The cellular and molecular responses of *Aspergillus fumigatus* to the antifungal drug caspofungin

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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ABBREVIATIONS

ABPA: Allergic bronchopulmonary aspergillosis
ACM: *Aspergillus* complete medium
AIDS: Acquired immune deficiency syndrome
AMM: *Aspergillus* minimal medium
Amp: Ampicillin
ATCC: American type culture collection
BLAST: Basic local alignment search tool
Ca²⁺: Calcium
CAS: Caspofungin
CFU: Colony forming units
CFW: Calcofluor white
CHS: Chitin synthase
CO₂: Carbon dioxide
CPA: Chronic pulmonary aspergillosis
DEPC: Diethylpyrocarbonate
DIG: Digoxigenin
DMEM: Dulbecco modified eagle medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
dNTP: Deoxynucleotide
EDTA: Ethylenediaminetetraacetic acid
eEF2: Elongation factor 2
EUCAST: European committee on antimicrobial susceptibility testing
FBS: foetal bovine serum
FCS: foetal Calf serum
FDA: Food and drug administration
Fwd: Forward
GAG: Galactosaminogalactan
GFP: Green fluorescent protein
GRASS: Generally regarded as safe
HIV: Human immunodeficiency virus
HOG: High-osmolarity glycerol
hph: Higromycin
HR: Homologous recombination
HSP90: Heat Shock Protein 90
IA: Invasive aspergillosis
IgM: Immunoglobulin M
IPTG: Isopropyl β-D-1-thiogalactopyranoside
K+: Kalium (potassium)
Kan: Kanamycin
Kg: Kilogram
L: Litters
LB: Luria Bertani
M: Molar
MEC: Minimal effective concentration
MFG: Micafungin
Mg2+: Magnesium
mg: Milligrams
MIC: Minimal inhibitory concentration
ml: Milliliters
MOPC: Mineral oil-induced plasmacytomes
MOPS: morpholinepropanesulfonic acid
NA: numerical apertura
Na+: Sodium (Natrium)
NaAc: Sodium acetate
GlcNAc: N-acetyl-glucosamine
NaCl: Sodium Chloride
NET's: Neutrophil extracellular traps
ng: nanograms
NK: Natural killers
nm: Nanometres
Nstd: Nested
NT: No treatment
ORF: Open reading frame
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PDA: Potato dextrose agar
PE: Paradoxical effect
PEG: Polyethylene glycol
PGE: Paradoxical growth effect
pH: Potential of hydrogen
phleo: Phleomycin
PI: Propidium iodide
PKC: Protein kinase C
PMN: Polimorphonuclear
pmol: Picomolar
Prom: Promotor
ptrA: Pyrithiamine
RAS: Rat Sarcoma
Rcf: Relative centrifugal force
Rev: Reverse
RFP: Red fluorescent protein
RNA: Ribonucleic acid
Rpm: Revolutions per minute
RPMI: Roswell park memorial institute
RT: Room temperature
SAB: Sabouraud
SD: Standard deviation
SDS: Sodium dodecyl sulfate
SOC: Super optimal broth with catabolite repression
TAE: Tris-acetate-ethylenediaminetetraacetic acid
Taq: *Thermus aquaticus*
TE: Tris-ethylenediaminetetraacetic acid
Tm: Melting temperature
UTR: Untranslated region
UV: Ultra violet
V: Volts
VM: Vogel’s medium
X-Gal: 5-bromo-4-chloro-indolyl-β-D-galactopyranoside
µl: Microliters
µm: Micrometers
µM: Micromolar
ABSTRACT

The University of Manchester
Sergio David Moreno Velásquez
Doctor of Philosophy

The cellular and molecular responses of *Aspergillus fumigatus* to the antifungal drug caspofungin

2017

The opportunistic fungus *Aspergillus fumigatus* has emerged as one of the most common fungal human pathogens, causing severe and usually fatal systemic infections that account for more than 200,000 cases annually with mortality rates usually exceeding 50%. During infection, the virulence of *A. fumigatus* highly depends on its capacity to rapidly respond to external stress encounters in the human niche, such as the host immunological response and the activity of antifungal drugs. The echinocandin, caspofungin, is one of most commonly used antifungal drugs to treat intolerant or refractive patients suffering from invasive aspergillosis. Caspofungin disrupts the catalytic subunit of the β-1,3-glucan synthase complex, Fks1, resulting in the reduced production of the main cell wall component of *A. fumigatus*, the polysaccharide β-1,3-glucan. Despite its clinical relevance in patients with aspergillosis, caspofungin displays attenuated activity at high concentrations, a phenomenon known as ‘the paradoxical effect’. Little is known about the paradoxical growth of *A. fumigatus* during caspofungin treatment. Therefore, in this thesis, I investigated the key cellular and molecular responses of *A. fumigatus* upon caspofungin treatment, particularly during paradoxical growth by live-cell imaging.

High-resolution confocal live-cell microscopy revealed that treatment with either low (0.5 µg/ml) or high (4 µg/ml) concentrations of caspofungin for 36 h caused similar abnormalities in *A. fumigatus*, including wider, hyperbranched hyphae, increased septation and repeated hyphal tip lysis. Regenerative intrahyphal growth occurred as a rapid adaptation to the lytic effects of caspofungin on hyphal tips and the dynamic relocation of Fks1 to vacuoles was a key feature observed in response to caspofungin treatment. The reduced amount of β-1,3-glucan resulting from caspofungin treatment was compensated by increased α-1,3-glucan and chitin content in mature hyphal tips. Interestingly, all lysed cells recovered by regenerative intrahyphal growth. However, after 48 h treatment, only cells exposed to high caspofungin concentrations developed paradoxical growth in leading hyphae. This response was associated with a relocalization of Fks1 at hyphal tips.
Consistently, cells undergoing paradoxical growth showed normal morphology and ceased to undergo cell lysis, as well as having a normal content of β-1,3-glucan and α-1,3-glucan but not chitin, which remained high. Notably, the localization of the regulatory subunit of the β-1,3-glucan synthase complex, Rho1, was unaffected by caspofungin, but it was required for the development of paradoxical growth. Interestingly, the gene expression of the β-1,3-glucan synthase complex was downregulated by caspofungin treatment. In addition, caspofungin activity induced the nuclear translocation of the Ca^{2+} regulated transcription factor CrzA to nuclei and only hyphal tip cells in which this translocation occurred underwent cell lysis. Finally, similarly high concentrations of caspofungin also induced paradoxical growth of *Aspergillus fumigatus* during human A549 alveolar cell invasion. This thesis outlines several critical adaptations that occur at the cellular, subcellular and molecular levels at different times during exposure to high and low concentration of caspofungin.
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OVERVIEW OF THE THESIS

This thesis has been written in the ‘alternative format’. Chapter 1 provides an overall introduction and biological context of the study, including a statement of the aims of the research I have carried out. Chapter 2 provides the materials and methods used in this whole study. Chapter 3 has been accepted for publication to Antimicrobial and Agents and Chemotherapy. Chapter 4 has been submitted to Fungal Genetics and Biology as a Video article. Chapter 5 further describes the morphological adaptations of A. fumigatus to the antifungal drug caspofungin, with particular emphasis on cell-wall remodeling, and the dynamic response of the Ca\(^{2+}\)-regulated transcription factor, CrzA. It is aimed that the data presented in chapter 5 will be published as part of a future article. Finally, chapter 6 highlights the main conclusions and findings from the research described in the thesis and defines future work that should lead on from these key findings. The format of chapters 3 and 4 is the same as the manuscripts from which they have been derived except that all the Figures and Tables have been placed near to where they have been cited and Supplementary Figures from the papers have been removed and have been treated as normal Figures in the many bodies of these chapters. The Supplementary materials now solely represent movies of high resolution, live-cell imaging time courses of different aspects of the cellular and molecular responses of Aspergillus fumigatus to the antifungal drug caspofungin.

The contribution of each author to the manuscripts is listed below:

Chapter 3: Caspofungin-mediated growth inhibition and paradoxical growth in Aspergillus fumigatus involve fungicidal hyphal tip lysis coupled with regenerative intrahyphal growth and dynamic changes in β-1,3-glucan synthase localization

Sergio D. Moreno Velásquez, Constanze Seidel, Praveen R. Juvvadi; William J. Steinbach, Nick D. Read.

Target Journal: Antimicrobial Agents and Chemotherapy
Abstract:
Caspofungin targets cell wall β-1,3-glucan synthesis and is guideline-recommended to treat invasive aspergillosis as salvage therapy. Although caspofungin is inhibitory at low concentrations, it exhibits a ‘paradoxical effect’ (reversal of growth inhibition) at high concentrations by an undetermined mechanism. Treatment with either growth inhibitory (0.5 µg/ml) or paradoxical growth-inducing (4 µg/ml) caspofungin concentrations for 24 h caused similar abnormalities, including wider, hyperbranched hyphae, increased septation, and repeated hyphal tip lysis followed by regenerative intrahyphal growth. By 48 h, only hyphae at the colony periphery treated with the high caspofungin concentration displayed paradoxical growth. Similar high concentrations of caspofungin also induced paradoxical growth of Aspergillus fumigatus during human A549 alveolar cell invasion. Localization of the β-1,3-glucan synthase complex (Fks1 and Rho1) revealed significant differences between cells exposed to growth inhibitory and paradoxical growth inducing concentrations of caspofungin. At both concentrations, Fks1 initially mislocalized from hyphal tips to vacuoles. However, only continuous exposure to 4 µg/ml of caspofungin for 48 h led to recovery of normal hyphal morphology with renewed localization of Fks1 to hyphal tips. Rho1 remained at the hyphal tip under both caspofungin concentrations but was required for paradoxical growth. Farnesol blocked paradoxical growth and relocalized Fks1 and Rho1 to vacuoles. Our results highlight the importance of regenerative intrahyphal growth as a rapid adaptation to the fungicidal lytic effects of caspofungin on hyphal tips and the dynamic localization of Fks1 as part of the mechanism for the caspofungin-mediated paradoxical response in A. fumigatus.

Authors’ Contributions:
Sergio D. Moreno Velasquez: Design of experiments; live-cell microscopy, image processing and analysis; bioinformatics analysis, phenotypic analysis; virulence studies; generation of mutants; writing and preparation of manuscript.
Constanze Seidel: Bioinformatics analysis; generation of mutants; and microscopy analysis.
Praveen R. Juvvadi: Design of experiments; generation of mutants; writing of manuscript.
William J. Steinbach: Design of experiments; writing of manuscript.
Nick D. Read: Project supervisor; design of experiments; writing of manuscript.
Chapter 4: Live-cell imaging of the dynamics of caspofungin-induced paradoxical growth in the human pathogen, *Aspergillus fumigatus*

**Sergio D. Moreno Velásquez**, Constanze Seidel, Nick D. Read.

**Target Journal:** *Fungal Genetics and Biology*

**Abstract:**
The antifungal drug caspofungin exhibits attenuated activity at high concentrations against *Aspergillus fumigatus*, a phenomenon known as ‘paradoxical growth’. Despite its clinical relevance, this cellular response remains little understood. Recently, it was shown that treatment for 36 h with either low or high caspofungin concentrations results in hyphal tip lysis, regenerative intrahyphal growth, hyperbranching and increased septation. Here, we shown by live-cell imaging that after ~ 40 h hyphae treated with high caspofungin concentrations switch to normal growth. However, gene expression of the β-1,3-glucan synthase complex remains downregulated during paradoxical growth.

**Authors’ Contribution:**
*Sergio D. Moreno Velásquez:* phenotypic analysis, microscopy analysis.

*Constanze Seidel:* Microscopy analysis.

*Nick D. Read:* Project supervisor; design of experiments; writing of manuscript.
Chapter 5: Caspofungin treatment induces the recruitment of the transcription factor CrzA to nuclei and the reorganization of the main cell-wall components during paradoxical growth in *Aspergillus fumigatus*

*Sergio D. Moreno Velásquez, Nick D. Read.*

**Abstract:**
Caspofungin targets the cell wall synthesizing enzyme β-1,3-glucan synthase and is used as second-line therapy to treat invasive aspergillosis. Despite its clinical importance, caspofungin exhibits attenuated activity at high concentrations, a phenomenon known as the ‘parado... . Short periods of treatment (30 min) with either growth inhibitory (0.5 µg/ml) or paradoxical growth (4 µg/ml) concentrations of caspofungin in mature hyphae caused similar morphological defects including loss of polarity and hyperbranching, as occurred with cells continuously exposed to caspofungin from the stage of initial inoculation. Treatment with caspofungin at low and high concentrations was associated with the mislocalisation of Fks1 in mature hyphae after 30 min. Concomitantly, we observed a reduced β-1,3-glucan, but increased chitin content at mature hyphal tips. Furthermore, caspofungin treatment induced nuclear translocation of the Ca²⁺-regulated transcription factor CrzA. Paradoxical growth in *A. fumigatus* CEA10 is observed at the colony level by 48 h. This response was observed in several azole resistant mutants but in some of these clinical strains azole resistance was found to be independent of the paradoxical effect. Paradoxical growth was associated with the normalized content of β-1,3-glucan, α-1,3-glucan, galactomannan but not chitin, which remained high. Finally, it was demonstrated that caspofungin remains active and stable in media after 48 h after paradoxical growth has been initiated. These results further illustrate the dynamic, multiple early and late cellular and molecular responses that *A. fumigatus* develop in association with paradoxical growth, of which some may play role in overcoming caspofungin treatment in the clinical situation.

**Authors’ Contribution:**
*Sergio D. Moreno Velásquez:* Design of experiments; live-cell microscopy, image processing and analysis; phenotypic analysis; virulence studies; writing and preparation of the text.
*Nick D. Read:* Project supervisor; design of experiments; revision of the text.
Chapter 1

1. Introduction

1.1 The fifth kingdom

The fifth kingdom, the group that comprises all kinds of fungi, is one of the most unique and diverse group of living systems. Fungi like all eukaryotes possess a nucleus enveloped by a membrane and containing chromatin, vacuoles, mitochondria, and a cytoskeleton (Bennett, 2010). Previously, fungi were characterized as the cryptogams (plants without flowers or seeds) derived by spontaneous generation. However, their lack of chlorophyll, ability of nutrition by absorption, the presence of chitin in the cell wall (i.e. in the exoskeleton of insects) and their phylogenetic characteristics have revealed that fungi are more similar to animals than plants (Baldauf and Palmer, 1993).

Fungi inhabit a wide range of environmental niches spanning a broad spectrum of food sources, temperature, and moisture content. They spread their offspring primarily by spore dispersal, providing a highly adapted means to colonize almost every environmental niche on the planet, including extreme habitats, such as tundra, aquatic zones, soda lakes, geothermal regions, desert soils and acidic copper mines (Newsham, 2012).

One of the most relevant features of fungi is their ability to use a wide range of nutritional sources. Fungi have a unique metabolic arsenal required to perform several essential roles in the ecosystem, including the recycling of carbon, nitrogen, sulphur and phosphate (Hawksworth, 1991). Indeed, fungi associated with plants can store 70 % more atmospheric carbon than plants alone making them major regulators of the global climate (Averill et al., 2014). On the other hand, due to their filamentous or unicellular growth, fungi can coexist with a myriad of living systems, including bacteria, plants and animals. For instance, fungi undergo symbioses with cyanobacteria or algae in the formation of lichens, but also are
associated with roots of most plants as mycorrhizae and endophytes. In animals, fungi form several symbioses, such as mutualistic associations in the rumen of herbivorous mammals (McLaughlin et al., 2009).

The exact number of fungal species is unknown but it has been estimated to reach about 5.1 million species (Xu et al., 2013). So far, 100,000 species of fungi have been described and the number of new species is increasing at a rate of 1.2 %, annually (Hawksworth et al., 1996). Such a diverse group impacts significantly on our society, yet less than 5 % of known fungi are of economic interest.

This versatile group of microorganisms is superior to bacterial in terms of metabolic versatility, robustness and secretory capacity. Some of their current commercial uses include: fermentation of cheese, wine, beer, soy sauce, bread, paper, alcohol and dyes (Bennett, 2010; Read and Boddy, 2010). The biotechnology industry uses fungi in the production of enzymes, organic acids, herbicides, pesticides, antibiotics, statins, steroids, ethanol and biogas. Notably, the majority of enzymes used worldwide are produced by filamentous fungi (Meyer et al., 2016). Furthermore, some fungi are directly harvested as edible mushrooms and even others are used recreationally due to their hallucinogenic properties.

Although most fungi are beneficial in the regulation of the biosphere, many species are pathogens of plants and animals. In agriculture, pathogenic fungi are the main cause of crop diseases causing significant economic losses (Agrios, 2005). Furthermore, some species can contaminate post-harvested crops by producing mycotoxins, which following ingestion can cause disease and even death in vertebrates, including humans (Bennett and Klich, 2003). Additionally, some fungal species can directly infect animals. Although the number of medically important fungi species is low, the increasing number of invasive infections by fungi has become a very significant problem.
**1.2. Fungal invaders**

Fungi occupy virtually every inhabitable ecological niche on earth, exhibiting the broadest spectrum of hosts for any group of pathogens, thus infecting most organisms, including animals and plants (Agrios, 2005; Brown et al., 2012; Cantrell et al., 2011). These plant invaders are not a new problem and about 30% of crop diseases are caused by fungi with a global demand for fungicides of €10 billion in 2014 (Fisher et al., 2012; Meyer et al., 2016). Several fungal pathogens can cause diverse plants diseases, which are responsible for the annual crop yield reduction of at least 10% (Table 1.1). Remarkably, plant fungal pathogens destroy enough food to feed approximately 600 million people annually, which represent roughly 8.5% of the total population (Fisher et al., 2012).

So far, over 8,000 fungal species are known to cause disease in plants. The most destructive fungal plant pathogen is the ascomycete *Magnaporthe oryzae*, responsible for rice blast, the most devastating rice disease worldwide (Ou, 1980). This fungus can affect a variety of grasses including millet, barley and wheat (Couch et al., 2005). Another devastating species of economical concern is the grey mould *Botrytis cinerea*. This fungus is considered a typical necrotroph, but its particular way of activating programmed cell death in their hosts allows this fungus to infect more than 200 plant species (Dean et al., 2012). *Fusarium oxysporum* is a ubiquitous plant pathogen that causes vascular wilt ending in the leaf of the host (Agrios, 2005). *F. oxysporum* species comprises several formae speciales which can infect more than 100 different plants of economic importance, including tomato, banana, melon and cotton (Michielse and Rep, 2009). Despite the development of novel controls against such pathogens their impact on agriculture is still immeasurable.
Outbreaks of fungal pathogens can also indirectly affect the annual crop production by threatening species that maintain crop health. The ascomycete *Pseudogymnoascus destructans* is responsible for the large reduction of bat populations in North America and subsequent increases in crop-destroying insects, a feeding source for these bats. Indeed, some bat species are threatened with extinction (Frick *et al.*, 2010). Moreover, a high incidence of deaths in at least 500 species of amphibians in 54 countries has been caused by the oomycete *Batrachochytrium dendrobatidis* (Fisher *et al.*, 2009). This large loss of amphibian species has generated a significant ecosystem change in Central America (Colón-Gaud *et al.*, 2009). Furthermore, recent reports shown the emergence of the oomycete *Pythium insidiosum* as a

<table>
<thead>
<tr>
<th>Rank</th>
<th>Fungal pathogen</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Magnaporthe oryzae</em></td>
<td>Rice, barley, wheat, and millet</td>
</tr>
<tr>
<td>2</td>
<td><em>Botrytis cinerea</em></td>
<td>More than 200 plants, mostly dicotyledonous</td>
</tr>
<tr>
<td>3</td>
<td><em>Puccinia</em> spp.</td>
<td>Wheat, barley and triticale</td>
</tr>
<tr>
<td>4</td>
<td><em>Fusarium graminearum</em></td>
<td>All cereal species</td>
</tr>
<tr>
<td>5</td>
<td><em>Fusarium oxysporum</em></td>
<td>Crops, melon, tomato, cotton and banana</td>
</tr>
<tr>
<td>6</td>
<td><em>Blumeria graminis</em></td>
<td>Grasses, mostly wheat and barley</td>
</tr>
<tr>
<td>7</td>
<td><em>Mycosphaerella graminicola</em></td>
<td>Wheat</td>
</tr>
<tr>
<td>8</td>
<td><em>Colletotrichum</em> spp.</td>
<td>Bananas, cassava and sorghum</td>
</tr>
<tr>
<td>9</td>
<td><em>Ustilago maydis</em></td>
<td>Corn</td>
</tr>
<tr>
<td>10</td>
<td><em>Melampsora lini</em></td>
<td>Flax rust</td>
</tr>
</tbody>
</table>

Table 1.1. Top 10 plant fungal pathogens. Modified from Dean *et al.* (2012).
new pathogen, affecting the respiratory tract of horses in Brazil (Souto et al., 2016). Fungi as ubiquitous organisms can damage invertebrate animals as well. Bumble bees are essential pollinators of crops of economic interest, and a global reduction in Bumble Bee populations of almost 96% has been noted in recent years, mainly due to infections with the intracellular fungus *Nosema bombi* (Cameron et al., 2016). Another example of invertebrate fungal infections occurs in the sea fan corals *Gorgonia ventalina* off the coast of Florida, and is caused by the filamentous fungus *Aspergillus sydowii* (Kim and Harvell, 2004). The emergence of new fungal diseases is threatening a remarkable number of individuals in many animal populations and this could affect directly the global ecosystem (Day, 2016).

1.3. **Fungal infections in humans**

In the competition of survival, fungi can become pathogens against susceptible human hosts (Fisher et al., 2012). These unique adaptations have not been induced by fungal species acquiring novel and unique virulence factors, but primarily due to the changing host landscape in response to the appearance of AIDS, widespread use of antibiotics, foreign body catheters, organ transplantation, aggressive treatments against cancer, immunosuppression treatments, and the high-risk intensive care unit environment (Badiee and Hashemizadeh, 2014). Thus, several fungi have now ascended from saprotrophs to pathogens.

Fungi are ubiquitous in the environment and each human breath on average contains up to 10 fungal spores (Fröhlich-Nowoisky et al., 2009). Fungi are an important part of the mammalian microbiome and dysbiosis of the fungal populations is associated with a range of diseases (Carding et al., 2015; Huffnagle and Noverr, 2013). Human fungal infections widely vary from non-lethal mycoses (e.g., athlete’s foot) through to serious life-threatening diseases such as invasive aspergillosis. Annually, human pathogenic fungi are responsible for about 1.5 million deaths (Brown et al., 2012). Most costs result from treating non-lethal fungi,
particularly those responsible for superficial diseases of the nails, skin, and hair (Havlickova et al., 2008). Mucosal infections of the genitalia are common, as well. It has been estimated that roughly 75 million women suffer from episodic of infections of vaginal thrush, annually (Sobel, 2007). Furthermore, oral infections caused by fungi are common in denture wearers and babies, which are common in patients treated with steroids for asthma, in leukemia and transplants patients (Brown et al., 2012).

Fungi can contribute other notable diseases, including blindness and disfiguring chronic subcutaneous infections. For instance, fungal keratitis, caused by several fungi including Fusarium sp., Aspergillus sp., and Candida sp., are a leading cause of ocular morbidity worldwide and affect around 1 million of people per annum. This is the leading cause of blindness globally and is a particular problem in Asia and Africa (Ansari et al., 2013; Srinivasan, 2004).

Besides their high potential as superficial invaders, fungi have been recognized as one of the main group of organisms responsible for respiratory allergies worldwide. More than 80 genera of fungi, including Alternaria, Cladosporium, Epicoccum, Ganoderma and Aspergillus, are associated with respiratory allergies (Gravesen, 1979; Horner et al., 1995). In fact, fungal allergic sensitivity in industrialized countries can affect around 13 to 20 % of the population, ranging from significant to severe allergic diseases, such as asthma and rhinitis (Gergen et al., 1987). In recent years, invasive fungal infections have become an unprecedented problem in society. Indeed, more people died from the Top 10 of invasive fungal diseases (Table 1.2) than from malaria and similar to tuberculosis.
<table>
<thead>
<tr>
<th>Fungal disease agent</th>
<th>Location</th>
<th>Life-threatening infections cases</th>
<th>Mortality rates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>Worldwide</td>
<td>&gt;200,000</td>
<td>30-95</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>Worldwide</td>
<td>&gt;400,000</td>
<td>46-75</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Worldwide</td>
<td>&gt;1,000,000</td>
<td>20-70</td>
</tr>
<tr>
<td><em>Pneumocystis jirovecii</em></td>
<td>Worldwide</td>
<td>&gt;400,000</td>
<td>20-80</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>Worldwide</td>
<td>&gt;10,000</td>
<td>30-90</td>
</tr>
<tr>
<td><em>Blastomyces dermatitidis</em></td>
<td>Midwestern and United States</td>
<td>~3,000</td>
<td>&lt;2-68</td>
</tr>
<tr>
<td><em>Coccidioides immitis</em></td>
<td>United States</td>
<td>~25,000</td>
<td>&lt;1-70</td>
</tr>
<tr>
<td><em>Histoplasma capsulatum</em></td>
<td>United States</td>
<td>~25,000</td>
<td>28-50</td>
</tr>
<tr>
<td><em>Paracoccidioides brasiliensis</em></td>
<td>Brazil</td>
<td>~4,000</td>
<td>5-27</td>
</tr>
<tr>
<td><em>Penicillium marneffei</em></td>
<td>Southeast Asia</td>
<td>&gt;8,000</td>
<td>2-75</td>
</tr>
</tbody>
</table>

Table 1.2. 10 most significant invasive fungal infections in humans (Brown et al., 2012).
Despite the existence of approximately 600 species of human invasive pathogenic fungi, over 90% of all the deaths are due to species belonging to four genera: Candida, Cryptococcus, Pneumocystis, and Aspergillus (Brown et al., 2012). These fungi are opportunistic agents that cause diseases in patients with compromised immune systems (HIV, cancer and trauma) or patients undergoing clinical procedures (stem cell, bone and organ transplants), where the mortality rates range from 50 to 95% (Gow et al., 2012).

*Candida* is the most common cause of invasive fungal infection worldwide and is the fourth most common cause of nosocomial bloodstream sepsis in developed countries (Wisplinghoff et al., 2004). More than 17 *Candida* species have been reported as etiologic agents (Hazen, 1995), whereas the most common infections are caused by five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei* (Pappas et al., 2009). Members of the *Candida* group can easily disseminate by catheters during neonatal intensive care, gut surgery, and liver transplantation. It has been estimated that 400,000 cases of *Candida* infections occur per year and the mortality rates can reach up to 46-75% (Brown et al., 2012; Morgan, 2005).

Other fungi of particular concern belong to the *Cryptococcus* genus. In recent years, the incidence of the pathogenic yeasts *C. neoformans* and *C. gatti*, have become more frequent worldwide (Srikanta et al., 2014). Such fungi are responsible for over one million cases of cryptococcal meningitis infections per year. In addition, mortality rates caused by *Cryptococcus* in AIDS sufferers are estimated to be around 20% in the United States and 55-70% in Latin America (Park et al., 2009). Furthermore, recent studies have reported the presence of cryptococcal infection even in patients without immunodeficiency problems, which usually result in pulmonary illnesses (Chen et al., 2008).
*Pneumocystis jirovecii* is one of the most prevalent opportunistic infection agents in people with T-cell immunodeficiency (Castro and Morrison-Bryant, 2010), including patients with haematological malignancies, transplant patients, and people on prolonged immunosuppressive therapy such as HIV and AIDS patients. This fungus is ubiquitous and human beings appear to provide a reservoir for it. The incidence of *Pneumocystis* infections reach around 400,000 cases per annum with mortality rates ranging from 20 to 80% (Brown *et al.*, 2012). The impact of this pathogenic fungus only in the United States has resulted in >52,000 deaths per annum (D’Avignon *et al.*, 2008).

While most invasive fungal diseases result from the fungus taking advantage of a malfunctioning of the host immune system, none of them have the broad infection impact of the species *Aspergillus*.

### 1.4. Aspergillus

The genus *Aspergillus* comprises one of the most successful groups of moulds with a worldwide distribution. This group, obtained its name due to its characteristic spore-bearing structure, which resemble a device used by the Roman clergy to sprinkle holy water during a part of the liturgy, the ‘asperges’ (Ainsworth, 1976). Since its discovery (Micheli, 1729), *Aspergillus* has been recognized as one of the largest genera of moulds, comprising around 260 described species (Samson and Varga, 2009).

The *Aspergillus* group is essentially a primary consumer, driving natural cycling of chemical elements, primarily carbon and nitrogen (Haines, 1995). Most of the *Aspergillus* species are saprotrophs that grow on decaying vegetation such as mouldy hay, organic compost piles and leaf litter (Bennett, 2010). Thus, *Aspergilli* play an indispensable role in plant decomposition by digesting some components, such as cellulose, hemicellulose, starch, pectin, lignin, and proteins (de Vries *et al.*, 2001).
Interestingly, this group can invade a plethora of substrates as diverse as dung, paper, textiles, and even antique parchments (Polacheck et al., 1989). Indeed, the mould *A. versicolor* has been reported as the primary cause of damage of the Ghirlandaio fresco in the Ognissanti church that occurred after Florence was flooded in 1969 (Ciferri, 1999).

Due to its good fermentation capabilities and high level of protein secretion, several species of *Aspergillus* are of biotechnological interest. In the biotechnology industry, *Aspergillus* spp. are involved in important processes including the production of enzymes (e.g. amylases), commodity chemicals (e.g. citric, itaconic, and kojic acid), and food products (rice wine, miso and soy sauce) (Bennett, 2010). Remarkably, annual worldwide production of citric acid using *A. niger* is about 1.6 million metric tons, which by far exceeds the commercial production of any other organic acid made by microbial fermentation (Meyer et al., 2016). Furthermore, some species of *Aspergillus* have high commercial impact in the biomedical industry, mainly in the production of secondary metabolites such as neurokinin antagonists, ion channel ligands, antifungal drugs, and statins (Bennett, 2010).

Besides its significant contribution to the biotechnology industry, some species of *Aspergillus* are important spoilage agents of food and recently have become a major life-threatening pathogen in animals, including humans. The continuous exposure to *Aspergillus* spores can cause disease in three major ways: through the production of mycotoxins, by inducing allergic responses, and via systemic infections (Bennett, 2010). Constant exposure to high concentrations of conidia is responsible for several hypersensitivities such as farmer’s lung, bird fancier’s lung, compost lung, malt worker’s lung and ABPA. The most severe *Aspergillus* allergic pulmonary complication caused by *Aspergillus* species is the allergic bronchopulmonary aspergillosis (ABPA), a bronchial asthma triggered by a hypersensitivity to *Aspergillus* antigens (Latgé, 1999; Kousha et al., 2011). Moreover, the production of mycotoxins (aflatoxin, ochratoxin, fumigillin and patulin) by some species of
Aspergillus has been an increasing problem in the food industry, mainly in the contamination of all major cereal crops, peanuts and even marijuana. One of the most potent carcinogens known is aflatoxin, a secondary metabolite produced by A. flavus and A. parasiticus (Bennett et al., 2003).

To date, about 40 of the 260 described species of Aspergillus have been reported to be harmful to human beings, triggering the disease known as “aspergillosis” (Klich, 2006). First described as an avian disease, aspergillosis covers different syndromes that are grouped in three categories: allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA), and invasive aspergillosis (IA). Despite the large number of Aspergillus species that elicit invasive aspergillosis, over 90% of all cases in Europe are caused by the mould A. fumigatus, followed by A. flavus, A. terreus and A. niger (Latgé, 1999; Richardson and Lass-Flörl, 2008).

1.5. The opportunistic fungus Aspergillus fumigatus

The ascomycete Aspergillus fumigatus is a filamentous fungus with a broad distribution, ranging from the northern tundra to the tropics (Pringle et al., 2005). This saprotrophic mould (Fig. 1.1), whose genome consists of 9,926 predicted genes (29.4 megabases) distributed in 8 chromosomes (Fedorova et al., 2008; Nierman et al., 2005) is typically found in compost heaps as an essential recycler of plant material (Tekaia and Latgé, 2005).

Due to a complex sporulation machinery capable of disseminating small spores into the environment, the mould A. fumigatus is one of the most abundant species in the airborne mycoflora and is one of the most ubiquitous aeroallergens worldwide (Mullins et al., 1984; Mullins et al., 1976).
The airborne conidia are usually less abundant in indoors, rather than outdoors, in which they rarely exceed 100 CFU/m² (Torpy et al., 2013). *A. fumigatus* spore concentration also fluctuates during every season with higher levels during the autumn and winter (Mullins et al., 1976). *A. fumigatus* spores can survive in several home reservoirs including dust, flowers, soil of ornamental plants or even in certain foods such as, tea, biscuits, fruits, and spices (Bock et al., 1989; Bouakline et al., 2000; Staib et al., 1978).

