The regulatory mechanism involved in this process remains unclear so far.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

MICONAZOLE: REVIVAL OF AN OLD DRUG?

The story of miconazole started in 1969, when researchers of Janssen Pharmaceutica developed this promising broad-spectrum antifungal drug. Due to the low gastrointestinal absorbance of miconazole, related to its highly lipophilic character, intravenous administration was required for successful treatment of invasive candidiasis (1). However, toxicity, partially due to the carrier solution of the intravenous formulation, restricted its use for treatment of systemic infections (2). In contrast, the lipophilic properties of miconazole make it very suitable for topical preparations (Table 6.1).

Table 6.1. Overview of topical preparations containing miconazole available in Belgium (3).

Product	Use	Dosage form	Concentration
Gyno-Daktarin	vaginal	vaginal capsule	200 mg or 1200 mg/capsule
Gyno-Daktarin	vaginal	cream	20 mg/g
Daktarin-Cilag	oral	gel	20 mg/g
Loramyc	oral	muco-adhesive tablet	50 mg/tablet
Tibozole	oral	muco-adhesive tablet	10 mg/tablet
Daktarin-Consumer	cutaneous	cream	20 mg/g
Daktarin-Consumer	cutaneous	powder	20 mg/g
Daktarin-Consumer	cutaneous	powder spray	20 mg/g
Daktarin-Consumer	cutaneous	tincture	20 mg/ml
Daktozin (combination with zinc oxide)	cutaneous	paste	2.5 mg/g
Daktacort (combination with hydrocortisone)	cutaneous	cream	20 mg/g
Acneplus (combination with benzoylperoxide)	cutaneous	cream	20 mg/g

The discovery of triazole antifungals during the nineties was very important because they showed a potent fungistatic activity and because they had a very low toxicity profile compared to all other available antifungals at that time (4). Despite the great success of azoles against *Candida* spp., resistant clinical isolates appeared. Furthermore, biofilm-related infections became increasingly important and these sessile

cells showed a very high resistance to azoles. Consequently, the search for new fungicidal antifungals continued, and the echinocandins were introduced in 2001 (4). However, the extensive experience gained with 'older' antifungal drugs (like miconazole), may contribute to developing new treatments of biofilms. In this regard, the reintroduction of miconazole may be an attractive alternative in the battle against fungal biofilms and associated resistance.

In a first study (Chapter 3), we show that miconazole possesses fungicidal activity against mature biofilms of various *C. albicans* strains and strains belonging to other *Candida* spp., including *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. As these four *Candida* spp. are the leading cause of candidiasis, our results indicate a promising broad-spectrum activity of miconazole in the treatment of biofilm-related infections. Although we observed a drastic increase in the production of ROS when *Candida* biofilms were treated with miconazole, this is probably not the main cause for its fungicidal activity.

The *in vitro* concentrations of miconazole used in our studies can never be achieved *in vivo* for treatment of biofilm-related candidemia or invasive candidiasis. Many biofilm-related infections are caused by colonization of medical devices, such as catheters. This often leads to the necessity to replace the medical device, thereby increasing the medical costs and patients' discomfort (5). A valuable alternative may be found in antifungal lock therapy (ALT). This offers the possibility to use very high drug concentrations to sterilize the catheter and avoid further biofilm dissemination. Due to limited clinical data, this method is not yet used on a large scale to eradicate *Candida* biofilms from catheters. However, ALT with amphotericin B and echinocandins seems promising (6). Based on our data, miconazole could have potential for ALT, and it would be useful to include this antifungal in future ALT studies.

