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Host-Fungal Interactions: Pathogenicity versus Immunity

Guest Editors: Arianna Tavanti, Julian R. Naglik, and Nir Osherov

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Editorial

Host-Fungal Interactions: Pathogenicity versus Immunity

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Fungal pathogens represent a leading cause of life-threatening infections in debilitated patients and exert a substantial toll on health care resources. A major development in the past few years, resulting from extremely rapid technological advances, has been the elucidation of the genome sequences of all of the major human fungal pathogens. Together with the development of transcriptomics and proteomics, fungal pathogen research has entered the so-called "omics" era. However, despite the remarkable acceleration in the understanding of many molecular mechanisms underlying phenomena such as drug resistance, fungal virulence, and pathogenesis, many key aspects of the host-fungal interactions are still not fully understood.

This current issue provides in-depth review articles drafted by international experts tackling different perspectives of the host-pathogen interplay. Their contributions offer insights into aspects related to fungal virulence and drug resistance of both established and emerging fungal pathogens, as well as models to study their pathogenicity.

The paper by G. P. Moran and colleagues explains why *Candida albicans* has evolved to become such a successful opportunistic pathogen, by comparative global gene expression analysis performed on *C. albicans* and its most closely related species *C. dubliniensis*.

The epidemiology of invasive fungal infections has evolved over the past twenty years. Yeasts other than *C. albicans* and moulds other than *Aspergillus fumigatus* have emerged as significant causes of invasive mycoses in severely immunocompromised patients. Tools allowing a prompt diagnosis for these emerging pathogenic fungi are lacking, often leading to a delay in effective treatment and high mortality rates. This is the case for invasive mucormycosis, a rapidly progressing infection often refractory to antifungal therapy, with a negative prognosis in individuals with impaired immune defence. The key role of iron uptake, angioinvasion, and neurotropism in the virulence repertoire of this fascinating group of fungi is reviewed by G. Morace and E. Borghi.

Chitin is one of the most abundant biopolymers in nature and comprises part of the fungal cell wall structure. Although humans do not biosynthesize chitin, they do express chitin degrading enzymes. The targeted recognition of fungal chitin by the host may have implications for diagnostic assays, as well as for potential new therapeutic approaches. This emerging topic is reviewed by K. Vega and M. Kalkum. Another major process that influences fungal cell wall structure is protein glycosylation, which is essential for eukaryotic cells. However, the mechanisms by which carbohydrates play a role in the development of fungal diseases are still poorly characterised. In this context, C. Jin provides an overview on the contribution of protein glycosylation to growth, cell wall synthesis and development in *A. fumigatus*, possibly providing a novel strategy for drug development.

Histoplasma capsulatum is a major fungal pathogen endemic to the Americas, exhibiting a broad variety of clinical presentations, ranging from mild lung infection to life-threatening systemic infections. The severity of infection and its outcome result from a complex interaction between the virulence repertoire of the pathogen and the host's immune defences. The paper by M. R. Mihu and J. D. Nosanchuk

focuses on the dynamic host-pathogen interplay in the context of the virulence arsenal displayed by the fungus and the innate and adaptive immune responses of the host.

The molecular mechanisms underlying the ability of dermatophytes to establish infection and persist in the host are reviewed by R. R. Achterman and T. C. White. Despite the fact that this group of filamentous fungi are the most common cause of cutaneous mycoses, very little is known about dermatophyte pathogenesis. The paper presents the current research status on dermatophyte virulence factors.

The ability of fungal cells to chemically communicate with each other and with other microorganisms has only recently been discovered. The paper by F. Cottier and F. A. Mühlschlegel depicts a fascinating picture of a closely connected microbial community in the environment, where multispecies communication takes place. Versatile fungal communicative competences are thought to have a significant impact on several fungal biological functions such as mating, growth, or the regulation of virulence factor expression. Among the latter, hyphal formation has been widely recognised to significantly contribute to the pathogenesis of several fungal species, by promoting adhesion to biotic or abiotic surfaces and tissue penetration. The paper by A. Brand presents the current knowledge on *C. albicans* hyphal growth during infection, addressing the issue of specific functions and mechanical/structural properties conferred by hyphal formation during infection.

Fungal pathogens have evolved complex mechanisms of resistance to antifungal drugs. The nature, frequency, and molecular mechanisms of resistance to currently used antifungals are the focus of the review by P. Vandeputte and colleagues, who also describe the latest approaches to develop new antifungal strategies.

A growing number of fungal pathogens have been associated with life-threatening infections resulting from biofilm-related infections that are particularly difficult to treat. Indeed, biofilm-embedded fungal cells are far more resistant to antifungal agents than their corresponding planktonic cells. The molecular basis of this remarkable resistance to antifungals has yet to be fully elucidated. In this respect, G. Ramage and coworkers review the latest findings on this topic and shed new light on the multifactorial nature of biofilm antifungal resistance. From a different perspective, H. Tournu and P. Van Dijck describe some of the most recent approaches shown to eradicate *Candida* biofilms, based on novel strategies to evaluate biofilm formation in vitro and in vivo.

The choice of the most appropriate experimental infection model to use for fungal pathogenesis-, virulence-, immunology-, and therapy-based studies is critical. In this issue, several contributions were made towards this topic. Mucosal and systemic models of *Candida* infection and virulence are discussed in depth by D. M. MacCallum, while the range of experimental models currently available for cryptococcosis is reviewed by W. Sabiiti and coworkers. G. Hamilos and colleagues offer a critical analysis of the use of *Drosophila melanogaster* as a surrogate model to study the immunopathogenesis of fungal infection and illustrate the recent advances in the study of medically important filamentous fungi in *Drosophila*.

Bioluminescent imaging is a powerful tool to perform real time evaluation of temporal and spatial progression of infections. Mainly used for the study of bacterial infection process, M. Brock discusses the application of bioluminescent imaging to the study of mycoses, summarising the key features of different luciferase systems and providing suggestions for future applications.

We believe that the collection of reviews authored by experts in the field presented here will provide a timely update of recent rapid developments in the field of medical mycology. We hope that the reviews will be helpful in assembling the interlocking pieces of the complex jigsaw that is the fungal-host interplay, as well as provide a basis for the generation of novel ideas and hypotheses for future experimentation.

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Review Article

Recent Advances in the Use of *Drosophila melanogaster* as a Model to Study Immunopathogenesis of Medically Important Filamentous Fungi

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Airborne opportunistic fungi, including *Aspergillus* and other less common saprophytic molds, have recently emerged as important causes of mortality in immunocompromised individuals. Understanding the molecular mechanisms of host-fungal interplay in robust experimental pathosystems is becoming a research priority for development of novel therapeutics to combat these devastating infections. Over the past decade, invertebrate hosts with evolutionarily conserved innate immune signaling pathways and powerful genetics, such as *Drosophila melanogaster*, have been employed as a means to overcome logistic restrains associated with the use mammalian models of fungal infections. Recent studies in *Drosophila* models of filamentous fungi demonstrated that several genes implicated in fungal virulence in mammals also play a similarly important pathogenic role in fruit flies, and important host-related aspects in fungal pathogenesis are evolutionarily conserved. In view of recent advances in *Drosophila* genetics, fruit flies will become an invaluable surrogate model to study immunopathogenesis of fungal diseases.

1. Introduction

In recent years, opportunistic fungi have emerged as leading causes of morbidity and mortality in immunocompromised individuals [1–3]. *Aspergillus* is by far the most common of these molds, and mortality rates for invasive aspergillosis exceed 90% in hematopoietic stem cell transplant recipients [4, 5]. Even more concerning, however, is that infections caused by other difficult-to-treat opportunistic molds, such as Mucorales species, are increasingly being observed in several cancer centers [6–8]. The increase in the frequency and spectrum of invasive fungal infections in immunocompromised patients underscores the need for expanding our knowledge of the pathogenesis of opportunistic fungal infections and developing novel therapeutic approaches.

The versatility and complexity of virulence mechanisms and predisposing host conditions that lead to development of invasive mold infections [9, 10] necessitate understanding the nature of host-fungal interactions at the cellular and molecular levels in order to identify host immune pathways and pathogen determinants involved in disease progression [11, 12]. Pioneering studies over the past decade demonstrated that a variety of opportunistic fungi can invade and cause fatal infection in a variety of simple invertebrate hosts, such as the fruit fly *Drosophila melanogaster*, and the roundworm *Caenorhabditis elegans* [13–20]. Also, it has become evident from these studies that important aspects of innate immunity have been evolutionarily conserved across phylogeny. Thus, because of their simplicity, well characterized innate immune signaling pathways, and because both the host and pathogen are amenable to genetic analysis and high-throughput screening in each of these pathosystems, the use of invertebrate models has accelerated studies of microbial virulence and host immunity [21–24]. In addition,

because of their low cost, small size, and short generation time, invertebrate hosts have been used in mass screening assays for selection of antimicrobial compounds with novel mechanisms of action. In this review, we outline recent advances in the study of medically important filamentous fungi in *Drosophila* model and discuss future implications and challenges in the use of this elegant pathosystem.

2. Antifungal Innate Immune Pathways in *Drosophila melanogaster*

2.1. Humoral Antifungal Immune Responses. Although lacking adaptive immunity, invertebrates are capable of having efficient innate immune responses against an array of pathogens in their natural environments. Two major pathways orchestrate innate immune responses in *D. melanogaster*, the immune deficiency (*imd*) pathway that confers protection against gram-negative bacteria, and the *Toll* pathway that is critical for immunity against gram-positive bacteria and fungi [25]. Detection of invading microorganisms by host receptors of the peptidoglycan recognition protein (PGRP) or gram-negative binding protein (GNBP) families triggers the activation of signal transduction pathways in the fat body (liver analogue) via the *Toll* receptor leading to a systemic humoral response characterized primarily by massive synthesis and release of potent antimicrobial peptides. Despite the broad spectrum of antimicrobial peptides, some specificity exists upon their induction following infection by various microbial pathogens. For example, in *D. melanogaster*, fungi and gram-positive bacteria mainly induce the production and release of drosomycin and metchnikowin via the *Toll* pathway, whereas gram-negative microbes induce the production and release of diptericin, attacin, and cecropin via the *imd* pathway [25]. The predominant role of the *Toll* pathway in *Drosophila* immunity against *Aspergillus* was first demonstrated in a landmark study by Lemaitre et al. [17], who found that *Toll* mutant flies, in contrast to wild-type flies, were highly susceptible to *Aspergillus* infection.

In both insects and mammals, the interaction of immunostimulatory cell wall molecules of invading fungi with *Toll* receptor(s) leads to activation of intracellular phosphorylation cascades, with subsequent translocation of nuclear factor κB-like transcriptional factors to the nucleus and induction of antimicrobial peptide-encoding genes [10, 25]. However, in contrast to mammalian *Toll* receptors, there is no direct interaction between microbial ligands and *Drosophila Toll* receptor. Instead, activation of the *Toll* signaling cascade is mediated by GNBP-3, a soluble pattern recognition receptor that senses long-chain fungal b-(1–3)-glucans and triggers a serine protease cascade leading to the processing of a small cytokine-like molecule, *Spätzle*, which comprises the functional ligand of the *Toll* receptor [25]. Importantly, GNBP3 mutant flies are highly susceptible to opportunistic fungi, including *Candida* and *Aspergillus* while retain functional *Toll* pathway activity, implying *Toll*-independent immune-related functions of this receptor. Indeed, GNBP3 has been implicated to play a role in pathogen agglutination, and activation of the melanization reaction at the early stages of fungal invasion [26]. Importantly, parallel to GNBP3, a second detection system senses the activity of proteolytic virulence factors that are released in the fly hemolymph during invasive fungal growth and redundantly activates *Toll* pathway via the protease *Persephone* [27].

2.2. Cellular Antifungal Immune Responses. When compared to humoral immune responses, *D. melanogaster* cellular immune responses are less well characterized. Notably, recent studies in insects challenge the importance of humoral immunity in pathogen clearance, demonstrating that the vast majority of bacteria (99.5%) are rapidly eliminated from the haemolymph well before the induction of antimicrobial peptides [28]. Hence, cellular immune responses seem to play instrumental roles in early recognition and elimination of microbial pathogens. The key transcription factor downstream of the *Toll* pathway, the nuclear factor-κB homologue Dif, is required for regulation of both humoral and cellular immunity in flies [25]. Phagocytosis is a hallmark of the cellular immune response and exhibits considerable similarity across phylogeny. Hence, opsonization and recognition by specific receptors mediate the initial stages of phagocytosis in both invertebrates and mammals. For example, in *Drosophila* peptidoglycan, recognition proteins (PGRPs) such as PGRP-LC and Croquemort (a human CD36 homologue) participate in the recognition and phagocytosis of gram-negative bacteria [29, 30], whereas the transmembrane scavenger receptor *eater* has been shown to recognize bacteria and fungi (*Candida silvata*) and play a *Toll*-independent role in antifungal immunity [23, 31]. Of interest, thioester-containing proteins with a complement-like activity against invading pathogens have been identified in many insects, including fruit flies [25, 32]. A high-throughput screen in *Drosophila melanogaster* S2 RNAi library identified a novel protein, macroglobulin complement related (*Mcr*), that exerts opsonizing activity specifically against *Candida albicans* [33]. In addition, S2 *Drosophila* cells efficiently eliminate *C. albicans* yeast cells and induce significant damage to the hyphae of filamentous fungi, including *Aspergillus* and the *Mucorales*, in a way that resembles the antifungal effector function of human phagocytes [23, 34].

The molecular mechanisms of intracellular elimination of pathogens by *Drosophila* phagocytic cells are less well characterized. Thus, insect phagocytic cells are also capable of generating an oxidative burst of oxygen radical intermediates, whereas induction of nitric oxide synthase has been shown to protect against bacterial infection in *Drosophila* larvae [35]. Furthermore, numerous antimicrobial peptides contained within human neutrophil granules, such as lysozyme, lipases, metalloproteases (like the mammalian gelatinases or collagenases), and nucleases, are similarly produced by the phagocytic hemocytes of most insects in response to infection [11, 25]. Although little is known about the molecular mechanisms of intracellular elimination of pathogens in fruit flies, recent studies demonstrate that the evolutionarily conserved autophagy pathway is important

for immune surveillance and clearance of intracellular pathogens that escape into the cytoplasm, including *Cryptococcus* [36]. On the other hand, unique cellular responses against larger invading pathogens (e.g., parasites), such as encapsulation and melanization mediated by specialized immune effector cells, are seen in *Drosophila* and other insects [11, 25].

2.3. Epithelial Immune Responses. In *D. melanogaster*, antimicrobial peptide-encoding genes are constitutively expressed in epithelia that are in direct contact with the external environment. However, in contrast with the systemic immune responses mediated by the fat body, where the *Toll* pathway modulates immune responses against gram-positive bacteria and fungi, epithelial immune responses in *D. melanogaster* appear to be partially controlled by the *Imd* pathway [25]. Furthermore, recent evidence indicates that genes involved in oxidative stress and/or detoxification of reactive oxygen species are critical for epithelial defense [37]. In addition, recent studies demonstrated a major role for the Janus kinase- (JAK-) signal transducer and activator of transcription (STAT) signaling pathway in epithelial host defense via regulation of stem cell proliferation and epithelial cell homeostasis [38].

A recent study in a gastrointestinal infection (GI) model of candidiasis in *Drosophila* larvae demonstrated an important role of normal gut flora in epithelial immunity by preventing colonization and invasive infection by *Candida*, which resembles the increasingly appreciated regulatory role of human epithelial microbiota in shaping epithelial immune responses [39]. Of interest, activation of JNK signaling during *Candida* infection accounted for extensive epithelial cell death in the gut and mortality of *Drosophila* larvae. In parallel, *Candida* infection triggered a systemic protective immune response that was mediated by NO release from larvae hemocytes and the parallel activation of the *Toll* pathway by pathogen-secreted aspartyl proteinases.

2.4. Toll-Independent Innate Immune Pathways in Drosophila. The complexity of the immune defenses in insects is much higher than initially perceived, and cross-talk between the *Imd* and *Toll* pathways takes place in response to both gram-negative and gram-positive microbes [25]. Furthermore, besides the *Toll* and *Imd* signaling cascades, other pathways associated with developmental or stress resistance processes are induced in response to infections in both invertebrates and mammals. For example, a pioneer study in *Drosophila* demonstrated that antimicrobial peptide activation can be achieved independently of classic immunoregulatory pathways by the transcription factor FOXO, a key regulator of stress resistance, metabolism, and aging [40]. In uninfected animals, antimicrobial peptide genes are activated in response to nuclear FOXO activity when induced by starvation or by using insulin-signaling mutants, revealing a new mechanism of cross-regulation of metabolism and innate immunity that has proven to be functional in humans as well [40]. Furthermore, investigators showed that the activation of the evolutionarily conserved p38 MAPK pathway is important for resistance to infection by bacteria and fungi; of interest, in contrast to the mammalian homologue, activation of p38 MAPK occurs independently of the *Toll* signaling [41].