![Image of Aspergillus fumigatus colony](image)

**Figure 1.1.** The mould *Aspergillus fumigatus*. Typical *A. fumigatus* colony after 3 days of incubation on Vogel’s medium. The enlarged region highlights the presence of an asexual spore producing structure (conidiophore).

This successful fungus produces green pigmented, uninucleated conidia of roughly 2 µm diameter extruded as chains from aerial hyphae localised above the mature mycelial mass. However, under specific conditions *A. fumigatus* is also able to produce sexual meiotic ascospores (Bennett, 2010; O’Gorman et al., 2009).
The spores of *A. fumigatus* are extremely well-suited for air dispersal because of their small size and high hydrophobicity that allows spores to remain airborne for long periods (Fig. 1.2). Furthermore, spores possess an echinulate surface that increases their air resistance, enhancing dispersion (O’Gorman, 2011). Once the spores of *A. fumigatus* establish contact with appropriate nutrients, spore metabolism is activated breaking dormancy and triggering isotropic growth (Harris, 1997). After the first nuclear division, an axis of polarity is established and a germ tube begins to emerge. This axis is maintained as the germling elongates by tip growth until subsequent branches emerge leading to the formation of a colony (Harris *et al*., 1999; Momany and Taylor, 2000).

Despite its simple life cycle and humble role in the tropic chain as an organic matter recycler, *A. fumigatus* possesses an exquisite combination of genomic and cellular traits that allow it to be named as a primary mould pathogen in immunocompromised patients and several animals including dogs, cows and birds (Abad *et al*., 2010; Tell, 2005).

Among the human pathogenic species of *Aspergillus*, *A. fumigatus* is the best equipped to survive the mammalian environment followed by *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans* (Denning, 1998; Morgan *et al*., 2005). The virulence of *A. fumigatus* is multifactorial and is due to a combination of biological traits, the fast growth and adaptation to stress conditions, mechanisms for evading the immune system and the ability to cause damage to the host (Abad *et al*., 2010).
Figure 1.2. Life cycle of *Aspergillus fumigatus*. The fungal mycelium is a network of branched filaments of connected compartments, which each contain several nuclei. This mycelium can develop either conidiophores or cleistothecia for the production of asexual or sexual spores, respectively (reproduced from Casselton and Zolan, 2002).

One of the first virulence traits in *A. fumigatus* is the size and shape of its spores, which provides *A. fumigatus* with a dispersal structure ideal for infiltrating deep into the alveoli, whereas larger spores of other human fungal pathogens, including *A. niger* and *A. flavus* are more easily removed by mucocilliary clearance of the upper respiratory tract (Dagenais and Keller, 2009). Furthermore, *A. fumigatus* conidia possess an extremely protective cell envelope, possessing rodlet and melanin layers that provide a resistant mechanism to avoid the immune system recognition (Jahn *et al.*, 1997; Thau *et al.*, 1994). Additionally, conidia of this fungus bind better than those of other *Aspergillus* species to intact lung cell basal lamina
(WasylInka et al., 2001). Besides these features, *A. fumigatus* conidia can remain viable for many months even under dry conditions.

The fungus *A. fumigatus* is a thermophilic fungus able to growth at 37 °C, 55 °C and survive more than 75 °C, a necessary ability in order to thrive in decaying vegetation and to infect a mammalian host (Beffa et al., 1998; Ryckeboer et al., 2003). Indeed, *A. fumigatus* germinates and grows more efficiently than *A. flavus* and *A. niger* at human body temperatures (Araujo and Rodrigues, 2004). Interestingly, human sera, particularly albumin, enhance mycelial growth of several *Aspergillus* species and specifically promote conidial germination in *A. fumigatus* (Rodrigues et al., 2005).

In addition to its structural features, *A. fumigatus* is well equipped with a myriad of digestive enzymes necessary to obtain essential nutrients from the surrounding human environment milieu. Although in the environment *A. fumigatus* secretes extracellular proteinases that degrade and recycle organic matter, during infection these proteases breakdown several human structural barriers in order to obtain nutrients (Abad et al., 2010). This is likely facilitated by some of the 99 putative secreted proteases encoded by the genome of *A. fumigatus*, which can degrade collagen, elastin, fibrinogen, and casein (Birch et al., 1996; Ibrahim-Granet and Denfert, 1997; Kolattukudy et al., 1993; Nierman et al., 2005; Reichard et al., 2000). Furthermore, *A. fumigatus* inhibits and reduces the complement activation of serum proteins by the production of a soluble complement-inhibitory factor, the binding of an alternative complement inhibitor factor H and the reduced binding of C3 molecule per unit of conidia surface (Behnsen et al., 2008; Vogl et al., 2008; Washburn et al., 1990).

Healthy individuals inhale approximately between 10,000 to 15,000 L of air each day and are continuously exposed to fungal conidia (Balloy and Chignard, 2009). The inhalation of *A. fumigatus* airborne spores can lead to the deposition in the bronchioles or alveolar spaces.
Furthermore, the small conidia of *A. fumigatus* can also gain access through broken skin, eyes, ears and the gastrointestinal tract (Denning, 1998).

Figure 1.3. Diagrammatic representation of model of invasive aspergillosis development in immunocompromised individuals. Magnified region shows the interaction between macrophages, neutrophils and dendritic cells. Macrophages detect and ingest swollen conidia and germlings. Conidia can prevent phagolysosomal fusion and grow out of macrophages. Neutrophils detect and immobilize conidia and outgrown hyphae by the formation of neutrophil extracellular traps (NET’s). Dendritic cells ingest conidia and hyphae; transport them to draining lymph nodes in order to activate the adaptive immune response. PMN: Polymorphonuclear leukocytes. NK: Natural killer cells (adapted from Dagenais and Keller, 2009).
Once in the lungs, conidia have the ability to bind to several structures allowing greater adhesion in the airways and subsequent dissemination to different organs, such as kidneys, liver, and spleen (Denning, 1998; Latgé, 1999).

Aspergillosis covers different syndromes that are grouped in three categories: allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA), and invasive aspergillosis (IA). Invasive aspergillosis (IA) is the most severe infection caused by Aspergillus species. Firstly described in 1953, IA is considered as a the leading cause of death, particularly in individuals with haematological malignancies and undergoing hematopoietic stem cell transplants (Del Bono et al., 2008). This life-threatening form of aspergillosis is characterized by hyphal invasion and destruction of pulmonary tissue. Estimates have shown that there are more than 200,000 cases of IA annually, resulting in an overall 50% mortality rate even if diagnosed and treated. However, if the diagnosis is missed or delayed, the mortality rate can reach 100% lethality (Brown et al., 2012).

The severity and increased incidence of invasive aspergillosis relies on the interplay between host and A. fumigatus. This well-equipped fungus is inherently an opportunistic pathogen and human disease pathology and progression are result of both the host response and the multiple cellular adaptations of A. fumigatus, which in most cases results in the reorganization of the cell wall.

1.6. The cell wall of Aspergillus fumigatus

The mould A. fumigatus inhabits diverse and adverse niches such as the human body, and these challenge its life cycle. The ability of A. fumigatus to survive environmental stresses such as osmotic stress, oxidative stress, mechanical stress, nutrient limitations, pH and temperature changes is highly dependent on the cell wall (Lee and Sheppard, 2016). Initially considered as an inert exoskeleton, the fungal cell wall is a dynamic organelle that
continuously changes in response to the surrounding environment. The cell wall plays an essential role in fungal growth and morphogenesis, it determines organism osmotic integrity, acts as a molecular sieve allowing the selective passage of molecules into and out the cell; and in pathogenic fungi, mediates antigenicity and adherence to various host surfaces (de Nobel et al., 2000; Lee and Sheppard, 2016; Smits et al., 1999). Notably, the hyphal cell wall accounts for 20-40% of the mycelial dry weight (Gastebois et al., 2009; Mouyna and Fontaine, 2009).

The composition of the cell wall varies among fungal species, but mainly comprises three components: glucans, chitin and mannoproteins (Arana et al., 2009). The cell wall in *A. fumigatus* comprises various proteins, carbohydrates, melanin and other pigments, which changes during different developmental stages.

Conidia of *A. fumigatus* contain an inner cell wall composed of β-1,3-glucans (38%), galactomannan (26%) and chitin/chitosan (5.6%). The β-1,3-glucans form branches and cross-link with chitin, galactomannans and proteins in order to provide structure and rigidity to the cell wall (Mouyna and Fontaine, 2009). The outer cell wall contains α-1,3-glucans (14%), galactomannan (13%), β-1,3-glucans (5%), and chitin/chitosan (0.5%). The outer cell wall polysaccharides are non-covalently associated and form a weaker network of macromolecules (Fig. 1.4, Table 1.3).

Once in a favorable environment, resting conidia of *A. fumigatus* break dormancy, undergo isotropic growth and germinate in order to produce polarized filamentous hyphae. These processes require the remodeling and reshaping of the cell wall. The cell wall of *A. fumigatus* hyphae is surrounded by an amorphous extracellular matrix, which comprises galactosaminogalactan, galactomannan and α-1,3-glucan (Fontaine et al., 2010; Gravelat et al., 2013). Similarly to resting conidia, the cell wall of *A. fumigatus* hyphae is composed of
two layers. The inner cell wall fraction contains β-1,3-glucans (30%), β-1,3/1,4-glucans (3%), chitin/chitosan (17%), galactomannan (5%) and galactosaminogalactan (4%), while the outer cell wall fraction contains α-1,3-glucans (42%), galactomannan (1.4%), and galactosaminogalactan (2.3%) (Mouyna and Fontaine, 2009).

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Conidia</th>
<th>Hyphae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkali-insoluble</td>
<td>Alkali-soluble</td>
</tr>
<tr>
<td>α-1,3-glucan</td>
<td>ND</td>
<td>14</td>
</tr>
<tr>
<td>β-1,3-glucan</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td>β-1,3/1,4-glucan</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Chitin</td>
<td>1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Chitosan</td>
<td>3.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Galactomannan</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td>Galactosaminogalactan</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1.3. Cell wall composition of A. fumigatus. The composition is showing in percent. ND: not detected. NA: not available (reproduced from Mouyna and Fontaine, 2009).
Figure 1.4. Comparison of the cell wall components of *A. fumigatus* resting conidium and hypha. The conidial spore wall consists of an immunologically inert outer hydrophobin rodlet layer and includes an inner melanin layer, whereas hyphae outer cell wall composition consist of α-1,3-glucan, galactomannan and galactosaminogalactan (adapted from Erwig and Gow, 2016; Latgé, 2010).
The major component of *A. fumigatus* cell wall is β-1,3-glucan (Mouyna and Fontaine, 2009). This homopolymer is composed of D-glucose residues linked by β-1,3-glucosidic bonds. β-1,3-glucan is responsible for the wall mechanical rigidity and serves as the main structural constituent to which other components are covalently attached (Bowman and Free, 2006). Polymers of β-1,3-glucan are synthesized by the β-1,3-glucan synthase complex, which is localized at zones of continuous growth such as the hyphal tips. This complex uses UDP-glucose as a substrate and promotes the association of nascent glucan chains within the cell wall space. In *A. fumigatus*, this complex is composed of a catalytic subunit (Fks1) and a regulatory subunit (Rho1 GTPase) (Beauvais *et al.*, 2001, 1993). The *fks1* and *rho1* genes are highly conserved in fungi. Fks1 is an integral plasma membrane protein, which comprises 16 putative trans-membrane helices. Rho1 is a key regulator of β-1,3-glucan synthase cell wall integrity pathway (CWI), cytoskeleton, endocytosis and polarized secretion (Dichtl *et al.*, 2012; Kwon *et al.*, 2011; Levin, 2011; Park and Bi, 2007). The genome of *A. fumigatus* encodes only one copy of *fks1* and *rho1*. While the deletion *fks1* results in defective growth and swelling of the hypha, the deletion of *rho1* is lethal in *A. fumigatus* (Dichtl *et al.*, 2012, 2015).

During hyphal growth of *A. fumigatus*, the roughly 1,500 residues of extruded β-1,3-glucan are processed and modified by several classes of enzymes including endo-β-1,3-glucanases (ENG), β-glucanosyltransferases (GEL), and branching enzymes (BGT). These enzymes are responsible for the cutting, extruding and joining of fragments of glucose to an existing chain (Lee and Sheppard, 2016). The glycosylphosphatidylinositol (GPI)-anchored proteins (ENG), cleave internal residues of soluble β-1,3-glucans (Hartl *et al.*, 2011). *A. fumigatus* encodes six putative ENG proteins and the single deletion of *eng1* or *eng2* are dispensable (Hartl *et al.*, 2011; Mouyna *et al.*, 2013). GEL enzymes are glucanosyltransferases that mediate the elongation and reorganization of β-1,3-glucan chains.
These proteins cleave β-1,3-glucan molecules internally and transfer the newly generated reducing end to the nonreducing end of another β-1,3-glucan molecule. GEL proteins are attached to the cell wall by a glucosylphosphatidylinositol (GPI) anchor. *A. fumigatus* harbours seven *gel* homologues (Mouyna *et al*., 2013).

Chitin is the second most abundant polysaccharide of the cell wall of fungi. Chitin is localized within the inner cell wall, close to the plasma membrane and it is distributed throughout the whole cell wall of hyphae in which it provides tensile strength (Lenardon *et al*., 2010). This carbohydrate is a long homopolymer composed of N-acetyl-glucosamine (GlcNAc) with residues linked by β-1→4 linkages. In yeast, chitin constitutes 1-2% of the cell wall, while in filamentous fungi it comprises 10-20% of the mycelial dry weight (Bartnicki-García, 1968). Unlike β-1,3-glucan, chitin is synthesized by multiple chitin synthases (CHS), which are membrane proteins catalyzing the polymerization of GlcNAc using uridine diphosphate-GlcNAc as substrate (Lenardon *et al*., 2010).

The genome of *A. fumigatus* encodes eight putative chitin synthase genes (*chsA, B, C, D, E, F, csmA* and *csmB*) (Gastebois *et al*., 2009). The function of the eight individual CHS is regulated spatially and temporally, and has key roles during hyphal extension, septal formation, and conidium formation (Horiuchi, 2009). *ChsA, ChsB, ChsC, ChsD, ChsE*, or *ChsF* are dispensable for chitin synthesis and growth in *A. fumigatus* (Mellado *et al*., 1996a, 1996b). However, *ChsG* and *CsmA* are required for the proper production of chitin and growth in *A. fumigatus* (Aufauvre-Brown *et al*., 1997; Mellado *et al*., 1996a). Mature chitin can be modified by chitinases responsible for the hydrolysis of chitin. Chitinases cleave the β-1,4 linkages between N-acetylglucosamine residues in the chitin polymer. *A. fumigatus* contains 14 chitinases with compensatory activity among them (Alcazar-Fuoli *et al*., 2011; Jaques *et al*., 2003).
Chitin in *A. fumigatus* can be deacetylated to chitosan within the cell wall and extracellular matrix through the activity of chitin deacetylases (CDA), which results in only glucosamine residues (Lee and Sheppard, 2016). In *A. fumigatus*, two chitin deacetylases are responsible for the production of chitosan, which confers solubility and rigidity to the cell wall (Arbia *et al*., 2013; Gastebois *et al*., 2009). In addition, *A. fumigatus* encodes four chitosanases capable of hydrolysing chitosan. The role of chitosan in *A. fumigatus* remains unknown, but is important for growth in *C. neoformans* and virulence in other fungal plant pathogens (Baker *et al*., 2007; El Gueddari *et al*., 2002).

Localised exclusively in the outer cell wall of several clinically relevant fungi including *H. capsulatum*, *C. neoformans* and *A. fumigatus*, α-1,3-glucan plays an important role in cell wall morphology and virulence (Rapleye *et al*., 2007; Reese *et al*., 2007). This component is a homopolymer of glucose residues linked by α-1,3 linkages, which differs from β-1,3-glucan in the orientation of its axial linkage. In *A. fumigatus*, α-1,3-glucan are a major amorphous cell wall polysaccharide, which accounts for 35 to 40% of the mycelial cell wall and 20 to 25% of the conidial cell wall (Maubon *et al*., 2006). This polysaccharide has a major adhesive role in the interactions between hyphae or germinating conidia and in biofilm formation (Beauvais *et al*., 2007; Fontaine *et al*., 2010). Additionally, the external α-1,3-glucan layer contributes to pathogenesis of *A. fumigatus* by concealing immunostimulatory β-1,3-glucans from detection by host phagocytic cells, thus conferring long term survival in invasive aspergillosis (Beauvais *et al*., 2013; Bozza *et al*., 2009).

The genome of *A. fumigatus* encodes three α-1,3-glucan synthases: Ags1, Ags2 and Ags3. α-1,3-glucan synthases are transmembrane proteins with a large molecular mass of approximately 200 kDa responsible for the continuous synthesis of α-1,3-glucan (Henry *et al*., 2012). All three synthases display a functional redundancy in the production of α-1,3-glucan. However, the lack of α-1,3-glucan caused by mutations triggers changes in the cell
wall organization, such as increased exposure of β-1,3-glucans and presence of an extracellular amorphous glycoprotein matrix over the surface of conidia (Beauvais et al., 2013). Currently, inhibitors of α-1,3-glucan are absent and due to the important role of this polysaccharide in the conidial cell-wall organization the development of future antifungal drugs may be restricted to prophylaxis, rather than the treatment of an established infection (Lee and Sheppard, 2016).

Galactomannans are the main mannose-containing cell wall polysaccharides in the cell wall of A. fumigatus. These are localized in the outer layer of the cell wall and are comprised of mannose and galactofuranose chains. The mannan chain provides the backbone of galactomannan with mannose residues linked in a α1→2 or α1→6 fashion (Latgé et al., 1991). Furthermore, this polysaccharide is embedded with β-1,3 and β-1,6 branching side of four to five β-1,5 linked galactofuranose residues (Latgé et al., 1994, 1991). Galactomannans in A. fumigatus are the only cell wall polysaccharides that are synthetized in the Golgi, where the transport of mannose into the Golgi is mediated by the GDP-mannose transporter, GmtA (Engel et al., 2012). Lack of mannan can affect the cell wall and impairs growth and sporulation. Although the mannan component of this polysaccharide is important for cell wall maintenance and growth, the role of A. fumigatus galactomannans in virulence remains unknown.

Galactosaminogalactans (GAG) are localized in the inner, outer and as part of the extracellular matrix produced by A. fumigatus hyphae. This irregular heteropolymer is a partially deacetylated glycan composed of galactose and N-acetyl-galactosamine linked by a α1→4 linkages (Mouyna and Fontaine, 2009).
The formation of GAG is mediated by the products of five-gene clusters of *A. fumigatus*. The synthesis of GAG is coordinated by the activity of cytosolic epimerases Uge3 and Uge5, which mediate the conversion of UDP-galactose from UDP-glucose and of UDP-N-acetyl-galactosamine from UDP-N-acetyl-glucosamine (Lee *et al*., 2014). Galactosaminogalactans are extruded through the plasma membrane by transmembrane transferases and subsequently the emergent polymer undergoes partial deacetylation by deacetylases (Lee *et al*., 2016).

GAG is a multifactorial virulence factor in *A. fumigatus* involved during biofilm adhesion, concealing cell wall β-1,3-glucans, mediating resistance to neutrophil extracellular traps (NETs) and suppressing host inflammatory responses (Gravelat *et al*., 2013; Lee *et al*., 2015). Although GAG plays multiple roles during pathogenesis in *A. fumigatus*, there are currently no antifungal drugs available to target the synthesis of GAG.

Due to the essential role, unique biochemistry, structural organization and exclusive components that are not present in mammalian cells, the cell wall of *A. fumigatus* is an exquisite target either for the development of novel or the enhancement of the already available antifungal drugs (Lee and Sheppard, 2016). However, despite the unique advantages that targeting the cell wall offer, most of the antifungal drugs available to treat aspergillus infection target the cell membrane and only one class of antifungal drug targets the cell wall.
1.7. Antifungal drugs

In recent years, the global number of clinical cases caused by fungal infections has increased significantly impacting the drug market. Novel antifungal drugs are expensive and long-period therapy is commonly required to achieve a successful curative result, principally in patients with immunosuppressive problems (Allen, 2010).

The number of therapeutic options for the treatment of fungal infections is limited when compared to those used to treat bacterial or viral infections (Roemer and Krysan, 2014). This is mainly because fungi are similar to mammalian cells in terms of their cellular and metabolic nature, thus there are limited pathogen-specific targets available. In the last 40 years, only three major classes of antifungal drugs have been used against invasive and systemic infections: polyenes, azoles, and echinocandins (Table 1.4). The mode of action of these antifungal drugs relies on targeting the cell envelope by disrupting either the cell membrane or the cell wall of fungi (Fig. 1.5).

Although, with the exception of 5-flucytosine, which inhibits the synthesis of nucleic acids, the targets of licenced antifungal drugs are restricted to the plasma membrane and cell wall, certain unconventional fungal agents target fungal virulence factors such as the heat shock protein 90 (Hsp90), translation elongation factor 2 (eEF2), calcineurin, chitin synthases and microtubules (Botet et al., 2008; Lamoth et al., 2013). These include sordarins, tracolimus, polyoxins and griseofulvin (Scorzoni et al., 2017).
<table>
<thead>
<tr>
<th>Group</th>
<th>Mode of action</th>
<th>Drug name</th>
<th>Uses</th>
<th>Dosage</th>
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<tr>
<td>Polyene</td>
<td>Interferes with fungal plasma membrane</td>
<td>Amphotericin B</td>
<td>Most fungal infections</td>
<td>0.5–1.0 mg/kg IV once/day</td>
</tr>
<tr>
<td>Azoles</td>
<td>Inhibition of ergosterol byosynthesis</td>
<td>Fluconazole</td>
<td>Mucosal and systemic candidiasis</td>
<td>100–800 mg po or IV daily</td>
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<td></td>
<td></td>
<td></td>
<td>Cryptococal meningitis</td>
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<td></td>
<td></td>
<td>Coccidioidal meningitis</td>
<td></td>
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<td></td>
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<td>Isavuconazole Aspergillosis</td>
<td>372 mg po or IV q 8 h (6 doses) initially,</td>
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<td>Mucormicosis</td>
<td>then 372 mg po or IV, daily</td>
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<td></td>
<td></td>
<td>Itraconazole Dermatomycosis</td>
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<td>Disruption of the cell wall synthesis</td>
<td>Caspofungin</td>
<td>Aspergillosis, Candidiasis</td>
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<td>70 mg IV on day 1, then 50 mg IV once/day</td>
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<tr>
<td>Anidulafungin</td>
<td>Candidiasis</td>
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</tr>
</tbody>
</table>

Table 1.4. Most common antifungal drugs used to treat systemic fungal infections (Allen, 2010). bid: twice a day. IV: intravenous. P: by month. q: every. tid: three times a day.
Figure 1.5. Mode of action of the most commonly used antifungal drugs: polyenes, azoles and echinocandins. Polyenes and azole target the cell membrane integrity by the formation of pores or toxic sterols, respectively. Echinocandins target production of the cell wall by inhibition of the β-1,3 glucan synthase complex (adapted from Scorzoni et al., 2017).

Polyenes are the oldest known antifungal compounds, of which amphotericin B, first isolated from *Streptomyces nodosus* is the most commonly used antifungal drug against systemic infections (Blum et al., 2013). This natural agent is a macrolide antibiotic, which consists of 7 conjugated double bonds, an internal ester, a free carboxyl group, and a glycoside chain with a primary amino group (Walsh et al., 2008). Amphotericin B binds to ergosterol, the main sterol in the fungal cell membrane, and triggers the formation of aqueous pores in the lipid bilayers. This induces intracellular acidification by impaired ion efflux, which in turn leads to the disruption of the membrane proton gradient and subsequent cell death (Finkelstein and Holz, 1973; Reeves et al., 2004). Furthermore, amphotericin B causes oxidative damage of the cell through lipoperoxidation of the cell membrane (Walsh et al., 2008).
Amphotericin B exhibits fungicidal activity against both yeast and filamentous fungi, including some Zygomycetes (Moen et al., 2009). Interestingly, amphotericin B is also used as an effective treatment against the protozoan *Leishmania*, the causative agent of the visceral leishmaniasis in immunocompetent individuals, which affects over 2.5 million individuals, globally (Coukell and Brogden, 1998; WHO, 2016).

The broad antifungal spectrum of the polyenes has contributed to them being considered the gold standard of antifungal therapy for several decades. However, prolonged use of polyenes is associated with nephrotoxicity, hypokalaemia and infusion-related reactions (Cornely, 2008). Indeed, prolonged administration of polyenes can induce the permeability of K\(^+\) and Na\(^+\) in several human cells, and as a consequence the duration of the antifungal therapy must be limited (Joly et al., 1994). Additionally, polyenes are expensive and unavailable in some regions (Roemer and Krysan, 2014).

Resistance to amphotericin B is rare, but it has become more evident due to the increased incidence of *Candida* species and emerging invasive mould infections (Blum et al., 2013; Ellis, 2002). Resistance is caused by decreased ergosterol content, changed lipid composition, large discharge of intracellular components, less absorption of amphotericin, defence mechanisms against oxidative damage and alterations in sterol to phospholipid ratio, which in turn leads to a reduced amphotericin B binding (Gray et al., 2012; Seo et al., 1999; Sokol-Anderson et al., 1988). The main alterations associated with polyene resistance are enzymes involved in the ergosterol biosynthesis such as sterol isomerases and desaturases, via the reduced oxidative damage by catalases and by inhibition of pores formation by activation of the Ras signalling pathway (Blatzer et al., 2015; Blum et al., 2013; Kanafani and Perfect, 2008; Sanguinetti et al., 2015; Sokol-Anderson et al., 1988).
Resistance to polyenes tend to be species-dependent and emerge slowly in isolates from patients treated with amphotericin B subjected to infections caused by *Candida* spp. *Cryptococcus* sp. *Trichosporon beigeli*, *Aspergillus terreus*, *Scedosporium apiospermum*, *S. prolificans*, *Sporothrix schenckii* and *Fusarium* spp. (Blum et al., 2013; Ellis, 2002).

Since the early 1980s, the azoles have become the most broadly used class of antifungal agents (Lass-Flörl, 2011). Azoles are synthetic compounds that comprise an azole ring attached to an isobutyl core (e.g., voriconazole, isavuconazole) or to an asymmetric carbon atom with a lipophilic complex mixed functional aromatic chain (e.g., posaconazole and itraconazole) (Walsh et al., 2008). Azoles inhibit ergosterol biosynthesis by the inhibition of the 14-α-demethylase (lanosterol demethylase), a fungal cytochrome P450 dependent enzyme (Lamb et al., 1997). Thus, depletion of the ergosterol from the fungal cell membrane impairs membrane fluidity, leading to the accumulation of toxic demethylated sterols, which in turn alters the morphology, arrests the growth and induces fungal cell death (Sheehan et al., 1999; Tatsumi et al., 2013). Azoles are the largest class of antifungal agents and because of the different characteristics in their activity, pharmacodynamics, pharmacokinetics, safety profiles, and species-specific activities; each azole is used in different clinical settings (Table 1.4). Unlike voriconazole and itraconazole, most azoles are fungistatic antifungal drugs (Mohr et al., 2008; Warrilow et al., 2010). In general, fluconazole has broad activity against pathogenic yeast such as *Candida* spp. and *Cryptococcus*, whereas itraconazole, voriconazole and posaconazole exhibit broader activity against yeast and moulds (Lass-Flörl, 2011).

Despite the high activity against fungal invasive infections, resistance to azoles has emerged (Bueid et al., 2010). There are several mechanism of resistance against azoles including activation of efflux pumps, qualitative changes in the target, quantitative changes caused by overexpression of ERG11 and alterations in the cell wall composition (Scorzoni et al., 2017). Typically, azole resistance is associated with either mutations in the cyp51A gene,
which encodes the drug target lanosterol 14-\textit{\textalpha} -demethylase (cyp51A gene) or mutations in the promoter region of the cyp51A gene (Snelders \textit{et al.}, 2008).

The current levels ofazole-resistance worldwide are due to two factors, prolonged exposure of patients to azoles and the increasing use of azoles in agriculture (Bromley \textit{et al.}, 2014; Snelders \textit{et al.}, 2008). Genetically resistant strains have been found in soil and compost due to the high use of azoles in the environment (Verweij \textit{et al.}, 2009). Furthermore, 10\% azole resistance has been reported in The Netherlands (van der Linden \textit{et al.}, 2011). Similarly, the UK National Aspergillosis Centre reported that 20\% of patients isolated are resistant to at least one azole with 10\% being multi-azole resistant (Bueid \textit{et al.}, 2010). In addition, the presence of \textit{A. fumigatus} strains with mutations in the cyp51A gene have been found in Belgium, Denmark, Germany, China, India, Tanzania, Kuwait, and Iran (Denning and Bromley, 2015).

More recently, the introduction of a new class of antifungal drugs, the echinocandins, has provided a new tool for combating invasive fungal diseases. In the period between 2001 and 2006, caspofungin, micafungin and anidulanfungin were developed as fungal cell wall disrupting agents against pathogenic fungi (Scorzoni \textit{et al.}, 2017). Echinocandins are fungal secondary metabolites, which comprise cyclic hexapeptides N-linked to a fatty acyl side chains (Figure 1.6) (Kurtz and Rex, 2001), that disrupt the synthesis of \textbeta}-1,3-\textalpha-glucan (the main component of the fungal cell wall) by non-competitive inhibition (Sawistowska-Schröder \textit{et al.}, 1984). In comparison with the standard antifungal drugs (polyenes and azoles), echinocandins exhibit several attractive attributes such as rapid onset of action, limited cytotoxicity against mammalian cells, minimal drug-drug interaction and activity against fluconazole resistant \textit{Candida} strains (Al-Badriyeh \textit{et al.}, 2009; Lewis and Graybill, 2008).
Figure 1.6. Chemical structures of the licenced echinocandins for the treatment of invasive fungal infections (reproduced from Denning, 2003).

Echinocandins are licenced for intravenous treatment and prevention of invasive *Candida* infections and serve as an alternative choice to the triazoles as salvage, empirical, prophylactic, as well as adjunctive therapy for invasive aspergillosis (Pappas *et al.*, 2016; Roemer and Krysan, 2014). However, echinocandins have low oral bioavailability and are unable to penetrate the central nervous system or eye (Pappas *et al.*, 2009).
Although echinocandins represent the most recent class of antifungal drugs, resistant isolates to all echinocandins are increasingly reported (Garcia-Effron et al., 2010). Echinocandin resistance typically emerges after prolonged therapy, but has been noted shortly after the onset of therapy (Thompson et al., 2008). Echinocandin resistance is due to mutations in the fks genes, activation of cell wall recovery pathways and species-specific intrinsic resistance, overall resulting in therapeutic breakthrough infections (Perlin, 2007). Mutations in the fks genes cause the modification of the encoded enzyme, leading to lower affinity between echinocandins and the catalytic subunit of the β-1,3-glucan synthase complex, the protein Fks. (Gonçalves et al., 2016).

Another form of resistance to echinocandins is associated with a cell wall recovery pathway in which an increased production of usually chitin compensate the lack of β-1,3 glucan caused by echinocandin treatment (Fortwendel et al., 2010; Rueda et al., 2014; Walker et al., 2015). Moreover, several fungal species are intrinsically resistant to echinocandins via several cellular and molecular mechanisms. For instance, in C. neoformans the interaction of echinocandins with the target Fks is avoided by either the wide capsule of polysaccharides that protects the cell wall or via lipid flipases, which mediate echinocandin resistance by blocking drug penetration or enhancing the drug exocytosis (Huang et al., 2016).

1.8. The echinocandin caspofungin

Caspofungin was the first echinocandin to be licensed for clinical use in the United States, marketed as Cancidas® in 2001 (Kartsonis et al., 2003). Clinical use of caspofungin is approved in over 80 countries for treating one or more of the following: oesophageal candidiasis, invasive candidiasis, invasive aspergillosis in patients refractory or intolerant to standard therapy; and empirical therapy of suspected fungal infections in individuals with
neutropenia and persistent fever (McCormack and Perry, 2005; Ngai et al., 2011). Currently, caspofungin is the only echinocandin licensed for treatment of invasive aspergillosis in Europe. Of significance is that caspofungin is cheaper than other conventional antifungal drugs and is as effective as amphotericin B or fluconazole against invasive candidiasis (Bruynesteyn et al., 2007; Mora-Duarte et al., 2002; Villanueva et al., 2002).