The concentration of miconazole in topical formulations for treatment of superficial infections is often higher than the one used in our study (Table 6.1). The concentration of miconazole in the available creams, gels and powders for topical administration (oral, vaginal and cutaneous) is 20 mg/g in Belgium. Single unit doses for vaginal candidiasis contain 200 or 1200 mg miconazole (3). In this way it is possible to reach very high local concentrations of miconazole. Based on our results, this should lead to an efficient killing of the *Candida* cells, even when present in a biofilm. A recently developed new delivery system for miconazole demonstrates the continued interest in

the fungicidal properties of this substance (7). Mucoadhesive tablets for treatment of oropharyngeal candidiasis have been developed and are sufficiently dosed (10 mg or 50 mg) for treatment of *Candida* biofilms. These tablets have no improved action compared to the oral gels, but are user-friendly, as the administration of only a single daily dose is required.

It is likely that the fungicidal activity of miconazole against *Candida* biofilms can be further improved in combination with other compounds. Similar tests have been performed already *in vitro* with other antifungals, but not specifically with miconazole. Glucanases may enhance the penetration of miconazole within the biofilm to increase its local concentration. Furthermore, it would be interesting to investigate whether the combination of miconazole with an antifungal of another class would result in an additional or synergistic effect. However, combinations of antifungals are not a preferred treatment strategy and are reserved for persistent candidemia originating from a biofilm-related infection (8). Since resistance to antifungals is an increasing clinical problem, it would be useful to have reliable data on combination therapy with miconazole.

THE FORMATION AND RESISTANCE OF FUNGAL BIOFILMS

Notwithstanding the fungicidal activity of miconazole against *Candida* spp. biofilms, we noticed a resistant fraction (1% - 10%) of sessile cells that were able to survive a high dose of miconazole (Chapter 3). To improve antifungal therapy, it is important to obtain more insights in the mechanisms of resistance. To this end, we used a *S. cerevisiae* deletion mutant bank (Chapter 4). Simultaneously, we investigated the mechanisms contributing to biofilm formation in *S. cerevisiae*.

The results of the screening highlighted the multi-factorial complexity of biofilm formation and resistance, which is consistent with many previous observations (9, 10) (Table 6.2). An in depth analysis of the results revealed that two major biological processes play an important role in *S. cerevisiae* biofilm formation and the susceptibility

to miconazole, i.e. the organization of two types of organelles, the mitochondria and the peroxisomes.

Table 6.2. Comparison between results of our screening study with previous observations.

Our study		Function	Previously observed	
Gene	**Changed phenotype**		**Organism**	**Reference**
SUN4	biofilm formation	cell wall organization	*C. albicans* (*SUN41*)	(11, 12)
SUV3	biofilm formation	helicase	*C. albicans* (*SUV3*)	(13)
LCB4	miconazole susceptibility	sphingolipid biosynthesis	*C. albicans* (*LCB4*)	(14)
ERG2	miconazole susceptibility	ergosterol biosynthesis	*C. albicans* (*ERG2*)	(15)
ERG4	miconazole susceptibility	ergosterol biosynthesis	*C. albicans* (*ERG4*)	(15, 16)
ERG24	miconazole susceptibility	ergosterol biosynthesis	*C. albicans* (*ERG24*)	(17)
VPS1	miconazole susceptibility	actin cytoskeleton organization	*S. cerevisiae* (*VPS1*)	(18)
SOD1	miconazole susceptibility	superoxide dismutase	*C. albicans* (*SOD4*, *SOD5*)	(19)
MXR1	miconazole susceptibility	methionine sulfoxide reductase	*S. cerevisiae* (*MXR1*)	(20)
TPS2	miconazole susceptibility	trehalose biosynthesis	*C. albicans* (*TPS1*, *TPS2*)	(21, 22)

The production of ATP is dependent on functional mitochondria. An increase in cellular metabolic activity during biofilm development was previously reported (23), indicating that biofilm formation is an energy dependent process. When genes involved in mitochondrial organization are affected, this probably leads to an ATP depletion which slows down or counters biofilm formation. This means that colonization of a substrate by yeast cells and the further development into a dense biofilm may depend on functional mitochondria. However, this does not open new perspectives for treatment, as mitochondrial inhibitors are not specific for yeast cells, and will likely interfere with human mitochondria, leading to toxic side effects. Furthermore, our study shows that mutants affected in genes in mitochondrial organization also exhibit a miconazole resistant phenotype, probably by mimicking a dormant state. In this way, it

is possible that mitochondrial inhibitors would enhance the resistance of yeast biofilms to miconazole.