3. Modeling Microbial Infection in *Drosophila melanogaster*

In *D. melanogaster,* the pathogen of interest is typically injected into the dorsal thorax via either needle pricking or microinjection [11]. In regard to fungal pathogens, the injection assay is technically a more standardized and reproducible method of infection and allows for a more precise estimation of fungal inoculums. Nonetheless, parenteral inoculation by passes the physiologic route of entry of the pathogen of interest and results in a more overwhelming infection that may not be suitable for pathogenesis studies. Thus, other more physiologic methods of infection are also used. For example, the *alb1 Aspergillus fumigatus* mutant, which is hypovirulent in mice, exhibited attenuated virulence in *Toll*-deficient flies only when introduced by feeding or rolling [20]. These infection methods are typically achieved by feeding insects in a lawn of yeast or molds or rolling insects over a fresh carpet of fungal spores. However, standardization of the infecting inocula is difficult with natural infection methods such as ingestion. Furthermore, infection with molds other than *Aspergillus by* feeding and rolling is difficult to perform because of the distinct pattern of growth of fungal colonies.

Female flies are typically used in infection experiments because of their larger size and relative resistance to injection injury when compared with male flies. Because wild-type *Drosophila* is resistant to most pathogenic fungi and bacteria, mutants deficient in various components of the *Toll* cascade are frequently employed to model infections. In most cases, crossing different loss-of-function alleles is required to generate homozygous *Toll*-mutant flies [11]. Nonetheless, the need for crossing of fly strains is a limitation for high throughput screening assays. Of note, microinjection introduces significantly higher inoculums within *Drosophila* hemolymph than needle pricking that allowed for establishment of invasive *Candida* infection in wild-type *Drosophila melanogaster* flies [42].

A major advantage of *Drosophila* in comparison to all other model host organisms is its genetic tractability, well-characterized immune system, and remarkable degree in conservation of biochemical pathways that control fundamental physiologic processes such as cell proliferation, differentiation, and tissue homeostasis. Furthermore, the innate susceptibility of *Drosophila Toll* mutant strains to fungal infections obviates the need to use immunosuppressive agents, thus eliminating the host variability inherent in the use of immunosuppressive regimens. In particular, *Drosophila* strains are amenable to both forward and reverse genetics, and large collections of *Drosophila* mutants and transgenic cell lines are commercially available (http://flybase.net/). Also, the *Drosophila* genome sequence

was one of the first to be completed and is probably one of the most fully annotated eukaryotic genomes found in a database (http://flybase.net/annot/). As a result, double-stranded RNA has been synthesized for each of the *Drosophila* genes (http://www.flyrnai.org/) and recently lines expressing RNAi have become available, which allow for conditional inactivation of every single gene at a whole animal or tissue level (http://www.vdrc.at/).

4. Virulence Studies of *Filamentous Fungi* in *Drosophila melanogaster*

4.1. Aspergillus. Since filamentous fungi have been in existence for about 1 billion years, the fly immune system evolved in the face of continued exposure to airborne conidia. Thus, *Drosophila* immune system has developed highly sophisticated and efficient strategies to combat infection caused by *Aspergillus* and other filamentous fungi. In fact, only a few entomopathogenic fungi are able to infect fruit flies in nature, via penetration of fly exoskeleton. Even when fungal pathogens are experimentally introduced directly into the fly hemolymph, wild-type flies are still capable of effectively eliminating infection. Lemaitre and colleagues were the first to demonstrate that *Aspergillus fumigatus* was able to infect and kill flies carrying mutations in various aspects of the *Toll* pathway [17]. *Toll*-deficient flies have been since implemented as a model to study immunopathogenesis of infections caused by *Aspergillus* and other medically important filamentous fungi. Several virulence attributes of *Aspergillus* pathogenicity in mammals have been tested in the fly model [20, 43]. With the exception of virulent factors that are important for microbial survival at mammalian temperature [44], most other virulence attributes that are important for mammalian pathogenicity of *Aspergillus* were equally important for successful infection in *Toll*-deficient fruit flies. In particular, *Aspergillus* mutants that are defective in siderophore biosynthesis (DeltasidA, DeltasidD), PABA metabolism (H515), starvation stress response, secondary metabolite production (DgliP), or melanin biosynthesis were attenuated in both *Drosophila and mouse* models of invasive aspergillosis [20, 43]. Notably, fungal cell wall melanin was dispensable for *Aspergillus* virulence when fungal spores were injected into the fly hemolymph but was important for establishment of invasive infection though *Drosophila* epithelia [20]. Hence, the tempo and site of infection as well as differences in local host defense mechanisms may influence expression of virulence factors of fungi in the fly model. Evermore, similar to recent findings with the Δ *CgrA* mutant [44], putative virulent factors of *A. fumigatus* with a role in thermotolerance may not be encountered in *Drosophila* or other invertebrate models because infection in these mini-hosts takes place at temperatures much lower (25°C) than the mammalian physiologic temperature (37°C). Despite these limitations, accumulating experimental evidence suggests that *Drosophila* is a relevance model to study *Aspergillus* virulence.

The interstrain and interspecies variations in virulence for a collection of *Aspergillus fumigatus* and *Aspergillus terreus* clinical isolates were recently studied in *Toll*-deficient fruit flies [45]. Although there was no significant difference in the survival of flies infected with *A. fumigatus* versus *A. terreus* or flies infected with colonizing versus invasive isolates of either species, two dominant A. fumigatus clades identified by rep-PCR were associated with significantly different survival rates in *Toll*-deficient flies. Therefore, the fly model of aspergillosis could detect subtle changes in virulence and uncover distinct *A. fumigatus* clades that differ in their pathogenicity. Of interest, a similar pathogenicity study of *Candida albicans* clinical isolates that were previously ranked for virulence in mice was recently performed in wild-type *Drosophila* flies infected by microinjection [42]. Of interest, there was a significant correlation in virulence of *C. albicans* strains between the fly and the mouse model of disseminated candidiasis. Nonetheless, differences in virulence were not evident using immune-deficient spatzle$^{-/-}$ flies, suggesting that *Toll* signalling might actually be required to predictably differentiate virulence.

The recent completion of the sequencing of the *A. fumigatus* genome and the development of molecular toolsets to study the biology of *A. fumigatus* is expected to lead to the generation of multiple *Aspergillus* mutants and creates a need for high-throughput strategies capable of assessing the contribution of individual genes to *Aspergillus* virulence [46]. Validation of *Drosophila* as a suitable model for large-scale virulence studies was provided by a recent screen of 34 *Candida albicans* mutants defective in putative transcription factor genes. This study identified a novel transcriptional regulator of cell wall integrity, CAS5, which proved to be important for virulence in both *Drosophila* and the mouse model of invasive candidiasis; a parallel screen in *C. elegans* subsequently confirmed the role of CAS5 in *Candida* virulence [47].

4.2. Mucorales (Formerly Zygomycetes). Mucorales species have recently emerged as an important cause of serious angioinvasive infections in immunocompromised individuals [6–8]. *Rhizopus* species accounts for majority of cases of mucormycosis in humans [7]. Few animal models of mucormycosis exist, and the immunopathogenesis of this infection is largely unknown. However, sequencing of *Rhizopus oryzae* genome has been completed and genetic tools are available (http://www.broad.mit.edu/annotation/fungi/rhizopus_oryzae/). In immunocompetent individuals, blood and tissue phagocytes efficiently eliminate *Mucorales* spores and hyphae by oxidative and nonoxidative killing mechanisms. Quantitative (i.e., neutropenia) or qualitative (i.e., associated with glucocorticoids, hyperglycemia, and/or acidosis) defects in phagocytic cell activity permit unrestricted growth of the hyphal form and invasive infection. Iron metabolism has a central role in pathogenesis of mucormycosis [6–8]. Thus, patients with iron overload states, including individuals undergoing chelation therapy with deferoxamine, are uniquely predisposed to mucormycosis. Of interest, deferoxamine acts as a siderophore for *Mucorales* species and promotes *in vitro* fungal growth. Similarly, the increased availability of serum iron in patients

with diabetic acidosis partially accounts for their unique susceptibility to mucormycosis. As opposite to deferoxamine, other iron chelators such as deferasirox lack xenosiderophore activity for *Rhizopus* induce an iron-starvation effect to the fungus and have shown to be protective in animal models of mucormycosis [6–8].

Although Mucorales have not be reported to be entomopathogenic, we recently found that as opposite to other medically important filamentous fungi, injection of different Mucorales species in wild-type *D. melanogaster* results in a hyperacute infection, with disseminated fungal proliferation and high mortality rates [23]. Several aspects of immunopathogenesis of mucormycosis in humans were modeled in *Drosophila*, including increased host susceptibility following administration of corticosteroids, and the iron chelator deferoxamine. Of interest, the use of another iron chelator, deferasirox, which induces iron starvation to Mucorales spp and protects mice and possibly humans from infection, also significantly protected *Drosophila* from mucormycosis. In addition, *Cunninghamella bertholletiae*, which appears to be the most virulent Mucorales species in humans, exhibited increased virulence in comparison to other Mucorales species in the fly model [23].

The fly model of mucormycosis has been established in wild-type *Drosophila*, which obviates the need for crossings and allows for simple and rapid assessment of research questions in *Mucorales* pathogenicity. Thus, flies were recently implemented to evaluate the role of endosymbiotic toxin-producing bacteria in the virulence of *Rhizopus* species. Although a significant number of clinical *Rhizopus* isolates were found to harbor rhizoxin-producing bacteria, there was no difference in fungal virulence following antibiotic mediated eradication of the endosimbionts in both *Drosophila* and mice [48]. In addition, the association of increased voriconazole use with the emergence of Mucorales infection in immunocompromised patients was recently tested in the fly model. Surprisingly, preexposure of Mucorales to this newer triazole dramatically increased susceptibility of fruit flies to mucormycosis in *Toll*-independent fashion, which was also observed in the mouse model [49]. Collectively, these studies demonstrate that Mucorales species have developed common virulence strategies to invade evolutionarily disparate organisms such as *Drosophila* and humans.

Of interest, virulence of *Cunninghamella* in the fly model is significantly affected by the composition of fungal culture media, possibly reflecting differences in acquisition of iron or other nutritional factors [50]. In addition, because innate immunity in *Drosophila* is under circadian regulation, the timing of infection has significant impact in host defense against various pathogens, including filamentous fungi. In fact, genes involved in circadian rhythm regulation were significantly induced following infection with Mucorales species in *Drosophila* [23]. Furthermore, starvation of flies prior to infection confers protection against bacterial infection via release of NO [51], and possibly via regulating other immune-related pathways, such as FOXO signaling [40] and the autophagy response. Therefore, all these parameters need to be considered in virulence testing of *Mucorales* and other filamentous fungi in *Drosophila*.

Gene expression profiling in human monocytes and in immunocompromised mice infected with *Rhizopus* versus *Aspergillus* demonstrates a differential induction of immune-related genes during mucormycosis [52], which likely reflects unique virulence traits of Mucorales species. Similarly, transcriptional profiling at early time points of infection in wild-type fruit flies infected with *Rhizopus* (pathogenic) versus *Aspergillus* (nonpathogenic) indicated distinct sets of genes that were selectively regulated in response to mucormycosis [23]. These genes could represent molecular targets for drug development aiming at modulating host immune response during infection. Of interest, a similar transcriptome profiling in *Drosophila* infected with two strains of *Pseudomonas* with different pathogenic properties revealed common groups of genes with those identified during *Rhizopus* infection of flies [53]. Notably, a group of genes down regulated following infection with the pathogenic strain in both studies included a skeletal muscle gene regulatory network under the control of cJun-N-terminal Kinase (JNK) pathway. Notably, activation of this pathway promoted local resistance to *P. aeruginosa* in flies and mice [54].

4.3. Other Emerging Filamentous Fungi. *Fusarium* and *Scedosporium* species are ubiquitous, saprophytic molds that are notoriously resistant to conventional antifungal agents [2]. These fungi have been increasingly reported causes of invasive, frequently fatal infections in immunosuppressed hosts. Occasionally, these opportunistic pathogens can cause difficult-to-treat localized infections in immunocompetent individuals with certain predisposing conditions, including onychomycosis, fungal keratitis, skin and soft tissue infection, and rarely brain abscesses [2]. Furthermore, as opposite to other filamentous fungi, *Fusarium* species have a unique predisposition for development of fungemia and disseminated necrotic skin lesions in severely immunocompromised patients [2]. These features suggest the existence of uncharacterized, unique virulence factors of these organisms. *Drosophila melanogaster* wild-type flies were recently found to be resistant to infection by different clinical isolates of *Scedosporium*, whereas *Toll*-deficient flies were highly susceptible to these fungi [24]. Of interest, *Fusarium* species caused lethal infection in wild-type flies although in a less acute mode of infection than in *Toll* deficient flies, an observation consistent with the ability of these fungi to infect a broad range of phylogenetically disparate hosts, ranging from plants to mammals. Although the lack of genetic tools currently precludes comprehensive analysis of virulence factors in these fungi, comparative analysis of host defense mechanisms during infection with these and other filamentous fungi in the *Drosophila* model could provide valuable information on the pathogenesis of these emerging infections.

5. Antifungal Drug Efficacy Studies in *Drosophila* Models of Filamentous Fungi

Drosophila has proven to be a reliable model for testing orally absorbed compounds with antifungal activity. In particular, voriconazole conferred significant protection in

Toll-mutant flies infected with *A. fumigatus* [20]. Furthermore, the combination of voriconazole and terbinafine, two drugs that block sequential steps in the ergosterol pathway and show synergy *in vitro* against *Aspergillus*, was synergistic in the *Drosophila* model of aspergillosis [20]. Similarly, voriconazole preexposure was protective in flies infected with *Fusarium moniliforme* and *S. apiospermum*, but not in flies infected with *S. prolificans*, a finding that is consistent with *in vitro* susceptibilities of these species and *in vivo* studies in mice [24]. Besides conventional antifungal agents, administration of deferasirox, an iron chelator that induces iron starvation and exerts selective antifungal activity against Mucorales, significantly increased survival of flies in *Drosophila* model of mucormycosis [23].

Nonetheless, there are important limitations in the use of *Drosophila* and other invertebrate models in drug efficacy studies. Thus, precise estimation of the dose of a pharmacologic compound that is orally administered in flies is challenging. A more accurate way of drug delivery can be achieved by microinjection; however, this method is time consuming and requires technical training and specialized equipment in fruit flies. In addition, measurement of drug levels for pharmacokinetic analysis in *Drosophila* requires HPLC or bioassay methods that are more cumbersome, imprecise, and technically demanding in this model than in mammals [11]. For all these reasons, pharmacodynamic studies, which typically require multiple dosing of antifungal agents for long periods of time, are not feasible in *Drosophila*. Finally, the metabolism and elimination pathways of drugs and the potential for drug-drug interactions are largely unknown in *Drosophila* for most existing compounds.

Despite their limitations, *Drosophila* and other invertebrates are attractive models for mass-screening candidate antifungal compounds that will require subsequent validation in mammalian systems [55]. Such approaches have been used successfully in *Drosophila* to select life-extending compounds [56] and recently in *C. elegans* to identify compounds with novel mechanism of antifungal activity against *Candida* [57]. In the *C. elegans* study, thousands of synthetic and natural molecules were screened in a 96-well plate liquid culture system and several compounds that exhibited *in vivo* activity without significant *in vitro* effect were selected, proving the benefits of this strategy. Notably, two of the 15 selected compounds identified in this screening exhibited potent antifungal activity in the mouse model of invasive candidiasis [57]. Overall, the simplicity, low cost, small size, and short generation time of invertebrate hosts make them ideal for high-throughput screening. As a proof of principle, many pharmaceutical and biotechnology companies are increasingly using minihost models for drug discovery. For example, Exelixis, Inc. (South San Francisco, CA) has created an extensive collection of *Drosophila* gene disruption strains for use in drug-target identification. Similarly, larger pharmaceutical companies such as Novartis (Basel, Switzerland) have created *Drosophila* functional genomics departments dedicated to the study of disease-related pathways and discovery of novel drug targets. Nonetheless, *D. melanogaster* models of infectious diseases are less amenable to automated mass screening for antimicrobial agents than are *C. elegans* models because of technical limitations associated with the size of the animals, methods of infection, frequent need for fly crosses to generate the desired mutants, and inability of adult flies to propagate in liquid culture systems.