Caspofungin acetate, previously known as L-743872 and MK-0991, is a semisynthetic water-soluble amphipathic lipopeptide with a molecular mass of 1213 Da, derivative of pneumocandin Bo, a fermentation product of the fungus Glarea lozoyensis (Bills et al., 1999).

Like other echinocandins, caspofungin is only available for intravenous application. The drug is highly protein bound and shows linear pharmacokinetics (Maertens and Boogaerts, 2003). Caspofungin is applied in daily doses of 50 mg after an initial 70 mg loading dose, which maintains a mean concentration of > 1 µg/ml in individuals with underlying Candida or Aspergillus infections (Stone et al., 2002). Caspofungin undergoes spontaneous peptide hydrolysis and N-acetylation, resulting in the production of an inactive ring-opened peptide and subsequently the drug is excreted in faeces and urine (Deresinski and Stevens, 2003).

Although caspofungin can be effective and safe at doses up to 100 mg daily, several adverse events can develop during treatment, such as flushing, nausea, headache, vomiting, and infusion related phlebitis (Cornely et al., 2007; Mayr et al., 2012).

In yeast, caspofungin acts as a fungicidal drug and shows potent in vitro and in vivo activity against Candida sp., including azole and amphotericin B-resistant strains (Espinell-Ingroff, 1998; Keating and Figgitt, 2003; Swinne et al., 2009). Furthermore, caspofungin is active against C. albicans catheter-associated biofilms (Cateau et al., 2008).
Caspofungin exposure in *Candida* sp. results in a lack of filamentation, leading to clumping and enlarged cells with abnormal septa, as well as lower β-1,3-glucan and higher chitin content in the cell wall (Bizerra *et al*., 2011).

In filamentous fungi, caspofungin has broadly been described as a ‘fungistatic’ antifungal drug (Chen *et al*., 2011; Mayr *et al*., 2012; Sakagami, 2010). Strains of *A. fumigatus* exposed to inhibitory concentrations of caspofungin exhibit several morphological defects, such as abnormal swollen germ tubes, as well as short, stubby and highly branched hyphae with thick cell walls (Kurtz *et al*., 1994). However, caspofungin is unable to totally abolish *A. fumigatus* growth (Bartizal *et al*., 1997). In some cases, caspofungin has preferential effects causing the bursting of the tip of hyphae and inducing hyperbranching in *A. fumigatus* (Bowman *et al*., 2002).

Despite its potent activity against invasive fungal diseases (Table 1.5), caspofungin displays limited activity against some pathogenic fungi such as *Cladophialophora bantiana*, *Pseudallescheria boydii*, *Scedosporium apiospermum*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Acremonium strictum*, *Penicillium* spp., *Bipolaris* spp. (Espinel-Ingroff, 1998; Pfaller *et al*., 1998). Caspofungin is inactive against *C. neoformans*, *Fusarium* spp. and *Trichosporon* spp. (Bal, 2010; Maligie and Selitrennikoff, 2005).
**In vitro activity of Candida spp.**  
Active against most *Candida* spp. Fungicidal against *C. albicans, C. glabrata, C. tropicalis, C. kefyr* and *C. pelliculosa*. Also active against *C. parapsilosis, C. krusei, C. guilliermondii* and *C. lusitaniae*.

**In vitro activity of Aspergillus spp.**  
Generally fungistatic against *A. fumigatus, A. flavus, A. niger, A. versicolor* and *A. terreus*.

**Animal studies in candidiasis**  
Displayed fungicidal activity, reduced fungal burden and prolonged survival in immunocompetent and severely immunocompromised models of invasive candidiasis, including infections caused by azole-resistant *Candida* spp.

**Animal studies in aspergillosis**  
Prolonged survival in experimental models of disseminated or pulmonary aspergillosis, but with varying effects on fungal burden in tissues.

**Pharmacokinetic/pharmacodynamics data**  
|$C_{\text{max}}$ : MEC ratio most closely associated with reductions in fungal burden in murine model of invasive pulmonary aspergillosis and the AUC : MIC ratio in a non-neutropenic murine model of candidiasis

Table 1.5. Pharmacodynamic properties of caspofungin. $C_{\text{max}}$: Maximum plasma concentration; MEC: Minimal effective concentration; AUC: area under the plasma concentration-time curve; MIC: Minimal inhibitory concentration (reproduced from McCormack and Perry, 2005).
The efficacy of caspofungin in animal models was the most important step towards introducing this compound into clinics for the treatment of fungal infections. However, despite the clinical benefits against fungal infections, caspofungin at high concentrations shows an attenuated activity in some species of *Candida* and *Aspergillus* (Fortwendel *et al*., 2010; Stevens *et al*., 2004).

1.9. The paradoxical effect of caspofungin

Typically, the activity of an antibiotic or antifungal drug is proportional to drug concentration, and thus it is expected to have an increased effect with higher drug concentrations. In 1948 it was reported that *Staphylococcus aureus*, β-hemolytic *Streptococci* and *Enterococcus faecalis* were killed slower by high concentrations of benzylpenicillin, a response called the ‘paradoxical zone phenomenon’ (Eagle and Musselman, 1948). This phenomenon, known as the ‘Eagle effect’, also occurs in several other bacterial species treated with quinolone antibiotics, nalidixic acid, ciprofloxacin, norfloxacin and sparfloxacin (Cantón *et al*., 1993; Carret *et al*., 1991; Crumplin and Smith, 1975; Diver and Wise, 1986; Piddock *et al*., 1990).

In 1988, a similar phenomenon to the ‘Eagle effect’ was demonstrated in echinocandin treatment of fungi. Cilofungin (LY121019), the precursor of all echinocandins, displayed a growth inhibition effect at low concentrations against various isolates of *C. albicans* and *C. tropicalis*. However, in some isolates this inhibitory effect was disrupted at higher concentrations, similar to that observed with untreated isolates (Hall *et al*., 1988). The clinical relevance was unexplored because cilofungin was not approved for clinical use. Similarly, a paradoxical attenuation of the antifungal activity of caspofungin against *C. albicans* spp. and *Aspergillus* spp. was reported (Petraitis *et al*., 1998; Ramage *et al*., 2002; Stevens *et al*., 2004).
The paradoxical effect (PE) in both *Candida* and *Aspergillus* spp. displays a quadriphasic nature as follows: growth of the fungal pathogen below the MIC (Phase 1); inhibition of growth above the MIC (Phase 2); reduction of growth inhibition at higher concentrations (Phase 3); total inhibition of growth at the highest concentration of antifungal drug (Phase 4) (Fig. 1.7) (Stevens et al., 2004). Significantly, this quadriphasic phenomenon is echinocandin-specific, species-specific and strain-specific (Jacobsen et al., 2007; Mariné et al., 2009).

Figure 1.7. Quadriphasic nature of the paradoxical growth effect of caspofungin on the growth of *Candida* and *Aspergillus* species. Phase 1: Subinhibitory phase; Phase 2: Inhibition of growth above the MIC; Phase 3: Attenuation of activity at higher concentrations; Phase 4: Inhibition of growth at the highest drug concentration. PGE: Paradoxical growth effect (reproduced from Stevens et al., 2004).
The paradoxical effect is not a consequence of a resistant sub-population because of mutations in the gene encoding target Fks, upregulation of the glucan synthase complex or drug degradation (Rueda et al., 2014; Stevens et al., 2004). Rather it depends on several cellular mechanisms responsible for the fungal cell adaptation to environmental stresses, such as the protein kinase C (PKC) cell wall integrity pathway, the high-osmolarity glycerol response (HOG) pathway, the heat shock protein 90 (Hsp90) and the protein calcineurin (Kaneko et al., 2009; Popolo et al., 2001; Wiederhold et al., 2005). Remarkably, these pathways regulate the cell wall response, resulting in the increased synthesis of cell wall chitin (Munro et al., 2007).

The PE occurs in approximately 15-30% of Candida isolates (Fleischhacker et al., 2008; Stevens et al., 2004). Isolates from cancer patients display a greater percentage of strains that develop PE than previously reported, including 90% of 20 C. albicans isolates against caspofungin treatment (Chamilos et al., 2007). In C. albicans in the presence of high doses of caspofungin, β-1,3-glucan and β-1,6-glucan are decreased, whereas chitin content is increased (Stevens et al., 2006). Furthermore, the cell wall ultrastructure of C. albicans, C. tropicalis, C. orthopsilosis and C. parapsilosis during PE displayed budding cells without clear rings around constrictions between mother and daughter cells, with abnormal thickness of the septa, enlarged yeast forming clumps, and the absence of filamentation (Bizerra et al., 2011).

The in vivo development of PE in animal models of candidiasis is unclear. Isolates of C. albicans that develop paradoxical growth in vitro are unable to show a clear paradoxical growth in a murine model of invasive candidiasis by either survival rate or kidney colony forming unit recovery (Clemons et al., 2006). Furthermore, C. albicans pre-exposed to high-dose caspofungin showed less virulence in the wax moth larvae Galleria mellonella.
Those larvae developed rapid melanisation and a decreased number of hyphae in tissue (Rueda et al., 2014). However, C. albicans and C. tropicalis, with or without the paradoxical effect, show no difference in virulence in a Drosophila model of candidiasis infection (Zanette and Kontoyiannis, 2013).

Although less well understood, PE also occurs in several Aspergillus species. The in vitro concentration-dependent effects of caspofungin, micafungin and anidulafungin against germinated and non-germinated A. fumigatus, A. terreus, and A. flavus conidia revealed paradoxical increases in metabolic activity at higher concentrations (Antachopoulos et al., 2008). Notably, caspofungin triggers the greatest number of strains to display PE at lower concentrations. In A. fumigatus, the presence of high doses of caspofungin during 24 h induces cell abnormalities including irregular, short, hyperbranching hyphae. However, normal hyphae growth develops after 72 h in presence of high-dose caspofungin (Loiko and Wagener, 2016). This cellular response is dependent on the expression of the glucan synthase Fks1 (the target of caspofungin) and seems to be independent of the chitin compensatory mechanism.

Evidence about paradoxical effect in vivo in Aspergillus sp. is more limited than in Candida sp. The first mention of paradoxical effect in A. fumigatus was in a rabbit model of invasive aspergillosis, wherein rabbits administered with high doses of caspofungin display improved survival, reduced organism mediated injury but with no fungal burden reduction or galactomannan antigenemia (Petraitiene et al., 2002). In murine model(s) of invasive aspergillosis caspofungin and micafungin each exhibited dose-dependent pharmacodynamic activity and reduction in fungal burden, but caspofungin shows a steeper dose-response curve and a modest paradoxical increase in fungal burden (Lewis et al., 2008).
Furthermore, studies of murine invasive aspergillosis models using increasing doses of caspofungin, therapeutic efficacy was dose-dependent at 0.1 mg/kg/day and 1 mg/kg/day, but there was a paradoxical increase in pulmonary fungal burden and inflammation at 5 mg/kg/day (Moretti et al., 2012). Interestingly, higher doses of caspofungin enhance inflammatory pathology by recruiting more neutrophils.

Based on the indirect evidence of the development of PE in animals, the possible occurrence of PE in patients is intriguing. A multicentre, trial of standard-dose caspofungin (50 mg/day) vs high-dose caspofungin (150 mg/day) showed similar favourable responses between treatments (Betts et al., 2009). Similarly, a second multicentre clinical trial evaluated micafungin at 2 doses (100 mg and 150 mg) vs caspofungin at standard maintenance dosing (50 mg/day) with invasive candidiasis and both treatments showed similar patient responses with no differences in adverse events (Pappas et al., 2007).

The discovery of the PE in vitro in the most pathogenic fungi in humans, arouse concerns about diminished antifungal activity of higher echinocandin doses, particularly in patients infected with less sensitive fungi or infections at location difficult to reach in the human body. Thus, the study of the main cellular and molecular responses in vitro of A. fumigatus to caspofungin are pivotal in order to understand the adaptations that trigger the paradoxical effect.
2. Aims of the research described in this thesis

Caspofungin has been shown to disrupt the β-1,3-glucan synthase in pathogenic fungi. However, little is known about the cellular and molecular responses and adaptations that A. fumigatus develop in order to overcome caspofungin activity. Therefore, the overall objective of my PhD research was to investigate experimentally the cellular and molecular basis of the morphological responses of Aspergillus fumigatus upon caspofungin treatment with a particular emphasis on the paradoxical effect. In order to do this I have combined advanced live-cell imaging techniques with mutant analysis and the generation of various reporter strains in which I have labelled specific proteins as using previously generated reporter strains. I have also performed Real Time PCR to analyse global changes in gene expression associated with responses to caspofungin.

The primary aims of the research described in each of my thesis chapters were:

1. To study the major morphological responses of Aspergillus fumigatus upon caspofungin treatment (Chapters 3 and 4).
2. To understand the fundamental underlying cellular and molecular process of caspofungin’s limited fungicidal activity at high concentrations (Chapter 3).
3. To investigate the timing and development of the development of the paradoxical growth caused by caspofungin in Aspergillus fumigatus at high resolution at the subcellular and cellular levels (Chapters 3 and 4).
4. To analyse the subcellular dynamics and global changes in gene expression of the primary target and catalytic subunit, Fks1, and the regulatory subunit, Rho1of the β-1,3-glucan synthase complex in response to caspofungin treatment (Chapters 3 and 4).
5. To analyse the dynamic response of the Ca^{2+}-related transcription factor, CrzA, and cell-wall adaptations of A. fumigatus to caspofungin treatment (chapter 5).


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Chapter 2

4. Materials and methods

2.1. Materials

Chemicals were purchased from Roche (UK), Merck (UK), Sigma (UK), Difco (UK), and Gibco (UK), unless otherwise stated. Restriction enzymes were obtained from New England Biolabs (NEB, UK). Enzymes for standard PCR were purchased from TAKARA (Clontech, Madison, USA) or New England Biolabs (NEB, UK). Enzymes for real-time PCR were purchased from Bioline (ThermoFisher Scientific, UK).

Nitrocellulose membranes (Amersham, Hybond™-N+, UK) for Southern blots were purchased from GE Healthcare Life Science, chromatography paper from Whatman (UK), filter paper (Miracloth) from Calbiochem (UK) and the Blocking-Reagent from Roche (UK). Protein Marker was obtained from (BioRad, UK). For DNA-isolation and amplicon observation agarose molecule grade (Bioline, UK) was used routinely. DNA was stained with Safe View (NBS Biologicals, UK). The 1 kb-DNA-marker was purchased from Biolabs (NEB, UK).

With the exception of Alexa fluor 488 goat anti-mouse IgM (Life technologies, UK), all fluorescent dyes used in the present study (Table 2.1) were purchased from Sigma (UK). Solutions, dyes, inhibitors and buffers were prepared using double distilled water (ddH2O), ethanol (Fisher Scientific, UK) or DMSO (Thermo Scientific, USA) according to the manufacturer’s instructions. Solutions referred to as sterile were autoclaved for 15 min at 121 °C, 15 psi, unless otherwise stated. All the kits used in this study are shown in Table 2.2.
<table>
<thead>
<tr>
<th>Dye</th>
<th>Target</th>
<th>Final concentration</th>
<th>Ex/Em&lt;sup&gt;*&lt;/sup&gt; (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline blue</td>
<td>Cell wall β-1,3-glucan</td>
<td>100 µg/ml</td>
<td>405/450-550</td>
</tr>
<tr>
<td>Alexafluor 488 goat anti-mouse IgM</td>
<td>Cell wall α-1,3-glucan</td>
<td>10 µg/ml</td>
<td>488/520-570</td>
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<tr>
<td>Fluorescent brightener 28</td>
<td>Cell wall chitin</td>
<td>1 µg/ml</td>
<td>405/450-550</td>
</tr>
<tr>
<td>Cell mask deep red</td>
<td>Plasma membrane</td>
<td>1:1000 dilution</td>
<td>660/670-790</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>α-mannans</td>
<td>5-10 µg/ml</td>
<td>488/505-520</td>
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<tr>
<td>7-amino-4 chloromethyl- coumarin (CMAC)</td>
<td>Vacuoles</td>
<td>5-10 µM</td>
<td>405/450-550</td>
</tr>
<tr>
<td>FM4-64</td>
<td>Membranes</td>
<td>2.5-5 µM</td>
<td>515/&gt;550</td>
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<tr>
<td>Nile Red</td>
<td>Lipid droplets</td>
<td>1-10 µg/ml</td>
<td>563/&gt;590</td>
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<tr>
<td>Propidium iodide</td>
<td>Cell death marker</td>
<td>5 µg/ml</td>
<td>543/&gt;550</td>
</tr>
<tr>
<td>WGA-Alexa Fluor 488</td>
<td>Cell wall chitin</td>
<td>0.5 µg/ml</td>
<td>488/520-570</td>
</tr>
</tbody>
</table>

Table 2.1. Fluorescent dyes used in the present study for confocal microscopy.

<sup>*</sup> Abbreviations: Ex, excitation; Em, emission. Excitation and fluorescent emission maxima in nm.
Table 2.2. Kits used in this study.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Manufacturer</th>
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<td>PCR DIG Probe synthesis kit</td>
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<tr>
<td>DNeasy® Plant Mini kit</td>
<td>QIAGEN, Germany</td>
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<tr>
<td>QIAquick® PCR Purification kit</td>
<td>QIAGEN, Germany</td>
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<tr>
<td>QIAquick® Gel Extraction kit</td>
<td>QIAGEN, Germany</td>
</tr>
<tr>
<td>RNeasy® Plant Mini kit</td>
<td>QIAGEN, Germany</td>
</tr>
</tbody>
</table>

2.1.2. Primers

All primers used in this study were dissolved in sterile water to a final concentration of 10 µM (Table 2.3) and 5 µM (Table 2.4) for standard PCR and real-time PCR, respectively. Primers were designed using serial cloner (2.6.1), purchased from Eurofins (UK) and stored at -20 ºC.

<table>
<thead>
<tr>
<th>VAM3-GFP amplification</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1_Vam3_fwd</td>
<td>GACCACCCCTGATCATTCTG</td>
</tr>
<tr>
<td>P2_Vam3_rev</td>
<td>TAGTTCTGTACCAGGACGGATAGGTATGTTGCGG</td>
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<tr>
<td></td>
<td>CAGGA</td>
</tr>
<tr>
<td>P3_Vam3_ORF_Fd</td>
<td>GGAGCTGGTGCAAGGCCAGCTGGAGGCCGTGCATGTC</td>
</tr>
<tr>
<td></td>
<td>CTTCGATCGTTTGAG</td>
</tr>
<tr>
<td>P4_Vam3_ORF_Rev</td>
<td>AGCAGCAACTATCCTCGGTGT</td>
</tr>
<tr>
<td>Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>P5_Vam3_nstd_Fd</td>
<td>GAATAGTCCGGCAGAATCCA</td>
</tr>
<tr>
<td>P6_Vam3_nstd_Rv</td>
<td>GACCACCCCCTGATCATTTGTG</td>
</tr>
<tr>
<td>tagRFP-T_out_fwd</td>
<td>ATACTGCGACCTCCCTAGCA</td>
</tr>
<tr>
<td>vam3_check_rev</td>
<td>GCGTGTTGAGTACGTCATTG</td>
</tr>
<tr>
<td>Vam3 Fwd probe</td>
<td>ATGTCCTTCGATCGTTTGAG</td>
</tr>
<tr>
<td>Vam3 Rev probe</td>
<td>CTATCCGAGTGTGACTGCTAGG</td>
</tr>
<tr>
<td>Rho1-GFP amplification</td>
<td></td>
</tr>
<tr>
<td>P1 Fwd LB Rho1</td>
<td>GTAATGCGCATGGTTTTAAG</td>
</tr>
<tr>
<td>P2 Rev LB Rho1</td>
<td>TAGTTCTGTACCGAGCCGGGATACACACTAGTACATGGCCA</td>
</tr>
<tr>
<td>P3 Fwd ORF Rho1</td>
<td>GGAGCTGGTGCGGCGCTGGAGCCGTCATGG</td>
</tr>
<tr>
<td></td>
<td>CTGAAATCCGCC</td>
</tr>
<tr>
<td>P4 Rev ORF Rho1</td>
<td>GAAGACAACGCCTCCCTAAAG</td>
</tr>
<tr>
<td>P5 Fwd prom Rho</td>
<td>GCTCTGAACGATATGCTCCCTAAATGGAGTAGGA</td>
</tr>
<tr>
<td></td>
<td>AATTGGGG</td>
</tr>
<tr>
<td>P6 Rev prom Rho</td>
<td>ATCCACTTAACGTTACTGAAATCCTTGGAAGGAGA</td>
</tr>
<tr>
<td></td>
<td>CAATGATCA</td>
</tr>
<tr>
<td>P7 PptrA_rev</td>
<td>GTTTCAAGTTGCAAATGACTATCATC</td>
</tr>
<tr>
<td>P8 hph_ATG_fwd</td>
<td>TAGTCATTGCAAACCTTTGAAACATGCTGAACCTACC</td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>P9 phleo_ATG_fwd</td>
<td>TAGTCATTGCAACTTGAAGACTAGTCATTGCAACTT</td>
</tr>
<tr>
<td></td>
<td>GAAAC</td>
</tr>
<tr>
<td>P10 phleo_rev</td>
<td>GGGAGCATATCGTTCAGAGCTCAGTCTGCTCCTC</td>
</tr>
<tr>
<td></td>
<td>GGCC</td>
</tr>
<tr>
<td>P11 nest LB Rho</td>
<td>CTTCTGTTCGTCCTTTTGCTCTG</td>
</tr>
<tr>
<td>P12 nest RB Rho</td>
<td>CAGATAATTCCCATGAAGCTC</td>
</tr>
<tr>
<td>P14 Fw Rho1 HR</td>
<td>GGACTCGTGAGGAAGATTAA</td>
</tr>
</tbody>
</table>

Table 2.3. Primers for PCR used in this study. Fwd: forward primer; Rev: reverse primer; ORF: open reading frame; Nstd: nested primer; Prom: promoter; ptrA: pyrithiamine; hph: hygromycin B; phleo: phleomycin; HR: homologous recombination; LB: left border; RB: right border.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>fks1</td>
<td>AFUB_078400</td>
<td>Fwd: ACGACAGATCCGGGTACTAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: GATTATCGTAGCCCTGCTG</td>
</tr>
<tr>
<td>rho1</td>
<td>AFUB_072830</td>
<td>Fwd: GTCTTCTGATTGTCTTCTCCAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: GACGGTCATAATCTTCTCTGA</td>
</tr>
<tr>
<td>β-tubulin</td>
<td>AFUB_010330</td>
<td>Fwd: AACGAGGCTCTGTACGACAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: AACTTGCAGCATCAGAGTT</td>
</tr>
<tr>
<td>Gpda</td>
<td>AFUB_050490</td>
<td>Fwd: GAGCTCAAAAAACATCCTCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: GACGAGCTTGACGAAGTTG</td>
</tr>
<tr>
<td>Actin</td>
<td>AFUB_093550</td>
<td>Fwd: TAACATTGTCATGTCTGTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: AACCACCAATCCACACAGA</td>
</tr>
<tr>
<td>pma1</td>
<td>AFUB_041460</td>
<td>Fwd: CTGGTGATATTATCGTTGTTGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: TCATCGTCCTTCTCTTTGA</td>
</tr>
</tbody>
</table>

Table 2.4. Primers used for real-time PCR.
2.1.3. Organisms and plasmids

The human pulmonary carcinoma alveolar cell-line A549 used for infection assays was obtained from ATCC® (CCL-185™).

The following *Aspergillus fumigatus* (Table 2.5) and *Escherichia coli* (Table 2.6) strains were used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Function</th>
<th>Genotype</th>
<th>Parental strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA10</td>
<td>Wild-type</td>
<td>-</td>
<td>-</td>
<td>Monod <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Af293</td>
<td>Wild-type</td>
<td>-</td>
<td>-</td>
<td>Bowyer, P., Gift</td>
</tr>
<tr>
<td>ΔakuB</td>
<td>Ku DNA helicase</td>
<td>akuBΔ::pyrG</td>
<td>CEA17</td>
<td>da Silva Ferreira <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>Fks1-GFP</td>
<td>1,3-β-glucan</td>
<td>fks1::gfp::hph&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A1160</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>synthase</td>
<td>catalytic subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-Rho1</td>
<td>1,3-β-glucan</td>
<td>gfp::rho1::pyrG&lt;sup&gt;-&lt;/sup&gt;</td>
<td>A1160</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>synthase</td>
<td>regulatory subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFP-pyrG</td>
<td>Cytoplasmic GFP</td>
<td>βtub::gfp::pyrG&lt;sup&gt;-&lt;/sup&gt;</td>
<td>A1160</td>
<td>Bromley, M Gift</td>
</tr>
<tr>
<td>RFP-VAM3</td>
<td>Vacuoles</td>
<td>gfp::vam3::ptrA&lt;sup&gt;R&lt;/sup&gt;</td>
<td>A1160</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2.5. Strains of *A. fumigatus* used in this study. CEA17: uracil auxotroph of *A. fumigatus* clinical isolated CEA10. ΔakuB: mutant deficient for nonhomologous end joining recombination.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F' φ80, lacZ(M15 Δ(lacZYA-argF), U169, recA1, endA1, hsdR17, (rk-, mk+) phoA, supE44, λ–, thi-1, gyrA96, relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Top10</td>
<td>F' mcrA Δ(mrr-hsdRMS-mcrBC), φ80, lacZ ΔM15 ΔlacX74, nupG, recA1, araΔ139 Δ(ara-leu) 7679, galE15 galK16 rpsL(StrR) endA1 λ-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Top10F'</td>
<td>F'[lacQ, Tn10 TetR]) mcrA, Δ(mrr-hsdRMS-mcrBC), φ80, lacZ ΔM15 ΔlacX74, deoR, nupG, recA1, araD139 Δ(ara-leu) 7679, galU, galK, rpsl(StrR) endA1, λ-</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 2.6. *E. coli* strains used in this study.

2.2. Methods

2.2.1. Growth and maintenance of *Aspergillus fumigatus*

All manipulations of *A. fumigatus* were carried out in a Class II microbiological safety cabinet Bio2+ (ENVAIR, UK). The work surface of the cabinet was cleaned thoroughly before and after each experiment by the addition of 1 % (v/v) CHEMGENE (Medimark Scientific, UK) followed by 70 % (v/v) of industrial methylated spirit (Fischer Scientific, UK).

Minimal and complete media for *A. fumigatus* were prepared accordingly (Pontecorvo *et al.*, 1953; Vogel, 1956) (Table 2.7). The *A. fumigatus* strains were grown on minimal or complete medium plates and supplemented with vitamins, amino acids and nucleotides when necessary (Table 2.8). *Aspergillus fumigatus* isolates were grown in tissue culture flasks containing 10 ml of ACM or Sabouraud dextrose agar (OXOID, UK), and inoculated spores were incubated at 37 °C for 5 days or until confluent growth was obtained. Subsequently,
spore suspensions were prepared by adding 10 ml of sterile 0.05% (v/v) Tween 20, tightening the lid of the tissue culture flask and shaking vigorously to dislodge the spores. Spore suspensions were filtered through a sterile filter paper (Miracloth), and then the suspension was centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was removed and spores were re-suspended in 1 ml of sterile 0.05% (v/v) Tween 20. For each spore suspension, the concentration was determined by counting using a Haemocytometer chamber (Superior, DE). Spore suspensions were adjusted in 0.05% Tween 20 to 2x10^7/ml and 1x10^6/ml for colony growth and microscopy analyses, respectively.

For short term storage, tissue culture flasks with confluent mycelium or spore suspension were stored at 4 °C. For long-term storage, 500 µl of spore suspension was mixed with 500 µl of 80 % glycerol in sterile CryoTubes (Nunc, Thermo Fisher Scientific, UK) and stored at -80°C.

<table>
<thead>
<tr>
<th>Media or stock</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Minimal medium (MM)</strong> (1 L)</td>
<td>50 ml 20x salt solution; 1 ml 1000x Trace elements; 20 g glucose; pH 6.5 with 10 N NaOH; 15 g agar when solid medium required.</td>
</tr>
<tr>
<td><strong>Complete medium (CM)</strong> (1 L)</td>
<td>MM with 2 g peptone; 1 g yeast extract; 1 g casamino acids; 1 ml vitamin solution; 1 ml trace elements; pH 6.5 with 10 N NaOH; 15 g agar when needed.</td>
</tr>
<tr>
<td><strong>20x Salt solution</strong> (1 L)</td>
<td>120 g NaNO₃; 10.4 g KCl; 10.4 g MgSO₄·7H₂O; 30.4 g KH₂PO₄</td>
</tr>
<tr>
<td><strong>1000x Trace elements</strong></td>
<td>22 g ZnSO₄·7H₂O; 11 g H₃BO₃; 5 g MnCl₂·4H₂O; 5 g FeSO₄·7H₂O; 1.6 g CoCl₂·5H₂O; 1.6 g CuSO₄·5H₂O; 1.1 g</td>
</tr>
</tbody>
</table>
(1 L) \( (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}; 50 \text{ g Na}_4\text{EDTA}; \text{pH 6.5-6.8 with KOH} \)

**1000x Vitamin solution** (1 L)

0.1 g D-biotin; 0.1 g pyrodoxin-HCl; 0.1 g thiamin-HCl; 0.1 g riboflavin; 0.1 g p-aminobenzoic acid; 0.1 g nicotinic acid

**Vogel’s media (VM)** (1 L)

20 ml 50 x Vogel’s salts; 15 g sucrose; 15 g agar when needed

**50x Vogel’s salts** (1 L)

150 g \( \text{Na}_3\text{Citrate-5.5 H}_2\text{O} \); 250 g \( \text{KH}_2\text{PO}_4 \), anhydrous; 100 g \( \text{NH}_4\text{NO}_3 \), anhydrous; 10 g \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \); 5 g \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \); 5 ml Vogel’s trace elements; 2.5 ml biotin Solution (100 \( \mu \text{g/ml} \)). Add 2 ml of chloroform as preservative. Store at room temperature.

**Vogel’s trace elements**

In 95 ml distilled water, dissolve successively with stirring:

(100 ml)

5 g Citric acid·1 H2O; 5 g \( \text{ZnSO}_4 \cdot 7\text{H}_2\text{O} \); 1 g \( \text{Fe(NH}_4)_2\text{(SO}_4)_2 \cdot 6\text{H}_2\text{O} \); 0.25 g \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \); 0.05 g \( \text{MnSO}_4 \cdot 1\text{H}_2\text{O} \); 0.05 g \( \text{H}_3\text{BO}_3 \), anhydrous; 0.05 g \( \text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O} \); 2 ml chloroform.

---

Table 2.7. Growth media and stock solutions for *A. fumigatus*. 

---

110
<table>
<thead>
<tr>
<th>Components</th>
<th>Stock concentration</th>
<th>Volume or weight per litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>0.05%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Nicotinamid</td>
<td>0.5%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Pyridoxin-HCl</td>
<td>0.1%</td>
<td>1 ml</td>
</tr>
<tr>
<td>p-Aminobenzoic acid (PABA)</td>
<td>0.1%</td>
<td>1 ml</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.25 %</td>
<td>1 ml</td>
</tr>
<tr>
<td>Arginine</td>
<td>-</td>
<td>1.2 g</td>
</tr>
<tr>
<td>Uracil</td>
<td>-</td>
<td>1 g</td>
</tr>
<tr>
<td>Uridine</td>
<td>-</td>
<td>1.2 g</td>
</tr>
<tr>
<td>Methionine</td>
<td>1 %</td>
<td>3 ml</td>
</tr>
</tbody>
</table>

Table 2.8. Vitamins, amino acids and medium components.