The peroxisomes carry out the β-oxidation of fatty acids and have mechanisms to detoxify toxic oxygen radicals formed during this process (24). Consequently, a decreased activity of peroxisomes may lead to the accumulation of long chain fatty acids and an increase of intracellular ROS-levels. As long chain fatty acids are known to have a signaling function and are part of the cytoplasmatic membrane (25) and as elevated ROS-levels may have a fungicidal effect (26), it is not unexpected that biofilm formation and susceptibility to miconazole are influenced by changes in the activity of peroxisomes. However, a direct connection between peroxisomal activity and the phenotypes we have studied has never been made. Strains affected in the peroxisomal matrix protein import showed decreased biofilm formation, whereas those affected in general peroxisomal organization showed a hypersusceptible phenotype to miconazole. Decreasing the peroxisomal activity would possibly increase the impact of miconazole treatment on yeast biofilms. Furthermore, the possibility of peroxisomes themselves as antifungal target should be considered. The first obstacle for this is oviously the similarity between yeast and human peroxisomes, which has consequences for the selectivity of molecules that would interfere with the activity of peroxisomes. Several genetic defects in human peroxisomes cause very severe disorders, which constitutes a second obstacle for the concept of peroxisomes as antifungal target. The prognosis for newborns with the Zellweger syndrome, being the most severe human peroxisomal disorder, is bad, as these children do not survive the first year. Other variants of peroxisomal disorders have a slightly better prognosis, but can also not be cured (27). Both the lack of specificity and the severe effects of deficient peroxisomes in humans, do not make peroxisomes a very attractive antifungal target at first sight. However, there are good reasons to further investigate the possibility of targeting fungal peroxisomes. In yeast cells, β-oxidation of fatty acids exclusively takes place in peroxisomes, while in human cells this also occurs in mitochondria (28). So, it is likely that the impact of molecules interfering with the peroxisomal activity on human cells would be lower than on yeast cells, as the latter have no alternative pathway for β-oxidation of fatty acids. Furthermore, our screening revealed the involvement of several peroxisomal genes in resistance to miconazole, that have not (yet) been associated with human peroxisomal

disorders. Possibly, the selective inhibition of these genes could result in a decreased resistance to miconazole without affecting the functionality of human peroxisomes.

It is possible that the observed miconazole hypersusceptibility of strains affected in peroxisome functioning is related to changes in the intracellular lipid composition, consequently leading to an altered signaling. A direct connection between the signaling function of intermediates in the sphingolipid pathway and the susceptibility to miconazole has been demonstrated (Chapter 5). Phytospingosine-1-phosphate seemed to be crucial in the resistance to miconazole as it acts as a signaling molecule enhancing the export of miconazole. There are similarities between the sphingolipid metabolism in yeast and human cells, but the part that plays a role in miconazole resistance is yeast specific (29). This offers interesting perspectives, as interference with this part of the pathway would theoretically not affect the human sphingolipid metabolism. Compounds inhibiting the production of phytosphingosine-1-phosphate, or increasing its degradation, may possibly enhance the fungicidal activity of miconazole against *Candida* biofilms.

THE BATTLE CONTINUES

Researchers continuously try to find new drugs or to improve existing ones to treat and beat microorganisms. Once a potent therapy has been developed, we know with near certainty that sooner or later microorganisms will develop resistance. At that moment it is crucial to have knowledge based on fundamental research to find new ways to overcome this resistance. This doctoral research has revealed the fungicidal capacities of miconazole against mature *Candida* biofilms, but has also unraveled the mechanisms of resistance of yeast biofilms to miconazole. This information will be useful for a battle that will certainly continue in the future.