6. Implementing RNAi Screens to Identify Host and Pathogen Determinants of Immunopathogenesis of Fungal Diseases

Over the past few years, *Drosophila melanogaster* S2 cells and RNAi technology have been successfully implemented to identify host factors implicated in pathogenesis of infections caused by intracellular pathogens [29, 30, 58]. There are many features of the *Drosophila* cell system that make it an attractive tool for these studies. Hence, the fly genome is highly annotated and fundamental innate immune pathways are evolutionarily conserved in *Drosophila* S2 macrophage-like cells. Furthermore, gene silencing is easier to perform in a high-throughput basis in *Drosophila* cell lines when compared to mammalian macrophage cell lines. Finally, *Drosophila* S2 cells have a successful track record in identifying novel host factors involved in phagocytosis and killing of many intracellular microbial pathogens, which have been subsequently validated in their mammalian cell counterparts [29, 30, 58]. In regard to fungal pathogens, investigators recently used an RNAi library of S2 cells to study genes involved in phagocytosis of *C. albicans* and identified novel genes encoding for proteins that specifically recognize and promote phagocytosis of *Candida* yeast cells [33]. Another RNAi screen in S2 cells was designed to select host factors that restrict intracellular survival and proliferation of the pathogenic fungus, *Cryptococcus neoformans* [36]. This study identified novel host genes implicated in *Cryptococcus* pathogenesis and revealed that proteins of the autophagy pathway are important for intracellular elimination of the fungus both in *Drosophila* S2 cells and mammalian macrophages.

In vitro, high-throughput screening strategies using phagocytic *D. melanogaster* cell lines have certain limitations. First, only host factors important for the intracellular life cycle of a pathogen can be tested. This approach is well suited for intracellular pathogens but not for extracellular organisms such as filamentous fungi. Thus, in contrast with bacteria, fungi have distinct replication stages (e.g., spore to hyphal transition) and relatively slow growth rates, which make difficult the establishment of reliable high-throughput phagocytosis and/or killing *in vitro* assays. In addition, silencing of important innate immune-related pathways may be missed in an *in vitro* screen because it may result in nonviable phenotypes, which can only be assessed using tissue-specific silencing *in vivo*. Finally, the complexity and dynamics of *in vivo* host-pathogen interplay, including tissue-specific host immune responses, cannot be reliably evaluated using an *in vitro* culture system.

Studies using conditional RNAi in *D. melanogaster* to analyze gene function in real time and a tissue-specific manner could overcome limitations of *in vitro* large-scale screening. In fact, the *in vivo* RNAi library for *Drosophila* flies

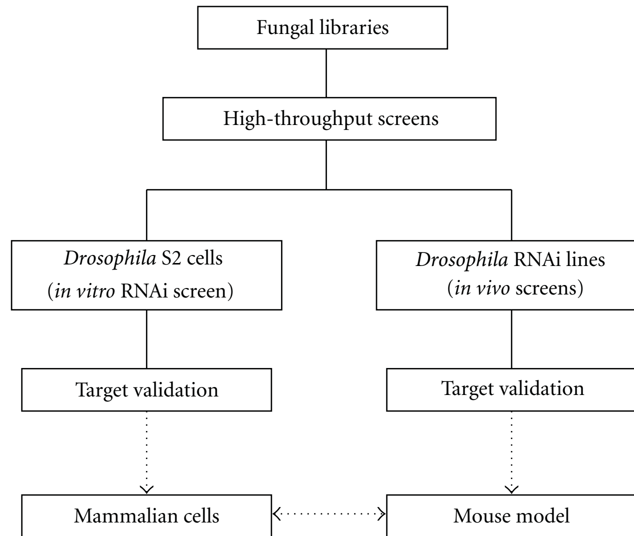

Figure 1: Prioritizing use of *Drosophila* model in selection of novel determinants of fungal pathogenicity in humans.

Table 1: Important research questions on antifungal immunity in *Drosophila*.

Which host defense mechanisms in *Drosophila* are important for fungal clearance before the induction of humoral immune responses? What is the contribution of cellular immunity in defense against fungi?
How *Drosophila* immune system discriminates opportunistic from entomopathogenic fungi?
Which fungal molecules other than b-glucans trigger activation of host immune responses in *Drosophila*?
Are there any fungal virulence factors that exert immunosuppressive effects on *Toll* signaling or other immune signaling pathways? Is there any role of pathogen or self-nucleic acid sensing in *Drosophila* host defense?
How *Drosophila* discriminates sensing of self- from non-self-immune activating molecules? Is there any evidence of the presence of endogenous ligands for *Drosophila* pattern recognition receptors?
Are there any immune modulating properties of the antimicrobial peptides in *Drosophila?*
Is there any role for antimicrobial peptide-DNA complex formation in insect immunity against fungi?
Is there any cooperative activity between different *Drosophila* immune receptors?

has became commercially available [59], and a pilot genome-wide *in vivo* screen in *D. melanogaster* designed to identify genes involved in epithelial host defense against an intestinal bacterial pathogen was recently completed [38]. For the first time, this study showed that the JAK-STAT signaling pathway has an important role in host defense against infections with bacterial pathogens in the gut by regulating epithelial cell homeostasis.

7. Limitations of *Drosophila* Model of Fungal Infections

Drosophila offers unique advantages in dissecting immuno-pathogenesis of fungal diseases because of its powerful genetics and highly conserved immune pathways. Nonetheless, the fly model also has some obvious limitations. For example, implementing *Toll*-deficient flies as model for virulence testing in a Mycology laboratory requires some degree of training for proper maintenance and crossing of *Drosophila* stocks, and basic equipment for manipulating, anesthetizing, and infecting the animals. Alternatively, use of larger in size invertebrates, such as *Galleria mellonella*, which are easier to infect and allow for infection at mammalian temperatures, could overcome some technical difficulties of the *Drosophila* model [60]. However, because in *Galleria mellonella* genetic tools are not available and innate immune pathways are less well characterized, this model is not suitable for in-depth analysis of host-related factors mediating fungal pathogenesis.

When compared to conventional animal models, the considerable difference in the anatomic structures of invertebrates and mammals raises questions on the pathophysiologic relevance of some *D. melanogaster* infection models. This may be particularly true for pathogens with life cycles adapted to mammalian hosts, or those that express their virulence mechanisms in a tissue-specific environment. For example, establishing a model of *Pneumocystis jirovecii* in

invertebrate hosts [61] is not feasible. Nonetheless, even in mammalian hosts, some virulence attributes of pathogenicity may be dispensable to certain pathophysiologic settings or infection sites. For example, researchers recently showed that gliotoxin production was required for *A. fumigatus* pathogenicity in corticosteroid-immunosuppressed mice but not in neutropenic mice [62]. Furthermore, *D. melanogaster* lacks important constituents of human immunity, including a functional adaptive immune response, highly specialized innate immune cell subsets (e.g., dendritic cells, natural killer cells), and a complex network of cytokines, chemokines, and other effector molecules that have critical roles in orchestrating cell communication and regulation of inflammation and tolerance during infection. Overall, despite the considerable similarities in innate immune mechanisms, invertebrate models are not directly comparable with mammalian models. Thus, it is reasonable to speculate that some of the virulence attributes of *Aspergillus* and other filamentous fungi that affect mammals might not be important in invertebrate minihost models. Therefore, *Drosophila* must be viewed as a complementary, high-throughput genetic model, which could accelerate identification of novel host and pathogen determinants with a relevant role in development of fungal diseases in humans (Figure 1).

8. Future Directions in Fungal Immunology Research in *Drosophila*

The identification of the *Drosophila melanogaster Toll* signaling cascade and the subsequent characterization of mammalian *Toll*-like receptors (TLRs) have fundamentally altered our understanding of innate immunity. However, much remains to be learned on evolutionarily conserved antifungal immune defense mechanisms in *Drosophila* (Table 1). For example, whether immunostimulatory molecules of fungi other than b-glucans trigger immune recognition in *Drosophila* has not been elucidated. In addition, it is unknown whether and how *Drosophila* discriminates between pathogenic and opportunistic fungi. Similarly, the contribution of cellular immunity and *Toll*-independent mechanisms of antifungal host defense in fruit flies remains to be explored. Since the identification of mammalian TLRs, it became evident that nucleic acid sensing is an important aspect in pathogen recognition. Hence, dedicated endosomal TLRs and cytoplasmic pattern recognition receptors are specialized in sensing bacterial and viral nucleic acids and trigger robust inflammatory responses. Recent studies also demonstrate an important role for DNA neutrophil extracellular trap (NET) formation during bacterial and fungal infections [63]. In *Drosophila*, the role of nucleic acid sensing in immunity is largely unknown. Nonetheless, recent studies in other insects suggest that DNA NET formation is important for innate antibacterial immunity [64]. Finally, in humans, evolutionarily conserved antimicrobial peptides exert important immunomodulatory properties besides their direct effector function, by acting on various chemokine and signaling receptors [65, 66]. Therefore, whether *Drosophila* antimicrobial peptides retain a similar role is an important research direction in understanding the evolution of mammalian immune system.

Conflict of Interest

D. P. Kontoyiannis has received research support and honoraria from Schering-Plough, Pfizer, Astella Pharma US, Inc., Enzon Pharmaceuticals, and Merck & Co., Inc. G. Samonis has received honoraria from Schering-Plough, Astella Pharma US, Inc., Enzon Pharmaceuticals, and Merck and Co., Inc. G. Hamilos and G. Samonis have no conflicts of interest.

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Review Article

Fungal Biofilm Resistance

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Fungal biofilm infections have become increasingly recognised as a significant clinical problem. One of the major reasons behind this is the impact that these have upon treatment, as antifungal therapy often fails and surgical intervention is required. This places a large financial burden on health care providers. This paper aims to illustrate the importance of fungal biofilms, particularly *Candida albicans*, and discusses some of the key fungal biofilm resistance mechanisms that include, extracellular matrix (ECM), efflux pump activity, persisters, cell density, overexpression of drug targets, stress responses, and the general physiology of the cell. The paper demonstrates the multifaceted nature of fungal biofilm resistance, which encompasses some of the newest data and ideas in the field.

1. Clinical Significance of Fungal Biofilms

Fungi represent a significant burden of infection to the hospital population. The use of broad-spectrum antibiotics, parenteral nutrition, indwelling catheters, or the presence of immunosuppression, or disruption of mucosal barriers due to surgery, chemotherapy, and radiotherapy are among the most important predisposing factors for invasive fungal infection [1]. *Candida* bloodstream infection is the third most common cause of nosocomial bacteremia in patients requiring intensive care and the most common etiologic agent of fungal-related biofilm infection. *C. albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is most frequently associated with biofilm formation. Indwelling medical devices, such as intravascular catheters, can become colonized with *Candida* spp. allowing the development of adherent biofilm structures from which cells can then detach and cause an acute fungemia and/or disseminated infection. It has recently been shown that the cells that detach from the biofilm have a greater association with mortality than equivalent planktonic yeasts [2]. These implant-associated infections are inherently difficult to resolve and may require both long-term antifungal therapy and the physical removal of the implant to control the infection. Other non*albicans Candida* species associated with biofilm formation and catheter-related bloodstream or device-related infections include *C. glabrata*, *C. parapsilosis*, *C. dubliniensis*, *C. krusei*, and *C. tropicalis* [3–5].

Yeasts and filamentous fungi biofilm-related infections have also been increasingly described [6], including *Pneumocystis* [7], *Coccidioides* [8], *Aspergillus* [9], *Zygomycetes* [10], *Blastoschizomyces* [11], *Saccharomyces* [12], *Malassezia* [13], *Trichosporon* [14], and *Cryptococcus* [15]. *Cryptococcus neoformans* has been shown to colonize and subsequently form biofilms on ventricular shunts [15], peritoneal dialysis fistulas [16], prosthetic hip joints [17], and cardiac valves [18]. Different *Trichosporon* species can cause disseminated life-threatening infections associated with biofilm-related infections [14, 19, 20], including cardiac grafts [21], catheters [22], and breast implants [23]. *Malassezia pachydermatis* has been isolated from patients undergoing parenteral nutrition [13], *Blastoschizomyces capitatus* has been associated with catheter-related fungemia [11], *Saccharomyces cerevisiae* has been detected from dentures of stomatitis patients [24], and recurrent meningitis has been associated with a *Coccidioides immitis* biofilm at the tip of a ventriculoperitoneal shunt tubing [8].

There are also growing reports of the filamentous mould *Aspergillus fumigatus* being involved in biofilm infections. For example, in the respiratory tract, it can cause an aspergilloma, which is a localised infection consisting of a spherical mass of hyphae. Aspergillary bronchitis has also been reported, which is characterized by bronchial casts containing mucus and mycelia [25]. Bronchopulmonary lavage (BAL) of patients with aspergillosis may also reveal the presence of numerous hyphae in the form of a complex multicellular mycetoma structure samples when examined histologically [26]. In addition to this, it has been reported to cause serious biomaterial-related infections of joint replacements, catheters, heart valves, cardiac pace makers, and breast augmentation implants [27–30]. The urinary tract, whilst less frequently associated with *A. fumigatus*, has been reported to support an aspergilloma [31, 32]. It is also frequently associated with complex sinus infections, which in canines have been described as superficial mucosal fungal plaque [33–36].

It is increasingly clear that a diverse panel of fungi have the capacity to form biofilms, and as such our knowledge of fungal biofilms has improved dramatically. Through work primarily with *C. albicans*, we now have a clearer perspective on the molecular characteristics of fungal biofilm development [3, 6, 37, 38]. Clinically, these are important as they are refractory to antifungal treatment, which poses a major problem to clinicians as the dose required to eradicate the biofilm can exceed the highest therapeutically attainable concentrations of antibiotics [39]. The focus of this paper is to provide an up-to-date understating of the key factors responsible for the failure of antifungal agents against fungal biofilms.

2. Biofilm Basics

Microbiologists have historically studied planktonic (free floating and homogeneous cells) in pure culture. However, there has been a paradigm shift as the link between sessile (surface attached and heterogeneous cells) and microbial pathogenesis and human infection is now widely accepted [40]. It is apparent that a wide range of bacteria and fungi are able to alternate between planktonic growth and sessile multicellular communities, commonly referred to as biofilms. Estimates suggest that up to 80% of all microorganisms in the environment exist in biofilm communities [41].

Biofilms are defined as highly structured communities of microorganisms that are either surface associated or attached to one another and are enclosed within a self-produced protective extracellular matrix (ECM) [6]. The advantages to an organism of forming a biofilm include protection from the environment, resistance of physical and chemical stress, metabolic cooperation, and a community-based regulation of gene expression. In recent years, there has been an increased appreciation of the role that fungal biofilms play in human disease as microbes growing within biofilms exhibit unique phenotypic characteristics compared to their planktonic counterpart cells, particularly increased resistance to antimicrobial agents [6]. In addition to providing safe sanctuary for microorganisms, biofilms may also act as reservoirs for persistent sources of infection in a patient and as such adversely effect the health of an increasing number of individuals, including patients with HIV-infection, cancer, transplants, patients requiring surgery or intensive care, and newborn infants [3, 42].

The adhesion and colonization of complex fungal populations onto biological and innate surfaces, such as the oral mucosa or denture acrylic substrates, is commonplace for clinically relevant fungi [43–46]. A wide variety of environmental factors contribute to the initial surface attachment, including the flow of the surrounding medium (urine, blood, saliva, and mucus), pH, temperature, osmolarity, bacteria, presence of antimicrobial agents, and host immune factors [47–52]. Fungal biofilms have defined phases of development that have been described through the use of defined model systems [51, 53–58]. These key phases include arriving at an appropriate substratum, adhesion, colonisation, extracellular matrix (ECM) production, biofilm maturation, and dispersal [37, 55, 59]. Understanding this entire process has enabled us to begin to unravel how some of the mechanisms are involved in resistance.

3. Studying Fungal Biofilm Resistance

Initial studies that began to investigate biofilm resistance were basic, investigating antifungal effects purely at the phenotypic level through descriptive analyses. The pioneering work by Julia Douglas's group working on *C. albicans* biofilms utilised some of the earliest models, from which quantitative assessment using dry weight measurements, tetrazolium salt (MTT) reduction assays, and incorporation of [^{3}H] leucine were described [60, 61]. These simple static models were expanded to include flow, which was shown to alter antifungal susceptibility [47]. However, typically these models were cumbersome, requiring expert handling, longer processing times, and the use of specialized equipment not generally available. Therefore, methods for rapid high-throughput testing were preferable, and around this time, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium hydroxide (XTT) colorimetric method was described for investigating yeast adhesion and susceptibility [62, 63]. This assay measures the collective metabolic activity of the cells within biofilm and is used as the basis for developing a standardized high-throughput susceptibility screen on *Candida* biofilms, which is now widely used by the research community [55, 58]. The XTT assay is noninvasive and nondestructive, requiring minimal postprocessing of samples compared to other methods, such as viable cell counts [64]. Using this technique, multiple microtiter plates can be processed simultaneously without compromising accuracy. A caveat to its use is that it does not quantify biofilm-dependant characteristics, such as biomass or morphological status, and caution must be exercised when evaluating XTT data from different isolates as there is often dramatic variability between strains [65]. Therefore, it should only be used for direct comparison of a treated isolate to an untreated control rather than absolute quantification of biofilm formation *per se*. The next breakthrough in

high-throughput biofilm testing has recently been described where nanoproduction of *C. albicans* biofilms is achievable, creating 768 equivalent and spatially distinct nanobiofilms on a single glass microarray [66]. However, it remains to be determined whether there is an assay system sensitive enough to quantify the metabolic activity of each nanobiofilm.