2.2.2. Growth and maintenance of *Escherichia coli*

Media for *E. coli* (Table 2.9) were prepared and appropriately supplemented with antibiotics (Table 2.10), as previously described (Sambrook and Russell, 2001). All ingredients were added to ddH₂O, poured into bottles with loosened caps and autoclaved. For solid media, agar (15 g/L) was added. Heat-sensitive solutions, such as antibiotics, amino acids and vitamins were filter-sterilized with 0.22 μm pore filter membrane (ELKay, UK) and subsequently added to the media when they were at roughly 50 °C.
**Medium** | **Ingredients for 1 litter**
---|---
Luria-Bertani (LB) | 10 g tryptone; 10 g yeast extract; 5 g NaCl, pH 7.5
SOC | 20 g tryptone; 5 g yeast extract; 0.58 g NaCl; 0.185 g KCl; 2.03 g MgCl$_2$ · 7H$_2$O; 2.46 g MgSO$_4$ · 7H$_2$O; 3.6 g glucose

Table 2.9. Media used for *E. coli*.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Stock solution</th>
<th>Final concentration</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (Amp)</td>
<td>50 mg/ml in ethanol</td>
<td>100 µg/ml</td>
<td>-20 ºC</td>
</tr>
<tr>
<td>Kanamycin (Kan)</td>
<td>10 mg/ml in water</td>
<td>50 µg/ml</td>
<td>-20 ºC</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 mg/ in water</td>
<td>50 µg/ml</td>
<td>-20 ºC</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5 mg/ml in ethanol</td>
<td>25 µg/ml</td>
<td>-20 ºC</td>
</tr>
<tr>
<td>X-Gal</td>
<td>25 mg/ml in DMF</td>
<td>40 µg/ml-25 µl/plate</td>
<td>-20 ºC</td>
</tr>
<tr>
<td>IPTG</td>
<td>24 mg/ml in water</td>
<td>8 µg/ml-40 µl/plate</td>
<td>-20 ºC</td>
</tr>
</tbody>
</table>

Table 2.10. Antibiotics and supplements for *E. coli* media. For the storage of *E. coli* strains, freshly grown suspensions of bacteria were adjusted with 15% of sterile glycerol (final concentration) and frozen at -80 ºC.
2.2.3. Growth and maintenance of the alveolar epithelial cell line, A549

The human pulmonary carcinoma alveolar cell line A549 was maintained by serial passage in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 X foetal bovine serum, 1x L-glutamine, and 1x penicillin/streptomycin and maintained at 37°C in the presence of 5% CO₂. Cell viability, morphology and confluence were observed by light microscopy. When ≥ 90% confluence was observed, cells were split into more flasks at 1:2 split ratio. For this purpose, the medium was removed and cells were rinsed with 10 ml of Ca²⁺ and Mg²⁺ free DPBS followed by addition of 5 ml of 1x trypsin-EDTA solution and incubation for 10 min at 37°C in the presence of 5% CO₂. Trypsin was inactivated by adding an equal volume of DMEM supplemented with serum and the cellular suspension was centrifuged at 400 rcf for 10 min at room temperature. The supernatant was removed and the resulting pellet was suspended in fresh medium and subsequently stored in a Galaxy 170 S carbon dioxide humidifier incubator (Eppendorf, UK) at 37°C in the presence of 5% CO₂.

Culturing was routinely carried out in 75 cm² tissue culture flasks in a total volume of 15 ml per flask and sub-cultured twice per week. All tissue culture maintenance and experiments were performed in a Class II microbiological safety cabinet BioMAT-2 (CAS, UK). Cells were monitored daily by microscopy for bacterial/fungal infection by observing the status of the medium and the morphology of the human cells. Cells used were 30 passages old after which cultures were discarded. For long term storage, A549 cells were stored in liquid nitrogen to maintain a maximum possible viability. For this purpose, cells were harvested by addition of trypsin and centrifugations, as previously described.

The pellet was re-suspended in 6 ml of freezing medium (50% FCS, 20% DMSO and 30% unsupplemented DMEM). Aliquots of 2 ml of cell suspension were added to CYRO.S cryo vials. Vials were transferred to a Mr. Frosty freezing chamber (Merck, UK) containing pure isopropanol for 24 h and subsequently transferred to a liquid nitrogen container.
2.3. Bioinformatics

2.3.1. BLAST search analysis

BLAST searchers (Altschul et al., 1997, 1990) were used in order to identify homologues of known proteins in the sequenced genome of the *A. fumigatus* strain A1163. Proteins of interest were identified in *A. fumigatus* and *A. oryzae* by literature searches. Subsequently, the sequences of the proteins were obtained from www.EnsemblFungi.org, www.aspergillusgenome.org and the Broad Institute (www.broad.mit.edu/). The sequences of interest were used in order to design primers for the amplification of DNA fragments used for mutations or for the design of exon-exon primers for the measurement of gene expression by real-time PCR.

2.4. Molecular methods

2.4.1. Plasmid DNA preparation of *Escherichia coli*

Isolation of plasmid DNA was done by using the alkali-lysis method, as previously described (Sambrook and Russell, 2001). Briefly, 2 ml of LB broth containing ampicillin (100 µg/ml) and a single colony of transformed bacteria were incubated overnight at 37 ºC with vigorous shaking. The suspension was centrifuged at 13,000 rpm for 1 min. The medium was removed by aspiration and the bacterial pellet was re-suspended in 200 µl of Tris-EDTA buffer, 200 µl of alkali-lysis buffer were added and incubated for 5 min at room temperature, followed by the addition 200 µl of neutralization buffer and incubation for 5 min on ice.

The suspension was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to a new tube. Following the addition of 500 µl of isopropanol, the suspension was centrifuged at 14,000 rpm for 20 min. The supernatant was removed and 500 µl of ethanol (70%) were added, then the suspension was centrifuged at 14,000 rpm for 10 min. The supernatant was removed and the pellet was air dried at room temperature.
Finally, the pellet was dissolved in 20 µl of TE buffer and incubated for 10 min at 68 °C. The concentration of plasmid DNA was calculated using a Synergy2 microplate reader (BioTek). The DNA solution was stored at -20 °C for subsequent analyses. The solutions for plasmid isolation and the plasmids used in this study are shown in the Table 2.11 and Table 2.12, respectively.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA buffer</td>
<td>25 mM Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>10 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>100 µg/ml ARNase, pH 8</td>
</tr>
<tr>
<td>Alkali-lysis buffer</td>
<td>0.2 M NaOH</td>
</tr>
<tr>
<td></td>
<td>1% SDS</td>
</tr>
<tr>
<td>Neutralization buffer</td>
<td>1.5 M Potassium acetate, pH 4.8</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA pH 8</td>
</tr>
</tbody>
</table>

Table 2.11. *E. coli* plasmid isolation solutions.
Plasmid | Construction | Source |
---|---|---|
PpCR2.1-TOPO | Cloning vector | Invitrogen |
PpSK379 | gpdA::ptrA::amp | (Bertuzzi et al., 2014) |
PpAN.7.1 | gpdA::hph::amp | FGSC |
PpCoS230 | pyrG::amp | Seidel, unpublished |
PpCoS234a | pyrG::gpdA::gfp::amp | Seidel, unpublished |
PpSDM1 | pyrG::gpdA::gfp::rho1 | This study |

Table 2.12. Plasmids used in this study. FGSC: Fungal Genetics Stock Center

2.4.2. Genomic DNA extraction of *Aspergillus fumigatus*

Fungal genomic DNA was extracted from mycelium grown for 16-18 h in complete medium. Mycelium was harvested with a sterile spatula, washed twice with distilled water and pressed briefly between paper towels until dried. The biomass was either immediately used for DNA extraction or stored at -80°C until required. For immediate DNA extraction, mycelium was frozen in liquid nitrogen and grounded to a powder in a mortar and pestle pre-cooled to -20°C or freeze-dried by lyophilisation for 24 h.

For fast and unclean extraction of *A. fumigatus* DNA, standard protocols were performed (Timberlake and Marshall, 1989). Briefly, freshly harvested conidia were inoculated in a 9 cm plastic Petri dish containing roughly 20 ml of fresh ACM medium, which was subsequently incubated for 16 h at 35 °C in static conditions. The ground biomass was placed in a 2 ml Eppendorf tube and mixed with 1 ml of extraction buffer (50 mM EDTA, 0.2 % SDS), followed by 2 h incubation at 68 °C. The suspension was centrifuged at 13,000 rpm for 5 min; the supernatant was placed in a new Eppendorf tube followed by the addition of 80 µl
of 8 M potassium acetate (pH 4.2) and incubation on ice for 5 min. DNA was precipitated with the same volume of isopropanol, washed twice with 500 µl 70 % ethanol and air dried. Finally, the dried pellet was re-suspended in TE buffer or 68 °C-pre-heated water.

In order to collect highly pure DNA, the chloroform-isoamyl-alcohol extraction method was used (Sambrook et al., 1989). Briefly, ground mycelium was collected in a 2 ml Eppendorf tube containing an equal volume (0.8- 1 ml) of extraction buffer C (Table 2.13) and phenol, chloroform, isoamyl-alcohol (25:24:1) (Applichem, DE), and incubated at room temperature for 30 min (every 10 min the solution was mixed thoroughly with a vortex).

The suspension was centrifuged at 12,000 rpm for 15 min and the water phase was transferred to a new 2 ml eppendorf tube. Subsequently, 500 µl of chloroform was added and the suspension was mixed by inversion several times, followed by centrifugation at 10,000 rpm for 10 min. The water phase was transferred to a new 2 ml Eppendorf tube and 800 µl of isopropanol was added. The solution was further mixed and centrifuged at 10,000 rpm for 10 min, the supernatant was removed and the pellet was air-dried for 20 min. The pellet was dissolved in 400 µl of TE containing ARNase A (10 µg/ml) and incubated at 37 °C for 15 min followed by the addition of 8 µl of 5 M NH₄Ac and 1 ml of 96% ice-cold ethanol. The sample was mixed by inverting, incubated for 20 min at -20 °C and centrifuged at 10,000 rpm for 10 min. Finally, the resulting pellet was air-dried and dissolved in 40 µl pre-heated water followed by incubation at 68 °C for 10 min. The concentration and integrity of DNA was checked in a 1 % agarose gel; subsequently the purity was determined using a Synergy2 microplate reader (BioTek, UK) and stored in aliquots at 4 °C until required.
Table 2.13. Buffers used for *A. fumigatus* pure DNA extraction. For ultrapure genomic DNA isolation and purification the DNeasy Plant Mini Kit was used according to the manufacturer’s instructions.

### 2.4.3. DNA precipitation

Contamination by small nucleic acid fragments, proteins and salts can be reduced by precipitation of DNA. Therefore, in order to purify DNA, ethanol precipitation was used. Briefly, 2.5 volume of ethanol and 1/10 volume of 3 M NaAc (pH 5.2) were added to the DNA solution. The sample was mixed, kept at -80 °C for 15 min and centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol,

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction buffer C</td>
<td>1 % SDS</td>
</tr>
<tr>
<td></td>
<td>0.024 g/ml p-aminosalicylic acid</td>
</tr>
<tr>
<td></td>
<td>0.2 ml 5x RNB</td>
</tr>
<tr>
<td>5x RNB</td>
<td>1 M Tris/HCl</td>
</tr>
<tr>
<td></td>
<td>1.25 M NaCl</td>
</tr>
<tr>
<td></td>
<td>0.25 M EGTA</td>
</tr>
<tr>
<td></td>
<td>pH 8.5, autoclave</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris/HCl</td>
</tr>
<tr>
<td></td>
<td>1 mM Disodium EDTA</td>
</tr>
<tr>
<td></td>
<td>pH 4</td>
</tr>
</tbody>
</table>

Table 2.13. Buffers used for *A. fumigatus* pure DNA extraction. For ultrapure genomic DNA isolation and purification the DNeasy Plant Mini Kit was used according to the manufacturer’s instructions.
followed by centrifugation at 13,000 rpm for 10 min. The pellet was dried at 37 °C for 10 min and dissolved in sterile ddH₂O or TE buffer.

2.4.4. Isolation of total RNA of *Aspergillus fumigatus.*

Fresh spores were harvested, adjusted to 5x10⁸ conidia/ml in ACM (supplemented when needed), and incubated at 37 °C at 180 rpm for 14-16 h. Mycelium was filtered through a sterile funnel lined with a double layer of filter paper and washed twice with cold sterile water and frozen immediately in liquid nitrogen. The biomass was ground to a powder with a mortar and pestle and used immediately for RNA extraction or stored at 80 °C until needed.

Approximately 50 mg of ground mycelium was transferred to an Eppendorf tube and RNA extraction was carried out with TRIzol. 1 ml of cold TRI reagent® (Ambion, USA) was added to the ground mycelium, mixed by pipetting (until it become clear) and incubated at room temperature for 10 min. The suspension was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was placed into a new Eppendorf tube. 200 µl of chloroform was added, mixed and incubated at room temperature for 5 min followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and 250 µl of 0.8 M sodium citrate/1.2M NaCl was added. Subsequently, 250 µl of isopropanol 100% was added, the suspension was mixed by vortexing and incubated at room temperature for 10 min followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was removed and 300 µl of cold ethanol 70% was added, and the solution was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was dried out at room temperature for 5 min. The pellet was further dissolved in 100 µl cold RNase-free water. For long term storage, 50 µl of RNA extract was incubated at 80 °C.
In order to remove traces of DNA, the RNA extract was treated with RQ1 RNase-free DNase (Promega, UK), following the instructions of the manufacturer. Briefly, 50 µl of fresh RNA extract was incubated at room temperature for 20 min with 6 µl of 10 X buffer and 4 µl of DNase solution. After the incubation period, 1 µl of RQ1 DNase stop solution was added.

RNA purification was carried out using the Qiagen RNeasy Plant mini kit, according to the manufacturer’s instructions. Briefly, the fresh RNA extract free of DNA was dissolved with 40 µl of RNase-free water, 350 µl of buffer RLT and 250 µl of absolute ethanol, each component was gently mixed by inversion. The solution was then transferred to a QIA shredder spin column, centrifuged at 13,000 rpm for 30 s at room temperature and the supernatant discarded. 500 µl of RPE buffer was added to the column, centrifuged at 10,000 rpm for 2 min and the supernatant discarded. The last step was repeated. The column was transferred to a new 2 ml collection tube and centrifuged at 14,000 rpm for 1 min at room temperature. The column was transferred to a 1.5 ml RNAse-free tube. 40 µl of RNase free-water was added to the column and further incubated for 5 min at room temperature. The tube was then centrifuged at 10,000 rpm for 1 min at room temperature. Finally, the column was discarded and the purity and concentration of the RNA was determined using a Synergy2 microplate reader (BioTek, UK). Aliquots of 20 µl were stored at -80°C until required.

2.4.5. Gel electrophoresis

The separation and identification of DNA fragments was done through electrophoresis gels, as previously described (Sambrook and Russell, 2001) (Table 2.14). Electrophoresis gels were prepared by melting agarose into 1x TAE buffer (0.8 or 1 % final concentration) containing DNA dye safe view (1:1000). 2 µl of DNA samples were mixed with 1/10 10x DNA loading buffer and were loaded onto the gel. A 1 kb ladder was used as a standard DNA marker.
The ladder used depended on the size of the products expected. Gels were run at a constant voltage of 110V in 1x TAE buffer for 50 min in an electrophoresis chamber (Horizon 58, Gibco). Gels were visualized and photographed under UV light using a transilluminator and imaging system ChemiDoc™. (BIORAD, UK) to detect each DNA amplicon and size.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>50 x TAE buffer</strong></td>
<td>40 mM Tris-acetate</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>pH 8</td>
</tr>
<tr>
<td><strong>10 x loading buffer</strong></td>
<td>15% Ficoll 400</td>
</tr>
<tr>
<td></td>
<td>5 mM EDTA (pH 8)</td>
</tr>
<tr>
<td></td>
<td>1 % SDS</td>
</tr>
<tr>
<td></td>
<td>1.5 M Bromophenol blue</td>
</tr>
</tbody>
</table>

Table 2.14. Components for electrophoresis gel.

For isolation of DNA fragments, 0.8%-1% low melting agarose gel was routinely used. The appropriate DNA bands were cut out under UV light and purified with the QIAquick® gel extraction kit or with freeze and squeeze method, in which the agarose band was frozen for 10 min at -20°C, and subsequently melted gently with one’s fingers.
2.5. Polymerase chain reaction (PCR)

2.5.1. Standard polymerase chain reaction

Polymerase chain reaction (PCR) was performed either with the high-fidelity phusion (Table 2.15) or the long-amplification Taq (Table 2.16) polymerases according to the manufacturer’s protocols. As DNA template plasmid (0.2-10 ng) and genomic DNA (50-10 ng) were used. The PCR reactions were carried out in either an Eppendorf master cycler gradient (for small amplicons 0.5 to 2 kb) or a Quanta Biotech S-96 (for long amplicons, 2-7 kb). The polymerization duration and annealing temperatures varied dependent on each application, but PCR programs were generally used with 30 cycles, at a denaturation temperature of 95°C, and a polymerization temperature of 72°C. In the case of oligonucleotides containing restriction sites, the PCR reaction was carried out for 4-5 cycles at a lower annealing temperature then the melting temperature of the primers.

<table>
<thead>
<tr>
<th>Phusion polymerase components</th>
<th>(50 μl reaction)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Phusion HF or GC buffer</td>
<td>10 μl</td>
<td>1 X</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 μl</td>
<td>200 μM</td>
</tr>
<tr>
<td>10 μM Forward Primer</td>
<td>2.5 μl</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>10 μM Reverse Primer</td>
<td>2.5 μl</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.5 μl</td>
<td>3%</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>to 50 μl</td>
<td></td>
</tr>
<tr>
<td>Phusion DNA polymerase</td>
<td>0.5 μl</td>
<td>1.0 units/50 μl PCR</td>
</tr>
<tr>
<td>DNA Template</td>
<td>Variable</td>
<td>50-100 ng</td>
</tr>
</tbody>
</table>

Table 2.15. Standard PCR reaction components.
### Table 2.16. PCR reaction components for long fragments.

<table>
<thead>
<tr>
<th>Long-amplification polymerase components</th>
<th>(50µl reaction)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X LA PCR Buffer ll (Mg$^{2+}$ free)</td>
<td>5 µl</td>
<td>1 X</td>
</tr>
<tr>
<td>25 mM MgCl$_2$</td>
<td>5 µl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>2.5 mM dNTPs</td>
<td>8 µl</td>
<td>400 µM</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>2 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>2 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>Up to 50 µl</td>
<td></td>
</tr>
<tr>
<td>TaKaRa LA Taq</td>
<td>0.5 µl</td>
<td>2.5 units/50 µl PCR</td>
</tr>
<tr>
<td>DNA template</td>
<td>1 µl</td>
<td>&lt; 1 µg</td>
</tr>
</tbody>
</table>

2.5.2. Touch-down PCR (TD-PCR)

In order to overcome problems associated with high annealing temperatures, extensive secondary structures, high % G+C islands and targets with > 60% G+C content; touch-down PCR was used as described (Korbie and Mattick, 2008). The key principle is to employ successively lower annealing temperatures, beginning first with an annealing temperature above the $T_m$, subsequently transitioning to a lower temperature over the course of the initial 10-15 cycles. This robust method, increases the stringency of the reaction without the need to screen different salt concentrations, enhancers or annealing temperatures. Touch-down PCR was performed with the long-amplification Taq polymerase according to manufacturer’s protocols in a Quanta Biotech S-96 thermocycler as shown in Table 2.17.
<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Denature</td>
<td>95 °C</td>
<td>3 min</td>
</tr>
<tr>
<td>2</td>
<td>Denature</td>
<td>95 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>3</td>
<td>Anneal</td>
<td>T&lt;sub&gt;m&lt;/sub&gt;+10 °C</td>
<td>45 s</td>
</tr>
<tr>
<td>4</td>
<td>Elongate</td>
<td>72 °C</td>
<td>60 s</td>
</tr>
</tbody>
</table>

Repeat steps 2-4 (10-15 times)

<table>
<thead>
<tr>
<th>Phase 2</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Denature</td>
<td>95 °C</td>
<td>30 s</td>
</tr>
<tr>
<td>6</td>
<td>Anneal</td>
<td>T&lt;sub&gt;m&lt;/sub&gt; or (T&lt;sub&gt;m&lt;/sub&gt;-5 °C)</td>
<td>45 s</td>
</tr>
<tr>
<td>7</td>
<td>Elongate</td>
<td>72 °C</td>
<td>60 s</td>
</tr>
</tbody>
</table>

Repeat steps 5-7 (20-25 times)

<table>
<thead>
<tr>
<th>Termination</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Elongate</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>9</td>
<td>Halt reaction</td>
<td>4 °C</td>
<td>15 min</td>
</tr>
<tr>
<td>10</td>
<td>Hold</td>
<td>23 °C (room temperature)</td>
<td>Until removed</td>
</tr>
</tbody>
</table>

Table 2.17. Touch-down PCR reaction parameters.

For the cloning of PCR products, restriction enzyme sites were added to both primers, or TA cloning was used. For TA cloning, the PCR products amplified with Expand (Roche, Mannheim) or other proof reading polymerases were incubated with Taq polymerase and then cloned into pCR2.1 TOPO (Invitrogen, NV Leek, The Netherlands).
2.5.3. Fusion PCR

Fusion PCR is a very rapid and versatile technique compared to conventional procedures. The molecules generated by this approach can be used to replace genes, replace promoters, remove genes and tag proteins. Additionally, this approach produces linear fragments which cannot be easily excised once integrated into the genome (Kuwayama et al., 2002; Nayak et al., 2006; Yang et al., 2004; Yu et al., 2004; Zarrin et al., 2005).

For the production of additional fragments, replacements and homologous recombination, the selectable markers for pyrG (orotidine-5’-phosphate decarboxylase) hygromycin B or pyrithiamine genes were used (Table 2.18). Three overlapping amplicons were designed for the generation of cassettes consisting of the sequence for the selectable marker, the upstream and the downstream sequence of the target gene and the final construct was carried out by phusion PCR using nested primers (Szewczyk et al., 2006) (Fig. 2.1). The final construct consisted of the selectable gene flanked by homologous sequence upstream and downstream of the gene of interest enabling targeted gene replacement by homologous recombination following fungal transformation.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ptrA</td>
<td>Pyrithiamine resistance</td>
<td>Goda et al., 2005</td>
</tr>
<tr>
<td>pyrG</td>
<td>Uracil auxotrophy (orotidine-5’-phosphate decarboxylase)</td>
<td>Ballance et al., 1983</td>
</tr>
<tr>
<td>hph B</td>
<td>Hygromycin resistance</td>
<td>Cullen et al., 1987</td>
</tr>
</tbody>
</table>

Table 2.18. Selectable markers for A. fumigatus.
Figure 2.1. Gene tagging, gene replacement and promoter replacement by fusion PCR products. A) C-terminal tagging of Histone H1 with RFP. The flanking DNA (green and
are from the coding sequence of the gene to be tagged (histone H1) and the 3’ untranslated region (3’ UTR). The central region is from a cassette and contains the mRFP coding sequence and the *A. fumigatus* gene (AfpyrG). Homologous recombination during transformation leads to the integration of the fragment into the genome. B) Replacement of the ORF of a hypothetical gene. The cassette is a selectable marker such as pyrG. Homologous recombination leads to the replacement of the target gene with AfpyrG. C) Substitution of the endogenous promotor with a regulatable promoter. The cassette consists of a selectable marker (AfpyrG) upstream of the regulatable promotor (Szewczyk et al., 2006).

In all cases, the DNA of the strain Af1163 was used to prepare upstream and downstream amplicons of the gene of interest, while the middle part of the cassette was formed from the selectable marker of interest. The whole amplified products were run on agarose 1 % (w/v) and visualized using a system ChemiDoc™. (BIORAD, UK). A surgical blade was used to excise the relevant band followed by gel extraction using QIAquick® Gel Extraction according to the manufacturer’s instructions.

### 2.5.4. Quantitative gene expression by real-time PCR

For real-time PCR analysis, one-step quantitative reverse transcription-PCR kit (SensiFAST SYBR®, Bioline) was used. The sequences for the genes of interest were designed using the software Serial Cloner (2.6.1). Controls with DEPC water and no reverse transcriptase were included in order to discard contamination with genomic DNA. Each reaction was carried out in a 10 µl reaction volume containing 5 µl of 2x quantitative PCR master mix, 25 pmol of each corresponding primer, and 10 ng of total RNA. Real-time PCRs from each independent experiment were performed in triplicate using the 7,500 fast real-time PCR system (Applied Biosystems®) according to the manufacturers conditions (Table 2.19).
Melting curves for each reaction were conducted at 60-90 °C. The data were analysed with the software real-time 7,500 (V2.0.1) and presented as the mean. The $2^{-\Delta \Delta CT}$ method (Livak and Schmittgen, 2001) normalized to actin, gpda, pma1 or β-tubulin was used to measure the changes in gene expression.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reverse transcription</td>
<td>45-50 °C</td>
<td>10 min</td>
</tr>
<tr>
<td>2</td>
<td>Polymerase activation</td>
<td>95 °C</td>
<td>2 min</td>
</tr>
<tr>
<td>40</td>
<td>Denaturation</td>
<td>95 °C</td>
<td>5 s</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>60 °C</td>
<td>10-30 s</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72 °C</td>
<td>5-30 s</td>
</tr>
</tbody>
</table>

Table 2.19. Real-time PCR conditions used in this study.

2.5.5. PCR from Aspergillus fumigatus spores

Extraction of DNA of filamentous fungi for PCR analysis is usually time consuming and expensive, especially when it is necessary to check a large number of transformants for different mutations. To avoid this, conidia of A. fumigatus were used directly for PCR analysis. The PCR assay was performed with conidia obtained from freshly grown colonies on agar plates, at 37°C or 30°C for 2 days. The spores were harvested by gently scraping the colony surface with a sterile wire and transferred to an Eppendorf tube filled with 100 µl sterile ddH₂O. Samples were vigorously vortexed and the appropriate spore concentration was adjusted in a reaction tube ($10^4$-10$^6$ spores per reaction), followed by freezing them for 10-15 min at -80°C. The mix was added proportionally to the samples and they were generally subjected to the following PCR conditions: denaturation at 95°C for 5 min, 30
cycles of 95°C for 18 s, appropriate annealing temperature and time, 72°C for 1-2 min, followed by 72°C for 5-10 min.

2.6. Transformation

2.6.1. Transformation of *Escherichia coli*

The transformation of chemically competent cells of the *E. coli* strain TOP10 F’ was performed according to the manufacturer’s instructions (Invitrogen). Briefly, frozen competent cells were thawed and mixed to ensure a homogeneous suspension. Followed the addition of 5 µl of the fragment of interest, cells were incubated for 30 min on ice. Subsequently, cells were treated by heat-shock at 42 ºC for 30 sec in a water bath. Cells were placed on ice and suspended in 300 µl of SOC (Super Optimal Broth with Catabolite Repression) medium (pre-heated at 37 ºC). Cells were incubated at 37 ºC for 1 h at 180 rpm on a rotary shaker. Finally, 80 µl of the suspension was spreaded onto LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37 ºC.

Transformed cell were selected by the blue/white screening method (Vieira and Messing, 1982). Transformation of the vector into competent cells disrupts the lac operon and after induction with IPTG (Isopropyl ß-D-1-thiogalactopyranoside) in the presence of the substrate X-Gal (5-bromo-4-chloro-indolyl-ß-D-galactopyranoside) remains white, while untransformed cells that retain a functioning lac operon turn blue due to the production of 5,5’-dibromo-4,4’-dichloro-indigo. 40 µl of X-Gal/IPTG stock solution (400 µg/ml in formamide) was gently spread over the surface of an LB plate containing 100 µg/ml ampicillin and air-dried for 10 min. 100 µl of transformed cells were spread gently across the surface of the plate and allowed to dry for 5 min before incubating at 37°C for 24 h. Finally, plates were transferred to 4°C for 3 h to enable colour development and white colonies selected with sterile toothpicks for further analysis.
2.6.2. *Aspergillus fumigatus* transformation

Standard procedures and solutions (Table 2.20) for *A. fumigatus* protoplast transformation were used as described (Yelton *et al.*, 1984). Fresh spores were harvested, adjusted to $5 \times 10^8$ conidia/ml in ACM (supplemented when needed), and incubated at 30 °C for 14-16 h at 180 rpm. Mycelium was filtered through a sterile funnel lined with a double layer of filter paper and washed twice with sterile water. The washed mycelium was incubated at 30 °C for 4 h at 80 rpm in a falcon tube containing 10 ml of ACM and 10 ml of enzyme solution. Then, protoplasts were separated from the undigested mycelium through a 40 µm filter (Falcon, USA). Protoplasts were suspended in 20 ml of solution protoA and centrifuged at 180 rpm for 10 min.

The supernatant was carefully removed and the pellet was gently re-suspended in 2 ml of solution protoA and transferred to a 2 ml Eppendorf tube; and centrifuged at 4000 rpm for 3 min. The supernatant was removed and the pellet was slowly re-suspended in 2 ml solution protoB, followed by a centrifugation at 4000 rpm for 3 min. Subsequently, the pellet was gently dissolved by pipetting in 1 ml of STC solution. The number of protoplasts was adjusted to $2 \times 10^7$ cells in STC solution.

For transformation, 15 μl (~2 μg) of the extracted plasmid, linearized plasmid or PCR amplified cassette was added to 100 μl of protoplasts gently mixed and incubated at room temperature for 30 min. Then, 1 ml of PEG solution at room temperature was slowly added and incubated at room temperature for 5 min to catalyse protoplast fusion and DNA uptake. Finally, the protoplast solution was gently mixed with 20 ml of medium, poured into a Petri dish and allowed to solidify. Plates were incubated at 37°C until growth was detected. If growth was undetected after 7 days the transformation was regarded as negative. Positive colonies were picked and transferred onto Sabouraud dextrose agar containing hygromycin B (200 μg/ml) or pyrithiamine (100 μg/ml).
Solutions | Weight
--- | ---
1.1 M KOH (100 ml) | 6.17 g
1 M Tris-HCl (100 ml) | 15.76 g/ autoclave
1 M Tris-base (100 ml) | 12.11 g/autoclave
PEG solution (100 ml) | 4.47 g KCl
 | 0.74 g CaCl$_2$·2H$_2$O
 | pH 7.5 (1 ml 1M Tris-HCl + 196 µl 1 M Tris-base)
 | 25 g PEG-3350/ sterilize using 0.22 µm filter
Proto A (100 ml) | 4.47 g KCl/ autoclave
Proto B (100 ml) | 4.47 KCl
 | 0.74 g CaCl$_2$ 2H$_2$O/ autoclave
STC (100 ml) | 24.2 g sorbitol
 | 0.74 g CaCl$_2$ 2H$_2$O
 | 0.16 g Tris-HCl
 | pH 7.5
Enzyme solution (40 ml) | 3.3 g KCl
 | 0.84 g citric acid monohydrate
 | pH 5.8 with 1.1. M KOH
 | 5.12 g Vinoflow FCE (Novo Nordisk)
 | Sterilize using 0.45 µm filter
<table>
<thead>
<tr>
<th>RM medium (400 ml)</th>
<th>136.9 g Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 g glucose</td>
</tr>
<tr>
<td></td>
<td>0.4 g ammonium tartrate</td>
</tr>
<tr>
<td></td>
<td>8 ml <em>Aspergillus</em> salt solution</td>
</tr>
<tr>
<td></td>
<td>4 ml biotin (0.1 mg/ml)</td>
</tr>
<tr>
<td></td>
<td>pH 6.5</td>
</tr>
<tr>
<td></td>
<td>6 g Agar</td>
</tr>
</tbody>
</table>

Table 2.20. Solutions used for *A. fumigatus* transformation.

2.7. Single and homologous integration into the genome of *A. fumigatus*

2.7.1. Homologous integration screening

DNA was extracted from putative transformants and screened by PCR to determine if the construct had integrated into the fungal genome through homologous recombination. The screening PCR reaction contained 1.5 μl of each appropriate fusion primer, with 1X Phusion™ polymerase Master Mix in HF buffer in a 25 μl final volume mix. Tubes were heated for 30 s at 98°C to activate the polymerase, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 59 to 61°C for 30 s and elongation of 72°C for 20 s per expected Kb of predicted product size. The reactions terminated at 72°C held for 10 min. Products were run on a 1% (w/v) agarose gel and visualized under UV light. The positive transformants were further analysed by Southern blot in order to determine single integration into the genome of the construct of interest.
2.7.2. Southern blot (DNA-DNA hybridization)

DNA-DNA hybridization was performed using DIG labelled DNA-PCR probes, amplified using the PCR DIG Probe Synthesis Kit from Roche (Mannheim), according to the manufacturer’s protocols.

Standard procedures for DNA-DNA hybridization were used as previously described (Southern, 2006). The genomic DNA sample was digested for 16 h with the appropriate restriction enzymes. Subsequently, the digested DNA was separated at 50 V for 3-4 h through 1.2% agarose gel electrophoresis, until the bromophenol blue band was weakly visible in the lower third of the gel (Sambrook and Russell, 2001). The gel was stained with safe view for 30 min and the bands were marked by making little holes with a 1 μl pipette tip. In order to induce the proper DNA transfer, gels were washed with standard buffers (Table 2.21). The gel was washed for 10 min in depurination solution, the solution was removed and the gel was further incubated for 15 min in denaturation solution, twice. After rinsing in water, the gel was equilibrated twice with neutralization solution for 15 min and was further equilibrated for 10 min in 20x SSC.