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SUMMARY / SAMENVATTING

SUMMARY

The interest in fungal biofilm formation and in the resistance of fungal biofilms against antimicrobial agents has increased due to the expanding problem of biofilm-related infections. In this doctoral research the activity of miconazole against biofilms of *Candida* species has been investigated. Furthermore, we have focused on the molecular mechanisms involved in fungal biofilm formation and in resistance to miconazole.

Although azole antifungals are considered fungistatic, miconazole has fungicidal activity against planktonic *C. albicans* cells, presumably associated with the induction of reactive oxygen species (ROS) production. Only few data are available concerning the effect of miconazole against sessile *C. albicans* cells. The effect of miconazole on mature biofilms formed by 10 *C. albicans* strains and 5 strains from other *Candida* species has been evaluated (Chapter 3). Miconazole showed a significant fungicidal effect against all mature *Candida* biofilms tested and caused elevated ROS levels, both in planktonic and sessile cells. Addition of ascorbic acid drastically reduced these levels. While ROS quenching decreased the susceptibility to miconazole of planktonic cells of most *Candida* strains, no reduced fungicidal activity of miconazole against biofilms was observed, indicating that ROS induction was not the only factor underlying its fungicidal activity. Miconazole did not cause a significant increase in apoptosis.

Notwithstanding the fungicidal activity of miconazole against *Candida* spp. biofilms, we noticed that a resistant fraction (1% - 10%) of sessile cells was able to survive a high dose of miconazole. We further investigated the molecular mechanisms involved in fungal biofilm formation and unraveled the mechanisms of resistance to miconazole. To this end, a screening of a *Saccharomyces cerevisiae* deletion mutant bank was carried out (Chapter 4). The results revealed that genes involved in peroxisomal transport and the biogenesis of the respiratory chain complex IV play an essential role in biofilm formation. On the other hand, genes involved in transcription and peroxisomal and mitochondrial organization influence the susceptibility to miconazole of yeast biofilms. Additionally, our data confirm previous findings on genes involved in biofilm formation and in general stress responses.

Based on the results of the screening of the *S. cerevisiae* deletion mutant bank, the role of sphingolipid biosynthetic intermediates in the resistance to miconazole was investigated (Chapter 5). We found that the *LCB4* gene, coding for the enzyme that catalyzes the phosphorylation of dihydrosphingosine and phytosphingosine, is important in governing the miconazole resistance of sessile *S. cerevisiae* and *C. albicans* cells. The addition of 10 nM phytosphingosine-1-phosphate (PHS-1-P) drastically reduced the intracellular miconazole concentration and significantly increased the miconazole resistance of a hypersusceptible *C. albicans* heterozygous *LCB4/lcb4* mutant, indicating a protective effect of PHS-1-P against miconazole-induced cell death in sessile cells. At this concentration of PHS-1-P, we did not observe any effect on the fluidity of the cytoplasmatic membrane. The protective effect of PHS-1-P was not observed when the efflux pumps were inhibited or when tested in a mutant without functional efflux systems. Also, the addition of PHS-1-P during miconazole treatment increased the expression levels of genes coding for efflux pumps, leading to the hypothesis that PHS-1-P acts as a signaling molecule and enhances the efflux of miconazole from sessile *C. albicans* cells.

SAMENVATTING

De interesse in biofilmvorming door fungi en de resistentie van biofilms tegen antimycotica is toegenomen door de groeiende problematiek omtrent biofilm-gerelateerde infecties. In deze dissertatie werd de activiteit van miconazol tegen *Candida* biofilms onderzocht. Er werd ook aandacht besteed aan de moleculaire mechanismen die betrokken zijn bij de biofilmvorming van fungi en de resistentie tegen miconazol.