The recent interest in *Aspergillus* and *Cryptococcus* biofilms, as well as others like *Pneumocystis*, has led to further modifications of these, but in essence the platforms and techniques remain largely similar [67]. For example, Martinez and Casadevall (2006) developed a microtiter plate biofilm assay for *C. neoformans* to determine the susceptibility profiles of *in vitro* sessile structures [68]. Similarly, a 96-well-based biofilm model for *A. fumigatus* has been described and used to determine the susceptibility profiles and resistance mechanisms of conidia and adherent hyphal biofilms using an XTT-based reduction and Alamar blue assays [69–71]. The oxidation reduction indicator Alamar blue has also been shown to be a reproducible and cheaper alternative to XTT in recent years, which merits further study [72, 73].

As mentioned, the presence of flowing liquid over the biofilm can alter antifungal sensitivity [47]. There is a growing range of flow systems utilized to model biofilm development [51, 56, 74–76]. For example, a "seed and feed" modified Robin's device that permits multiple biofilms to be formed under constant flow conditions, cylindrical cellulose filters, constant depth film fermenters, perfusion fermenters, flow chambers, and a Robbin's device have all been described [51, 52, 54, 55, 77, 78]. The Lopez-Ribot group recently described a simple flow model based on a gravity-fed flow method that enabled the group to demonstrate that biofilms were thicker and more resistant to polyenes and echinocandins by 4- and 2-fold, respectively [79]. Interestingly, perfusion of biofilms created under flow with these two antifungal agents showed time- and dose-dependant activity, which were potent against dispersed cells [80]. These systems will prove useful for future investigations of invasive candidiasis where biofilms are common, particularly for catheter-related infections in the ICU, where there is a growing interest in catheter-lock therapy [81, 82]. In addition, there are now also a significant number of biofilm models available for *in vivo* investigations, and many of these have been utilised to elucidate biofilm resistance mechanisms [83], including an implanted chamber under the skin [71], catheter models [84, 85], vaginal model [86], and denture model [87].

4. Fungal Biofilm Resistance Mechanisms

One of the defining characteristics of biofilms is their increased resistance to antimicrobial agents. Fungi have been reported to be up to 1000-fold more resistant to antifungal agents than planktonic free-floating cells, yet this recalcitrance to antimicrobial therapy has yet to be fully elucidated [14, 58, 88]. Despite some antifungal agents being efficacious against fungal biofilms, particularly the echinocandins and liposomal amphotericin B formulations, the intrinsic resistance exhibited by these complex structures has promoted detailed investigation [70, 89–92].

Antifungal resistance is both complex and multifaceted. It can be inducible in response to a compound, or an irreversible genetic change resulting from prolonged exposure. Specifically, these include alterations or overexpression of target molecules, active extrusion through efflux pumps, limited diffusion, tolerance, and cell density, which are all characterised mechanisms utilised by fungi to combat the effects of antifungal treatment [93]. Planktonic cells generally rely on irreversible genetic changes to maintain a resistant phenotype, whereas biofilms are able to persist due to their physical presence and the density of the population, which provides an almost inducible resistant phenotype irrespective of defined genetic alterations. The following section will now describe some of the pivotal factors that play a role in fungal biofilm resistance, which are summarised in Figures 1 and 2.

4.1. Physiological State. The general physiological state of cells in sessile populations has also been implicated to influence the susceptibility profiles of biofilms. Metabolic dyes assays (e.g., XTT-based assays) confirm that cells within biofilms are undergoing mitochondrial respiration during development [55, 58, 62, 65, 70]. Other factors including the effect of growth rate on *C. albicans* biofilm resistance have also been studied, where varying the rates was shown to play no role in resistance to amphotericin B [54]. Similarly, biofilms of *C. albicans* grown under glucose- and iron-limited conditions were shown to both be highly resistant to amphotericin B [94]. Furthermore, studies of biofilms grown under anaerobic conditions demonstrated that *C. albicans* biofilms were resistant to the high levels of amphotericin B and different azole antifungals [95]. Nevertheless, factors including pH, temperature, oxygen availability, and other environmental stresses will alter the biofilm architecture, and possibly antifungal sensitivity [96, 97]. Therefore, whilst the physiological state of the cell may have a minor role in resistance (e.g., dormancy), it is more likely that more complex factors are involved.

4.2. Cell Density. The architecture of biofilms is highly ordered to enable the perfusion of nutrients and expulsion of waste products. Mature biofilms, whilst densely populated, exhibit spatial heterogeneity with microcolonies and water channels being present, and features are common to both bacterial and fungal biofilms [55, 98, 99]. Cell density is therefore an important resistance factor within complex biofilm populations of yeast and filamentous fungal biofilms, particularly towards azoles. It was demonstrated that both planktonic and resuspended biofilm cells exhibited azole sensitivity at low cell numbers (10^3 cells/ml), which became increasingly resistant as the density of the cells increased tenfold [100], a phenomenon also been demonstrated in *A. fumigatus* [101]. Both our group and others have shown phase-dependant increased antifungal resistance in *A. fumigatus* and *C. albicans*, respectively [70, 102], which support the idea that the physical density of the cells within the biofilm produces recalcitrance to antifungal agents.

Within dense biofilms, there is cooperation between individual cells through quorum sensing processes, which

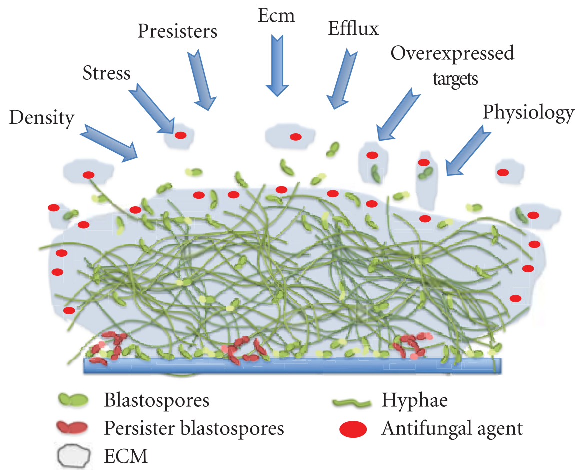


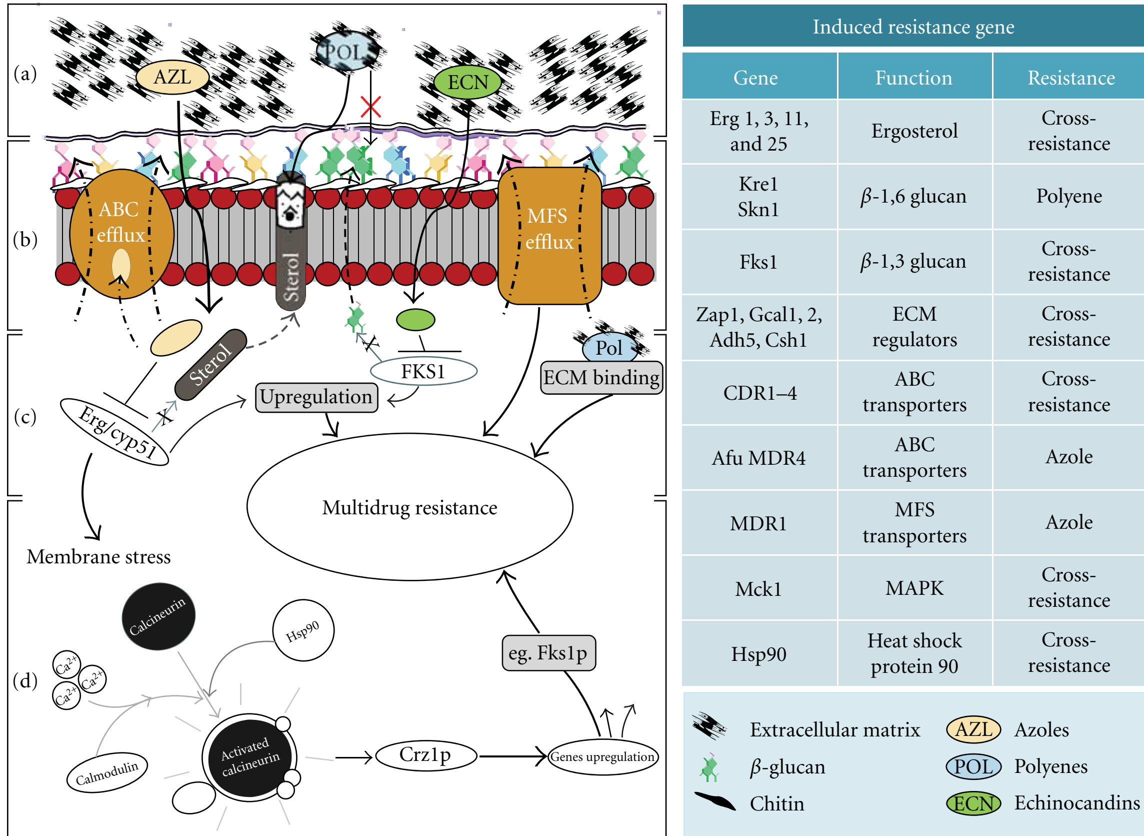
FIGURE 1: Schematic overview of fungal biofilm resistance mechanisms. Generic overview of key biofilm resistance mechanisms associated with *C. albicans*, but which are likely to be common to other fungi. This figure illustrates the density and complexity of the *C. albicans* biofilm, with different morphotypes present surrounded by ECM. The arrows represent the different factors that drive antifungal resistance within the biofilm, including density, stress, persisters, ECM, efflux, overexpressed targets, and the general physiology of the biofilm. These have been placed according to their contribution to resistance, with those that have a greater effect closer to the middle and those with less impact at the edges.

provides the ability of microorganisms to communicate and coordinate their behaviour via the secretion of signalling molecules in a population-dependent manner [103]. In fungi, this was first described in *C. albicans* when Hornby and colleagues identified farnesol *trans*, *trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol [104]. Exposing *C. albicans* to exogenous farnesol results in genome wide responses, including activation of genes involved in drug resistance (Ca*FCR1* and Ca*PDR16*) [105, 106]. It has now been shown that quorum sensing in *C. albicans* is likely driven by the two-component regulatory system of Chk1p [107]. However, when deleted the Δ*chk1* strain shows a similar azole resistance profile to that of wild type [100], indicating that the regulatory circuit controlling biofilm resistance may be yet to be discovered, or cell density is not a defined biofilm resistance factor. However, given that echinocandins are highly effective against biofilms suggests cell density has a limited effect against this compound [92]. In addition, previous work has shown that disrupted biofilms that are resuspended and tested using the CLSI methodology in comparison to planktonic cells retain a resistant phenotype [71, 108], indicating alternative mechanisms of resistance.

4.3. Overexpression of Drug Targets. The azoles are generally fungistatic against yeasts, including *Candida* species, and fungicidal against moulds, such as *Aspergillus* species. The fungistatic nature of the azoles towards *C. albicans* induces a strong directional selection on the surviving population to evolve drug resistance [109, 110]. In fact, high levels of azole resistance in *C. albicans* clinical isolates often accumulate through multiple mechanisms including the alteration of Erg11 [109]. Azoles actively target the 14α-demethylase enzyme encoded by ERG11, blocking ergosterol biosynthesis and leads to depletion of the ergosterol content of membranes and results in the accumulation of toxic sterol pathway intermediates, such as 14 α-methylergosta-8,24(28)-dien-3b,6a-diol, which inhibits growth [111, 112]. The principle drug target, Erg11p, can develop point mutations or be overexpressed [111–113]. Common mutations in the Erg11p that confer moderate azole resistance are S405F, Y132H, R467K, and G464S [114–116].

Given the importance of ergosterol as a target of azoles and the high level resistance exhibited by these structures, then the sterol composition of *C. albicans* biofilms has been investigated. Sterol analyses showed that ergosterol levels were significantly decreased in intermediate (12 h) and mature phases (48 h), compared to those in early-phase biofilms (6 h) [102]. In contrast, in one of the first *C. albicans* biofilm studies to use microarray analysis, overexpression of Ca*ERG25* and Ca*ERG11* was reported [56]. Alteration of ergosterols in biofilm membranes may explain their resistance to both azole and polyene-derived antifungal agents. For example, *C. albicans* biofilms cultured in a flow cell for 36 h were compared to planktonic cells, where it was shown that a subpopulation of blastospores from the biofilm were 10 times more resistant to amphotericin B than planktonic populations [76]. Transcriptional analysis of this biofilm subpopulation for genes from the beta-1,6-glucan pathways indicated a possible association between the high level of resistance and upregulation of Ca*SKN1*, and Ca*KRE1* in the biofilm blastospore population compared to exponential and stationary phase planktonic *C. albicans* cells. Therefore, changes in both the cell membrane and the cell wall may be important determinants of resistance in the biofilm. Subsequent work in *C. albicans* has shown that transcriptional responses in young and mature biofilms after exposure to high doses of fluconazole or amphotericin B demonstrated differential antifungal drug responses [117]. Exposure of both young and mature biofilms to fluconazole induced upregulation of genes encoding enzymes involved in ergosterol biosynthesis (Ca*ERG1*, Ca*ERG3*, Ca*ERG11*, and Ca*ERG25*), particularly biofilms exposed for longer periods (22 h), whereas treatment of both young and mature biofilms with amphotericin B resulted in an overexpression of predominantly Ca*SKN1*, with a modest upregulation of Ca*KRE1*. Removal of the antifungal in this study depleted further transcriptional changes, except for Ca*SKN1*, which was impacted by prior fluconazole exposure. It was speculated that this is related to biofilm regrowth. Increased ergosterol genes have also been reported *in vivo* in a *C. albicans* central venous catheter biofilm model, demonstrating the importance of the molecule within the biofilm [118].

Induction of ergosterol genes has also been described in *C. dubliniensis*, where incubation with fluconazole and formation of biofilm was coupled with upregulation of the Cd*ERG3* and Cd*ERG25* [119]. Moreover, upregulation of genes involved with ergosterol biosynthesis has been described in *C. parapsilosis* biofilms [120], which are also resistant to azole antifungal therapy [121]. Overall, these data



Induced resistance gene		
Gene	Function	Resistance
Erg 1, 3, 11, and 25	Ergosterol	Cross-resistance
Kre1 Skn1	β-1,6 glucan	Polyene
Fks1	β-1,3 glucan	Cross-resistance
Zap1, Gcal1, 2, Adh5, Csh1	ECM regulators	Cross-resistance
CDR1–4	ABC transporters	Cross-resistance
Afu MDR4	ABC transporters	Azole
MDR1	MFS transporters	Azole
Mck1	MAPK	Cross-resistance
Hsp90	Heat shock protein 90	Cross-resistance

Figure 2: Molecular mechanisms of fungal biofilm resistance. Antifungal drug resistance in fungal biofilms is both complex and multifactorial. The diagram illustrates the mechanisms of different class of antifungal agent action (azoles [AZL], polyenes [POL], and echinocandins [ECN]) and resistance: (a) the layer of ECM present in the biofilm shields the cells from antifungal agents by binding and reduced penetration; (b) the membrane transporter system ABC and MFS efflux pumps extrude antifungal molecules and reduce the intracellular concentration; (c) mutation in *ERG*, *Cyp51,* and *FKS1* genes alters the drug target leading to cross-resistance; (d) antifungal pressure induces stress responses, such as the calcineurin signalling pathway, which is activated, and coping responses occur through upregulation of various signal transducers. On the right hand side, the table lists different resistance genes and their functions, and antifungal agents affected.

highlight the importance of ergosterol in biofilm resistance, particularly with respect to azoles, which indirectly inhibit their biosynthesis. Recent studies have shown that simvastatin, which impairs cholesterol metabolism in humans, is capable of inhibiting *C. albicans* biofilms, thus providing a potential novel strategy of combating these tenacious infections [122].

4.4. Efflux-Pump-Mediated Resistance. The primary molecular mechanism leading to high-level azole resistance in *C. albicans,* that is, increased efflux of drug mediated mostly by the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters [123–125]. The ABC transporters in *C. albicans* constitute a multigene family, which includes several CDR genes (CDR1-4) [126, 127]. The ABC transporters include a membrane pore composed of transmembrane segments and two ABCs on the cytosolic side of the membrane, which provide the energy source for the pump [128, 129]. Importantly, multiple antifungal agents can be substrates for these transporters, and thus, their overexpression can lead to cross-resistance among different drugs, particularly azoles. Among members of the MFS, which are secondary transporters and use proton-motive force across the plasma membrane, the MDR1 gene encodes a major facilitator that has been implicated in *C. albicans* azole resistance, and its overexpression leads to exclusive fluconazole resistance [46, 113]. Echinocandin sensitivity is unaffected by efflux pumps [130].