The DNA bands were transferred overnight at RT by capillary forces to a neutral nitrocellulose membrane settled up (bottom to top) overnight as followed: A bridge of Whatman paper (presoaked in 20x SSC and making contact with 20x SSC reservoirs at both ends), Gel (upside down), membrane (presoaked in 20x SSC), 3 layers of Whatman paper (presoaked in 20x SSC), several layers of tissue and a glass plate. The membrane was subsequently cross-linked with UV radiation (254 nm, 1.200 X 102 μJ for each side). Then the membrane was pre-hybridized in hybridization solution for 1 h at 68 °C and then further hybridized for 12 h with the specific probe at 68 °C, followed by stringent washing. Initially the membrane was washed twice for 15 min at room temperature in 2x washing solution, followed by 2 times washing for 15 min at 68 °C in 0.5x washing solution. After 5 min at
room temperature in washing buffer, the membrane was incubated for 1 h at room
temperature in blocking buffer followed by 30 min incubation with 2 µl anti-DIG-Antibody-
AP (Roche; 11093274910) in 20 ml new blocking buffer. Antibodies were washed away for
15 min in washing buffer at room temperature, twice. Finally, the membrane was equilibrated
in AP-buffer for 5 min at room temperature and transferred to a plastic film with AP-
substrate (5 µl CDPStar, Roche in 500 µl AP-buffer). Detection was carried out by using the
transilluminator and imaging system ChemiDoc™. (BIORAD, UK). Whether the membrane
was reused, stripping was done in stripping buffer, two times for 15 min at 37 °C, followed
by equilibration for 5 min in 2x SSC.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hybridization solution</strong></td>
<td>5x SSC; 0.02% SDS; 1 % Blocking reagent (Roche); 0.1 % N-Laurylsarcosin</td>
</tr>
<tr>
<td><strong>Depurination Solution</strong></td>
<td>0.25 M HCl</td>
</tr>
<tr>
<td><strong>Denaturation solution</strong></td>
<td>0.5 M NaOH; 1.5 M NaCl</td>
</tr>
<tr>
<td><strong>Neutralization solution</strong></td>
<td>1.5 M NaCl; 0.25 M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td><strong>20x SSC</strong></td>
<td>3 M NaCl; 0.3 M Na citrate, pH 7</td>
</tr>
<tr>
<td><strong>2x Washing solution</strong></td>
<td>2x SSC; 0.1 % SDS</td>
</tr>
<tr>
<td><strong>0.5x Washing solution</strong></td>
<td>0.5x SSC; 0.1% SDS</td>
</tr>
<tr>
<td><strong>Washing buffer</strong></td>
<td>100 mM Maleic acid; 150 mM NaCl; 0.3 % Tween 20</td>
</tr>
<tr>
<td><strong>Blocking buffer</strong></td>
<td>100 mM Maleic acid; 150 mM NaCl; 1 % Blocking reagent</td>
</tr>
</tbody>
</table>
**AP-buffer** 0.1 M Tris-HCl; pH 9.5; 0.1 M NaCl; 50 mM MgCl₂

**Stripping buffer** 0.2 M NaOH; 0.1 % SDS

Table 2.21. Solutions used for Southern blot.

### 2.8. Microscopy

#### 2.8.1 Confocal laser-scanning microscopy (CLSM)

Live-cell imaging of fungal cells at high resolution was performed using a laser scanning confocal microscope (Leica, TCS SP8X), which was mounted on an inverted microscope and equipped with a blue diode 405 laser and argon ion lasers (457 nm, 476 nm, 488 nm and 514 nm). Simultaneous brightfield images were captured with a transmitted light detector. The laser intensity and laser exposure of the cells were kept to a minimum to reduce photobleaching and phototoxic effects. A 40x/0.85 NA dry and a 63x/1.4 NA water objective lens were used. Imaging was carried out at 37°C in a microscope temperature controlled chamber (Cube & Box, CH). Confocal images were captured using the Leica microsystem CMS software (v. 3.3, LAS AF). Images were processed using ImageJ (v. 1.44, MacBiophotonics) and Adobe Photoshop (v. 13, Adobe).

#### 2.8.2. Immunostaining of α-1, 3-glucans

In order to study the localization and content of α-1, 3-glucans in the cell wall, the fluorescently labelled antibody, MOPC-104E, which specifically recognizes α-1,3-glucans in the cell wall was used. Cells were prepared, with some modifications, as previously described (Fujikawa et al., 2009). 500 conidia exposed to caspofungin were inoculated in minimal medium at 37°C for 24 and 48 h. The medium was removed and fungal cells were fixed with 3% (v/v) formaldehyde solution in distilled water for 1 h. Cells were washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) for 5 min. Cells were infiltrated in 1% (v/v) Tween 20 in PBS buffer (PBS-T). The samples were
incubated with MOPC-104E IgM (λ light chain) to a final concentration of 4 µg/ml in PBS-T overnight at room temperature in darkness. Cells were washed three times with PBS and re-incubated for 3 h in dark with Alexa Fluor 488 goat anti-mouse IgM (µ chain) to a final concentration of 10 µg/ml in PBS buffer. The secondary antibody was removed by washing cells three times with PBS. After rinsing with PBS, the samples were imaged (Ex/Em 488/520 nm). Fixed samples were kept at 4°C for three weeks.

2.9. Clinical approaches

2.9.1. Antifungal susceptibility testing

The minimal effective concentration of echinocandin was adapted according to the Antifungal Susceptibility Testing Subcommittee of EUCAST by using the broth microdilution method (EUCAST, 2015). Briefly, a final inoculum concentration of 500 CFU/ml was inoculated in RPMI 1640 medium supplemented with 2% glucose at pH 7 buffered with morpholinepropanesulfonic acid (MOPS) in a 96 well-plate containing two-fold serial dilutions of echinocandins for 24 h at 37 ºC under static conditions. Echinocandins were used at a concentration range of between 0.03 to 16 µg/ml. The MEC was the lowest echinocandin concentration that resulted in the growth of small, rounded, compact microcolonies compared to those untreated (echinocandin-free RPMI-1640).

2.9.2. Aspergillus fumigatus infection of alveolar cells

The human pulmonary carcinoma alveolar cell line A549 was maintained by serial passage in Dulbecco Modified Eagle Medium (DMEM) supplemented and maintained at 37°C in the presence of 5% CO₂. Cells were seeded at a concentration of 4 x 10^5 cells/ml in a 2-well µ-slide culture chamber (Ibidi®) and incubated at 37°C and 5% CO₂ to ≥ 90% confluence. The old medium was removed and fresh unsupplemented DMEM was added for an additional 24 h. Epithelial monolayers were challenged with 500 conidia/well and
incubated with either 0.5 or 4 µg/ml of caspofungin in a SANYO carbon dioxide humidifier incubator (InCu saFe, JP) at 37°C/5% CO₂ for 72 h.

Infected epithelial cells were fixed with 3% formaldehyde as previously described (Mason et al., 1985). Briefly, infected cells were incubated with the plasma membrane-selective CellMask™ deep red dye in PBS (1:1000) at 37 °C in the presence of 5% CO₂ for 15 min, cells were washed twice with pre-warmed PBS and fixed with 3% formaldehyde in PBS for 10 min. Cells were rinsed twice with PBS before imaging. Fixed cells were kept at 4 °C for one week in PBS for subsequent analysis.

2.10. Statistical analysis

To analyse each set of data and compare data between groups, a 1-way analysis of variance and Bonferroni’s multiple comparison tests with 99% confidence intervals were used. For real-time PCR the non-parametric, Wilcoxon signed-rank test, was used. All the statistical analyses were performed using IBM SPSS software (version 20.0). Comparisons were deemed significant with a p value of 0.05. All experiments were conducted in triplicate.

2.11. References


CHAPTER 3

3. Caspofungin-mediated growth inhibition and paradoxical growth in *Aspergillus fumigatus* involve fungicidal hyphal tip lysis coupled with regenerative intrahyphal growth and dynamic changes in β-1,3-glucan synthase localization

Sergio D. Moreno-Velásquez, Constanze Seidel, Praveen R. Juvvadi, William J. Steinbach, Nick D. Read

**Target Journal:** *Antimicrobial Agents and Chemotherapy*

3.1. Abstract

Caspofungin targets cell wall β-1,3-glucan synthesis and is guideline-recommended to treat invasive aspergillosis as salvage therapy. Although caspofungin is inhibitory at low concentrations, it exhibits a ‘paradoxical effect’ (reversal of growth inhibition) at high concentrations by an undetermined mechanism. Treatment with either growth inhibitory (0.5 µg/ml) or paradoxical growth-inducing (4 µg/ml) caspofungin concentrations for 24 h caused similar abnormalities, including wider, hyperbranched hyphae, increased septation, and repeated hyphal tip lysis followed by regenerative intrahyphal growth. By 48 h, only hyphae at the colony periphery treated with the high caspofungin concentration displayed paradoxical growth. Similar high concentrations of caspofungin also induced paradoxical growth of *Aspergillus fumigatus* during human A549 alveolar cell invasion. Localization of the β-1,3-glucan synthase complex (Fks1 and Rho1) revealed significant differences between cells exposed to growth inhibitory and paradoxical growth inducing concentrations of caspofungin. At both concentrations, Fks1 initially mislocalized from hyphal tips to vacuoles. However, only continuous exposure to 4 µg/ml of caspofungin for 48 h led to recovery of normal hyphal morphology with renewed localization of Fks1 to hyphal tips. Rho1 remained at the
hyphal tip under both caspofungin concentrations but was required for paradoxical growth. Farnesol blocked paradoxical growth and relocalized Fks1 and Rho1 to vacuoles. Our results highlight the importance of regenerative intrahyphal growth as a rapid adaptation to the fungicidal lytic effects of caspofungin on hyphal tips and the dynamic localization of Fks1 as part of the mechanism for the caspofungin-mediated paradoxical response in *A. fumigatus*.

### 3.2. Introduction

Invasive aspergillosis (IA) caused by *Aspergillus fumigatus* is a major cause of death in immunocompromised patients, particularly in individuals with hematologic malignancy or following hematopoietic stem cell or solid organ transplant (Kontoyiannis et al., 2010; Pappas et al., 2010). Emerging resistance to triazoles (e.g., voriconazole), which is the international consensus guideline-recommended primary antifungal therapy against IA (Patterson et al., 2016), is a grave public health concern (Brown et al., 2012; Kousha et al., 2011; Vermeulen et al., 2013). Echinocandins (e.g., caspofungin, micafungin and anidulafungin) are recommended as second-line agents for salvage therapy to treat patients who are refractory or intolerant to conventional therapies (Patterson et al., 2016).

Caspofungin, the most commonly used echinocandin, disrupts the synthesis of the β-1,3-glucan in the cell wall, displaying what has been described as ‘fungistatic’ activity against filamentous fungi (Song and Stevens, 2016), including *A. fumigatus* (Espinel-Ingroff, 1998). However, caspofungin has also been reported to cause lysis of some hyphal cells, and thus to exhibit partial fungicidal effects, in *A. fumigatus* hyphae (Bowman et al., 2002; Ingham and Schneeberger, 2012; Loiko and Wagener, 2016; Walker et al., 2015). Treatment of *A. fumigatus* with caspofungin leads to swollen, short and highly branched hyphae with thicker cell walls due to the reduction of β-1,3-glucan (Kurtz et al., 1994). However, caspofungin exhibits reduced antifungal activity at high concentrations, a phenomenon known as the “paradoxical effect”, which was first observed in *Candida albicans* (Stevens et al., 2004).
The paradoxical effect has also been observed in other Candida spp. and in Aspergillus spp. (Antachopoulos et al., 2007; Chamilos et al., 2007). Although the exact mechanism of how the paradoxical response is elicited remains unclear, it has been shown to involve the calcium, calcineurin, CrzA, heat shock protein 90 (Hsp90) and cell wall integrity pathways (Ries et al., 2017; Fortwendel et al., 2010; Juvvadi et al., 2015; Kaneko et al., 2009; Wiederhold et al., 2005). In Candida spp., the paradoxical effect has been shown to be independent of mutated, or over expressed, β-1,3-glucan synthase, but is mainly dependent on chitin upregulation (Stevens et al., 2005). Using a conditional mutant, Loiko and Wagener (2016) provided evidence that the paradoxical effect in A. fumigatus relies on recovery of β-1,3-glucan synthase Fks1 activity.

Fungal cell wall β-1,3-glucan is synthesized at the plasma membrane by the largely conserved β-1,3-glucan synthase complex (Park and Bi, 2007). In A. fumigatus, this complex consists of a catalytic subunit, Fks1, and a regulatory subunit, Rho1 (Beauvais et al., 2001). While both Fks1 and Rho1 are consistently localized at sites of polarized growth (Beauvais et al., 2001; Dichtl et al., 2010), conditional deletion of fks1 or mislocalization of Rho1 leads to defective growth with hyphal tip lysis in A. fumigatus (Dichtl et al., 2010, 2015).

In the current study, we addressed important unanswered questions relating to the mode-of-action of caspofungin in connection with growth inhibition and paradoxical growth in A. fumigatus. We utilized quantitative, high resolution, confocal live-cell imaging to investigate the dynamic, concentration-dependent, caspofungin-induced changes that occur at cellular and subcellular hyphal levels.

Our first aim was to understand the fundamental process underlying caspofungin’s limited fungicidal activity (Bowman et al., 2002; Ingham and Schneeberger, 2012; Loiko and Wagener, 2016; Walker et al., 2015) in order to elucidate the basic cellular mechanism of growth inhibition caused by the antifungal. We demonstrate that caspofungin-mediated
growth inhibition involves a highly dynamic fungicidal process at the colony periphery through repeated apical hyphal compartment lysis followed by regenerative, intrahyphal growth, which occurs in combination with increased septation and subapical hyperbranching.

The second aim was to determine if paradoxical growth in *A. fumigatus* is only limited to *in vitro* fungal cell culture in the absence of host cells. Here, we show that it also occurs during the infection of human A549 lung epithelial cells.

The third aim was to analyse the dynamic subcellular localization of Fks1 and Rho1 during growth inhibition and paradoxical growth, because both are components of β-1,3-glucan synthase and thus relevant to the localization of β-1,3-glucan synthesis. We show that Fks1 (but not Rho1) exhibits relocalization from growing hyphal tips to vacuoles during growth inhibition and then returns to the tips during paradoxical growth. Furthermore, the quorum sensing molecule, farnesol, inhibited paradoxical growth and caused the mislocalization of both Fks1 and Rho1 in the presence of high caspofungin concentrations that would normally induce paradoxical growth. These results are consistent with the indispensable localization of both Fks1 and Rho1 at the hyphal tips for paradoxical growth to occur.

### 3.3. Materials and methods

#### 3.3.1. Strains, media and growth conditions

All the *A. fumigatus* strains (Table 3.1) were maintained at 37°C on Vogel’s medium (VM) containing 2% sucrose, 1.5% agar (w/v), and supplemented with uracil and uridine when required (Vogel, 1956). For infection assays, Dulbecco’s Modified Eagle Medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma) and 10% penicillin and streptomycin (Sigma) were used. Fresh conidia were harvested with 0.05% Tween 20 and the conidial concentration was determined by counting using a haemocytometer. To monitor and quantify the paradoxical effect in individual cells or at the
colony level, 500 conidia were incubated in 1.5 ml of liquid VM in a #1 German borosilicate 2-well slide culture chamber or 12-well chamber (Thermo Scientific), respectively. trans, trans-farnesol 96% (Sigma) was used when required. All the treatments were incubated at 37°C for 24 or 48 h in the presence of 0.5 or 4 µg/ml of caspofungin (Sigma).

Table 3.1. Aspergillus fumigatus strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Parental strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA10</td>
<td>Wild-type</td>
<td></td>
<td>Monod et al., 1993</td>
</tr>
<tr>
<td>ΔakuB</td>
<td>akuBΔ::pyrG-</td>
<td>CEA17</td>
<td>da Silva Ferreira et al., 2006</td>
</tr>
<tr>
<td>Fks1-GFP</td>
<td>fks1::gfp::hph</td>
<td>A1160</td>
<td>This study</td>
</tr>
<tr>
<td>Fks1-GFP/Rab5-RFP</td>
<td>fks1::gfp::hph::pyrG+::rfp</td>
<td>A1160</td>
<td>This study</td>
</tr>
<tr>
<td>GFP-Rho1</td>
<td>gfp::rho1::pyrG+</td>
<td>A1160</td>
<td>This study</td>
</tr>
<tr>
<td>GFP-pyrG</td>
<td>βtub::gfp::pyrG+</td>
<td>A1160</td>
<td>Gift M. Bromley</td>
</tr>
</tbody>
</table>

3.3.2. Construction of A. fumigatus fks1-gfp, rho1-gfp and rfp-rab5 expression strains

In order to construct the fks1-gfp expression strain, the C-terminal 2,148 bp portion of the genomic DNA (excluding the termination codon) encoding Fks1 was PCR-amplified using phusion high-fidelity DNA polymerase (NEB) and cloned at KpnI and BamHI sites in the GFP expression vector pUCGH (Langfelder et al., 2001). The 550 bp fks1 terminator was also PCR-amplified and cloned at the SbfI-HindIII sites on pUCGH. After confirming the accuracy of the fks1-gfp plasmid by sequencing, the plasmid was linearized by digesting with KpnI-HindIII. Transformants were selected by resistance to hygromycin B. For the construction of rho1-gfp, fusion PCR was used (Szewczyk et al., 2006) with pyrG as the selectable marker. For the construction of Rab5 expression strain, a 1000 bp rab5 promoter, the 789 bp rab5 encoding cDNA and 1000 bp rab5 terminator were cloned into a pJW-RFP-
NS vector containing pyrG marker gene. Rab5 encoding cDNA was cloned at the C-terminus of RFP encoding gene to express RFP-Rab5 under the control of its native promoter. The rfp-rab5 construct was then transformed into the fks1-gfp expression strain. Positive strains were verified by PCR for homologous integration. The homologous integrants were then checked for localization by fluorescence microscopy.

3.3.3. Confocal microscopy

Live-cell imaging of fungal cells at high resolution was performed using an inverted Leica laser scanning confocal microscope (TCS SP8X), equipped with blue diode, white light, and argon ion lasers. Simultaneous brightfield images were captured with a transmitted light detector. The laser intensity and laser exposure of the cells were kept to a minimum to reduce photobleaching and phototoxic effects. A 40x/0.85 NA dry and a 63x/1.4 NA water objective lens were used. Imaging was carried out at 37°C in a microscope temperature controlled chamber (Cube & Box, Switzerland). Confocal images were captured using the Leica microsystem CMS software (v. 3.3, LAS AF). Images were processed using ImageJ (v. 1.44, MacBiophotonics) and Adobe Photoshop (v. 13, Adobe).

To visualize GFP targeted to the cytoplasm or tagged to Fks1 or Rho1, excitation of the fluorescent protein was performed at 488 nm and the fluorescent signal emitted was detected over the range of 505 to 550 nm. To image vacuoles, 7-amino-4-chloromethylcoumarin (CMAC) from Sigma, at a final concentration of 5 µM, was used with excitation and emission wavelengths of 405 nm and 450-520 nm detected, respectively. For the observation of epithelial cells co-incubated with A. fumigatus, the plasma membrane-selective CellMask™ deep red dye (Ex/Em 649/660-750 nm), supplied by Thermo Scientific, was used. Treated cells were incubated for 30 min at 37°C before observation.
3.3.4. Human A549 epithelial cell line infection assay

The human pulmonary carcinoma alveolar cell line A549 (ATCC, CCL-185) was maintained by serial passage in Dulbeco Modified Eagle Medium (DMEM) supplemented and maintained at 37°C in the presence of 5% CO₂. Cells were seeded at a concentration of 4 x 10⁵ cells/ml in a 2-well µ-slide culture chamber (Ibidi®) and incubated at 37°C and 5% CO₂ to ≥ 90% confluence.

The old medium was removed and fresh unsupplemented DMEM was added for an additional 24 h. Epithelial monolayers were challenged with 500 conidia/well and incubated with either 0.5 or 4 µg/ml of caspofungin for 72 h. Infected epithelial cells were fixed with 3% formaldehyde as previously described (Mason et al., 1985). Briefly, infected cells were incubated with the CellMask™ Deep Red probe in PBS (1:1000) at 37°C in the presence of 5% CO₂ for 15 min, cells were washed twice with pre-warmed PBS and fixed with 3% formaldehyde in PBS for 10 min. Cells were rinsed twice with PBS before imaging. Fixed cells were kept at 4 °C for one week in PBS for subsequent analysis.

3.3.5. Statistical analyses

To analyse each set of data and compare data between groups, a 1-way ANOVA and Bonferroni’s multiple comparison tests with 99% confidence intervals were used. All the statistical analyses were performed using IBM SPSS software (version 20.0). Comparisons were deemed significant with a p value of ≤ 0.05. All experiments were conducted in triplicate.
3.4. Results

3.4.1. The switch to paradoxical growth is elicited in leading hyphae between 36 and 48 h after continuous exposure to a high caspofungin concentration

The paradoxical growth effect in liquid media has been shown to occur within 3 days in the continuous presence of caspofungin in several strains of *A. fumigatus* (Loiko and Wagener, 2016). We confirmed the development of paradoxical growth of the CEA10, Af293 (Juvvadi *et al.*, 2015), ΔKU80 and several azole-resistant strains cultured in *Aspergillus* minimal medium, glucose minimal media and RPMI 1640 (data not shown). However, the observation of paradoxical growth at the cellular level by live-cell imaging was impossible due to the high background autofluorescence in the media and the highly dense growth of *A. fumigatus* (data not shown). Therefore, to investigate the timing and development of the paradoxical effect in more detail at the hyphal level, we used a strain expressing cytoplasmic GFP over 48 h cultured in Vogel’s media which emits low background autofluorescence. For this purpose, we compared the cellular morphogenesis of *A. fumigatus* in untreated samples with those treated with either 0.5 or 4 µg/ml of caspofungin in liquid medium.

Conidia of *A. fumigatus* undergo a period of isotropic growth prior to germination (Momany and Taylor, 2000). Conidia that had been treated with either concentration of caspofungin for 24-48 h were much larger and of a similar size to each other (9.7-10.9 µm in diameter) compared with the much smaller untreated conidia (3.5-3.9 µm) (Table 3.2). These results indicate that caspofungin induces significantly greater isotropic growth of conidi
Table 3.2. Quantification of the main cellular and subcellular effects on leading hyphae of microcolonies treated with caspofungin (0.5 and 4.0 µg/ml) over 24, 36 and 48 h. Septation and branching frequency were quantified 0-70 µm from tip. Measurements were performed three times on three biologically samples (average ± standard deviation [SD], n = 30). The asterisks indicate significant differences between treated and untreated cells (p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>0.5 µg/ml</th>
<th>4 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>36 h</td>
<td>48 h</td>
</tr>
<tr>
<td><strong>Conidium width (µm)</strong></td>
<td>3.9 ± 0.7</td>
<td>3.6 ± 0.7</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td><strong>Hyphal width (µm)</strong></td>
<td>3.6 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td><strong>Branching (µm)</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Septation (µm)</strong></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Single untreated conidia following germination established a normal pattern of hyphal growth and branching by 12 h and thereafter up to 48 h developed into an extensive network of branched hyphae (Fig. 3.1).

Figure 3.1. Induction of paradoxical growth in microcolonies of *A. fumigatus* occurs by 48 h of caspofungin treatment. 500 conidia were inoculated in liquid VM in the absence or presence of caspofungin for 48 h at 37 °C. Scale bars: 100 µm.
When examined at higher magnification, the untreated hyphae exhibited a similar range of morphologies between 24 and 48 h (Fig. 3.2). In contrast, expansive growth of microcolonies derived from single conidia that were continuously exposed to either 0.5 or 4 µg/ml of caspofungin was similarly inhibited over the first 36 h of treatment (Fig. 3.1). However, besides being smaller, the caspofungin-treated microcolonies at 36 h were much more compact with more densely packed hyphae compared with the pattern of hyphal growth in the absence of caspofungin. At higher magnification, the hyphal morphology within these microcolonies continuously treated with either concentration of caspofungin for 24 or 36 h was similar; the treated hyphae were significantly wider, hyperbranched, and exhibited increased septation compared with the untreated ones (Fig. 3.2) and this was borne out by quantitative measurements of these parameters (Table 3.2).

However, in the presence of 4 µg/ml of caspofungin for 36-48 h, the leading hyphae at the periphery of the microcolony underwent a radical change in morphology to that of untreated hyphae (Fig. 3.2). Furthermore, the microcolony diameter was ~25% larger than microcolonies subjected to 0.5 µg/ml of caspofungin (Fig. 3.1) indicative of a switch to a faster rate of hyphal extension. Nevertheless, there were small changes in the morphology of the leading hyphae treated with 0.5 µg/ml of caspofungin for 48 h compared with those treated with the same concentration for 24 or 36 h with significant decreases in hyphal width, and the frequency of branching in the older colonies. However, these differences were small compared with the morphology of the leading hyphae that had switched to a ‘paradoxical growth’ pattern that strongly resembled the growth pattern of untreated hyphae (Figure 3.2; Table 3.2). Hyphal morphology in older regions of the colony remained the same as those treated with caspofungin for 24 h or 36 h (data not shown). Subsequent analysis showed that the paradoxical growth
pattern of leading hyphae treated with 4 \( \mu \text{g/ml} \) of caspofungin continued unabated even after 96 h of treatment (data not shown). Conversely, microcolonies exposed to 0.5 \( \mu \text{g/ml} \) caspofungin showed constant growth impairment over the same period (data not shown). These results are consistent with the paradoxical growth response being restricted to only the leading hyphae exposed to 4 \( \mu \text{g/ml} \) of caspofungin for between 36 and 48 h and once this response had been elicited it was constant.

![Figure 3.2](image)

**Figure 3.2.** Cellular development during paradoxical growth in leading hyphae of *A. fumigatus*. 500 conidia were grown in liquid VM in absence or presence of 0.5 or 4 \( \mu \text{g/ml} \) of caspofungin for (A) 24 h (B) 36 h (C) 48 h at 37°C. Scale bars: 10 \( \mu \text{m} \).
3.4.2. Caspofungin causes hyphal tip lysis followed by regenerative, intrahyphal growth

To investigate the influence of the limited hyphal tip lysis following caspofungin treatment on the pattern of growth of *A. fumigatus* hyphae, we used high resolution, time-lapse confocal live-cell imaging (Fig. 3.3; Movies 3.1-3.3). Only in this way was it possible to monitor in the same samples the dynamic process of germination, hyphal growth, branching and lysis of hyphal compartments during colony initiation in liquid medium in the absence of caspofungin (untreated control) or in the continuous presence of caspofungin at a low inhibitory concentration (0.5 µg/ml) or at a concentration that promoted paradoxical growth (4 µg/ml).

Single untreated conidia developed into a regular hyphal network in liquid cultures (Fig. 3.1; Movie 3.1), whereas individual conidia treated with a broad range of caspofungin concentrations, ranging from 0.06 (minimal effective concentration) to 16 µg/ml, initially showed a more hyperbranched and compact form of microcolony growth (Fig. 3.2). Untreated conidia typically formed a second germ tube several hours after the first one and at ~180° to this initial germ tube. Caspofungin treated conidia typically underwent bipolar germ tube formation similar to untreated ones (Movies 3.2 and 3.3), but the formation of the second germ tube was often followed by the emergence of further germ tubes at random over the conidium surface (Movie 3.2).

Constant exposure to either inhibitory (0.5 µg/ml) or paradoxical growth inducing (4 µg/ml) concentrations of caspofungin resulted in repeated lysis of hyphal tip compartments (Fig. 3.3; Movies 3.2, 3.3). Cytoplasmic leakage due to hyphal lysis was restricted to the peripheral microcolony zone by presumed septal pore plugging mechanism, which has been previously shown to occur rapidly following hyphal damage (Beck *et al.*, 2013; Jedd and Chua, 2000). In this way septal pore occlusion and isolation of the damaged hyphae did not extensively
compromise the integrity of the rest of the colony. However, within 1 h of the damage and presumed septal occurrence of pore plugging, a new hyphal tip had emerged within the damaged hyphal compartment and growth re-commenced by regenerative, intra-hyphal extension (Fig. 3.3C, D, G, H; Movie 3.2).

Interestingly, the intrahyphal tip could sometimes be observed to push out the previous remains of the lysed hyphal compartment and it was not uncommon to see the new regenerating hypha subsequently lyse and another new hypha to emerge within this second lysed hyphae (Figs. 3.3E-H; Movie 3.2). The caspofungin-treated hyphae underwent increased septation and branching (Table 3.2). Lateral hyphal branches commonly, but not always, emerged immediately adjacent to the septum on its distal side relative to the microcolony periphery (indicated by asterisks in Figs. 3.3A, C, E; Movies 3.2 and 3.3). The successive lysis of hyphal compartments, regenerative intrahyphal growth and lateral branch formation gave rise to the more compact nature of the microcolony compared that of the untreated colony (Fig. 3.1). Taken together these results revealed a rapid adaptation response of *A. fumigatus* against the partial fungicidal effects of caspofungin.
Figure 3.3. Intrahyphal growth caused by the tip-fungicidal effect of caspofungin. Live-cell imaging of leading hyphae of a microcolony of *A. fumigatus* expressing cytoplasmic GFP exposed to 0.5 µg/ml of caspofungin in VM for 14 h at 37°C. (A) Before extensive hyphal tip lysis. Note branch formation (asterisk) behind septum. (B) Lysis of apical compartments. Open circle indicates septa each with a septal pore that has probably become plugged preventing loss of GFP labelled cytoplasm. (C) Intrahyphal regenerative growth has been initiated from the septa indicated by asterisks in (B). New growing hyphal tips in lysed hyphal compartments (arrows). (D) Further extension of hyphal tips (arrows) undergoing regenerative, intrahyphal growth. (E) Presumed septal pore plugging (open circle) in a hypha that had previously undergone intrahyphal growth and then lysed. (F) Presumed septal pore plugging following lysis (open circle) and a growing regenerating intrahyphal tip (arrow). (G) Growing tips of regenerating hyphae within old lysed hyphal compartments (arrows) and new branch formation (asterisks). (H) Growing tip of regenerating hyphae within old lysed hyphal compartment (arrow). Scale bar: 5 µm.
3.4.3. Caspofungin-mediated paradoxical growth response occurs during the infection of human A549 alveolar epithelial cells

Human A549 lung epithelial cells have been previously used as a model to study *A. fumigatus* pathogenesis and antifungal drug degradation (Bertuzzi *et al.*, 2014; Escobar *et al.*, 2016; Foster *et al.*, 1998; Wasylnka and Moore, 2003). In order to explore the possible development of paradoxical growth caused by caspofungin exposed to host environmental conditions, we monitored the cellular response of *A. fumigatus* during infection of the alveolar cell line A549 under conditions of human cell culture. For this, monolayers of A549 lung epithelial cells were inoculated with the fungus in the absence (control) or presence of low or high concentrations of caspofungin. A confluent monolayer of A549 cells was observed after 72 h of treatment with caspofungin (0.5 and 4 µg/ml), which indicated that the integrity of the alveolar epithelial cell line is unaffected by this echinocandin on its own.

Previous studies analysing *A. fumigatus* pathogenesis during infection of A549 cells have shown that growth of the fungus results in detachment of the epithelial cells within 24 h of inoculation (Bertuzzi *et al.*, 2014; Wasylnka and Moore, 2003). However, these prior studies had used high concentrations of fungal spores (i.e. $10^5$-$10^6$ conidia per ml) as inocula. We found that the onset of epithelial cell detachment was delayed if we used much lower concentrations of conidia as inocula. With 500 conidia per ml, significant epithelial cell detachment was not observed until ~ 48 h following conidial inoculation. Furthermore, only when low spore concentrations were used with germlings widely spaced apart was it possible to observe discrete fungal microcolonies < 72 h following inoculation.
In the absence of caspofungin treatment, microscopic examination of epithelial cells co-cultured with 500 conidia per ml, showed rounding up and detachment of alveolar cells only after 48 h. After 72 h of co-culturing fungal and epithelial cells in the absence of caspofungin, *A. fumigatus* growth within the culture well was extensive and most of the alveolar cells were detached (Fig. 3.4). Continuous treatment of co-cultured cells with 0.5 or 4 µg/ml of caspofungin for 24, 48 or 72 h showed hyperbranched hyphae exhibiting increased septation (data not shown) and the manifestation of this was evident from the discrete, compact microcolonies observed after 72 h (Fig. 3.4A). However, after 72 h the microcolonies exposed to 4 µg/ml caspofungin possessed slightly larger diameters (1097 ± 133.6 µm) compared to those treated with 0.5 µg/ml caspofungin (810 ± 79.7 µm), which is indicative of the former starting to undergo paradoxical growth. These results indicate that the paradoxical growth response of *A. fumigatus* can be recapitulated in a human host cell environment.
Figure 3.4. *A. fumigatus* undergoes paradoxical growth during infection of the A549 alveolar cell line. (A) Live-cell confocal microscopy of *A. fumigatus* expressing cytoplasmic GFP co-cultured with A549 alveolar cells without or with 0.5 or 4 µg/ml caspofungin in DMEM at 37°C for 72 h showing microcolonies. In (A) extensive fungal growth has resulted in the amalgamation of adjacent microcolonies in the absence of caspofungin. (B) Leading hyphae. (C) Quantification of microcolony diameters at 24, 48 and 72 h post-inoculation.
3.4.4. The normal hyphal tip localization of Fks1 was switched to vacuoles following caspofungin treatment but restored to hyphal tips during paradoxical growth

Based on immunofluorescence staining of fixed hyphae, Beauvais et al. (2001) reported that the catalytic subunit of β-1,3-glucan synthase, Fks1, is predominantly localized at the hyphal tips of A. fumigatus. Because the hyphal growth pattern of A. fumigatus responds differently to low and high concentrations of caspofungin, we analysed the dynamic subcellular localization of Fks1 in living hyphae during continuous exposure to growth inhibitory and paradoxical growth inducing concentrations of the drug. For this purpose, we constructed a strain expressing Fks1-GFP in its native locus by labelling Fks1 with a GFP tag at its C-terminus and monitoring the localization in the leading hyphae in the absence or presence of the two concentrations of caspofungin. Fks1-GFP was expressed under the control of the fks1 native promoter.