Azolen werken fungistatisch, maar miconazol vertoont een fungicide werking tegen planktonische *C. albicans* cellen. Die is vermoedelijk gekoppeld aan een toename van de productie van zuurstofradicalen. Slechts weinig gegevens zijn beschikbaar over het effect van miconazol op *C. albicans* biofilmcellen. Het effect van miconazol op mature biofilms werd onderzocht op 10 *C. albicans* stammen en op 5 stammen van andere *Candida* species (hoofdstuk 3). Miconazol vertoonde een significant fungicide werking tegen alle geteste mature *Candida* biofilms en veroorzaakte een toename van zuurstofradicalen, zowel in planktonische als sessiele cellen. De toevoeging van ascorbinezuur zorgde voor een drastische reductie van de hoeveelheid zuurstofradicalen. In tegenstelling tot planktonische cellen, waarbij een daling van de miconazol gevoeligheid van de meeste *Candida* stammen optrad, bleef de fungicide werking van miconazol ten opzichte van biofilms behouden. Dit toont aan dat de inductie van zuurstofradicalen niet de enige factor is die bijdraagt tot de fungicide werking van miconazol tegen biofilms. Op basis van onze resultaten kon worden uitgesloten dat miconazol apoptose induceert.

Ondanks de fungicide werking van miconazol tegen biofilms van diverse *Candida* species, bleek een resistente fractie (1% - 10%) van biofilmcellen in staat om de blootstelling aan een hoge concentratie miconazol te overleven. Hierop werden de moleculaire mechanismen onderzocht die betrokken zijn bij de vorming van biofilms en hun resistentie tegen miconazol. Hiertoe werd een *Saccharomyces cerevisiae* deletiemutanten bank gescreend (hoofdstuk 4). De resultaten toonden aan dat genen betrokken bij peroxisomaal transport en de biogenese van complex IV van de

elektronen-transportketen een essentiële rol spelen in biofilmvorming. Anderzijds beïnvloeden genen betrokken bij de transcriptie en genen betrokken bij de organisatie van peroxisomen en mitochondriën de gevoeligheid van biofilms voor miconazol. De resultaten vertoonden bovendien overeenstemming met eerdere bevindingen omtrent genen die geassocieerd zijn met biofilmvorming en de respons op algemene stress.

Op basis van de resultaten van de screening van de *S. cerevisiae* deletiemutanten, werd de invloed op miconazol resistentie van intermediairen van de sfingolipiden biosynthese verder onderzocht (hoofdstuk 5). Het gen *LCB4*, dat codeert voor het enzyme dat dihydrosfingosine en fytosfingosine fosforyleert, bleek belangrijk te zijn voor miconazol resistentie van *S. cerevisiae* en *C. albicans* biofilms. De toevoeging van 10 nM fytosfingosine-1-fosfaat (FS-1-F) zorgde voor een sterke afname van de intracellulaire miconazol concentraties en voor een significante stijging van de miconazol gevoeligheid van een hypergevoelige heterozygote *LCB4*/*lcb4 C. albicans* mutant. Dit toont aan dat het FS-1-F biofilmcellen beschermt tegen de fungicide werking van miconazol. Bij deze concentratie FS-1-F werd geen verandering waargenomen in de stabiliteit van het cytoplasmatisch membraan. De beschermende werking van FS-1-F werd niet waargenomen wanneer de effluxpompen geïnhibeerd werden, noch bij mutanten waarbij alle effluxpompen werden uitgeschakeld. De toevoeging van FS-1-F tijdens de miconazol behandeling zorgde bovendien voor een overexpressie van genen die coderen voor effluxpompen. FS-1-F fungeert waarschijnlijk als een signaalmolecule die de efflux van miconazol bevordert in *C. albicans* biofilmcellen.

CURRICULUM VITAE

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Education

- **2007 -2013** Doctoral research at the Laboratory of Pharmaceutical Microbiology, Ghent University
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- **2007** Master Industrial pharmacist, Ghent University
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- **2006** Master Pharmaceutical Sciences – pharmacist, Ghent University
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- **2001** Graduated in Sciences-Mathematics (Broederschool, Sint-Niklaas)

Publications

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Oral presentations

- The role of sphingolipids in tolerance of *Candida albicans* biofilms against miconazole. *FWO workshop on bacterial and fungal biofilms*, Leuven, Belgium, 2010.
- The role of sphingolipids in tolerance of *Candida albicans* biofilms against miconazole. *Yeasterday*, Leuven, Belgium, 2010.
- The role of sphingolipids in the tolerance of *Candida albicans* biofilms to miconazole. *Biofilms in nosocomial fungal infections*, Paris, France, 2011.