Genes encoding for drug efflux pumps have been reported in biofilms to be differentially regulated during development and upon exposure to antimicrobial agents include Ca*CDR1, CaCDR2,* and Ca*MDR1* [102, 108, 131, 132]. In the first study to investigate the role of efflux pumps, it was demonstrated that expression of genes encoding both types of efflux pump were upregulated during the course of biofilm formation and development. Both Ca*CDR1* and Ca*CDR2* were upregulated in 24 and 48 h biofilms, whereas Ca*MDR1* was transiently upregulated at 24 h [108]. However, their contribution to resistance in the biofilm phenotype was placed in doubt when a set of *C. albicans* isogenic strains deficient in efflux pumps carrying single- and double-deletion mutations (Δ*cdr1*, Δ*cdr2*, Δ*mdr1*, Δ*cdr1*/Δ*cdr2*, and

Δ*mdr*/Δ*cdr1*) that rendered the cells hypersusceptible to fluconazole when planktonic retained the resistant phenotype during biofilm growth. In a subsequent investigation, *C. albicans* biofilms were formed through three distinct developmental phases that were associated with high fluconazole resistance. Again, the same set of isogenic *C. albicans* strains were utilised where it was shown that 6 h old biofilms formed by double and triple mutants were >4- to 16-fold more susceptible to fluconazole than the wild-type strain [102]. At 12 and 48 h, all the strains became highly resistant to this azole, indicating lack of involvement of efflux pumps in resistance at late stages of biofilm formation. In cell density studies of the efflux pump isogenic strains, these remain hypersensitive at low cell concentrations yet resistant at high cell concentrations and in biofilm, indicating a contributory resistance role of cell density [100]. Nevertheless, similar to the study by Ramage and coworkers [108], *C. albicans* biofilms were shown to express Ca*CDR* and Ca*MDR1* genes in all three phases (6, 12, and 48 h), whilst planktonic cells expressed these genes transiently. In fact, GFP promoter studies have shown induction of efflux pumps after 15 min adherence to provide a tolerant biofilm phenotype [132]. Animal studies have also shown that biofilms formed on implanted catheters express efflux pumps [84, 118]. Transcript upregulation of Ca*CDR2* at 12 h (1.5-fold) and Ca*MDR1* at both 12 h (2.1-fold) and 24 h (1.9-fold) was demonstrated [118]. In *C. glabrata,* similar results are reported, where expression of Cg*CDR1* and Cg*CDR2* was investigated during the early (6 h), intermediate (15 h), and mature (48 h) phases of biofilm development. At 6 h and 15 h, the biofilms exhibited approximately 1.5- and 3.3-fold upregulation of Cg*CDR1* and 0.5- and 3.1-fold upregulation of Cg*CDR2*, respectively, in comparison to planktonic cells [133]. Expression of Ct*MDR* in *C. tropicalis* biofilms has also been reported [134].

Collectively, these studies suggest that multifaceted, phase-specific mechanisms are functional in resistance of fungal biofilms. This is confirmed in studies of *A. fumigatus* biofilm resistance. Mutations within the *cyp*51A gene, which alters the ergosterol biosynthesis pathway, have been reported to cause azole resistance in *A. fumigatus* [135–138]. However, a recent study reported that 43% of azole-resistant isolates did not carry the cyp51A mutation, indicating that other mechanisms of resistance were responsible [139]. It was hypothesised that efflux-mediated mechanisms of resistance may explain this clinical resistance, which may also be important in biofilms. Sequence analysis suggests that *A. fumigatus* has 278 different MFS and 49 ABC transporters [140]. *A. fumigatus MDR* pumps have previously been shown to be associated with increased resistance to itraconazole [141, 142]. Currently, however, there is little evidence to suggest that these play an active role in clinical resistance [143]. Phase-specific analysis of resistance in *A. fumigatus* biofilms revealed increased resistance to azoles, polyenes, and echinocandins as each biofilm matured from 8 to 12 to 24 h [70, 71]. Biochemical analysis of efflux pump activity showed a significant increase in efflux pump activity in the 12 h and 24 h phases, and upregulation in 8 h germlings when treated with voriconazole. Moreover, inhibition of efflux pump activity with the competitive substrate (MC-207, 110) reduced the susceptibility to voriconazole by 5-fold. Quantitative expression analysis of Afu*MDR4* mRNA transcripts revealed a biphasic increase as the mycelial complexity increased (maximal at 12 h), which was coincidental with strain-dependant increase in azole resistance. Similar biphasic increases in *C. glabrata* CDR genes were also observed [133]. Voriconazole also significantly induced Afu*MDR4* expression, which was also detected *in vivo* [71]. Global transcriptional analyses of voriconazole-treated *A. fumigatus* mycelia of over 2000 genes were shown to be differentially expressed, and amongst these was a cluster of 15 different transporters mRNA at significantly increased levels, including MDR proteins of the ABC and MFS classes, such as AfMDR1 and AfMDR2 [144]. Also, unpublished microarray studies of the different phases of *A. fumigatus* biofilms from our group revealed a cluster of pumps and transporters, which is similar to studies of *C. albicans* [131].

Collectively, this data in addition to the available literature support the hypothesis that efflux pumps are an important, but not exclusive, determinant of fungal biofilm resistance to azoles [143, 145]. Their primary role may be for homeostasis within complex environments to protect themselves from acute toxicity [146], but within clinical environments exposure to azoles dugs may enhance the levels of efflux pump expression, therefore either contributing towards or inducing clinical resistance [139]. However, it is likely that they play a greater protective (resistance) role in the early phases of biofilm growth until the production of ECM, one of the primary mechanisms of biofilm resistance.

4.5. Extracellular Matrix. ECM is a defining characteristic of fungal biofilms, providing the cells protection from hostile factors such as host immunity and antifungal agents [6]. In some of the pioneering works by the Douglas group, *C. albicans* ECM was shown to increase when biofilms are grown under dynamic flow conditions [47, 48, 53]. However, subsequent work has shown that while diffusion is hampered by ECM, penetration of antifungal drugs is not thought to play a key role in biofilm resistance [53]. Recent studies have provided new insights that suggest the chemical composition of ECM and its regulation may play a central role in resistance.

The composition of the ECM of these biofilms in *C. albicans* and *C. tropicalis* is complex, comprising of protein, hexosamine, phosphorus, uronic acid, and carbohydrates [147]. Recently, it has been shown that extracellular DNA is another important component of the ECM in C. *albicans* [148], as the addition of DNase improves the efficacy of polyenes and echinocandins, but not to azoles [149]. One of the principle carbohydrate components is beta-1,3 glucans, as treatment of *C. albicans* biofilms with beta-1,3 glucanase helps detach biofilms from a substrate [147]. Its contribution is confirmed in a series of investigations by the Andes group where it was shown to increase in *C. albicans* biofilm cell walls compared to planktonic organisms and was also detected in the surrounding biofilm milieu and as part of the ECM [150]. Beta-1,3 glucans have also been shown to increase in investigations of three specific phases of biofilm

development grown on both denture acrylic and catheter substrates [151]. Its contribution to resistance was realized when it was also shown that biofilm cells walls bound 4- to 5-fold more azole than equivalent planktonic cells, and culture supernatant bound a quantifiable amount of this antifungal agent. Moreover, beta-1,3 glucanase markedly improved the activity of both fluconazole and amphotericin B. Addition of exogenous biofilm ECM and commercial beta-1,3 glucan also reduced the activity of fluconazole against planktonic *C. albicans in vitro* [150]. The group has recently shown that the ECM β-1,3 glucan is synthesised from Fks1p using a defined knockout and overexpressing strain [152]. This study demonstrated that beta-1,3, glucan is responsible for sequestering azoles, acting as a “drug sponge” and conferring resistance on *C. albicans* biofilms [152]. Further studies have shown that they are also responsible for sequestering echinocandins, pyrimidines, and polyenes [153]. This has been confirmed independently where AMB was shown to physically bind *C. albicans* biofilms and beta-glucans [154]. Subsequent studies have identified a role for the Ca*SMI1*, a gene involved in cell wall glucans, in biofilm ECM production and development of a drug-resistant phenotype, which appears to act through transcription factor Ca*Rlmp* and glucan synthase Fks1p [155].

In addition to Ca*FKS1,* a zinc-response transcription factor Ca*ZAP1* has been shown to be a negative regulator of ECM soluble beta-1,3 glucan in both *in vitro* and *in vivo C. albicans* biofilm models through expression profiling and full genome chromatin immunoprecipitation [156]. Conversely, two glucoamylases, Ca*GCA1* and Ca*GCA2*, are thought to have positive roles in matrix production. A group of alcohol dehydrogenases Ca*ADH5*, Ca*CSH1*, and Ca*LFD6* also have roles in matrix production, with Ca*ADH5* acting positively, and Ca*CSH1* and Ca*LFD6* acting negatively [156]. It is thought that these alcohol dehydrogenases generate quorum-sensing aryl and acyl alcohols, which coordinate biofilm maturation. Collectively, it appears that *C. albicans* ECM production is highly regulated and is a key resistance factor. It is also present on a number of other *Candida* spp., including *C. glabrata*, *C. parapsilosis*, *C. tropicalis,* and *C. dubliniensis* [157, 158].

Significantly less is known regarding the role of *A. fumigatus* biofilm ECM in antifungal resistance. In an aerial static model, the presence of extracellular hydrophobic ECM is composed of galactomannan, alpha-1,3 glucans, monosaccharides, polyols, melanin, and proteins, including major antigens and hydrophobins [159]. This study demonstrated that hydrophobic matrix cohesively bound hyphae together, and that the matrix increased with maturity of the developing structure. Further studies report that a new galactosaminogalactan and the galactomannan were the major polysaccharides of the *in vivo A. fumigatus* ECM [160]. Extensive ECM production was also reported as the maturity of the biofilm increases [9]. For *A. niger,* after germination upon a support, the new hyphae also produce an ECM [161]. The production of ECM has also been reported elsewhere, where it has been shown to be produced on both polystyrene and cystic fibrosis (CF) bronchial epithelial cells by *A. fumigatus*, that were resistant to antifungal agents [162].

Martinez and Casadevall (2006) reported that *C. neoformans* also have the ability to form biofilm structures *in vitro* and produce ECM [68]. In *Pneumocystis* spp., confocal microscopy has revealed organisms enmeshed in ECM. Intense monoclonal antibody staining to the major surface glycoproteins and an increase in (1,3)-beta-D-glucan content were also evident, suggesting that these components contributed to resistance [7]. *Blastoschizomyces capitatus*, *Malassezia pachydermatis*, *Saccharomyces cerevisiae*, *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus*, and *Apophysomyces elegans* are all reported to produce ECM in their biofilms [10, 11, 13, 163]. Therefore, ECM clearly plays a critical role in fungal biofilm resistance, particularly for *C. albicans*, from which we currently understand the greatest. It is one of the most significant and regulated resistance mechanisms utilized in the biofilm phenotype.

4.6. Persisters. Persister cells are an important mechanism of resistance in chronic infections [164], and a mechanism of resistance that has gathered some attention recently in fungal biofilms [165–167]. Persister cells are “dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics” [168]. In *C. albicans* biofilms, a small subset of yeast cells have been described that are highly resistant to amphotericin B following adhesion, which is independent of upregulation of efflux pumps and cell membrane composition [76, 166]. The first study in fungi was to describe persister cells in fungi, described as a subpopulation of highly tolerant cells. In this study, *C. albicans* persisters were detected only in biofilms and not in different planktonic populations [166]. Reinoculation of cells that survived killing of the biofilm by amphotericin B produced a new biofilm with a new subpopulation of persisters, suggesting that these were not mutants but phenotypic variants of the wild type, and that attachment to a substratum initiated dormancy. The presence of persisters in *C. albicans*, *C. krusei*, and *C. parapsilosis* biofilms treated with amphotericin B was also described [169]. It was further hypothesized that the periodic application of antimicrobial agents may select for strains with increased levels of persister cells, so 150 isolates of *C. albicans* and *C. glabrata* were obtained from cancer patients who were at high risk for the development of oral candidiasis and who had been treated with topical chlorhexidine once a day. It was shown that the persister levels of the isolates varied from 0.2 to 9%, and strains isolated from patients with long-term carriage had high levels of persisters, whereas those from transient carriage did not [170]. Therefore, in this clinically relevant scenario, prolonged and ineffectual antifungal treatment may be beneficial to the biofilm population, which may be responsible for antimicrobial drug failure and relapsing infections.

The role of reactive oxygen species (ROS) in sessile *C. albicans* cells was investigated as they are known to be induced by high concentrations of miconazole, allowing 1-2% of miconazole-tolerant cells to persist [171]. Superoxide dismutases (Sods) were found to be differentially expressed by miconazole-treated sessile *C. albicans* cells compared to untreated cells. Inhibition of superoxide dismutase resulted

in an 18-fold reduction of the miconazole-tolerant persister cells and increased endogenous ROS levels in these cells [165]. In biofilms from strains lacking Δsod4/Δsod5, at least 3-fold less miconazole-tolerant persisters were observed, and ROS levels were increased compared to the isogenic wild type. Therefore, miconazole-tolerant persisters are linked to the ROS-detoxifying activity of Sods. Whether this is the definitive molecular basis for *C. albicans* persister cells or a tolerance mechanism still remains to be determined, but these subpopulations are clearly another important fungal biofilm resistance mechanism.

4.7. Tolerance. Stress responses have become more fully realized as defined mechanisms of antifungal resistance. Pathogenic fungi encounter a range of physiological stresses from different environments, including temperature changes, ionic stress, changes in osmolarity, and oxidative stress, such as that experienced in the phagosomes of neutrophils [112]. These stresses are sensed through various receptors, which elicit responses through conserved signaling pathways. One of the most important is the mitogen-activated protein kinase (MAPK) signal transduction network, and the many others are subject to review [112]. It was first shown that the mitogen-activated protein kinase (MAPK) Mck1p, which is activated by contact stress, is involved in biofilm development. Moreover, the null mutant (*mck1*) biofilms were azole sensitive, in contrast to the sessile wild type and both planktonic strains. This indicates that Mck1p is involved in biofilm resistance through a stress pathway [172].

Calcineurin is a Ca^{2+}-calmodulin-activated serine/threonine-specific protein phosphatase that plays many critical stress roles in the fungal cell, including amongst other things antifungal drug responses [173]. In planktonic cells, calcineurin is critical for *C. albicans* survival during azole treatment [174]. Inhibiting calcineurin pharmacologically or impairing calcineurin function genetically has synergistic activity with fluconazole and renders the azoles fungicidal against *C. albicans* [175]. Calcineurin has also been implicated in mediating resistance to the azoles in both *in vitro* and *in vivo* models of biofilm formation [176]. *C. albicans* cells in biofilms are up to 1,000-fold more resistant to fluconazole than planktonic cells, indicating that inhibitors could be used in combinations as novel therapeutic interventions to treat or prevent biofilms, whereas *C. dubliniensis* calcineurin inhibitors were unable to form biofilms [177]. Similar studies have evaluated the efficacy of a voriconazole-micafungin combination against *C. albicans* biofilms. Voriconazole significantly antagonized the fungicidal effect of micafungin against biofilms. To investigate the mechanism of antagonism, an inhibitor of calcineurin was evaluated, which reversed the voriconazole-induced resistance to micafungin [178]. This study also suggested that heat shock protein 90 (Hsp90) molecular chaperone played a role in this antagonism. Hsp90 regulates complex cellular circuitry in eukaryotes and potentiates the emergence and maintenance of resistance to azoles and echinocandins in *C. albicans*, at least in part via calcineurin [179]. It physically interacts with the catalytic subunit of calcineurin, keeping it stable and poised for activation [180]. A recent study led by the Cowen group demonstrated that genetic depletion of Hsp90 reduced *C. albicans* biofilm growth and maturation *in vitro* and interestingly impaired dispersal of biofilm cells [181]. It also abrogated resistance of *C. albicans* biofilms to the azoles, which was also shown *in vivo*. Furthermore, depletion of Hsp90 led to reduction of calcineurin and Mkc1 in planktonic but not biofilm conditions, suggesting that Hsp90 regulates drug resistance through different mechanisms. A marked decrease in matrix glucan levels was observed, providing a mechanism through which Hsp90 might regulate biofilm azole resistance. In *A. fumigatus*, pharmacological depletion of Hsp90 led to reduced resistance to the echinocandins [181]. Moreover, a recent investigation of the *C. glabrata* biofilm proteome demonstrated upregulation of a heat shock protein (Hsp12p) and other stress proteins (Trx1p, Pep4p) [182]. Therefore, targeting Hsp90 may provide a novel strategy for treating fungal biofilm infections.