In the untreated control, the Fks1-GFP signal was mostly located at the growing hyphal tips with weaker staining in the cytoplasm (indicated by arrows in Fig. 3.5) and at the septa (not shown). Treatment with 0.5 or 4 µg/ml of caspofungin for 24 h caused Fks1-GFP to shift its localization from the hyphal tips to the cytoplasm, in which their morphology varied from being discrete structures that were rounded to more elongated and pleiomorphic in appearance (indicated by asterisks in Fig. 3.5). These cytoplasmic structures containing fluorescent Fks1-GFP resembled vacuoles (Hickey and Read, 2009) and were more abundant in the basal regions of apical hyphal compartments.
As indicated earlier (Figs. 3.1 and 3.2), the leading hyphae continuously exposed to 4 µg/ml caspofungin switched to paradoxical growth within 48 h. Strikingly, this was accompanied by the relocalization of Fks1-GFP back to the growing hyphal tips in contrast to the hyphae treated with the lower inhibitory concentration of caspofungin (0.5 µg/ml) in which the Fks1-GFP remained localized to the vacuoles (Fig. 3.5). The hyphae undergoing paradoxical growth with tip-localized Fks1-GFP had a normal morphology and did not undergo hyphal tip-lysis. Conversely, the hyphae treated with 0.5 µg/ml of caspofungin constantly showed vacuolar Fks1-GFP localization with minimal GFP signal at their hyphal tips (Fig. 3.5) and many of these underwent hyphal tip lysis accompanied by regenerative intrahyphal growth (data not shown).

These two patterns of Fks1-GFP localization were continuously observed following treatments with low or high concentrations of caspofungin, respectively, even after 96 h (data not shown). These results suggest that normal tip localization of Fks1 in growing hyphal tips is directly associated with the paradoxical response and the recovery of the normal hyphal morphogenesis following treatment with the high concentration of caspofungin.
Figure 3.5. The normal hyphal tip localization of Fks1 was relocalized to vacuoles following caspofungin treatment but restored to hyphal tips only during paradoxical growth. Conidia of *A. fumigatus* expressing Fks1-GFP were cultured in VM in the absence or presence of caspofungin for 24 and 48 h at 37°C. Arrows indicate hyphal tip localization of Fks1. Asterisks highlight the presence of Fks1 in vacuoles. Scale bars: 10 µm.

To verify the possible localization of Fks1-GFP to vacuoles, strains grown in 0.5 and 4 µg/ml of caspofungin were stained with the vacuole-selective dye, 7-amino-4-chloromethylcoumarin (CMAC). Treatment with either 0.5 or 4 µg/ml of caspofungin showed clear co-localization of Fks1-GFP with CMAC in the cytoplasmic organelles (Fig. 3.6), confirming the vacuolar localization of Fks1-GFP following treatment with the echinocandin.
Figure 3.6. Caspofungin relocalizes Fks1 to the vacuoles in *A. fumigatus*. 500 conidia were grown in liquid VM in absence or presence of caspofungin for 24 h at 37°C. (A) Untreated cells. (B) 0.5 µg/ml. (C) 4 µg/ml. White asterisks shows examples of colocalisation of GFP-Fks1 and CMAC in vacuoles. White arrows show that GFP-Fks1 in the hyphal tips in (A) are not labelled by CMAC. Scale bars 10 µm.
In order to confirm if the pattern of Fks1 localization during paradoxical growth is specific to caspofungin treatment, we examined the effect of another echinocandin, micafungin, on Fks1 localization. Micafungin contrasts with caspofungin in that it does not induce paradoxical growth in *A. fumigatus* (Fortwendel *et al.*, 2010; Juvvadi *et al.*, 2015). Micafungin treatment resulted in re-localization of Fks1 from the hyphal tips to cytoplasmic (Fig. 3.7), CMAC-labelled vacuoles after treatment with either 0.5 or 4 μg/ml micafungin for 72 h (Fig. 3.8), confirming that relocation of Fks1 to hyphal tips is specific to the paradoxical effect induced by caspofungin.

![Fks1 localization images](image)

**Figure 3.7.** Fks1 hyphal-tip localization is re-localized to vacuoles by the non-inducing paradoxical growth echinocandin, micafungin. Conidia of *A. fumigatus* expressing GFP-Fks1 were cultured in VM with or without micafungin for 24 and 72 h at 37°C. Asterisks highlight examples of Fks1 in vacuoles. White arrows indicate hyphal tip-localisation of Fks1. Scale bars: 10 μm.
Figure 3.8. Micafungin relocalized Fks1 to vacuoles. 500 conidia were grown in liquid VM in absence or presence of micafungin for 24 h at 37 °C. (A) Untreated cells. (B) 0.5 µg/ml. (C) 4 µg/ml. Microcolonies were stained with 5 µM 7-amino-4-chloromethylcoumarin dye (CMAC) for 30 min. Asterisks show examples of the colocalisation of GFP-Fks1 with CMAC in vacuoles. Note that the untreated control images are the same as used in Fig. 3.6 and have only been used for comparative purposes. Scale bars: 10 µm.
3.4.5. Fks1 co-localizes with the endocytic marker, Rab5, in vacuoles upon caspofungin treatment

In *A. nidulans*, mature endosome/vacuolar compartments are static and sizes of these organelles increase in retrograde direction towards basal compartments where storage, degradation and turnover of several molecules occurs (Armstrong, 2010; Peñalva, 2005). Thus, we speculated that the presence of caspofungin induces the trafficking of Fks1 to vacuoles via endosomes. Therefore, in order to further understand the possible link between the vacuole localisation of Fks1 and the endocytic response in *A. fumigatus*, we studied the subcellular localisation patter of the early endosomal marker, Rab5, during caspofungin treatment. To this purpose, the Fks1::eGFP strain was further labelled with Rab5-RFP under its endogenous promotor.

Untreated cells show tip localisation of Fks1, whereas Rab5 show punctate localization in subapical and apical compartments of mature hyphae (Fig. 3.9A). Notably, Fks1 and Rab5 co-localize at basal compartments, suggesting the normal recycling of Fks1-eGFP in vacuoles. Cells in presence of either 0.5 or 4 µg/ml of caspofungin showed a similar co-localization of Fks1 and Rab5 in basal compartments, particularly in the region close to the basal conidium (Fig. 3.9 B, C). Interestingly, no obvious localization of Rab5-RFP was noticed in apical compartments when exposed to caspofungin treatment, which suggests that caspofungin disrupts the proper endocytic activity of *A. fumigatus*. 
Figure 3.9. Caspofungin treatment induces the co-localization of Fks1 and Rab5 in *A. fumigatus*. Conidia of the strain Fks1::eGFP/Rab5::RFP were incubated in Vogel’s medium at 37 °C for 24 h in absence or presence of caspofungin. A) Untreated cells. B) 0.5 µg/ml of caspofungin. C) 4 µg/ml of caspofungin. Asterisks highlight the co-localization of Fks1 and Rab5 in basal compartments, presumably vacuoles. Scale bars: 10 µm.
The co-localization of Fks1 and Rab5 was further accentuated in vacuoles after 48 h in presence of caspofungin (Fig. 3.10). This is likely due to the increased amount of vacuoles in mature hyphae compared to young hyphae. Notably, several subapical compartments show the localization of Fks1 in vacuole-like structures, but not Rab5.

Figure 3.10. Caspofungin induces the co-localisation of Fks1 and Rab5 in *A. fumigatus* for long treatment periods. Conidia of the strain Fks1::eGFP/Rab5::RFP were incubated in Vogel’s medium at 37 °C for 48 h in presence of caspofungin. A) 0.5 µg/ml of caspofungin. B) 4 µg/ml of caspofungin. Asterisks highlight the co-localization of Fks1 and Rab5 in vacuoles in basal compartments. Scale bars: 10 µm.
3.4.6. Localization of the regulatory subunit of the glucan synthase, Rho1, is unaffected by caspofungin treatment but hyphal tip-localization of Rho1 is essential for paradoxical growth

Rho1, belonging to the Rho-GTPase family of proteins, is an important regulator of the glucan synthase complex in yeast and filamentous fungi (Beauvais et al., 2001; Sekiya-Kawasaki et al., 2002). Rho1 localizes at growing hyphal tips, where it is thought to activate Fks1, leading to the synthesis of β-1,3-glucan (Dichtl et al., 2010). In view of this link between Rho1 and Fks1, we also examined the localization of Rho1 during growth inhibitory and paradoxical growth concentrations of caspofungin and whether Rho1 plays a role in paradoxical growth. A strain expressing GFP-labelled Rho1 under the control of the gpdA promoter was constructed to image the protein in living hyphae. This strain had a normal cellular morphology and exhibited paradoxical growth on VM in the presence of 4 µg/ml of caspofungin (data not shown). Under control conditions (untreated), GFP-Rho1 was localized within the cytoplasm with a higher accumulation at hyphal tips and septa (Fig. 3.11A), as described previously (Dichtl et al., 2010). Negative staining or faint staining of organelles was also observed (Fig. 3.11A). The pattern of hyphal tip localization in the absence of caspofungin was similar to that of Fks1-GFP.

The localization pattern of GFP-Rho1 in the cytoplasm, septa or hyphal tips, whether they were growing or not, remained the same when incubated in the presence of either 0.5 or 4 µg/ml caspofungin for 24-96 h (Fig. 3.11B and 3.11C).
Figure 3.11. Tip-localization of Rho1 remains unaffected by caspofungin treatment. Conidia of *A. fumigatus* expressing GFP-Rho1 were cultured in VM in the absence or presence of caspofungin for 96 h at 37°C. (A) Untreated cells. (B) 0.5 µg/ml caspofungin. (C) 4 µg/ml caspofungin. The magnified inserts are of the hyphal tips labelled with asterisks. Note the presence of Rho1 (white arrowheads) at the septum after caspofungin treatment.
3.4.7. Farnesol blocks paradoxical growth and the relocalization of Fks1 and Rho1 to hyphal tips at high concentrations of caspofungin

Farnesol has been previously shown to cause the mislocalization of Rho1 in *A. fumigatus* (Dichtl *et al.*, 2010). While continuous treatment of hyphae for 96 h with 4 µg/ml caspofungin alone resulted in a delayed paradoxical growth response, co-treatment with 10 mM farnesol abolished this paradoxical effect (Fig. 3.12). The same treatment also prevented re-localization of Fks1 back to the growing hyphal tips; Fks1-GFP remained co-localized with the vacuolar marker CMAC although the farnesol combined with the caspofungin treatment caused significant reorganization of the intracellular membranes within the living hyphal compartments (Figs. 3.13 A-C). Taken together, these results indicate that although Rho1 is unaffected by caspofungin treatment, hyphal tip localization of Rho1 is necessary for the development of paradoxical growth and the concomitant relocalization of Fks1 to the tips of leading hyphae.
Figure 3.12. The tip-localization of Rho1 is essential for the development of paradoxical growth. (A) 500 conidia were spotted onto VM agar plates supplemented with 0, 0.5, or 4 µg/ml of caspofungin co-cultured with farnesol for 96 h at 37°C. (B, C) Quantification of radial growth in the presence of caspofungin and farnesol. Results and error bars represent the means ± SD for biological triplicates.
Figure 3.13. Co-treatment with farnesol and caspofungin exhibits continuous localisation of Fks1 in vacuoles of living hyphae. Conidia of *A. fumigatus* expressing Fks1-GFP were cultured in VM for 72 h at 37°C. (A) No caspofungin. (B) 0.5 µg/ml caspofungin. (C) 4 µg/ml caspofungin. Arrows indicate the vacuolar co-localization of Fks1-GFP1 and 5 µM 7-amino-4-chloromethylcoumarin (CMAC), the vacuole-selective dye. Note that caspofungin in the presence of farnesol causes significant re-organization of intracellular membranes within the living hyphal compartments. Scale bars: 5 µm.
3.5. Discussion

Increase in the emergence of azole-resistant strains of *A. fumigatus* which retain susceptibility to the echinocandin caspofungin (Lamoth *et al.*, 2013) makes it an important alternative therapy in patients. Although experimental insertion of point mutations in the *fks1* gene have resulted in caspofungin resistance (Gardiner *et al.*, 2005; Rocha *et al.*, 2007), unlike azole resistance, the development of caspofungin resistance in *A. fumigatus* clinical isolates has never been reported (Perlin, 2007). However, while caspofungin-mediated growth inhibition will affect cell wall integrity by reduction of β-1,3 glucan, the paradoxical reversal of the growth defect observed at high concentrations of caspofungin is puzzling (Fortwendel *et al.*, 2010; Rueda *et al.*, 2014).

In response to caspofungin, a compensatory increase in cell wall chitin concomitant with the upregulation of chitin synthases was previously reported in *A. fumigatus* (Fortwendel *et al.*, 2010; Walker *et al.*, 2015). At the morphological level, microcolony formation, hyphal lysis, intrahyphal development and thickened hyphae with increased chitin were seen as responses to caspofungin and potential components of rescue mechanisms against this drug (Bowman *et al.*, 2002; Ingham and Schneeberger, 2012; Walker *et al.*, 2015). However, detailed microscopic analyses of the initial response to caspofungin during growth inhibition and subsequent events involving microcolony formation, hyphal lysis and regenerative growth, have not been previously performed. In the current study, by using high resolution, live-cell imaging and fluorescent labeling techniques, we have demonstrated how hyphae adapt to growth inhibitory (0.5 µg/ml) and paradoxical concentrations (4 µg/ml) of caspofungin *in vitro* over different time periods. The recurrent hyphal lysis, septal occlusion, intrahyphal regenerative growth before the initiation of paradoxical growth with regular hyphal extension highlights the dynamics of morphogenetic adaptability of the fungus to high concentrations of caspofungin over time.
The development of the paradoxical growth in response to high concentrations of caspofungin seems to be unrelated to degradation or physical change in the state of the drug because some susceptible strains are unable to develop paradoxical growth and very high concentrations of caspofungin abolish this response (Stevens et al., 2004; Stevens et al., 2005; Rueda et al., 2014). Furthermore, the composition of the culture media may influence the glucan synthesis and consequently the antifungal properties of caspofungin, as previously described (Clavaud et al., 2012). However, we used several culture media including Aspergillus minimal media (AMM), glucose minimal media (GMM), RPMI 1640, VM and DMEM and we observed similar cellular responses including the development of paradoxical growth in several strains of A. fumigatus (data not shown). Thus, the development of paradoxical growth may be a complex cellular adaptation to long treatment periods. In agreement, several studies have implicated the role of key signaling pathways, including calcium, calcineurin, CrzA, the cell wall integrity (CWI) and the HOG (high osmolarity glycerol) pathways in regulating the caspofungin stress response in some strains of C. albicans and A. fumigatus (Ries et al., 2017; Fortwendel et al., 2010; Juvvadi et al., 2015; Munro et al., 2007; Wiederhold et al., 2005). Surprisingly, the onset of the paradoxical effect at cellular and subcellular levels and the impact of caspofungin subcellular dynamics to the β-1,3-glucan synthase complex, remains unknown.

Here, we monitored the localization pattern of the two major components of the β-1,3 glucan synthase complex, Fks1 encoding the β-1,3 glucan synthase catalytic subunit and the Rho1 GTPase regulatory subunit. Dynamic changes in Fks1 localization from hyphal tips to vacuoles following initial treatment (24 h) with growth inhibitory (0.5 μg/ml) and paradoxical concentrations (4 μg/ml) of caspofungin was accompanied by stunted hyphal growth, hyperbranching, increased septation and hyphal tip-lysis. Cytoplasmic leakage was prevented
through septal occlusion at the septal pores, presumably by Woronin bodies at the base of apical compartment, as previously described (Beck et al., 2013). Following hyphal tip-lysis though intrahyphal growth commenced further repeated hyphal tip-lysis occurred. Only between 36 and 48 h in the presence of paradoxical growth concentrations of caspofungin was hyphal tip relocation of Fks1 and resumption of active hyphal extension observed. These results are consistent with the recent evidence provided by Loiko and Wagener (2016), using a conditional fks1 mutant, that the paradoxical effect in A. fumigatus relies on recovery of the β-1,3-glucan synthase Fks1 activity.

We were able to demonstrate different patterns of Fks1 localization with respect to two echinocandins, caspofungin and micafungin. In contrast to caspofungin, high concentrations of micafungin have been previously shown not to induce paradoxical growth (Fortwendel et al., 2010; Juvvadi et al., 2015). In the present study we found that, similar to caspofungin treatment, exposure to micafungin resulted in vacuolar localization of Fks1. However, we did not observe the localization of Fks1 to hyphal tips over time or in the presence of higher concentrations of this drug. We argue that the localization of Fks1 in vacuoles after caspofungin contact may be due to degradation of the enzyme, which correlates with the reduced production of β-1,3-glucan and impaired morphology (Kurtz et al., 1994; Loiko and Wagener, 2017; Shoji et al., 2006). However, once paradoxical growth is triggered this process is abolished due to the renewed localization of Fks1 at the tip followed by the recovery of the basal content of β-1,3-glucan and normal hyphal morphology (Loiko and Wagener, 2017). Thus, this continuous accumulation of Fks1 in the vacuoles may be the reason for the absence of paradoxical growth with micafungin. Additionally, we were able to observe that Fks1 colocalizes with the early endosomal marker, Rab5, in vacuoles, suggesting that the vacuolar localization of Fks1 after caspofungin treatment
occurs via endocytosis. It is possible that Fks1 localized in the vacuoles may be subjected to degradation. Whether more rapid turnover at the plasma membrane occurs was not analysed in our study but could be addressed in the future by fluorescence recovery after photobleaching (FRAP) analysis.

We found that that the quorum sensing molecule, farnesol, inhibited paradoxical growth in A. fumigatus. Previously, treatment of A. fumigatus with farnesol has been shown to cause the mislocalization of Rho1 and Rho3 from growing hyphal tips as well as blocking the cell wall integrity pathway (Dichtl et al., 2010). Furthermore, farnesol treatment impairs the hyphal tip morphology as a result of an altered organization of the actin cytoskeleton (Dichtl et al., 2010). Given that Rho1 regulates F-actin organization, the cell wall integrity pathway and β-1,3-glucan synthase activity, we hypothesized that its correct tip-localization is required for the development of paradoxical growth. Thus, although Rho1 localization is unaffected by caspofungin treatment, hyphal tip localization of Rho1 is necessary for the development of paradoxical growth and the concomitant relocalization of Fks1 to the tips of leading hyphae.

The higher doses of echinocandins are very clinically relevant as their wide safety profile has led clinicians to escalate their doses in order to increase their potency, especially in complex patients with recalcitrant fungal disease. However, the true clinical implications of the activity of caspofungin at high concentrations in patients remain unclear. Caspofungin’s paradoxical effect, mostly reported and commonly observed in planktonic and biofilm systems of Candida spp. in vitro (Rueda et al., 2014; Melo et al., 2007), has also been reported to occur in murine model of invasive candidiasis and a rabbit model of invasive aspergillosis without significant change in survival rates (Clemons et al., 2006; Petraitiene et al., 2002). In a murine model of invasive aspergillosis, a paradoxical increase in lung fungal burden was noted at higher dose of
caspofungin (Moretti et al., 2012). However, this inconclusive phenomenon had remained unexplored during infection at cellular level in vitro. Here, we examined whether A. fumigatus is able to develop caspofungin-mediated paradoxical growth under a host-related condition that mimics aspects of physiological growth in tissue (Escobar et al., 2016; Hickey and Read, 2009; Sekiya-Kawasaki et al., 2002; Lamoth et al., 2013). Our live-cell imaging of A. fumigatus growth in human A549 lung epithelial cells, which attempted to mimic the components of the human lung host environment, potentially provided a more realistic view of microcolony differentiation and hyphal morphology at the growth inhibitory and paradoxical concentrations of caspofungin in the human host. Conversely to cells exposed to low inhibitory concentrations, caspofungin at paradoxical concentrations showed larger A. fumigatus microcolonies, similar to those observed in vitro. Although several host factors influence the growth of A. fumigatus and caspofungin activity, such as serum, hypoxia and immunomodulation (Ioannou et al., 2016; Odabasi et al., 2007; Warn et al., 2004; Hohl et al., 2008), we cannot rule out the possibility of similar adaptive mechanism against caspofungin treatment during A. fumigatus infection. Future studies at high resolution of infections using in vivo models may reveal new insights into cellular responses of A. fumigatus upon caspofungin treatment.

In summary, our results demonstrate two major findings: (1) leading hyphae of A. fumigatus rapidly overcome the partial fungicidal effect of caspofungin mainly by repeated cell lysis coupled with regenerative intra-hyphal growth. This, coupled with hyperbranching, is the main reason for the reduced colony size observed macroscopically during the first 36-48 h of treatment with caspofungin compared to untreated colonies; the reduced colony size during this period is thus not due to truly ‘fungistatic’ activity of caspofungin but rather the effects of repeated cell lytic and thus fungicidal activity. Therefore, it is clear that the use of the terms ‘fungistatic’ or
‘fungicidal’ provides an over simplistic view of fungal growth inhibition by caspofungin. (2) The onset of paradoxical growth in *A. fumigatus* caused by caspofungin requires the tip-localization of both the catalytic subunit (Fks1) and regulatory subunit (Rho1) of the β-1,3-glucan synthase complex, which are accompanied by normal hyphal tip morphogenesis. These findings are consistent with the morphological changes that occur during human alveolar infection *in vitro*.

Although the clinical relevance of the paradoxical growth remains unclear, we cannot exclude the possibility that this may be of significance during infection, particularly due to the increasing tendency to utilize higher doses of caspofungin over long periods to treat difficult infections (Steinbach *et al.*, 2015). These results highlight the need for utilizing high resolution live-cell imaging on fungal pathogens treated with other therapeutic antifungal drugs in order to gain further insights into their precise mode of action. Finally, further studies are required to decipher the specific trigger(s) for paradoxical growth initiation, and the mechanism behind Fks1 localization to the vacuoles.

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3.6. References


4. Live-cell imaging of the dynamics of caspofungin-induced paradoxical growth in the human pathogen, *Aspergillus fumigatus*

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**Target journal:** Fungal Genetics and Biology

4.1. Abstract

The antifungal drug caspofungin exhibits attenuated activity at high concentrations against *Aspergillus fumigatus*, a phenomenon known as ‘paradoxical growth’. Despite its clinical relevance, this cellular response remains little understood. Recently, it was shown that treatment for 36 h with either low or high caspofungin concentrations results in hyphal tip lysis, regenerative intrahyphal growth, hyperbranching and increased septation. Here, we shown by live-cell imaging that after ~ 40 h hyphae treated with high caspofungin concentrations switch to normal growth. However, gene expression of the β-1,3-glucan synthase complex remains downregulated during paradoxical growth.
4.2. Introduction

The opportunistic mould, *Aspergillus fumigatus*, causes life-threatening infections in immunocompromised patients resulting in mortality rates greater than 70%, in some cases (Brown *et al.*, 2012). The antifungal drug caspofungin, disrupts the production of β-1,3-glucan in the cell wall by inhibition of the catalytic subunit (Fks1) of the β-1,3-glucan synthase complex, which is particularly concentrated in hyphal tips (Beauvais *et al.*, 2001; Sánchez-León and Riquelme, 2016). Previously, treatment of *A. fumigatus* with caspofungin had been shown to cause hyphal tip lysis, generate swollen compartments, and increased septation and branching frequency (Kurtz *et al.*, 1994; Ingham and Schneeberger, 2012). However, recently high resolution, time-lapse imaging of living microcolonies of *A. fumigatus* showed that these processes were coupled with highly dynamic, repeated, regenerative intrahyphal growth from within lysed hyphal tips resulting in the compact growth pattern of young colonies over the first 36 h in the presence of both high and low caspofungin concentrations (Moreno-Velásquez *et al.*, 2017).

Despite its high antifungal activity, caspofungin is less effective at high concentrations after ~40 h, a phenomenon called the ‘paradoxical effect’ (Stevens *et al.*, 2004; Antachopoulos *et al.*, 2007). The exact cellular mechanisms responsible for the paradoxical effect are unknown but calcium, the heat shock protein 90 (Hsp90) and the cell wall integrity pathway have all been implicated (Kaneko *et al.*, 2009; Juvvadi *et al.*, 2015; Ries *et al.*, 2017). However, the dynamics in morphological change that occur during the switch from inhibitory to paradoxical growth are unknown. Here, we show the cellular response to growth inhibitory and paradoxical effect concentrations of caspofungin by using confocal live-cell imaging. We demonstrate that leading hyphae underlying paradoxical effect display normal growth and morphology.
4.3. Materials and methods

Aspergillus fumigatus strains (Table 4.1) were cultured on Vogel’s medium (VM) containing 2% sucrose (Vogel’s, 1956). To monitor and quantify the paradoxical effect in individual cells or at colony level, 1.5 ml of liquid VM in a #1 borosilicate 2-well slide culture chamber or 12-well chamber (Thermo Scientific), respectively, were inoculated with 500 conidia. All treatments were incubated at 37°C in the presence of either 0.5 or 4 µg/ml of caspofungin (Sigma).

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<td>A1160</td>
<td>Kind gift from M. Bromley</td>
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Table 4.1. Strains used in this study.

Live-cell imaging of fungal cells at high resolution was performed using a laser scanning confocal microscope (Leica, TCS SP8X), which was mounted on an inverted microscope and equipped with blue diode 405, white light, and argon ion lasers. Simultaneous brightfield images were captured with a transmitted light detector. Excitation/emission wavelengths 488nm/505-550 nm were used. A 40x/0.85 NA dry objective lens was used. Imaging was carried out at 37°C in a microscope temperature controlled chamber (Cube & Box, Switzerland). Confocal images were captured using the Leica microsystem CMS software (v. 3.3, LAS AF).
Total RNA of cells exposed to either 0.5 or 4 µg/ml of caspofungin was isolated from three independent cultures. RNA was isolated using the RNeasy® plant mini kit (Qiagen) according to the manufacturer's instructions. Following DNase treatment (Promega), the quantity and quality of RNA preparations were determined spectrophotometrically using a microplate reader (Synergy Z, BioTek). For real-time PCR analysis, one-step quantitative reverse transcription-PCR kit (SensiFAST SYBR®, Bioline) was used. The sequences for the genes fks1 (AFUB_078400), rho1 (AFUB_072830) and the putative β-tubulin (AFUB_010330) were designed using the software Serial Cloner (2.6.1). Real-time PCRs were performed in triplicate using the 7500 fast real-time PCR system (Applied Biosystems®). The 2^−ΔΔCT method normalized to β-tubulin was used to measure the changes in gene expression.

4.4. Results and discussion

The first objective of this study was to show the morphological changes that occur during the switch from growth inhibitory to paradoxical growth.

Microcolonies of A. fumigatus in the presence of either 0.5 or 4 µg/ml of caspofungin for 36 h exhibit similar morphologies, including hyperbranching, cell swelling, abnormal septal distribution and tip lysis (Moreno-Velásquez et al., 2017). Significantly, this cellular response was further observed over long periods (up to 96 h) in cells exposed to 0.5 µg/ml of caspofungin. Leading hyphae of microcolonies exposed to 0.5 µg/ml of caspofungin showed very dense growth subjected to continuous hyphal tip lysis (Movie 4.1). However, after 40-48 h, leading hyphae in 4 µg/ml of caspofungin showed a switch between highly affected to normal morphology and no further tip-lysis was noticed (Movie 4.2). The onset of this paradoxical growth observed in leading hyphae was not constant and varied among microcolonies. Previous
reports suggest that the degree of the paradoxical effect varies among *A. fumigatus* isolates and this may be directly linked to the growth rate of each strain (Forwendel *et al.*, 2010). Clearly, the timing of onset of the paradoxical effect correlates with the normal growth and abolition of hyphal tip-lysis of leading hyphae. This effect has been shown to be independent of the development of a resistant subpopulation or caspofungin degradation (Stevens *et al.*, 2004). However, this phenomenon is dependent on the expression of Fks1 and the proper tip localization of the β-1,3-glucan synthase complex (Loiko and Wagener, 2017; Moreno-Velásquez *et al.*, 2017).

This paradoxical response was also studied at the colony level (Figs 4.1 and 4.2). After 24 h, cells in the presence of either 0.5 or 4 µg/ml of caspofungin showed similar colony diameters but slightly smaller diameters compared to those untreated. After 48 h, however, cells exposed to 4 µg/ml of caspofungin showed larger colonies than cells exposed to 0.5 µg/ml. This paradoxical effect was more apparent after 72 h. In addition, we observed that 8 h old germlings and 16 h mature hyphae exposed to the same concentrations of caspofungin developed paradoxical growth after 48 h (Fig. 4.3 and 4.4). This suggests that *A. fumigatus* responds in a paradoxical manner upon caspofungin treatment independently of its developmental stage.
Figure 4.1. Colony morphology during paradoxical growth in A. fumigatus. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin for 72 h at 37 °C. The graph represents the colony diameter measured as the mean value ± standard deviation at each time point. The asterisks indicate significant differences (p < 0.05) from untreated cells.
Figure 4.2. Conidia production of *A. fumigatus* during caspofungin treatment. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin for 72 h at 37 °C. Each bar in the histogram represents the mean value ± standard deviation. NT: not treatment. The asterisks indicate significant differences (*p* < 0.05) from untreated cells.
Figure 4.3. Colony diameters of different developmental stages of *A. fumigatus* in presence of caspofungin. Fresh conidia, 8 h-old germlings and 16 h-old hyphae were cultured on Vogel’s medium at 37°C in presence of either 0.5 or 4 µg/ml of caspofungin for 96 h.
Figure 4.4. Colony diameter measurements of the major developmental stages in *A. fumigatus* during caspofungin treatment. Fresh conidia, 8 h-old germlings and 16 h-old hyphae were cultured on Vogel’s medium at 37ºC in presence of either 0.5 or 4 µg/ml of caspofungin for 96 h and the colony diameter was monitored every 24 h. A) Fresh conidia. B) 8 h-old germlings. C) 16 h-old hyphae. Results and error bars represent the means ± SD for biological triplicates. The asterisks indicate significant differences (*p* < 0.05) from untreated cells.
The second objective was to determine whether the inhibition of the conidial germination rate influenced subsequent paradoxical growth (Fig. 4.5). Whereas untreated conidia, exhibited 7% germination after 6 h, conidia treated with either 0.5 or 4 µg/ml of caspofungin exhibited reduced germination (2.3 and 0.8%, respectively, \( p < 0.05 \)). After 8 h, untreated conidia exhibited 72% germination, whereas conidia subjected to 0.5 or 4 µg/ml of caspofungin exhibited significantly lower germination (42.3 and 15%, respectively, \( p < 0.05 \)). Surprisingly, no cell abnormalities or cell lysis was observed in conidia or germlings exposed to caspofungin.

Figure 4.5. Germination of *A. fumigatus* upon caspofungin treatment. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin for 10 h at 37 °C. The graph represents the mean value ± standard deviation for biological triplicates. NT: not treatment. The asterisks indicate significant differences \( (p < 0.05) \) from untreated cells.
Among *Aspergillus* species, *A. fumigatus* has the highest germination rate, which depends on several factors including pH, temperature, and inoculum size (Araujo and Rodrigues, 2004). Here, we showed that caspofungin reduces the germination rate without affecting the morphology and final percentage of conidia that germinate (98%) in *A. fumigatus*. These observations suggest that neither reduced germination nor conidial or germling lysis are associated with the subsequent paradoxical growth effect observed at the colony level.