Poster presentations

- *In vitro* investigation of the activity of miconazole against biofilms of various *Candida* species. *FEBS Advanced lecture course on human fungal pathogens: molecular mechanisms of host-pathogen interactions and virulence*, Nice, France, 2009.
- *In vitro* investigation of the activity of miconazole against biofilms of various *Candida* species. *Yeasterday*, Amsterdam, the Netherlands, 2009.
- The role of sphingolipids in resistance of *Candida albicans* biofilms against miconazole. *ASM conference on Candida and candidiasis*, Miami, USA, 2010.
- PHS-1-P is a signaling molecule involved in tolerance to miconazole in sessile *Candida albicans* cells. *FEBS Advanced lecture course on human fungal pathogens: molecular mechanisms of host-pathogen interactions and virulence*, Nice, France, 2011.
- PHS-1-P is a signaling molecule involved in tolerance to miconazole in sessile *Candida albicans* cells. *Yeasterday*, Leiden, the Netherlands, 2011.
- Molecular basis of miconazole tolerance in *Candida albicans* biofilms. *ASM conference on Candida and candidiasis*, San Francisco, USA, 2012.

Courses

- Academic English: writing skills, Ghent University, 2008.
- Academic English: conference skills, Ghent University, 2009.
- FEBS Advanced practical and lecture course, Madrid, Spain, 2009.

Attendance to scientific meetings

- *FEBS Advanced lecture course on human fungal pathogens: molecular mechanisms of host-pathogen interactions and virulence*, Nice, France, 2009.
- *Yeasterday*, Amsterdam, the Netherlands, 2009.
- *ASM conference on Candida and candidiasis*, Miami, USA, 2010.
- *FWO workshop on bacterial and fungal biofilms*, Leuven, Belgium, 2010.
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- *Yeasterday*, Leiden, the Netherlands, 2011.
- *ASM conference on Candida and candidiasis*, San Francisco, USA, 2012.

Supervision of students

- Anneleen Spriet, Experimenteel onderzoek naar het effect van azolen op *Candida* biofilms en de betrokken resistentiemechanismen, 2008.
- Ewelina Kurtis, Screening of *Saccharomyces cerevisiae* deletion mutant bank for biofilm formation and sensitivity to miconazole, 2009.
- Karina Euting, Screening of *Saccharomyces cerevisiae* deletion mutant bank for biofilm formation and sensitivity to miconazole, 2009.
- Lies Van Bockxlaer, Moleculaire mechanismen van miconazoltolerantie in gistbiofilms, 2009.
- Valentina Coia, Screening of *Saccharomyces cerevisiae* deletion mutant bank for biofilm formation and sensitivity to miconazole, 2009.
- Bruno Stevens, Moleculaire resistentiemechanismen van *Candida albicans* biofilms tegen miconazol, 2010.
- Inge Van Acker, Experimenteel onderzoek naar resistentiemechanismen betrokken bij de celwand van *Candida albicans* biofilms na behandeling met miconazol, 2010.
- Joyce Slembrouck, Experimenteel onderzoek naar moleculaire resistentiemechanismen van *Candida albicans* biofilms tegen miconazol, 2010.

- Paloma Sastrón Toledo, Molecular mechanisms of resistance of fungal biofilms against miconazole, 2011.
- Evelien De Canck, Moleculaire resistentiemechanismen van *Candida albicans* biofilms tegen miconazol, 2011.
- Alicia Garcia Señan, Mechanisms of tolerance of *Candida albicans* biofilms against miconazole, 2012.
- Jolien De Sadeleer, moleculaire mechanismen van miconazol-resistentie in *Candida albicans* biofilms, 2012.

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