5. Conclusions

Fungal biofilm resistance is multifaceted, involving some basic physical barriers and some complex regulatory processes. The evidence that has been collected over the past decade would suggest that as the biofilm changes from an adherent phenotype into a complex biofilm, then different mechanisms of resistance are utilised, that is, phase-specific mechanisms. Clearly, efflux pumps are utilised during the early to intermediate phases of biofilm development but are relinquished towards maturity as ECM is produced to "soak" and deplete antifungal agents. The density of the mature biofilm may act as a physical barrier, and reduction of growth rates under different environmental pressures can benefit the biofilm. Moreover, during hyphal growth, ergosterol biosynthesis is regulated by antifungal treatment, the direct and indirect targets of polyenes and azoles, respectively. Within the dense biofilms, where penetration of antifungal drugs is possible, persister cells phenotypes ensure survival, and in these stressed environments, global stress proteins kick into action to protect and maintain. Overall, the evidence highlights that fungal biofilm resistance is an inducible phenotype that is a part of a highly evolved series of molecular pathways that regulate biofilm development and homeostasis.

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Review Article

Hosting Infection: Experimental Models to Assay *Candida* Virulence

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Although normally commensals in humans, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata,* and *Candida krusei* are capable of causing opportunistic infections in individuals with altered physiological and/or immunological responses. These fungal species are linked with a variety of infections, including oral, vaginal, gastrointestinal, and systemic infections, with *C. albicans* the major cause of infection. To assess the ability of different *Candida* species and strains to cause infection and disease requires the use of experimental infection models. This paper discusses the mucosal and systemic models of infection available to assay *Candida* virulence and gives examples of some of the knowledge that has been gained to date from these models.

1. *Candida* and Man

1.1. Carriage of Candida *Species.* In healthy individuals *Candida* species are harmless members of the normal gastrointestinal (GI), oral, and vaginal microbial flora. It is assumed that everyone carries *Candida* in their GI tract (reviewed in [1]), with *C. albicans* the species most frequently identified in faecal sampling, representing 40–70% of isolates [2–4]. Other isolates are usually identified as *C. parapsilosis, C. glabrata, C. tropicalis,* or *C. krusei* [2–4].

In comparison to GI carriage, oral carriage is observed in only ∼40% of healthy individuals, with considerable variation found between studies (reviewed in [1]). Higher carriage levels are generally associated with diabetes, cancer, HIV, or denture use (reviewed in [1]). Again, the majority of isolates (∼80%) are identified as *C. albicans*, with *C. glabrata* or *C. parapsilosis* making up the remainder [5–9].

Vaginal carriage occurs in an even smaller proportion of the healthy population, with only ∼20% of healthy women found to have vaginal *Candida* carriage [10–13]. *C. albicans* is again the most commonly identified species, with *C. glabrata* the only other species usually found [10, 12, 14–17].

Therefore, *C. albicans* is the major species found as a commensal in healthy individuals, with four other species, *C. tropicalis*, *C. parapsilosis*, *C. glabrata,* and *C. krusei*, also found.

1.2. Candida *and Disease.* *Candida* species, however, have an alternative lifestyle, causing opportunistic infection in hosts with altered physiological or immune response. The infections caused by *Candida* species range from self-limiting, superficial mucosal lesions (commonly referred to as thrush), chronic and/or recurrent mucosal, skin, and nail infections, through to life-threatening invasive or disseminated infection [1, 18–21].

In humans, the most common infections caused by *Candida* species are superficial infections of the mucosa, skin, and nails [20–24]. Pseudomembranous oral thrush is common in babies and in the elderly, but is also found in HIV-positive individuals and cancer patients (reviewed in [1, 25]). Denture stomatitis is also a significant infection, occurring in approximately 60% of denture wearers [26, 27]. In oral candidiasis most infections are caused by *C. albicans* (58%),

with the remainder caused by *C. parapsilosis, C. tropicalis, C. glabrata,* and *C. krusei* [28, 29].

Vaginal candidiasis, or thrush, another form of superficial infection, affects approximately 75% of women of childbearing age [30, 31]. *C. albicans* is most commonly isolated, with *C. glabrata* also found, but at a lower frequency [17, 30, 32–35], reflecting the species normally carried in the vulvovaginal area.

An additional form of candidiasis involving the mucous membranes, as well as the skin and nails, is chronic mucocutaneous candidiasis. Unlike other forms of candidiasis, there is evidence that this condition can be inherited or is associated with thymoma, with almost every infection caused by *C. albicans* [20–24, 36].

The most serious infections caused by *Candida* species, however, are invasive or disseminated infections. *Candida* species cause ~11% of all bloodstream infections and 20% of those occurring in the ICU population [37–39]. However, in comparison to bacterial infections occurring in the same patient population, these infections are much more serious as mortality rates remain high (~45%) [1, 40]. This is due, in part, to diagnostic difficulties and limited antifungal therapies. Invasive infections occur in those patients who are already seriously ill, with major risk factors including admission to ICU, surgery (especially abdominal surgery), and neutropenia (reviewed in [1]). The five *Candida* species commonly isolated from the human GI tract are also responsible for 90% of invasive *Candida* infections [1, 41]. Geographical variations in the epidemiology of these infections do occur, with *C. tropicalis* the most common cause of invasive *Candida* infection in both India and Singapore [42–44]. In addition, in patients with haematological malignancies and in young children and babies, there is increased incidence of *C. tropicalis* and *C. parapsilosis* [45–49].

Patients with invasive *Candida* infection usually present with clinical symptoms similar to those associated with invasive bacterial infection and can eventually develop sepsis [50]. From autopsy reports, it is evident that the lungs and the kidneys are the organs most commonly affected, with fungal lesions also found in the heart, liver, and spleen [51–55]. Infection most likely originates from the GI tract, as the majority of invasive infections show GI involvement (oesophagus, stomach, and intestines) [51, 53] and *Candida* isolates from the bloodstream are identical, or closely related, to isolates from nonsterile sites of the same patient [56].

Increasing numbers of patients suffering immunosuppression and undergoing invasive treatments, for example, for cancers and organ transplants, mean that there is an ever-increasing population at risk of invasive fungal infection. With a medical need for the development of new and more efficient diagnostics and therapies for fungal infection, we need a better understanding of *Candida* pathogenesis, that is, how do the major *Candida* species cause opportunistic infections?

2. Experimental Models of *Candida* Infection

Experimental infection models allow disease development to be followed from the moment that fungal cells are introduced into the host. To be a good model, a model should be reproducible, relatively easy to set up, and should reproduce the major clinical symptoms seen in the human disease. It is also an added advantage if the model is cost effective. Models which satisfy these conditions allow further in-depth investigation of *Candida* virulence to be carried out and, subsequently, allow inferences about *Candida* virulence in human disease to be made.

Although a great deal of preliminary research on virulence can be carried out by laboratory experiment, infection modelling requires the involvement of a host organism. It is only in a whole organism that the complex host-fungus interactions that determine whether or not disease will occur can be investigated. Although larger animals have been used to study *Candida* infections, for example, macaques [57, 58], piglets [59], rabbits [60–62], and guinea pigs [63, 64], the majority of *Candida* virulence studies use rodent infection models. This is due to economic factors, ease of handling, and the availability of genetically modified mouse strains, which allow human genetic conditions to be mimicked.

In this paper, experimental animal models that have been developed for *Candida* virulence assays are discussed. It should be noted that the majority of models focus on *C. albicans* as this is the major species associated with human *Candida* infections.

2.1. Mucosal Infection Models. To model *Candida* oral and vaginal infections, mucosal models have been developed mainly in rats and mice. The procedures used in rats and mice are generally similar. However, the larger animal has the added advantage that denture-associated fungal biofilms formation can also be studied in a host [65]. Establishment of infection at mucosal sites generally requires treatment with immunosuppressive agents, oestrogen, or antibiotics prior to infection, or the use of germ-free animals [66–68]. However, the nude (*Foxn1*^{nu}) mouse model of oral infection allows infection to be established without any immunosuppression or other pretreatment [69]. Greater detail can be found in more extensive reviews of these infection models [67, 68, 70, 71].

In order to assess virulence in mice using the oral infection model, mice are routinely pretreated with corticosteroids and *Candida* cells are administered into the oral cavity of anaesthetised animals either by applying a *Candida*-soaked cotton bud under the tongue or by applying the inoculum directly onto the teeth, gums, and oral cavity [67, 70, 72]. Virulence in this model is usually determined by fungal organ burden and histopathology.

Both rat and mouse models have been used to compare the virulence of *C. albicans* mutant strains and also clinical isolates [73–77]. Using these models, *C. albicans* mutant strains which are unable to switch between the yeast and hyphal growth forms were found to be unable to cause oral infection, demonstrating a requirement for yeast-hypha switching in oral infection [75]. In addition, protein kinase Ck2 was also shown to be required for oropharyngeal *C. albicans* infections [77].

Mouse and rat models have also been developed to assay *Candida* virulence in vaginal infection. In these models the rodents are maintained in oestrus in order to maintain

colonisation and infection, which probably mimics pregnancy-associated candidiasis [78–81]. In rats, this generally involves surgery to remove the ovaries, with subsequent administration of oestrogen [81]. Recently, however, a new rat model has been developed, similar to the mouse model, where oestrus is maintained merely through administration of oestrogen [82], which will increase the ease of setting up the infection model. Immunosuppression of the host can also prolong colonisation by *Candida* species [83]. These models allow us to examine single vaginitis episodes; however, a satisfactory model of recurrent, chronic vaginitis is not yet available.

The virulence of *C. albicans* clinical isolates has been compared in rodent vaginitis models, demonstrating that isolates have varying capacities to cause disease [84, 85]. This model has also been used to assess virulence of genetically modified *C. albicans* mutants [85–87].

In addition to assessing *C. albicans* virulence, this model can be used to examine virulence of other *Candida* species. As *C. glabrata* is also associated with human vaginal infection, researchers have used the rat vaginitis model to evaluate the virulence of a *C. glabrata* petite mutant, discovering than the mutant was more virulent that the parental strain [88]. In addition, *C. parapsilosis* isolates have also been assessed for their ability to cause vaginal infection in the rat model [80]. In this study only a single isolate, recently obtained from a woman with active vaginal infection, was capable of initiating infection [80].

A major development in *Candida* virulence testing at mucosal surfaces occurred recently with the development of a concurrent oral and vaginal infection model by Rahman et al. [72]. This mouse model allows both oral and vaginal infections to be initiated in the same host, greatly reducing the numbers of animals required for these virulence assays. A comparison of the virulence of three different *C. albicans* isolates in this model clearly demonstrated that *C. albicans* isolates were not equally virulent, with obvious differences in their ability to initiate mucosal infections [72].

2.2. Invasive Infection Models. Mouse models of invasive fungal infection have been the most popular methods to assess *Candida* virulence up until the present day, although assays have also been carried out in rabbits, guinea pigs, and rats also used in some studies. There are two major models of *Candida* invasive infection, the intravenous (IV) challenge model and the gastrointestinal (GI) colonisation with subsequent dissemination model. These models were recently reviewed [89].

2.2.1. Intravenous Challenge Model. The mouse IV challenge model has been used to study *Candida* virulence since the 1960s and is both well characterised and reproducible [90–92]. *Candida* cells are injected directly into the lateral tail vein, bypassing any requirement of the fungus to cross epithelial and endothelial barriers to gain entry into the bloodstream. In this mouse model, which is similar to human invasive infection occurring with catheter involvement, fungal cells are found in all organs, but disease progresses only in the kidneys and brain, which depends upon inoculum level and mouse strain [91–93]. Sepsis develops as invasive disease progresses, which eventually leads to the death of the mouse [92, 94, 95].

In these models of *Candida* invasive infection, virulence is determined by monitoring survival of infected mice and/or by quantifying fungal organ burdens at predetermined times after infection. Drug treatments can also be administered to the host to allow host conditions to be mimicked, for example, immunosuppression [88, 96–110] or diabetes [99], with greater *Candida* virulence in both of these treatments.

Using immunocompetent mice, the IV challenge model has been used to compare the virulence of different *Candida* species [97–99, 107, 111–114]. *C. albicans* is clearly the most virulent species [97, 98, 111, 112, 114], followed closely by *C. tropicalis* [97, 98, 111, 112, 114]. In contrast, *C. krusei* and *C. parapsilosis* were unable to kill the infected animals, even at high inoculum levels, and fungi were eventually cleared from the host [98, 111, 114].

In immunosuppressed mice, *C. tropicalis* showed greater virulence, with disease progressing in the kidneys, rather than infection being controlled which occurs in immunocompetent mice [96, 98, 99, 107, 115]. *C. parapsilosis* and *C. krusei* remained unable to initiate progressive infections, even with addition of immunosuppressive treatments [98, 107], although administration of a very high inoculum potentially allows some *C. parapsilosis* isolates to initiate disease [108, 110].

Within each *Candida* species, clinical isolates were found to show considerable virulence differences in the IV challenge model. This was true for *C. albicans* [97, 107, 116, 117], *C. tropicalis* [97, 99, 112, 115, 118], and *C. parapsilosis* [108, 119], with some isolates unable to initiate invasive infections. This raises questions as to whether virulence results found for a single strain or isolate are representative of the entire species. This could be of particular importance for *C. albicans* studies where the vast majority of gene disruption studies have been carried out in a single strain, SC5314, background.

Numerous studies have evaluated *C. tropicalis* clinical isolate virulence differences; however, there are very few studies published on the virulence of genetically modified *C. tropicalis* strains. One study which has been published was able to demonstrate that a secreted acid protease was required for full virulence of *C. tropicalis* in immunocompetent mice [120]. In contrast to *C. tropicalis*, vast numbers of studies have been published on the virulence of *C. albicans* mutants, with over 200 genes identified as contributing to the *C. albicans* virulence in this model (reviewed in [89]).

C. glabrata behaves very differently from the other *Candida* species in the mouse model of invasive infection. Although *C. glabrata* is maintained, or tolerated, at high levels in the kidneys of immunocompetent mice, the mice did not die and there was little inflammation associated with the fungal cells [113, 114]. Immunosuppression appears to increase virulence of *C. glabrata* in terms of higher fungal organ burdens, but mouse survival is only increased in some *C. glabrata* infections [100, 103–106]. However, because immunosuppression may allow invasive disease to develop in *C. glabrata*-infected mice, these treatments have been added

to an infection model used in some studies to compare the virulence of genetically modified *C. glabrata*, with fungal burdens used as the virulence estimate [88, 101, 102, 105]. The immunosuppressed mouse infection model has demonstrated the importance of hypertonic stress responses, the cell wall integrity pathway, and nitrogen starvation responses in *C. glabrata* virulence [103, 104, 106]. In addition, this model has identified a petite mutant, strains expressing hyperactive alleles of the transcription factor gene *PDR1* and the *ace2* null mutant as being more virulent than their parent strains [88, 105, 121]. However, it should be noted that the hypervirulent phenotype of the *C. glabrata ace2* null was completely lost in immunocompetent mice [122]. In other virulence experiments in immunocompetent mice, where virulence was determined from fungal organ burdens at day 7 after infection, researchers were able to demonstrate that the cell wall integrity pathway [123, 124] and oxidative stress response [125], as well as the transcription factor Pdr1p and some of the genes that it regulates [101, 121], contribute to *C. glabrata* virulence.

2.2.2. Gastrointestinal Colonisation and Dissemination Model. Gastrointestinal models can either be set up in neonatal or adult mice. Intragastric infection of neonatal mice leads to persistent colonisation, without any requirement for pretreatment of the mice. However, to obtain colonisation of adult mice, the natural mouse gastrointestinal flora must first be removed by treatment with broad spectrum antibiotics. Adult mice can either be infected by gavage (intragastrically) or orally via their chow or drinking water. Subsequent treatment of *Candida* colonised mice with immunosuppressants and/or drugs which damage the gut wall allow fungal dissemination to occur (reviewed in [70, 126]).

In the gastrointestinal models fungal colonisation is highest in the stomach, caecum, and small intestine [107, 127–129], reflecting some of the clinical findings seen in human invasive infection. During the model, persistent colonisation is routinely monitored by noninvasive faecal fungal counts, and after dissemination *Candida* cells can be cultured from the liver, kidneys, and spleen [128–130]. However, differences may be seen between mouse strains [131].

This murine model is believed to be a more accurate reflection of the events occurring in the human patient, with broad spectrum antibiotics allowing fungal overgrowth and later invasive therapies causing mucosal damage. Mucosal damage then allows *Candida* to enter the bloodstream and disseminate to the internal organs. In the mouse, similar to human patients, there is increased animal-to-animal variation compared to the intravenous challenge model, requiring higher numbers of animals per group to obtain statistically significant results [128–130].