The *third objective* was to analyse the relationship between hyphal tip lysis and the onset of paradoxical growth. Therefore, we quantified the percentage of leading hyphae lysed using the cell viability dye, propidium iodide (Fig 4.6). Minimal cell death was observed in untreated hyphae after 48 h. However, hyphae in presence of either 0.5 or 4 µg/ml of caspofungin for 24 h showed 54% and 65% tip lysis, respectively. Surprisingly, after 48 h, the number of lysed tip cells with the onset of paradoxical growth in the presence of 4 µg/ml of caspofungin was 3.5%, which was similar (*p >0.5*) from untreated cells (2.8%). In contrast, hyphae exposed to 0.5 µg/ml caspofungin for 48 h exhibited 42% tip lysis. Dose-dependent hyphal tip lysis during early colony development has been previously reported in *A. fumigatus* (Ingham and Schneeberger, 2012), but the fungicidal activity of caspofungin restricted to hyphal tips over long treatment periods has not been shown. We demonstrate that leading hyphae of colonies showing paradoxical growth do not suffer tip lysis. This limited fungicidal activity may explain the smaller colony diameters commonly observed on solid media (Fig.4.1) rather than a ‘fungistatic effect’ caused by caspofungin, as previously reported (Espinel-Ingroff. 1998; Chen *et al.*, 2011).
Figure 4.6. Quantification of the tip-lysis effect exerted by caspofungin treatment against *A. fumigatus*. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin at 37 °C. Cells were labelled with the cell death marker, propidium iodide (5 µg/ml). The graphic represents the mean value ± standard deviation for biological triplicates NT: not treated. The asterisks indicate significant differences ($p < 0.05$) from untreated cells.
Finally, we studied the changes in the expression of *fks1* and *rho1* genes (Fig. 4.7). Compared to the untreated control, cells exposed to 0.5 or 4 µg/ml of caspofungin for 24 h exhibited a 0.52 and 0.55-fold reduction in expression of *fks1*, respectively. Similarly, *rho1* showed a 0.53 and 0.5-fold reduced expression, respectively. Intriguingly, cells in the presence of 0.5 or 4 µg/ml of caspofungin for 48 h showed a 0.55 and 0.69 reduced expression of *fks1* and the expression of *rho1* 0.55 and 0.44 to 0.5 and 4 µg/ml of caspofungin, respectively. Alike, a reduced activity of the β-1,3 glucan synthase during paradoxical effect has been reported in *Candida albicans* (Stevens *et al.*, 2005). The unexpected down-regulation of the glucan synthase complex shows the inability of *A. fumigatus* to compensate the inhibition of the β-1,3-glucan by caspofungin. However, this may be compensated by the transcriptional up-regulation of chitin synthases during paradoxical effect, as a secondary mechanism (Fortwendel *et al.*, 2010).

Altogether, we demonstrated that paradoxical growth occurs only in leading hyphae exposed to high concentrations of caspofungin over long treatment periods. This results in hyphae with normal morphology and growth. The clinical relevance of paradoxical growth caused by caspofungin remains unclear. Therefore, understanding the morphological adaptations that allow *A. fumigatus* to overcome the exposure to antifungal drugs is necessary in order to improve standards or higher dosing schemes.
Figure 4.7. Quantification of the transcription of glucan synthase complex of *A. fumigatus* during caspofungin treatment by real-time PCR. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin at 37 °C. (A) *fks1* expression. (B) *rho1* expression. Results are presented as the mean fold change \(2^{-\Delta\Delta CT}\).
Acknowledgements

This work was financially supported by the Consejo Nacional de Ciencia y Tecnología-México, CONACyT grant 359173. C. Seidel was funded by the German Science Foundation (DFG, SE2405/1-1). Financial support was also provided by the European Union (grant PITN-GA-2013-607963) to N.D. Read. We thank Dr Mike Bromley for the GFP strain and Dr Sara Gago for her technical assistance.

4.5. References


CHAPTER 5

5. Caspofungin treatment induces the recruitment of the transcription factor CrzA to nuclei and the reorganization of the main cell-wall components during paradoxical growth in *Aspergillus fumigatus*

Sergio D. Moreno-Velásquez and Nick D. Read

5.1. Abstract

Caspofungin targets the cell wall synthesizing enzyme β-1,3-glucan synthase and is used as second-line therapy to treat invasive aspergillosis. Despite its clinical importance, caspofungin exhibits attenuated activity at high concentrations, a phenomenon known as the ‘paradoxical effect’. Here, we monitored the key morphological and cell-wall rearrangements caused by the impaired dynamics of the β-1,3-glucan synthase complex induced by caspofungin treatment in *A. fumigatus*. Short periods of treatment (30 min) with either growth inhibitory (0.5 µg/ml) or paradoxical growth (4 µg/ml) concentrations of caspofungin in mature hyphae caused similar morphological defects including loss of polarity and hyperbranching, as occurred with cells continuously exposed to caspofungin from the stage of initial inoculation. Treatment with caspofungin at low and high concentrations was associated with the mislocalisation of Fks1 in mature hyphae after 30 min. Concomitantly, we observed a reduced β-1,3-glucan, but increased chitin content at mature hyphal tips. Furthermore, caspofungin treatment induced nuclear translocation of the Ca^{2+}-regulated transcription factor CrzA. Paradoxical growth in *A. fumigatus* CEA10 is observed at the colony level by 48 h. This response was observed in severalazole resistant mutants but in some of these clinical strains azole resistance was found to be independent of the paradoxical effect. Paradoxical growth was associated with the normalized
content of β-1,3-glucan, α-1,3-glucan, galactomannan but not chitin, which remained high. Finally, it was demonstrated that caspofungin remains active and stable in media 48 h after paradoxical growth has been initiated. These results further illustrate the dynamic, multiple early and late cellular and molecular responses that *A. fumigatus* develop in association with paradoxical growth, of which some may play a role in overcoming caspofungin treatment in the clinical situation.

### 5.2. Introduction

Fungi have been increasing as the causative agents of life-threatening diseases, mainly in immunocompromised patients (Brown *et al*., 2012). The opportunistic fungus *Aspergillus fumigatus* has become one of the most common human fungal pathogens, causing severe and usually fatal systemic infections (Latgé, 1999). Echinocandins are the most novel class of antifungal drugs used to treat *Candida* spp. and *Aspergillus* spp. infections due to their rapid onset of action, limited human cytotoxicity, and high efficacy against azole resistant strains (Al-Badriyeh *et al*., 2009). Caspofungin is the most commonly used echinocandin in order to treat intolerant or refractive patients (Patterson *et al*., 2016). Like other echinocandins, caspofungin disrupts the production of the β-1,3 glucan, the main component of the cell wall in the major fungal pathogens (Mouyna and Fontaine, 2009; Ruiz-Herrera *et al*., 2006; Sawistowska-Schröder *et al*., 1984).

The cell wall β-1,3-glucan of *A. fumigatus* is elaborated by the β-1,3-glucan synthase complex (Park and Bi, 2007). In *A. fumigatus*, this complex is composed of two proteins, the catalytic subunit Fks1 and the regulatory subunit Rho1 (Beauvais *et al*., 2001). Fks1, the target of caspofungin, is an integral plasma membrane protein with 16 transmembrane helices, primarily
localized at the hyphal apex in proximity to the Rho-GTPase, Rho1 (Beauvais et al., 2001; Dichtl et al., 2010). Inhibition of the β-1,3-glucan synthase complex caused by caspofungin led to the lysis of C. albicans cells (Kurtz and Rex, 2001), whereas the presence of caspofungin induces the formation of short, swollen and highly branched hyphae in A. fumigatus (Kurtz et al., 1994).

Resistance to caspofungin has emerged at low frequency (Rueda et al., 2014). Point mutations in the Fks1 gene in C. albicans and A. fumigatus resulted in echinocandin resistant strains (Rocha et al., 2007). However, natural resistance of clinical isolates of A. fumigatus seems to be unrelated to mutations in the FKS1 gene (Mayr and Lass-Flörl, 2011). Intriguingly, caspofungin exhibits attenuated activity at high concentrations, a phenomenon known as the paradoxical effect, which seems to be independent of target modifications (Stevens et al., 2005). Morphological analysis of Candida spp during paradoxical growth showed morphological alterations, including absence of filamentation, abnormal septa, and loss of the β-1,3-glucan layer (Bizerra et al., 2011). Furthermore, the paradoxical effect in C. albicans and A. fumigatus led to up-regulation of the protein kinase C (PKC), Ca\(^{2+}\)-calcineurin-CrzA signalling, cell wall integrity (CWI) and high-osmolarity glycerol (HOG) pathways (Fortwendel et al., 2010; Juvvadi et al., 2015; Kaneko et al., 2009; Rueda et al., 2014; Reis et al., 2017) and this has been reported to be linked to a compensatory increase in cell wall chitin content (Fortwendel et al., 2010; Rueda et al., 2014, Reis et al., 2017).

Recently, we described that the tip localization of the β-1,3-glucan synthase complex is associated with the main morphological responses during paradoxical growth in A. fumigatus (Moreno-Velasquez et al., 2017). However, little is known about the initial cell morphological responses of mature hyphae, as well as the cell wall adaptations of A. fumigatus before and after paradoxical growth caused by caspofungin treatment. Therefore, by using live-cell imaging, we
investigated the subcellular dynamics of the Ca\textsuperscript{2+}-related transcription factor CrzA and the cell wall response of \textit{A. fumigatus} during paradoxical growth.

5.3. Materials and methods

5.3.1. Strains, media and growth conditions

All the \textit{A. fumigatus} strains (Table 5.1) were maintained at 37°C on Vogel’s medium (VM) containing 2\% sucrose, 1.5\% agar (w/v), and supplemented with uracil and uridine when required (Vogel, 1956). Fresh conidia were harvested with 0.05\% Tween 20 and the conidial concentration was determined by counting using a haemocytometer. To monitor and quantify the paradoxical effect in individual cells or at the colony level, 500 conidia were incubated in 1.5 ml of liquid VM in a \# 1 German borosilicate 2-well slide culture chamber or 12-well chamber (Thermo Scientific), respectively.

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Table 5.1. Strains used in this study.
5.3.2. **Antifungal susceptibility**

The minimal effective concentration of caspofungin (MEC) was determined according to the reference procedure for testing mould susceptibility established by the Antifungal Susceptibility Testing Subcommittee of EUCAST (EUCAST, 2015; Lass-Flörl et al., 2006). Caspofungin was dissolved in sterile water and used at a concentration range of between 0.03 to 16 µg/ml. Twofold serial dilutions in the assay medium were initially prepared in flat-bottom 96 well cell plates (Corning, USA) in order to obtain final concentrations ranging from 16 to 0.03 µg/ml of caspofungin in Vogel’s medium at a total volume of 200 µl. After the inoculation of 500 conidia per well and incubation for 24 h at 37 ºC, the MEC was defined microscopically as the lowest drug concentration that produced short, stubby and highly branched hyphae (Antachopoulos et al., 2007; Espinel-Ingroff, 1998, 2003).

5.3.3. **α-(1,3)-glucan staining in cell walls**

In order to study the localization and content of α-1,3-glucans in the cell wall, the fluorescently labelled antibody, MOPC-104E (Sigma), which specifically recognizes α-1,3-glucans in the cell wall, was used. Cells were prepared with some modifications as previously described (Fujikawa et al., 2009).

500 conidia exposed to caspofungin were inoculated in VM at 37°C. After 24 and 48 h, the medium was removed and fungal cells were fixed with 3% (v/v) formaldehyde solution in distilled water for 1 h. Cells were washed three times in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) for 5 min. Cells were infiltrated in 1% (v/v) Tween 20 in PBS buffer (PBS-T). The samples were incubated with MOPC-104E IgM (λ light chain) to a final concentration of 4 µg/ml in PBS-T overnight at room temperature in darkness. Cells were washed three times with PBS and re-incubated for 3 h in dark with Alexa Fluor 488
goat anti-mouse IgM (µ chain) to a final concentration of 10 µg/ml in PBS buffer. The secondary antibody was removed by washing cells three times with PBS. After rinsing with PBS, the samples were imaged (Ex/Em 488/520 nm). Fixed samples were kept at 4°C for three weeks.

5.3.4. Confocal microscopy

Live-cell imaging of fungal cells at high resolution was performed using an inverted Leica laser scanning confocal microscope (TCS SP8X), equipped with blue diode, white light, and argon ion lasers. Simultaneous brightfield images were captured with a transmitted light detector. The laser intensity and laser exposure of the cells were kept to a minimum to reduce photobleaching and phototoxic effects. A 40x/0.85 NA dry and a 63x/1.4 NA water objective lens were used. Imaging was carried out at 37°C in a microscope temperature controlled chamber (Cube & Box, Switzerland). Confocal images were captured using the Leica microsystem CMS software (v. 3.3, LAS AF). Images were processed using ImageJ (v. 1.44, MacBiophotonics) and Adobe Photoshop (v. 13, Adobe).

5.3.5 Measurements of different cell wall components

To visualize the localization and quantify the different cell wall components, such as chitin, β-1,3 glucan and galactomannans, staining was performed for 30 min at 37 °C with calcofluor white (Ex/Em 405/450-550 nm), aniline blue (Ex/Em 405/450-550 nm) and concanavalin A (Ex/Em 488/505-520), respectively, at final concentrations of 1 µg/ml, 100 µg/ml, and 7.5 µg/ml, respectively. Images were captured of optimally focused hypha tips with the same exposure time used for all the samples using the Leica Application Suite (LAS) program. Mean fluorescence intensities of leading hyphae (25 µm from the tip) were calculated for at least 20 individual cells under each condition by using ImageJ software (NIH).
5.4. Results

5.4.1. Minimal effective concentration of caspofungin

Because several *Aspergillus* species and isolates show different degrees of susceptibility to caspofungin, we determined the minimal effective concentration (MEC) of the strain CEA10 of *A. fumigatus*, according to the EUCAST protocol (EUCAST, 2015) (Fig. 5.1). The concentration of caspofungin was ranged from 0.03 to 16 µg/ml in Vogel’s medium. After 24 h of treatment, the minimal concentration of caspofungin that induced morphological damage such as short, stubby and lysis of several hyphal compartments was 0.5 µg/ml (Fig. 5.1B). Cell damage was clear after 48 h in the continuous presence of caspofungin.

![Figure 5.1. A. fumigatus morphology in response to the minimal effective concentration of caspofungin. Conidia of the strain CEA10 were inoculated in Vogel’s medium in absence or presence of caspofungin for 72 h at 37 °C. A) Untreated cells. B) 0.5 µg/ml of caspofungin (MEC). Scale bars: 10 µm.](image)
5.4.2. Caspofungin induces the mislocalization of Fks1 in young germlings and mature hyphae

In order to study the response of the β-1,3-glucan synthase complex following caspofungin treatment in mature hyphae, we examined the subcellular localization pattern of the catalytic subunit of the glucan synthase, Fks1, during initial exposure to caspofungin. A strain expressing GFP-labelled Fks1 under the control of its endogenous promoter was constructed to image the protein in living hyphae. Untreated cells showed Fks1 localized predominantly at the tips and septa (Fig. 5.2). However, the addition of caspofungin resulted in the loss of the GFP signal at tips after 30 min.

Concomitantly, the tip inhibition of Fks1 induced the cessation of hyphal elongation, as well as the polarity loss and dichotomous branching at hyphal tips. Surprisingly, this stress response was accentuated in cells exposed to lower concentration of caspofungin (0.5 µg/ml).

To further investigate the dynamics of Fks1 during early developmental stages in A. fumigatus, we studied the subcellular localization of Fks1 to continuous escalating doses of caspofungin in young germlings (Fig. 5.3). For this purpose, fresh conidia were cultured in Vogel’s medium with caspofungin for 16 h. Untreated cells, exhibited a tip localization of Fks1, whereas cells in the continuous presence of 0.5, 1 or 4 µg/ml of caspofungin showed a random localization of Fks1 to vacuole-like structures in apical and subapical compartments (Fig. 5.3 B,C,D). These observations were consistent with the aberrant morphology of A. fumigatus. Notably, no obvious differences were observed with different caspofungin concentrations. Interestingly, few bursting cells were noticed after 16 h of caspofungin treatment, suggesting that the hyphal tip lytic activity of caspofungin is cell developmental stage-dependent.
Figure 5.2. Caspofungin causes loss of the tip localization of Fks1 in mature hyphae of *A. fumigatus*. Conidia of the strain Fks1::eGFP were incubated in Vogel’s medium at 37 °C for 18 h in absence of any drug. Caspofungin was immediately added and the response of Fks1 was monitored by confocal microscopy. White arrows indicate the subcellular localization of Fks1. Scale bars: 5 µm.
Figure 5.3. Caspofungin causes a loss in the tip localization of Fks1 in young hyphae of \textit{A. fumigatus}. Conidia of the strain Fks1::eGFP were incubated in Vogel’s medium at 37 °C for 16 h in absence or presence of caspofungin. A) Untreated cells. B) 0.5 µg/ml of caspofungin. C) 1 µg/ml of caspofungin. D) 4 µg/ml of caspofungin. White arrows indicate the subcellular localization of Fks1. Scale bars: 10 µm.
5.4.3. The mislocalization of Fks1 caused by caspofungin blocks the tip production of β-1,3 glucan but the chitin production increases in mature hyphae of *A. fumigatus*

In order to study the β-1,3-glucan production in mature hyphae treated with caspofungin, after the tip disruption of Fks1, hyphae were stained with the cell-wall-selective β-1,3-glucan dye, aniline blue (Beauvais *et al.*, 2001). Untreated cells showed the most intense fluorescence signal at tips, indicating that the newly synthesized β-1,3-glucan occurs in hyphal tips (Fig.5.4). However, cells exposed to either 0.5 or 4 µg/ml of caspofungin showed no β-1,3-glucan signal at hyphal tips (Fig. 5.4 B, C). Although cells grown in the presence of caspofungin exhibited an aberrant apical morphology including tip lytic events in some hyphae, cellular extension still occurred.

The reduced content of β-1,3-glucan in the cell wall caused by caspofungin treatment resulted in it being compensated by increased chitin in *C. albicans* and *A. fumigatus* (Fortwendel *et al.*, 2010; Rueda *et al.*, 2014; Reis *et al.*, 2017). To investigate the *in vitro* response of the cell wall chitin following caspofungin treatment, we stained the cells with calcofluor (Fig.5.5). Untreated cells showed a normal distribution of chitin with stronger intensity in both the septa and hyphal tips. However, cells in the presence of either 0.5 or 4 µg/ml of caspofungin exhibited an increased intensity of calcofluor-labelled chitin throughout all the hyphae, particularly at their tips (Fig.5.5 B, C). Interestingly, hyphae exposed to 4 µg/ml of caspofungin exhibited stronger intensity of calcofluor-labelled chitin when compared to hyphae treated with 0.5 µg/ml of caspofungin. This increased concentration of chitin, as a compensatory mechanism for the reduced content of β-1,3-glucan, was unable to block hyphal bursting. This finding shows that chitin accumulation is insufficient to rescue *A. fumigatus* hyphae from the hyphal tip lytic activity of caspofungin.
Figure 5.4. Cell wall β-1,3-glucan content following disruption of Fks1 by caspofungin in mature hyphae in *A. fumigatus*. Conidia of the strain CEA10 were incubated in Vogel’s medium at 37 °C for 18 h in absence of any drug. Cells were treated with caspofungin for 30 min followed by aniline blue staining. A) Untreated cells. B) Cells treated 0.5 µg/ml of caspofungin. C) Cells treated with 4 µg/ml of caspofungin. Asterisks in A) highlight the normal tip signal of β-1,3-glucan. White arrows highlight the lack of β-glucan at the tips. Black arrow indicates tip bursting. Scale bars: 10 µm.
Figure 5.5. Cell wall chitin content followed by inhibition of Fks1 by caspofungin treatment mature hyphae of *A. fumigatus*. Conidia of the strain CEA10 were incubated in Vogel’s medium at 37 ºC for 18 h in absence of the drug. Cells were treated with caspofungin for 30 min followed by calcofluor white staining. A) Untreated cells. B) Cells treated with 0.5 µg/ml of caspofungin. C) Cells treated with 4 µg/ml of caspofungin. White arrows highlight the signal of chitin at the tips. Note the increased tip-chitin and lateral cell signal in hyphae exposed to caspofungin. Black arrow indicates tip bursting. Scale bars: 10 µm.
Measurements of the cell wall chitin in the first 25 μm region of mature hyphae following caspofungin treatment showed that the content of chitin after the disruption of Fks1 increased in a dose-dependent manner (Fig. 5.6). Cells exposed to either 0.5 or 4 μg/ml of caspofungin exhibited a significantly increased intensity of calcofluor labelled cell wall chitin at the hyphal tip (p <0.05; Fig. 5.5 B and-C), and lateral cell walls, and this increase was greater in cells subjected to 4 μg/ml of caspofungin (Fig. 5.5C) compared to the untreated control.

Figure 5.6. Cell wall chitin content in mature hyphae of A. fumigatus increases after the disruption of Fks1 by caspofungin. Conidia of the strain CEA10 were incubated in Vogel’s medium at 37 ºC for 18 h in absence of any drug. Cells were treated with caspofungin for 30 min followed by calcofluor white staining. Graphs represent the quantification of the first 25 μm region of mature leading hyphae. The cell wall chitin intensity was measured as arbitrary units (a.u.) of calcofluor white fluorescence intensity *p= 0.05 represents the significant increase of calcofluor white treatment following caspofungin treatment compared to the untreated control.
5.4.4. Caspofungin induces the nuclear translocation of the transcription factor, CrzA

Calcium signalling plays an essential role during antifungal therapy, such as echinocandin treatment (Fortwendel et al., 2010). Furthermore, paradoxical growth is associated with the activation of the Ca\(^{2+}\)-regulated proteins in A. fumigatus (Juvvadi et al., 2015). Therefore, in order to further understand the link between the Ca\(^{2+}\)-signalling and the stress response caused by caspofungin, we investigated the Ca\(^{2+}\)-related transcription factor CrzA in living hyphae. For this purpose, we used a CrzA::eGFP fusion strain under the control of its endogenous promotor.

In untreated cells, CrzA::eGFP was localized predominantly in the cytosol as has been previously shown to be recruited to nuclei in the presence of high extracellular CaCl\(_2\) (Karababa et al., 2006). This was used as a control in my experiments in which I was able to show that 100 mM CaCl\(_2\) also induced the nuclear recruitment of CrzA::eGFP after 10 min in non-caspofungin treated cells (Fig.5.7A). In cells exposed to high extracellular concentrations CaCl\(_2\), CrzA::eGFP localized in all nuclei within 5 min after the initial stimulus, remained in all nuclei until ~ 15 min after which CrzA::eGFP leaked out of nuclei. After ~ 40 min still retained CrzA::eGFP (Fig.5.7 B, C, D). Conversely to the CaCl\(_2\)-treated cells, the presence of either 0.5 or 4 µg/ml of caspofungin induced the translocation of CrzA::eGFP into nuclei in fewer cells in a dose-dependent manner (Fig. 5.8). The presence of 0.5 µg/ml of caspofungin induced the nuclear recruitment in ~15% of all treated cells observed with a maximum response after 10 min, whereas cells in the presence of 4 µg/ml of caspofungin caused nuclear recruitment of CrzA::eGFP in ~25% of cells after 15 min. Hyphal tip lysis, was observed after nuclear CrzA::eGFP recruitment 30-60 min later in apical and in subapical cells, which all formed branches to subsequently become apical cells. Interestingly, the presence of 4 µg/ml of
micafungin induced a similar CrzA::eGFP recruitment response (Fig. 5.7D), as in response to caspofungin.

Figure 5.7. Caspofungin and micafungin induce nuclear recruitment of the Ca\(^{2+}\)-regulated transcription factor, CrzA, in *A. fumigatus*. Conidia of the strain CrzA::eGFP were incubated in Vogel’s medium at 37 °C for 18 h in the absence of drug. Caspofungin was immediately added and the recruitment of CrzA-eGFP to nuclei was monitored by confocal microscopy. A) 100 mM CaCl\(_2\). B) 0.5 µg/ml of caspofungin. C) 4 µg/ml of caspofungin. D) 4 µg/ml of micafungin. White arrows highlight the CrzA::eGFP signal in nuclei. Scale bars: 10 µm.
Figure 5.8. Percentage of cells exhibiting CrzA-nuclear recruitment in response to caspofungin treatment. Conidia of the CrzA::eGFP strain were incubated in Vogel’s medium at 37 °C for 18 h in the absence of the drug. Caspofungin was immediately added and the recruitment of CrzA::eGFP to nuclei was monitored by confocal microscopy. Each point represents the mean value ± standard deviation. CaCl₂:100 mM. CAS: caspofungin.
5.4.5. The nuclear dynamics of *A. fumigatus* is affected by caspofungin treatment

The main morphological responses of *A. fumigatus* hyphae exposed to caspofungin have recently been linked to the expression and dynamic subcellular activity of the catalytic subunit of the β-1,3-glucan synthase complex, Fks1 (Loiko and Wagener, 2016; Moreno-Velásquez *et al.*, 2017). However, the subcellular and molecular dynamics associated with other organelles in response to caspofungin remains unknown. In order to elucidate if the mislocalization of Fks1 is coupled with cellular damage, nuclear localization was studied by imaging the protein histone H1 labelled with GFP.

Untreated cells exhibited nuclei uniformly distributed throughout the hyphal compartments of *A. fumigatus* (Fig. 5.9A). Leading hyphae showed the leading nucleus maintaining a constant spherical shape at a continuous distance from apex of roughly 10-13 µm (“nucleus exclusion zone”). The majority of nuclei were oriented in parallel to the growth axis. Untreated hyphae often showed a wave of nuclear division within a single leading hyphal compartment (data not shown) as shown previously in *A. nidulans* (Hickey and Read, 2009). Nuclei were observed moving in either direction or bi-directionally. Cells in the presence of either 0.5 or 4 µg/ml of caspofungin exhibited nuclei with a similar spherical shape to untreated ones (Fig. 5.9B, C). However, the distribution of nuclei was affected by caspofungin treatment. While untreated cells showed a large number of nuclei per apical compartment (not precisely quantified), cells treated with caspofungin clearly possessed fewer nuclei per apical cell compartment (typically 6-8 nuclei per compartment). Furthermore, cells exposed to caspofungin showed nuclei closer to the hyphal tip. Apical cells exposed to 0.5 µg/ml of caspofungin exhibited nuclei with distances of 1-4 µm from the apex, whereas cells exposed to 4 µg/ml of caspofungin exhibited nuclei distances of 3-7 µm from the apex.
Figure 5.9. The nuclear localization and dynamics in *A. fumigatus* hyphae treated with caspofungin. Conidia of the strain H1: GFP were incubated in Vogel’s medium at 37 °C for 24 h in the absence or presence of caspofungin. A) Untreated hyphae. B) 0.5 µg/ml of caspofungin. C) 4 µg/ml of caspofungin. Asterisks highlight the “nucleus exclusion zone”. Note the smaller exclusion zone in cells treated with caspofungin. Scale bars: 5 µm.
5.4.6. The vacuolar dynamics of *A. fumigatus* is unaffected by caspofungin

The vacuolar system in filamentous fungi is responsible for the osmoregulation, storage of molecules, solute transport along hyphae and removal of toxic components (Richards *et al.*, 2010; Veses *et al.*, 2008). In order to study the subcellular organization and dynamics of *A. fumigatus* vacuoles in response to caspofungin, a Ku80 strain expressing the syntaxin (VAM3) fused to RFP (VAM3::RFP) under the control of the *gpdA* promotor, was constructed (Fig. 5.10).

Untreated cells exhibited a gradient of different vacuolar morphologies back from the hyphal tip (Fig. 5.10A) as previously described for *A. oryzae* (Shoji *et al.*, 2006) and *A. nidulans* (Hickey and Read, 2009). While vacuoles close to hyphal tips were smaller, vacuoles localized in subapical compartments were larger. Notably, larger vacuoles were observed behind the first septum from apical hyphal cells. Often it was observed that different vacuolar compartments split apart and fused with each other. Furthermore, vacuoles randomly moved either towards the bases or tips of hyphal compartments and through septal pores between hyphal compartments (data not shown). Cells exposed to either 0.5 or 4 µg/ml of caspofungin exhibited a large accumulation of vacuoles in subapical compartments (Fig. 5.10B, C). However, no obvious differences in shape, number or dynamics of vacuolar organelles were observed when caspofungin-treated hyphae were compared to untreated control hyphae. These data indicate vacuole organization and dynamics seem to be unaffected by caspofungin treatment.
Figure 5.10. Vacuolar organization dynamics in *A. fumigatus* hyphae is unaffected by caspofungin. Conidia of the strain VAM3::RFP were incubated in Vogel’s medium at 37 °C for 24 h in the absence or presence of caspofungin. A) Untreated hyphae. B) 0.5 µg/ml of caspofungin. C) 4 µg/ml of caspofungin. Scale bars: 5 µm.
5.4.7. Colonies of A. fumigatus CEA10 exhibit the paradoxical effect by 48 h

The paradoxical growth in yeast and filamentous fungi is known to be echinocandin and species-specific (Chamilos et al., 2007; Fortwendel et al., 2010). Further to the description of the time course of microcolony growth described in chapter 4 (Fig. 4.1) in relation to the paradoxical effect in the A. fumigatus clinical strain CEA10, here we describe the same effect at the macroscopic colony level induced by caspofungin were examined during 96 h (Fig. 5.1). The untreated control showed faster radial growth than the colonies treated with 0.5 and 4 µg/ml of caspofungin. Initially, colonies subjected to either 0.5 or 4 µg/ml of caspofungin exhibited a similar morphology and colony size. However, after 48 h, the edges of the colonies treated with 4 µg/ml of caspofungin showed a slight recovery to the wild-type growth pattern, whereas the colonies subjected to 0.5 µg/ml of caspofungin continuously showed a smaller size, with a dense compact morphology. By 72 h, colonies exposed to 4 µg/ml of caspofungin had fully recovered the normal wild type morphology.

To further investigate the morphological effects of caspofungin on A. fumigatus, the colony diameters of the strain CEA10 was also measured (Fig. 5.12). Initially, colonies under caspofungin treatment showed similar radial growth ($p > 0.05$). By 48 h the radial growth of the colonies treated with 4 µg/ml of caspofungin was continuously faster than that of colonies treated with 0.5 µg/ml ($p < 0.05$) consistent with the earlier finding that colonies exposed to caspofungin develop paradoxical effect by 48 h following treatment.
Figure 5.11. Colony growth of *A. fumigatus* exposed to caspofungin. Comparison of CEA10 strain grown on Vogel’s medium in the presence of 0.5 or 4 µg/ml of caspofungin at 37 °C for 96 h.
Figure 5.12. Influence of caspofungin treatment on Colony diameters of *A. fumigatus* CEA10. Colonies were treated with 0.5 and 4 µg/ml of caspofungin on Vogel’s medium at 37 °C for periods up to 96 h. Colony measurements were performed three times on three biological samples (average ± standard deviation [SD], n = 9). The asterisks indicate significant differences ($p < 0.05$) from untreated cells.
5.4.8. Some clinical azole-resistant strains show paradoxical growth

Caspofungin has been widely used to treat both yeast and mould azole-resistant strains (Bennett, 2006; Denning, 2003; Morrison, 2006). However, the mechanism of caspofungin-mediated paradoxical growth in A. fumigatus azole resistant strains remains unknown. To investigate if azole resistant strains develop tolerance to increasing concentrations of caspofungin for long treatment periods, I monitored the onset of the paradoxical growth at colony level in azole resistant strains isolated from patients (Fig. 5.13).

The untreated azole resistant control showed faster radial growth than the strains in the presence of either 0.5 or 4 µg/ml of caspofungin. After 24 h, colonies subjected to either 0.5 or 4 µg/ml of caspofungin exhibited a similar morphology and colony size. With the exception of the strains 3AC and 19AK, after 48 h of treatment, the edges of the colonies treated with 4 µg/ml of caspofungin slightly recovered the wild-type growth pattern, whereas colonies subjected to 0.5 µg/ml of caspofungin showed continuously a smaller size, with a dense compact morphology as described for strain CEA10. With the exception of strains 3AC and 19AK, after 72 h colonies of each clinical strain exposed to 4 µg/ml of caspofungin recovered the wild-type morphology with fuzzy edges to their colonies. Independently of the concentration of caspofungin, these strains show similar colony morphology and size. These data indicate that the development of paradoxical growth caused by caspofungin in some strains is independent of the resistance to azoles.
Figure 5.13. Caspofungin treatment induces paradoxical growth in some *A. fumigatus* azole-resistant strains. 500 conidia of the indicated strains were spotted onto Vogel’s medium in presence of no, 0.5, or 4 µg/ml of caspofungin for 96 h at 37 °C. All the strains were isolated from patients.
5.4.9. Paradoxical growth in leading hyphae is associated with a normal content of α-1,3- and β-1,3-glucan, but not chitin

Previous studies showed the upregulation of chitin biosynthesis in response to growth inhibitory concentrations of caspofungin (Fortwendel et al., 2010; Rueda et al., 2014; Reis et al., 2017). Changes in hyphal growth at different concentrations of caspofungin and the intracellular dynamics of Fks1 would be expected to impact the synthesis of other major components of the cell wall. To further analyse this, we quantified the β-1,3-glucan, α-1,3-glucan and chitin contents in actively growing hyphal tips. Strains exposed to caspofungin were stained with aniline blue, calcofluor white and the primary antibody MOPC-104, which label β-1,3-glucan, chitin, and α-1,3-glucan, respectively. Quantification of the respective cell wall components was performed by measuring the signal intensity in the first 25 µm region from the tips of leading hyphae before (at 24 h) and during paradoxical growth (at 48 h).

While the untreated control strain exhibited an uniform distribution of β-1,3-glucan over the cell wall with the most intense signal in the apical zone (Fig. 5.14), treatment with caspofungin showed a reduction of β-1,3-glucan staining in a concentration-dependent manner at 24 h (Fig. 5.14D). Compared to untreated controls, hyphae exposed to either 0.5 or 4 µg/ml of caspofungin at 24 h showed 70% and 87% less β-1,3-glucan content in their apical regions, respectively (Fig. 5.14B, C, D). However, some cell wall β-1,3-glucan was still observed stained at the hyphal tips and septa under these conditions suggesting that caspofungin does not completely abolish the production of β-1,3-glucan.
Figure 5.14. Cell-wall β-1,3 glucan in leading hyphae is reduced by caspofungin, but restored during paradoxical growth. Conidia of *A. fumigatus* were inoculated in Vogel’s medium in absence or presence of caspofungin for 48 h at 37 °C. A) Untreated cells. B) 0.5 µg/ml. C) 4 µg/ml. D) Quantification of the leading hyphae cell wall β-1,3-glucan intensity measured as relative fluorescence units. Images show leading hyphae stained with aniline blue (100 µg/ml) for 30 min. Note the recovery of the β-1,3-glucan signal by 48 h. Scale bars: 10 µm. The asterisks indicate significant differences (*p* < 0.05) from untreated cells.
After 48 h of continuous caspofungin treatment, and following the initiation of paradoxical growth, leading hyphae exposed to either 0.5 or 4 µg/ml exhibited an increased content of β-1,3-glucan in their cell walls, which was particularly concentrated in the 25 µm region behind the tips (Fig. 5.14B, C, D). Only leading hyphae exposed to 0.5 µg/ml of caspofungin showed a slightly reduced amount (10%) of β-1,3-glucan compared to the untreated hyphae or those exposed to 4 µg/ml of caspofungin (Fig. 5.14D).

In contrast to the hyphal tip focused localization of β-1,3 glucan, imaging of α-1,3-glucan using fluorescently labelled with MOPC-104E IgM antibody revealed a homogeneous distribution throughout the cell wall during 24 and 48 h of growth under control (untreated) conditions (Fig. 5.15). However, although the distribution of α-1,3-glucan remained unaffected, hyphae exposed to either 0.5 or 4 µg/ml of caspofungin for 24 h showed a 2-fold increase in α-1,3-glucan content compared to the control (Fig. 5.15D). Surprisingly, after 48 h, hyphae exposed to caspofungin showed reduced α-1,3-glucan in the cell wall. While the leading hyphae exhibiting paradoxical growth (exposed to 4 µg/ml of caspofungin for 48 h) exhibited a similar α-1,3-glucan content compared to the untreated control, hyphae in presence of 0.5 µg/ml of caspofungin showed a slightly higher concentration (1.36-fold increase) of α-1,3-glucan. Of particular note, only the chitin content of cell walls remained higher than untreated controls during all the caspofungin treatment (Fig. 5.16).

Taken together, these results reveal that the initial reduction of β-1,3-glucan caused by caspofungin is compensated by the other major cell wall components, and with the exception of chitin, their original concentrations observed in untreated cell walls was restored during paradoxical growth.
Figure 5.15. Cell-wall α-1,3 glucan stained with the primary antibody MOPC-104 in leading hyphae is increased by caspofungin but restored to the untreated level during paradoxical growth after 48 h. Conidia of A. fumigatus were inoculated in Vogel’s medium in absence or presence of caspofungin for 48 h at 37 ºC. A) Untreated cells. B) 0.5 µg/ml. C) 4 µg/ml. D) Quantification of the leading hyphae cell wall α-1,3-glucan intensity measured as relative fluorescence units. Images show leading hyphae fluorescently labelled with MOPC-104E IgM antibody (4 µg/ml/PBS-T). Scale bars: 10 µm. * significant differences (p < 0.05) from untreated cells.
Figure 5.16. Cell-wall chitin in leading hyphae is increased by caspofungin treatment. Conidia of *A. fumigatus* were inoculated in Vogel’s medium in the absence or presence of caspofungin for 48 h at 37 ºC. A) Untreated cells. B) 0.5 µg/ml. C) 4 µg/ml. D) Quantification of the cell wall chitin in leading hyphae intensity measured as relative fluorescence units of the dye. Images show leading hyphae stained with calcofluor white (1 µg/ml). Scale bars: 10 µm. The asterisks indicate significant differences (*p* < 0.05) from untreated cells.
5.4.10. Paradoxical growth in *A. fumigatus* is not caused by caspofungin inactivity

In order to exclude, or demonstrate, the possibility that paradoxical growth in *A. fumigatus* is caused by the loss of caspofungin activity at high concentrations, we determined the stability and activity of caspofungin after the induction of paradoxical growth. For this purpose, we induced the development of paradoxical growth by collecting the media from the wells in which I had previously performed antifungal susceptibility testing with cells, as previously reported for *C. albicans* (Rueda *et al.*, 2014). Since the media used in this experiment was collected from wells previously incubated with *A. fumigatus* for 48 h, I had to also perform experiments to check that nutrient deprivation might not be influencing the activity of caspofungin. For this purpose I supplemented the media with fresh 2% glucose.

For new caspofungin, the EC$_{50}$ was 1.2 µg/ml ($p < 0.0001$) (Fig. 5.17). Strikingly, I found that the re-used medium containing caspofungin, and which had contained cells that had shown the paradoxical effect after 48 h, generated an EC$_{50}$ of 0.85 µg/ml ($p < 0.0001$) (Fig. 5.17B), showing a slightly higher antifungal activity ($p < 0.05$). Consistently, re-used medium containing caspofungin induced the typical microcolony growth with short and highly branched leading hyphae, as those cells exposed to fresh caspofungin (Fig. 5.17C). This data suggests that the paradoxical growth is not a response due to the lack of caspofungin activity.
Figure 5.17. EC$_{50}$ of new and re-used caspofungin. 500 conidia were inoculated in Vogel’s medium for 24 h at 37 °C. A) Fresh medium with new caspofungin. B) Re-used medium containing caspofungin after paradoxical growth. C) A. fumigatus exposed to the re-used medium containing caspofungin for 24 h. Absorbance was measured at 610 nm. Analyses were performed three times on three biological samples (average ± standard deviation [SD] n= 9).
5.5. Discussion

The research described in this chapter describes different cellular and molecular responses of 6-18 h old mature hyphae of *A. fumigatus* exposed for short periods (30 min) of low (0.5 μg./ml) and high (4.0 μg/ml) doses of caspofungin with a particular emphasis on monitoring the paradoxical effect. The responses analysed were: dynamic changes to hyphal growth and organization; Fks1 mislocalization; the compensatory effects of caspofungin on different cell wall components; recruitment of the Ca$^{2+}$-regulated transcription factor CrzA to nuclei; changes in nuclear and vacuolar organization; dependence of the paradoxical effect on azole resistance in some clinically isolated azole resistant strains; and the activity of caspofungin in media after 48 h once paradoxical growth has been initiated.

Surprisingly, at clinically relevant concentrations (e.g. 0.5-4.0 μg/ml) caspofungin is unable to cause a complete inhibition of *A. fumigatus* growth, but induces morphological changes. The minimum effective concentration (MEC), defined as the lowest drug concentration at which short, stubby and hyperbranching hyphae are visualized, has been introduced as a standard determination of caspofungin activity *in vitro* (Kurtz et al., 1994). In agreement with our results, previous reports in *A. fumigatus* and *A. terreus* showed the same MEC values using different media and caspofungin from different brands (Merck & Co. Inc. and Sigma) (Arikan et al., 2001). The MEC values showed here coincided with the MIC values for several *Candida* species, (Pfaller et al., 2006) and with different growth media, including Vogel’s medium. The latter minimal medium is an alternative cheap, easy to prepare growth medium to study *A. fumigatus* in the presence of caspofungin. In particular, it has the added advantage for live-cell fluorescence imaging of exhibiting low autofluorescence, which is a major problem with the
standard medium, RPMI 1640, used to determine MEC values (Moreno-Velasquez et al. 2017; chapter 3)

The catalytic subunit of the β-1,3 glucan synthase complex, Fks1, is the target of caspofungin (Douglas, 2001). Recently, Moreno-Velasquez et al. (2017) (also see chapter 3) showed that the presence of caspofungin induced the tip mislocalisation of Fks1 to vacuoles and this was consistent with the impaired growth of A. fumigatus. Furthermore, the expression of the β-1,3 glucan synthase has been shown to be an essential requirement for paradoxical growth (Loiko and Wagener, 2016). Here, we observed that in mature hyphae the mislocalisation of Fks1 caused by caspofungin occurs approximately within 30 min of exposure to the drug. The removal of Fks1 from the hyphal tip was consistent with the cessation of hyphal extension and disruption of polarized growth, lack of β-1,3-glucan and compensation by the increased formation of chitin in the cell walls of apical compartments, as previously reported (Loiko and Wagener, 2016; Rueda et al., 2014). Vacuoles are responsible for the storage of nutrients and degradation of toxic molecules (Richards et al., 2010; Veses et al., 2008). Results have been obtained which are consistent with Fks1 being endocytically internalized to the vacuole (Chapter 3; Moreno-Velasquez et al., 2017) where it probably undergoes degradation.

In filamentous fungi, the Ca^{2+}-dependent calcineurin pathway is essential to tolerate various stresses including temperature, pH, and antifungal drugs (Juvvadi et al., 2014). Calcineurin is the main regulator of the transcription factor CrzA, whose activities range from ion homeostasis to cell wall biogenesis (Thewes, 2014). Although, CrzA signaling is necessary during infection, it is unclear whether the transcription factor CrzA plays an additional role during antifungal treatment. Fortwendel et al. (2009) showed that A. fumigatus responds to caspofungin by the overexpression of chitin synthases, which in turn are regulated by the calcineurin pathway.
However, the direct connection between calcineurin-CrzA signaling during caspofungin treatment was not tested. Here, we showed that different concentration of caspofungin and micafungin induce the nuclear recruitment of CrzA in A. fumigatus. In agreement with my results, Ries et al. (2017) reported that a similar CrzA caspofungin-mediated nuclear localization to low concentrations of caspofungin (0.125 µg/ml). This localization was responsible for the regulation of several chitin synthases (chsA, chsC, chsG and csmB) via a direct promoter interaction, suggesting a direct role of the calcium-calcineurin-dependent CrzA during paradoxical growth (Ries et al., 2017). Interestingly, we observed several developmental and structural characteristics such as hyperbranching, higher chitin content, and hyphal lysis after the caspofungin-induced nuclear recruitment of CrzA. Altogether, these results strongly suggest a possible connection between the Ca²⁺ signaling and the response to echinocandins. Whether the nuclear recruitment of CrzA is involved in protection of the cells, preparing them for apoptosis or even regulating the paradoxical growth should be one focus of future research.

At the colony level, the growth rate of A. fumigatus in the presence of caspofungin was affected in a dose-dependent manner. During the early stages of colony development, the colonies exposed to caspofungin showed slower radial growth. Strikingly, after 72 h of incubation the colonies underlying higher concentrations of caspofungin showed an increased radial growth than the colonies subjected to lower concentrations of caspofungin. A similar dose-dependent effect of caspofungin has been shown in several pathogenic fungi, including C. albicans and A. fumigatus (Fortwendel et al., 2010; Stevens et al., 2004). This paradoxical effect is responsible of the attenuated activity of caspofungin at high concentrations (Wiederhold, 2009). We also, observed that this phenomenon in A. fumigatus is not due to the inactivity of caspofungin, as similarly reported for Candida albicans (Rueda et al., 2014). The onset of the
paradoxical growth phenomenon is characteristically delayed. In *C. albicans* it occurs after 30-35 h (Stevens *et al.*, 2005) whilst in *A. fumigatus* it occurs ~ 40 h, following incubation with caspofungin (Chapter 4; Moreno-Velasquez *et al.*, 2017). Additionally, we observed this caspofungin-mediated paradoxical response in azole-resistant strains. This response has been observed in several *Candida albicans* azole resistant strains. However, this paradoxical effect seems to be independent of azole-resistance (Stevens *et al.*, 2005).

The structure and composition of the cell wall varies with the developmental stage of a fungus and its environmental surroundings (Bartnicki-Garcia, 1968). Several studies suggest that different cell wall synthetic pathways have specific responses to compensate particular stresses (Lee and Sheppard, 2016). For instance, the loss of α-1,3-glucan was associated with an increase of β-1,3-glucan and chitin whilst the loss of β-1,3-glucan resulted in an increased production of chitin and galactosaminogalactan (Beauvais *et al.*, 2013; Henry *et al.*, 2012; Steinbach *et al.*, 2015). Therefore, it is conceivable that the reduced production of β-1,3-glucan as a consequence of caspofungin treatment may induce a specific cell wall response, which is not exclusive of chitin, as mainly reported by others (Fortwendel *et al.*, 2010; Rueda *et al.*, 2014; Reis *et al.*, 2017; Stevens *et al.*, 2005; Walker *et al.*, 2015). Of particular note was the observation that cells grown in the presence of caspofungin for 24 h showed a dose-dependent reduction of β-1,3-glucan throughout the cell walls of leading hyphae. My results indicated that this induced the overcompensation of the main cell wall components: α-1,3-glucans and chitin. It is highly likely that this altered ‘cell wall signature’ is associated with the main morphological changes including hyperbranching, increased septum formation, cell enlargement and subsequent lysis, as previously shown in *fks1* mutants (Dichtl *et al.*, 2015). Taken together, these results reveal that the initial reduction of β-1,3-glucan caused by caspofungin is compensated by the other major
cell wall components, and with the exception of chitin, their basal (i.e. normal) concentrations are restored to those of untreated cells during paradoxical growth.

Altogether, the results described in this chapter have highlighted several novel responses of *Aspergillus fumigatus* to caspofungin that may play roles be of clinical significance in an infected patient being treated with the drug.

5.6. References


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CHAPTER 6

6. Conclusions and future work

The overall and main objective of my PhD research was to investigate experimentally the cellular and molecular basis of the morphological responses of *Aspergillus fumigatus* upon caspofungin treatment with a particular emphasis on the paradoxical effect. The toxic profile of polyenes and the emergence of azole resistant strains have promoted the use of echinocandins, and particularly caspofungin, as an important second-line therapy against invasive aspergillosis (Bueid *et al.*, 2010; Lamoth *et al.*, 2013). However, the safety profile of caspofungin has raised the possibility of dosage escalation in order to treat difficult-infections (Steinbach *et al.*, 2015). However, although caspofungin is inhibitory at low concentrations, it exhibits a ‘paradoxical effect’ (reversal of growth inhibition) at high concentrations by an undetermined mechanism. The research described in this thesis analyses several cellular and molecular adaptations developed by *A. fumigatus* in response to escalating concentrations of caspofungin, and particularly those associated with the paradoxical effect. This final chapter describes the major conclusions and proposed future work from the research described in Chapters 3-5.

6.1. Chapter 3

The research described in Chapter 3 has been accepted for publication by *Antimicrobial Agents and Chemotherapy*. Two major findings discovered from the research described in chapter 3 were:

1) **Leading hyphae of *A. fumigatus* overcome the hyphal tip lytic effect of caspofungin by regenerative intra-hyphal growth.**
This is part of a highly dynamic fungicidal process at the colony periphery, which involves repeated apical hyphal compartment lysis followed by regenerative, intrahyphal growth that occurs in combination with increased septation and subapical hyperbranching. Intra-hyphal hyphae have been termed endohyphae by Kim and Hyun (2007). Filamentous fungi commonly form endohyphae in response to damage or lysis (Bowman et al., 2006; Horiuchi et al., 1999; Kim et al., 2001; Lowry and Sussman, 1966). The importance of intrahyphal growth for hyphal regeneration following hyphal damage seems clear. However, several reports suggest that endohyphae may enable fungi to survive against adverse conditions that may normally lead to the degeneration of hyphae (Calonge, 1968; Kim et al., 2001; Shankar et al., 1998). Although this cellular behaviour has been exclusively observed in response to mechanical damage or genetic mutations in filamentous fungi (Bowman et al., 2006; Lai et al., 2012; Takeshita et al., 2006), intrahyphal growth has never, to my knowledge, been investigated as a rapid response to antifungal drugs.

In my research I described that the mislocalization of Fks1 mediated by caspofungin treatment is coupled with cell lysis and regenerative intrahyphal growth in leading hyphae of A. fumigatus. Indeed, these repeated events at the colony periphery are the main reason for the reduced colony size observed macroscopically when compared to untreated colonies. Here, I have demonstrated that the reduced colony size during a long-therapy period is not due to a ‘fungistatic’ activity of caspofungin, as generally reported (Chen et al., 2011; Denning, 2003) but seems to be exclusively due to a fungicidal effect. Therefore, it is clear that the previous use of the terms ‘fungistatic’ or ‘fungicidal’ provides an over simplistic view of A. fumigatus growth inhibition by caspofungin. This has only become apparent as a result of the time-lapse, confocal
live-cell imaging that I performed to investigate the dynamic caspofungin-induced changes that occur at cellular and subcellular hyphal levels.

Future work following on from this novel discovery of regenerative hyphal growth overcoming hyphal tip lysis in response to caspofungin treatment should include: (a) a more detailed quantitative analysis and description of the regenerative growth using confocal live-cell imaging including applying fluorescent probes that label mitochondria; (b) labelling of the main protein HexA in Woronin bodies and live-cell imaging to study their behaviour during the blocking of septal pores which occurs during hyphal tip lysis and regenerative growth during growth inhibition by caspofungin.

2) The onset of paradoxical growth in *A. fumigatus* caused by caspofungin relies on the tip-localization of both the catalytic subunit (Fks1) and the regulatory subunit (Rho1) of the β-1,3-glucan synthase complex.

While caspofungin treatment induced the mislocalization of catalytic subunit of the β-1,3-glucan synthase complex, Fks1, from hyphal tips to vacuoles, I observed that the regulatory subunit, Rho1, remained at hyphal tips in the presence of the drug. Therefore, the subcellular mislocalization of Fks1 to vacuoles seems to be responsible for the impaired growth of *A. fumigatus*. Interestingly, fks1 is not an essential gene, and mutants that lack the fks1 gene behave in a similar manner to those exposed to caspofungin (Dichtl *et al.*, 2015). Furthermore, the proper expression of fks1 is necessary for the development of paradoxical growth in *A. fumigatus* (Loiko and Wagener, 2016). Thus, these data highlight that the proper tip-localization and expression of the catalytic subunit of the β-1,3-glucan synthase, Fks1, is required for the development of the paradoxical growth in *A. fumigatus*.
Although the role of Rho1 in *A. fumigatus* during caspofungin treatment is unknown, the fact that Rho1 regulates F-actin organization, the cell wall integrity pathway and the β-1,3-glucan synthase may reflect the importance of its proper tip-localization during echinocandin treatment (Beauvais *et al.*, 2001; Dichtl *et al.*, 2010; Park and Bi, 2007; Richthammer *et al.*, 2012). Hyphae grow by apical extension, which involves the spatial coupling of polarized secretion and compensatory endocytosis at the tip (Araujo-Bazán *et al.*, 2008; Taheri-Talesh *et al.*, 2008; Upadhyay and Shaw, 2008). It is likely that the subcellular mislocalization pattern of Fks1 is mediated by the endocytic machinery. Fks1 was shown to co-localize with the protein Rab5 which plays several roles in the organization of endosomal membrane domains including the regulation of early endocytosis (Pereira-Leal, 2008; Singer-Krüger *et al.*, 1994, 1995). This endosomal marker undergoes long distance bidirectional movement on microtubules via the motor dynein (Abenza *et al.*, 2009). The presence of Fks1 in vacuoles may result in caspofungin-mediated degradation of Fks1. Cessation of the Fks1 transport to the vacuole may allow the continued production and targeting of Fks1 to hyphal tips resulting in the normal morphology and growth of *A. fumigatus* during the onset of paradoxical growth.

Future studies following on from the roles of Fks1 and Rho1 in the onset of paradoxical growth should include providing stronger evidence for Fks1 being endocytically internalized and transported to the vacuole by: (a) co-localization with other endocytosis markers (e.g. FM4-64); and using non-lethal or conditional mutants compromised in different aspects of endocytosis such as actin patch formation and motor protein involvement in the process (Lara-Rojas *et al.*, 2016; Seidel *et al.*, 2013).
6.2. Chapter 4

The research described in Chapter 4 has been submitted as a Video Article to *Fungal Genetics and Biology*. Two major findings discovered from the research described in chapter 4 were:

1) **Hyphae undergoing caspofungin-induced paradoxical growth switch from repetitive hyphal tip lysis, regenerative hyphal growth, hyperbranching and increased septation and vacuolation to a normal (untreated) growth pattern.**

Building on results described in chapter 3, in chapter 4 the cellular morphological changes that occur during the onset and development of paradoxical growth in *A. fumigatus* was shown in more detail in the same microcolony by confocal live-cell imaging (Movie 4.2). Previously the onset and development of the paradoxical effect has not been reported in the same samples as achieved here and the behaviour of individual leading hyphae during paradoxical growth was unexplored. Only by continuous time-lapse imaging of the same sample over periods of 19 h has this been possible (Movies 4.1 and 4.2). With this approach it was possible to further confirm the results relating to regenerative hyphal growth described in Chapter 3. Furthermore, other experiments described in chapter 4 showed that caspofungin does not kill conidia or germlings of *A. fumigatus* although the rate of conidial germination was slightly delayed in the presence of caspofungin. Finally, it was clear from my data that cellular lysis only occurred in mature hyphae.

Future work should focus on identifying what the trigger is that initiates this caspofungin-induced growth switch. However, extensive analyses to identify a simple trigger initiating the onset of paradoxical growth have previously failed although evidence for various signaling pathways, including calcium, calcineurin, crzA, the cell wall integrity (CWI) and the HOG (high
osmolarity glycerol) pathways, have all been implicated in regulating the paradoxical effect in *C. albicans* and *A. fumigatus* (Wierhold *et al*., 2005; Munro *et al*., 2007; Fortwendel *et al*., 2010; Juvvadi *et al*., 2015; Reis *et al*., 2017). Thus, paradoxical growth may be a complex cellular adaptation to long treatment periods in response to the correct balance of multiple signals. Extensive whole genome transcriptional analysis that occurs during the 30 min period following caspofungin treatment of mature hyphae that induce Fks1 mislocalization followed by the period during which Fks1 is targeted back to the hyphal tip to initiate paradoxical growth may facilitate the identification of key players that trigger the paradoxical effect in *A. fumigatus*.

2) **Gene expression of both the main caspofungin target, the catalytic subunit as well as the regulatory subunit Rho1 of β-1,3-glucan synthase are downregulated during caspofungin treatment at growth inhibitory and paradoxical inducing concentrations.**

Surprisingly, the morphology and growth of *A. fumigatus* upon caspofungin treatment seem to be associated with downregulation of the β-1,3-glucan synthase complex (*fks1* and *rho1* genes). This shows the inability of *A. fumigatus* to compensate the inhibition of the β-1,3-glucan by caspofungin at the transcriptional level. Presumably, downregulation of the β-1,3-glucan synthase complex as a consequence of caspofungin treatment, results in a lower production of the target of caspofungin, the protein Fks1, thus the downregulation of *fks1* and *rho1* results in the reduced susceptibility of *A. fumigatus* to caspofungin, as a preventing mechanism. The incidence of resistance to echinocandins is low, but several breakthrough infections have emerged (Walker *et al*., 2008). To date, there is no clinical evidence that paradoxical growth contributes to resistance or breakthrough infections (Walker *et al*., 2010).
Future studies that identify the regulatory mechanism by which Fks1 is endocytically targeted to the vacuole and then back to the hyphal tip will be important for understanding the regulation of paradoxical growth. In particular, it will be important to determine whether the re-localization targeting of Fks1 to the hyphal tip is actually associated with the onset of paradoxical growth. Understanding this regulatory mechanism may provide novel drug targets to inhibit paradoxical growth.

6.3 Chapter 5

This Chapter describes a range of research that requires further experimentation before it can be published in one or more quality papers. Two important findings discovered from the research described in chapter 5 were:

1) **Caspofungin treatment induced the recruitment of the transcription factor CrzA to nuclei.**

Caspofungin-induced paradoxical growth bears the hallmarks of a classic Ca\(^{2+}\) stimulus-response signalling pathway initiated by a caspofungin stimulus and resulting in the translocation of the Ca\(^{2+}\)-regulated transcription factor CrzA to nuclei where it binds to specific promoter regions involved in regulating paradoxical growth. Evidence has been obtained that caspofungin activates either directly or indirectly a plasma membrane localized L-type Ca\(^{2+}\)-channel allowing Ca\(^{2+}\) to passively flow down a Ca\(^{2+}\) gradient from the external medium causing an increase in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)). This increase in [Ca\(^{2+}\)]\(_c\) and paradoxical growth were found to be inhibited by the Ca\(^{2+}\) chelator BAPTA and L-type Ca\(^{2+}\) channel blocker verapamil (Juvvadi *et al.*, 2015). The caspofungin-induced increase in [Ca\(^{2+}\)]\(_c\) is sensed by the primary intracellular Ca\(^{2+}\) receptor calmodulin, which undergoes a conformational change allowing it to interact with
important regulatory target proteins such as calcineurin. Calcineurin is composed of two subunits, a catalytic subunit, CnaA, and a regulatory subunit, CnaB, and is activated through phosphorylation at a unique serine-proline-rich region (SPR) on the CnaA subunit (Juvvadi et al., 2013, 2015).

Paradoxic growth in A. fumigatus has been shown to be associated with the activation of the Ca$^{2+}$-regulated proteins calcineurin and calmodulin in A. fumigatus (Fortwendel et al., 2009; Juvvadi et al., 2015) and calmodulin and calcineurin blockers have been found to block paradoxic growth (Juvvadi et al., 2015). Once activated, calcineurin can dephosphorylate the transcription factor CrzA in the cytoplasm allowing it to be translocates to nuclei where it binds to specific promoter regions involved in regulating paradoxic growth (Reis et al., 2017). Interestingly, I found that CrzA is not translocates to nuclei in every cell that caspofungin is exposed to and only those cells which exhibit nuclear recruitment are hyphal tip cells or subsequently produce hyphal tips as a result of branching. Hyphal tip lysis was found to only occur in these provide a caspofungin-induced response in addition to Fks1 mislocalization to the vacuole that can be monitored at the single cell level for the first time.

Future studies should focus on analysing the quantitative spatio-temporal dynamics of caspofungin-induced growth inhibition, Fks1 mislocalization, hyphal tip lysis and the onset of paradoxic growth at the single cell level using recently developed genetically encoded Ca$^{2+}$-sensitive probes (e.g. GCaMP6, Chen et al., 2013) which have been found to work extremely well in A. fumigatus (Muñoz, Bertuzzi, Bignell and Read, in prep). Previous measurements of [Ca$^{2+}$]$_c$ in response to caspofungin have been average measurements across whole cell populations using the genetically encoded, Ca$^{2+}$-sensitive, bioluminescent probe, aequorin (Juvvadi et al., 2015; Muñoz et al., 2015).
2) The main cell-wall components are reorganized during paradoxical growth in *Aspergillus fumigatus*.

The structure and composition of the cell wall vary with the developmental stages and environmental surroundings in order to adapt to particular stresses, such as antifungal agents (Bartnicki-García, 1968; Lee and Sheppard, 2016). Chapter 5 also described the cell wall signature of *A. fumigatus* in response to caspofungin treatment. Cells in the presence of caspofungin for 24 h showed a dose-dependent reduction of β-1,3-glucan along the length of the cell wall. This induced the overcompensation of the other main cell wall components, such as α-1,3-glucans, galactomannans and chitin. This particular cell-wall signature was associated with the main morphological changes including hyperbranching, impaired septa distribution, cell enlargement and subsequent lysis, as previously shown in *fks1* mutants (Dichtl et al., 2015). However, this cell wall overcompensation was insufficient to block the lytic effect caused by caspofungin. Surprisingly, after paradoxical growth the content of the main cell wall components, except chitin, was restored. This response correlates with the normal tip distribution of Fks1, normal morphology and end of hyphal tip lysis. Clearly, this cell-wall signature reveals several adaptations that *A. fumigatus* develops in order to survive caspofungin treatment. In the future, the discovery of new drugs could be developed that simultaneously block the synthesis of β-1,3-glucan, α-1,3-glucan, chitin and galactomannans as strategies to enhance the efficiency of caspofungin.

### 6.4 Model of the dynamics of the β-1,3-glucan synthase complex of *A. fumigatus* hypha in response to caspofungin treatment

Based on these observations we propose the following model (Fig. 6.1).
Figure 6.1. Model of the dynamics of the β-1,3-glucan synthase complex of *A. fumigatus* hypha in response to caspofungin treatment. After 24 h, growth inhibitory (0.5 µg/ml) and paradoxical growth (4 µg/ml) concentrations of caspofungin disrupt the tip-localization of the catalytic subunit of the glucan synthase, Fks1, to the vacuole, but the tip-localization of the regulatory subunit, Rho1, is unaffected. This localization pattern is associated with a reduced β-1,3-glucan content and an increased α-1,3-glucan and chitin content in the cell wall. Within ~ 40 h, the onset of the paradoxical growth is triggered by the re-localization of Fks1 to the hyphal tips and the recovery of β-1,3-glucan and α-1,3-glucan, to the normal levels with exception of chitin, which remains high.
This study and the lack of evidence about the paradoxical growth \textit{in vivo} raised the question of whether this phenomenon is only an \textit{in vitro} artefact observed in some pathogenic fungal species (Jacobsen \textit{et al}., 2007; Wiederhold, 2009). Intriguingly, some evidence suggests that the development of paradoxical growth \textit{in vivo} is questionable. Firstly, the caspofungin concentrations required to induce this adaptive responses are unlikely to be reached \textit{in vivo} (Rueda \textit{et al}., 2014). Secondly, the paradoxical growth induced by caspofungin against \textit{C. albicans} and \textit{A. fumigatus} is abolished by the presence of 50\% human serum \textit{in vitro}, possibly by protein binding or some other serum factor that tempers paradoxical growth (Elefanti \textit{et al}., 2013; Shields \textit{et al}., 2011). Thirdly, echinocandins used to treat either invasive candidiasis or invasive aspergillosis in patients often receive the calcineurin-mediated immunosuppressants, cyclosporine or tracolimus, which suppress the development of paradoxical growth \textit{in vitro} (Wiederhold \textit{et al}., 2005). Finally, high-doses of caspofungin increase the inflammatory response of murine macrophages (Moretti \textit{et al}., 2012; Rueda \textit{et al}., 2014) and thus, this strong immunological response in addition to the antifungal activity of caspofungin may abolish the development of \textit{A. fumigatus} in the human body.

Although we have observed several cellular and molecular adaptations, such as intrahyphal growth, the dynamic nature of the β1-3-glucan synthase complex and the development of paradoxical growth in response to caspofungin \textit{in vitro}, we cannot exclude the possibility that several of the responses described in this thesis may be of significance during infection, particularly in patients treated with high doses of caspofungin over long periods. Thus, the study of the main cellular and molecular responses \textit{in vitro} of \textit{A. fumigatus} to antifungal treatment currently remains highly valid in order to understand the rapid non-resistance related adaptations allowing \textit{A. fumigatus} to survive the onslaught of antifungal drugs.
Altogether, this the results presented in this thesis strongly support a mechanistic role for the β-1,3-glucan synthase in the induction of intrahyphal growth and the development of the paradoxical effect in *A. fumigatus*.

6.5. References


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