Comparison of *Candida* species virulence in this model demonstrated that *C. parapsilosis* had lower virulence compared to *C. albicans* and *C. tropicalis,* as there was little evidence of dissemination from the gut [107, 132]. However, *C. parapsilosis* was successful in establishing persistent colonisation of the GI tract [107]. In separate studies, *C. tropicalis* appeared to be more virulent than *C. albicans* in the gastrointestinal model, with greater dissemination to the internal organs [133, 134] and higher mortality rates [97, 134]. However, given the levels of variation observed in other models for the virulence of strains of different *Candida* species, further isolates will require to be assayed before a definitive conclusion on the relative virulence of the two species can be made.

To date, only a limited number of *C. albicans* mutant strains have been tested in the gastrointestinal colonisation and dissemination infection model, with only 6 mutants identified so far as contributing to virulence [89, 135]. However, this model has demonstrated that a constitutively filamentous *C. albicans* mutant was unable to disseminate, suggesting that the ability to switch between morphological forms may be more important for dissemination [136].

C. glabrata also behaved differently from the other four major *Candida* species in this model, being unable to colonise the oesophageal tissue in the neonatal mouse gastrointestinal colonisation and dissemination model [137]. Again, there was little host inflammatory response to *C. glabrata* [137], suggesting that *C. glabrata* virulence mechanisms may be quite different from those of the other species studied.

3. Beyond the Genome: Challenges of *Candida* Virulence Testing in the Postgenomic Era

The genome sequences of *C. albicans*, *C. glabrata*, *C. tropicalis,* and *C. parapsilosis* are now available [138, 139], encouraging the creation of large-scale mutant libraries. The challenge comes, however, when these large libraries are to be screened for genes involved in fungal virulence, with logistical, financial, and ethical issues to be considered.

In library screening programmes carried out to date different virulence testing strategies have been taken. Noble et al. [140] used signature-tagged mutagenesis to allow pools of mutants to be assayed in small numbers of animals, significantly reducing the animal numbers required for testing. By contrast, in order to screen a library of 177 *C. albicans* strains for altered virulence, Becker et al. [141] assayed each strain in 15 mice. From these two examples it is clear that traditional testing methods can lead to large numbers of mice being required to assay virulence. However, researchers have recently begun to address the issues of virulence testing large numbers of *Candida* strains by developing a range of minihosts, which are mainly based on invertebrate hosts.

Minihosts may not initially appear relevant to the human disease, but these hosts do possess an innate immune system and this is known to be critical in the development of *Candida* infections [142]. However, many of the minihosts do not possess an adaptive immune system, which may limit their usefulness. In addition, the majority of invertebrate models have the disadvantage that they must be kept at temperatures below normal human body temperature, with the exception of *Galleria* which can be incubated at 37°C. Potentially, incubation at lower temperatures may induce physiological changes in the fungus, affecting host-fungus interactions during disease development.

3.1. Wax Moth and Silk Worm Larval Models. The first minihost model developed for *Candida* virulence testing was

the *Galleria mellonella* (wax moth) larval model [143]. In this model fungi are injected into larvae, via a proleg, and survival is monitored over a short time period. The model is relatively cheap and has the added advantage that large numbers of larvae can be infected with each mutant strain, increasing the statistical power of the assay. The *Galleria* model has been successfully used to model *C. albicans* virulence, with results roughly similar to those found in mouse infection models [143–146]. A similar model has also been developed using the silk worm (*Bombyx mori*) [147, 148]. Both *C. albicans* and *C. tropicalis* are capable of killing silk worm larvae within two days [148], and *C. albicans* virulence differences were shown to correlate with results previously found in a mouse model [147].

3.2. Drosophila melanogaster. The fruit fly, *Drosophila melanogaster*, has also been used to assay *Candida* virulence [149–152]. The susceptibility of wild-type *D. melanogaster* continues to be debated; however, both Toll- and Spätzle-deficient fruit flies are susceptible to infection by *Candida* species when fungi are injected directly into the thorax [149–151]. Again, *D. melanogaster* models also have the advantage that large numbers of flies (>30 flies) can be infected with each *Candida* strain, increasing the statistical power of the assay.

In fruit flies, *C. albicans* was shown to be more virulent than other *Candida* species, confirming the results found in mammalian models (see above; [149]). In addition, virulence results for *C. albicans* clinical isolates and mutants were broadly similar to those found in the mouse systemic model [149–151]. However, differences do occur. In the fruit fly, CO_2 sensing is important for virulence, but this was not the case in the mouse IV challenge model [153]. This model has already been successfully used to screen a *C. albicans* mutant library, identifying Cas5, a transcription factor involved in cell wall integrity, as being required for full virulence [154].

In addition to the systemic *D. melanogaster* infection model, a new gastrointestinal infection model has also been developed recently, which should provide new options for virulence screening in a gastrointestinal model [152].

3.3. Caenorhabditis elegans. In addition to fly and larval models, the nematode *Caenorhabditis elegans* has also been evaluated as an infection model for assaying *Candida* virulence [155]. This model is particularly suited to high-throughput screening, as the *Candida* cells are fed to the nematodes in their food and assays are carried out in multi-well plates. This model has also been used successfully to screen a *C. albicans* transcription factor mutant library, allowing identification of transcription factor genes involved in hypha formation [155].

3.4. A Vertebrate Minihost: Zebrafish (Danio rerio). Zebrafish are the first vertebrate minihost model developed for virulence testing of *Candida*. This organism has the added advantage of having both innate and adaptive immune systems [156], and methods are also available to allow fish gene expression to be manipulated to mimic human genetic conditions [157].

The first virulence assay developed in zebrafish involved intraperitoneal injection of *C. albicans* into 7-month-old zebrafish [158]. In this model, similar to mouse models, progressive infection depends upon dose and is associated with increased proinflammatory gene expression. This model also allows increased group sizes, with group sizes of 20 fish being used to date. Using this model, researchers demonstrated that a clinical isolate with reduced virulence in a mouse model also showed reduced virulence in this model [158]. In addition, a *C. albicans* mutant (*efg1/cph1*) known to have attenuated virulence due to filamentation defects also had reduced virulence in this model [159, 160]. Of greater interest was the finding that, although these mutants were unable to form filaments *in vitro*, they clearly formed filaments when growing within fish. This model also allows interactions between zebrafish immune cells and *Candida* cells to be imaged, which will be made even easier in the future with the development of the new transparent adult (*casper*) zebrafish [161].

A second zebrafish infection model has also been described, where each fish larva (36 h after fertilization) is infected directly into the hindbrain ventricle with approximately 10 fungal cells [162]. In this model the *C. albicans efg1/cph1* mutant again demonstrated attenuated virulence, similar to results found in the mouse IV challenge model [162].

There are, however, disadvantages to the zebrafish infection models. One of the major drawbacks of this model, in common with the majority of other minihosts, is that the fish need to be kept at 28-29°C, which does not allow accurate mimicking of human infection.

4. Assaying Virulence in Experimental Models: Final Considerations

There are some important points to remember when evaluating *Candida* virulence in experimental infections. The first concerns the *Candida* species of interest. Although *C. albicans, C. tropicalis, C. glabrata,* and *C. krusei* are all associated with human carriage and infection, they are not natural mouse commensals or pathogens [163]. As such, there may be different interactions occurring between the fungus and the two different host species. This is of particular relevance when considering *C. glabrata* and its inability to initiate disseminated infection in the IV challenge models, especially when we know that *C. glabrata* can cause lethal infections in severely ill humans [164].

The second point to consider is that, although the immune systems of mice and men are similar, there are differences that could affect how the host and fungus interact [165–168]. Of particular relevance to *Candida* infections are differences in proportions of neutrophils and lymphocytes in the blood, complement receptor expression, and T-cell differentiation, to name but a few (reviewed in [168]). In addition, different mouse strains show differing susceptibility to infection, which could potentially alter virulence results [93, 169–172].

The third point to consider is which model should be used to evaluate *Candida* virulence. Some *C. albicans* isolates

exhibit virulence differences depending upon the infection model being used [72, 134, 173]. A good example is the *C. albicans* genome sequenced strain SC5314. In the IV challenge model, SC5314 is one of the most virulent *C. albicans* isolates, causing lethal infection in a relatively short time [92, 116]; however, in a vaginal infection model, SC5314 is a very poor coloniser of the vaginal mucosa [72]. In addition, a nongerminative *C. albicans* strain [173] and a *ura3* minus *C. albicans* strain [174], both of which were attenuated in systemic infection models [173, 175–177], successfully established mucosal infections [173, 174].

Only careful consideration of the above points will allow the *Candida* researcher to select the appropriate experimental *Candida* infection model to answer a particular research question. These models remain essential for increasing our understanding of fungal pathogenesis since both fungal attributes and host responses are known to contribute to the development of clinical disease.

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The author would like to apologise to those whose work could not be included in this review due to lack of space.

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Review Article

Chitin, Chitinase Responses, and Invasive Fungal Infections

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The human immune system is capable of recognizing and degrading chitin, an important cell wall component of pathogenic fungi. In the context of host-immune responses to fungal infections, herein we review the particular contributions and interplay of fungus and chitin recognition, and chitin-degrading enzymes, known as chitinases. The mechanisms of host chitinase responses may have implications for diagnostic assays as well as novel therapeutic approaches for patients that are at risk of contracting fatal fungal infections.

1. Introduction

Recipients of solid organs and hematopoietic cell transplants, AIDS patients, and burn victims are usually immunosuppressed for extended periods of time. Their prolonged immunosuppressive state is associated with a high risk of contracting invasive fungal infections (IFIs) [1, 2]. Most IFIs advance rapidly and are often not diagnosed early enough for antifungal drugs to function with full efficacy; therefore, the majority of these infections lead to death [1].

In contrast to immunosuppressed patients, immunocompetent individuals are protected from fungal infections by their functional innate immune system, which readily recognizes and eliminates fungal invaders. Recognition of fungal cellular features by the immune system appears to be a key component of the human antifungal defense [3]. For example, β-glucan on the fungal cell wall is recognized as a pathogen-associated molecular pattern (PAMP) by dectin-1 and activates pro- and anti-inflammatory cytokines in a myeloid-differentiation-primary-response-gene-88-(MYD88-) dependent signaling pathway [3–6]. An important component of the fungal cell wall that has not been fully explored as a PAMP is chitin, a polymer of *N*-acetylglucosamine [3, 7]. Chitin is one of the most abundant biopolymers, probably almost as abundant as cellulose [8, 9] and is found on fungal cell walls and exoskeletons of numerous organisms including parasitic worms (helminths) and arthropods. Although humans do not biosynthesize chitin, they do express chitin degrading enzymes, known as chitinases [10–12]. There are two known human chitinases that have chitinolytic activity, chitotriosidase (CHIT-1) and acidic mammalian chitinase (AMCase), as well as multiple noncatalytically active chitinases called chi-lectins [11–14]. The functions of CHIT-1 and AMCase are unknown, but they are thought to aid in the defense of chitin-containing pathogens. For instance, in guinea pigs, serum chitotriosidase levels increase in response to systemic fungal infection [15]. That chitinase levels can vary in response to fungal infections suggests the possibility of using host chitinase responses as a diagnostic. However, several other stimuli can also upregulate chitinase activity [16–19] and counterproductively, several polymorphisms in the *CHIT-1* and *AMCase* genes are known to decrease chitinase activity [20–24]. Thus, there are several challenges to be overcome if chitinase responses were to be used in the diagnosis of fungal infections. More recently, recombinant CHIT-1 was shown to have antifungal properties both *in vitro* and *in vivo*, suggesting the possibility of a gene therapy approach [25]. This paper will explore chitinase responses to fungal infections, current knowledge about the mechanism of chitin recognition by host-immune cells, and regulation of host-chitinase induction.

2. Invasive Fungal Infections (IFIs)

Fungal infections have become a major disease concern over the last three decades, in particular for recipients of solid organs and hematopoietic stem cells, AIDS patients, and burn victims, all of whom are usually immunosuppressed for extended periods of time [26–28]. Their prolonged immunosuppressed status leads to an increased risk of contracting opportunistic IFIs. IFIs are also on the rise in intensive care settings, likely due to a growing use of procedures with invasive medical devices and long-term use of antibiotics [29]. In all cases, the most common etiological agents are *Candida albicans* and *Aspergillus fumigatus* [27, 29].

Humans are exposed to hundreds of fungal spores each day, usually without a negative effect on their health. In the lungs of patients that lack sufficient pulmonary immune defenses, *A. fumigatus* fungal spores are able to swell, germinate, and branch into fungal hyphae. The infection can then disseminate to other organs through the bloodstream [26, 30]. Healthy individuals are able to eliminate fungal spores by mucociliary clearance, macrophages, and other primarily pulmonary defense mechanisms [26]. *C. albicans,* on the other hand, is a commensal organism residing in the gastrointestinal tract and oral, and vaginal mucosa of most healthy individuals, where it typically does not produce harmful side effects. However, *Candida* overgrowth can become symptomatic causing mucosal membrane infections, the most common being thrush and vaginal *candidiasis* [31–33]. Severe systemic *Candida* infections (*Candidemia)* and dissemination to internal organs can occur in immunocompromised patients [31–33].

Current methods for detecting IFIs are based on clinical signs and microbial examination. For example, pulmonary fungal infections are typically examined via CT scan, followed up with bronchoalveolar lavage (BAL) and biopsy [27, 30]. Systemic yeast infections such as candidiasis can be diagnosed by the blood culture [33]. However, current diagnostic methods usually do not detect fungal infections at early stages, and therefore, antifungal drug treatment is oftentimes inefficient or delayed. There are some serological tests that may be routinely performed assisting in the diagnosis of fungal infections via detection of fungal antigens in suspected patients [30]. For example, the galactomannan assay is sometimes used for the detection of *Aspergillus* in serum and BAL fluid. This assay works by detecting galactomannan released from the fungal pathogen by enzyme-linked immunosorbent assays [34, 35]. Elevated levels of galactomannan have been detected at early stages of fungal infections, however, the sensitivity and specificity of this assay has been criticized [36]. Moreover, the galactomannan assay is not useful for other fungal pathogens, including *Candida* [30, 37]. β-1,3-glucan serological detection assays are more widely used today because they can detect a wide range of fungi, including *Aspergillus* and *Candida,* but they do not detect zygo- or mucormycosis or cryptococcal disease [38]. The β-glucan assay works by detecting β-1,3-glucan, a major component of the fungal cell wall, circulating in the patient bloodstream [39, 40]. The assay has had great promise for fungal detection, especially when used to confirm galactomannan positive results, however, problems with false-positive (and false-negative) results have been reported [41, 42]. Despite the availability of such diagnostic tests, Garcia-Vidal et al. reported an increase in IFIs and lack of detection at an early stage, within 40 days after hematopoietic cell transplant in infected patients, demonstrating the ineffectiveness of present day diagnostic methods [27].

3. Fungal Cell Wall Components and Pattern Recognition Receptors

Generally, the innate immune system's response to PAMPs, which include glycosides, glycolipids, and carbohydrates, among others, involves pattern recognition receptors (PRRs) that are expressed by phagocytes. Pattern recognition then leads to a cascade of cellular signaling that activates phagocytes for defense [3, 26]. The recognition of fungal cellular features, in particular fungal cell wall components, by the immune system of the host is an important element for mounting an antifungal defense response [3, 5, 26, 43]. The fungal cell wall is composed of various mannoproteins, β-glucans as well as a thin, rigid layer of chitin (Figure 1). Many PRRs interact with fungal cell wall components. For example, mannoproteins with *O*-linked protein-carbohydrate conjugations are recognized by toll-like receptor (TLR)-4 [3, 44, 45], while mannoproteins that are N-linked can be recognized by dectin-2, mannose, and Fcγ receptors [3, 45–47] (Figure 2). The galectin-3 receptor recognizes β-mannosides [44, 48–50]. β-glucan is recognized as a PAMP by dectin-1 [4, 6] and when coated by phospholipomannan it is also recognized by both TLR-6 and TLR-2 [51–54]. Complement-coated β-glucan is recognized by complement receptor-3 [55, 56]. Dectin-1 recognition of β-glucans results in an MYD88-dependent pathway activation [3, 5, 44, 55, 57]. And finally, fungal CpG DNA is recognized by the intracellular receptor TLR-9 [58] (Figure 2). Recognition of fungal cell wall components by these PRRs generally leads to the nuclear factor kappa-lightchain enhancer of activated B cells (NF-κB) signaling; this results in the activation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, or anti-inflammatory cytokines, such as interleukin (IL)-10 (Figure 2). Whether chitin in the fungal cell wall is recognized as a PAMP, and if a specific chitin receptor exists as a PRR, remains unknown; yet it is a very likely possibility [3, 7].

4. Chitin as an Immune Modulator

Intranasal or intraperitoneal chitin administration to mice caused an immunological preactivation effect, called priming, in alveolar macrophages and natural killer (NK) cells [65]. Shibata et al. examined the effects of chitin particle sizes on cellular responses, in particular macrophage activation and priming. Balb/c mouse splenocytes that were cocultured

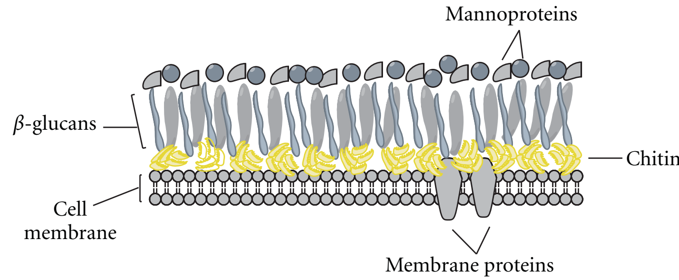

Figure 1: Fungal cell wall components. The fungal cell wall contains a cell membrane with various membrane proteins, a protective layer of chitin (yellow) as well as glucans (mostly beta), and mannoproteins on its surface. Different fungal cell walls contain different glucans. For example, the cell wall of *A. fumigatus* contains β-1,3- and β-1,4-glucan, and α-1,3-glucan [30], while *C. albicans* contains β-1,3- and β-1,6-glucan [44].

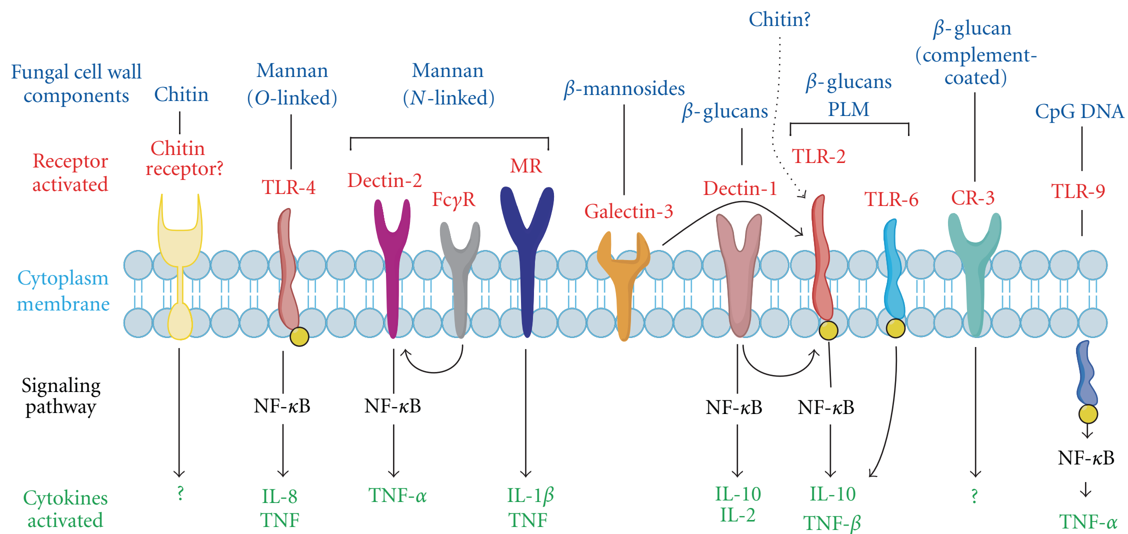

Figure 2: Fungal cell wall pathogen-associated molecular patterns (PAMPs) and their host-pattern recognition receptors (PRRs). Various fungal cell wall components are recognized by specific PRRs. Some PAMPs are recognized by multiple PRRs; for example, *N*-linked mannan is recognized by mannan receptor (MR), dectin-2, and FcγR [46, 47]. Phospholipomannan-(PLM-) coated β-glucans are recognized by both TLR-6 and TLR-2 [53, 54]. Other receptors may involve the signaling pathway of another PRR. For example, galectin-3, which recognizes β-mannosides, signals through TLR-2 (represented by a curved arrow) [47, 49] dectin-1, when activated by β-glucans can signal to activate the nuclear factor kappa-lightchain enhancer of activated B cells (NF-κB) on its own or with the help of TLR-2 [4, 45]. Fc gamma receptor (FcγR) may signal through dectin-2 when activated by *N*-linked mannan [3, 45, 47, 55]. Recognition of these fungal cell wall components mediates fungal recognition and defense by the host. Recognition by host PRRs usually involves signaling through NF-κB and activation of proinflammatory cytokines, such as TNF-α, or in some instances, and anti-inflammatory cytokines such as interleukin (IL)-10 [45]. The possibility of an alternative chitin receptor exists, activation of which leads to the recruitment of IL-4 producing cells [45, 59]. However, chitin has been shown to function as a T helper (Th)1 immune modulator, which stands in contrast an IL-4 associated Th2 response [60–64].

with chitin particles (50–100 μm), produced, and secreted IL-12, TNF-α, and IFN-γ [60]. However, intravenous injection of phagocytosable small chitin particles (1–10 μm) into C57 mice resulted in a macrophage priming that was dose dependent [61]. When utilizing a SCID mouse model instead of the C57 mice, the same chitin-macrophage priming effect was also found. Because SCID mice lack mature B and T cells, the authors concluded that neither T nor B lymphocytes were required for chitin-induced macrophage priming. An NK cell depletion experiment with anti-NK1.1 antibodies (anti-CD161c) then demonstrated a requirement for NK cells and NK-secreted IFN-γ in chitin-induced macrophage priming [61]. However, as we describe below, chitin particles can also be used to activate macrophages and monocytes directly in cell-culture experiments.

It should be noted that chitin can also serve as an immunoadjuvant [65]. Orally administered chitin suppressed the production of T helper (Th)2 cytokines and immunoglobulin (Ig)E in a ragweed allergy mouse model and induced IFN-γ instead [62]. In addition, when used as an adjuvant, chitin produced Th1 responses comparable to other adjuvants, including heat-killed *Mycobacterium bovis*, Freud's complete adjuvant, and the Bacillus Calmette-Guérin vaccine [63]. Chitin produced effects similar to those of

a Th1-promoting adjuvant in mouse models of ovalbumin-induced asthma and allergic hypersensitivities induced by the house dust mite *Dermatophagoides pteronyssinus* and by the fungal pathogen *A. fumigatus* [64, 66]. Chitin administration significantly reduced allergen-induced serum IgE levels and lung inflammation. Th1 cytokines IL-12, IFN-γ, and TNF-α were elevated, while IL-4 levels were decreased in mice-administered chitin as compared to controls [64, 66]. These and other studies strongly suggest that the immune system possesses a chitin recognition mechanism.

5. Mammalian Chitinases

Another immune response that may correlate with chitin recognition is the production of chitin-degrading enzymes, known as chitinases, by humans and other mammals. Chitinases belong to the glycosyl hydrolase 18 family, which is comprised of various proteins found in a wide range of organisms, including plants, bacteria, fungi, insects, protozoa, and mammals [13]. Six proteins with homology to chitinases have been identified in mammals. These include CHIT-1 and AMCase, which are the only two enzymatically active human chitinases able to hydrolyze chitin [11, 12, 14]. The other four of these highly homologous members of the chitinase family contain amino acid substitutions at their active sites, rendering these proteins noncatalytic. These noncatalytic chitinases are referred to as chi-lectins or chitinase-like proteins, and include chitinase-3-like protein 1 (CHI3L1, also known as YKL-40, Hcgp39, or GP39), stabilin-interacting chitinase-like protein (SI-CLP), YKL-39 (chitinase 3-like protein 2), and oviductin [13].

CHIT-1 is highly expressed by activated macrophages and is used as a marker for macrophage stimulation, suggesting a possible role in innate immunity [67, 68]. It was first discovered in the plasma of patients with Gaucher's disease; a disease characterized by the accumulation of lipid-laden macrophages [68–70]. The use of a chitinase detection assay, which measures the presence of chitinase activity via cleavage of the fluorogenic substrate 4-methylumbelliferyl chitotriosidase, showed that CHIT-1 levels were elevated several hundred-fold in the plasma of patients with Gaucher's disease. Therefore, CHIT-1 is now being used as a biomarker for the diagnosis of Gaucher's disease [68, 69, 71]. These findings drew attention to the cloning and further characterization of CHIT-1 [72, 73] and the discovery of the other enzymatically active human chitinase, AMCase. The sequence of AMCase is highly homologous to that of CHIT-1; however, AMCase is unique in that it functions strongest in acidic pH environments. Consistently, it was first found highly expressed in the stomach, intestinal tissue, and more recently is being studied as a biomarker for asthma and other hypersensitivities [11, 12, 27, 74].

Evolutionarily, chitinase production plays an important role in the life cycles of chitin-containing organisms such as fungi, insects and crustaceans, in which it is involved in either cell wall remodeling or molting. However, because mammals do not produce chitin, the physiological function of these chitinases and chi-lectins remains unclear, but various studies suggest that their function may lie in the digestion of chitin-containing foods and defense against chitin-containing pathogens and parasites [11, 13, 75].

6. Chitinases in Experimental Antifungal Therapy

Chitinases have also been investigated for their potential use in antifungal therapy. Low concentrations of recombinant human CHIT-1 degraded the cell walls of *Cryptococcus neoformans* and visibly inhibited its growth *in vitro* [67]. Morphological changes, such as atypical blebs, hyphal tip bursting, and restrictions of hyphal growth, were also observed for *Mucor rouxii* and *C. albicans* in the presence of recombinant CHIT-1 [67]. In addition, recombinant human CHIT-1 induced a dose-dependent improvement in the survival of mice with *C. albicans* and *A. fumigatus* infections [67]. Recently, it was shown that the culture medium conditioned by Chinese hamster ovary cells that had been retrovirally transfected with the human *CHIT-1* gene had antifungal activity [25]. These modified Chinese hamster ovary cells were then encapsulated in alginate microspheres and injected subcutaneously into BALB/c mice, where they continuously secreted active CHIT-1, and after infection with *C. neoformans*, mice harboring these cells had significantly lower fungal burden [25]. Therefore, the authors suggested that a continuous supply of active CHIT-1 should be explored in future gene therapies to prevent fungal infections.

7. Mammalian Chitinase Responses to Inflammation and Fungal Infections

Multiple stimuli, such as exposure to prolactin, interferon gamma (IFN-γ), lipopolysaccharides (LPS), and TNF-α can upregulate chitinase activity in human monocytes and macrophages, indicating a possible role for chitinase activity in inflammation [16–19]. Chitinase activity was reported to be upregulated as a result of various diseases, including candidiasis [76], *Wuchereria bancrofti* infections (filariasis) [21], and helminth infections [77, 78]. AMCase activity is highly upregulated in individuals suffering from asthma, chronic rhinosinusitis, or allergic bronchopulmonary aspergillosis [77, 78]. In addition, chitinase activity has been linked to fungal infections. In 1996, Overdijk et al. showed that, in guinea pigs, chitinase activity was induced after systemic infection with *A. fumigatus* [15, 79]. Furthermore, mice with pulmonary *C. neoformans* exposure had increased AMCase chitinase activities in the airways [80]. Intraperitoneal injections of zymosan, a yeast-cell wall-derived product that contains beta-glucans and small quantities of chitin, was shown to increase serum chitinase activity of rats [81].

Although chitinase activity does not appear to be specific for fungal infections, as it is also upregulated in other diseases, there appears to be a correlation between chitinase activity and inflammation as well as with disease induced by chitin-containing pathogens. These findings suggest that mammalian chitinase responses to fungal infections

and other parasitic infections may be triggered by the host response to a chitin-containing pathogen.

8. Chitinase Induction and Regulation

Little is known about how host chitinase activity is induced, but there is some indication that chitinase production and chitin recognition could be linked. Gorzelanny et al. used MALDI-TOF mass spectrometry to analyze the degradation of chitin by chitotriosidase and followed the stimulation of human monocyte/macrophage with a chitin hexamer [82]. These studies revealed that chitinases degrade chitin into smaller chitin-oligomers that in turn enhance the stimulation of macrophages, leading to more chitinase production [82]. However, the feedback mechanism of chitin recognition and chitinase secretion suggested by this study is still unclear and the signaling pathways involved are not fully understood.

Other chitin stimulation experiments revealed some aspects of the mechanism involved in the recognition of chitin and chitin-containing parasites by immune cells. Jumonji domain containing-3 (Jmjd3), a histone 3 Lys27 (H3K27) demethylase, along with Irf4 transcription factor, was determined to be essential for macrophage colony-stimulating factor (M-CSF)-bone-marrow-derived M2 macrophage polarization in response to *Nippostrongylus brasiliensis* helminth infection and chitin inoculation [59]. Another group found that mice exposed to *N. brasiliensis* helminth infection showed tissue invasion by macrophages and IL-4- and IL-13-producing immune cells as well as eosinophil recruitment [83]. Furthermore, transgenic mice that overexpressed *AMCase* in the lung, and were also exposed to *N. brasiliensis,* showed diminished infiltration of immune cells. The diminished infiltration of cells was likely due to *N. brasiliensis* chitin degradation and removal [83]. The same effect was observed when chitin alone was injected, and the effect was sustained, even in TLR-4-deficient animals [83]. The latter effect is interesting, because TLR-4, which recognizes LPS and leads to activation of the innate immune system, was previously considered a possible chitin PRR candidate [44]. The observed recruitment of IL-4 producing immune cells by chitin stands in stark contrast to the previously observed Th1 immune response induced by chitin when used as an adjuvant (see above). IL-4 is a typical Th2 response-inducing cytokine. It is possible though, that the IL-4 production by recruited immune cells is a secondary effect that requires other chemokines or other chemoattractants to be produced by primary chitin-sensing cells.

In contrast to TLR-4, TLR-2 and the IL17A receptor (IL-17AR) may at least be partially involved in chitin recognition. Da Silva et al. reported that mouse macrophages stimulated with chitin particles had increased levels of IL-17 protein and IL-17AR mRNA, and the increase in IL-17 was mediated via the TLR-2 pathway. *In vivo* investigations demonstrated that chitin induces acute pulmonary inflammation in wild-type mice, but not in TLR-2 knockout mice [84]. Therefore, it is possible that TLR-2 and IL-17AR are somehow involved in the recognition of chitin. TLR-2 is known to recognize bacterial particles, LPS, and more interestingly, zymosan, which contains chitin (see above) [44].

Portions of the downstream signaling pathway that leads to chitinase expression have been analyzed. *CHIT-1* mRNA expression in monocytes increases upon treatment with phorbol 12-myristate 13-acetate (PMA), which induces differentiation of monocytes into activated macrophages [85]. In addition, *CHIT-1* gene activation is accompanied by the binding of phosphorylated CCAAT-enhancer-binding protein (C/EBP)β and the transcription factor PU.1 to the promoter region of CHIT-1 (Figure 3) [85]. The upstream molecular signaling pathway leading to *CHIT-1* gene activation and chitinase induction has not been determined; however, roles for some key proteins involved in chitinase regulation have been noted [16–19]. Chitinase gene expression and activity was induced by human-monocyte-derived macrophages after prolactin stimulation in both a dose- and time-dependent manner [17]. Because prolactin has similar structural properties as some proinflammatory cytokines, alternative stimulations were preformed with IFN-γ, TNF-α, and LPS, and, as a negative control, with IL-10, which has anti-inflammatory properties. Chitinase activity was elevated in human monocyte-derived macrophages after stimulation with IFN-γ, TNF-α, and LPS and was significantly decreased after stimulation with IL-10 [16, 19]. These findings may indicate the involvement of chitinase activity induction during inflammatory conditions. Prolactin stimulation of human monocyte-derived macrophages was also performed in the presence or absence of specific kinase inhibitors [18]. The phosphatidylinositol 3-kinase (PI3-K) inhibitors wortmannin and LY-294002 reduced chitinase activity, as did the protein tyrosine kinase inhibitor genistein, the mitogen-activated kinase (MAPK) p38 inhibitor SB203580, and the MAPK p44/42 inhibitor U0126. No effect was observed on prolactin-mediated chitinase induction when the controversial protein kinase C inhibitor rottlerin was used, nor was an effect seen with PP2, a Src inhibitor, or AG490, a JAK2 inhibitor [18]. Accordingly, CHIT-1 induction can be mediated via a PI3-K/MAPK pathway (Figure 3).

9. Polymorphisms in Chitinase Proteins

The induction of chitinase activity as an immune response to various stimuli such TNF-α, prolactin, and chitin, and in response to fungal infections suggests that chitinases are indeed involved in the host's immune response to a pathogenic fungal invader. However, multiple known polymorphisms can affect chitinase activity, the most prominent being a 24-bp duplication in the *CHIT-1* gene. The *CHIT-1* gene is composed of 12 exons and the protein is secreted as two isoforms. The major isoform has a molecular mass of 50 kDa, undergoes posttranslational modifications, including *O*-linked glycosylation of the C-terminal region (which contains the chitin-binding domain) and is alternatively spliced into the 39 kDa minor isoform [20, 68]. Sometimes, a 24-bp duplication occurs in exon 10 of *CHIT-1* that causes a downstream cryptic 3′ splice site that generates mRNA with an in-frame deletion of 87 nucleotides (29 amino acids).

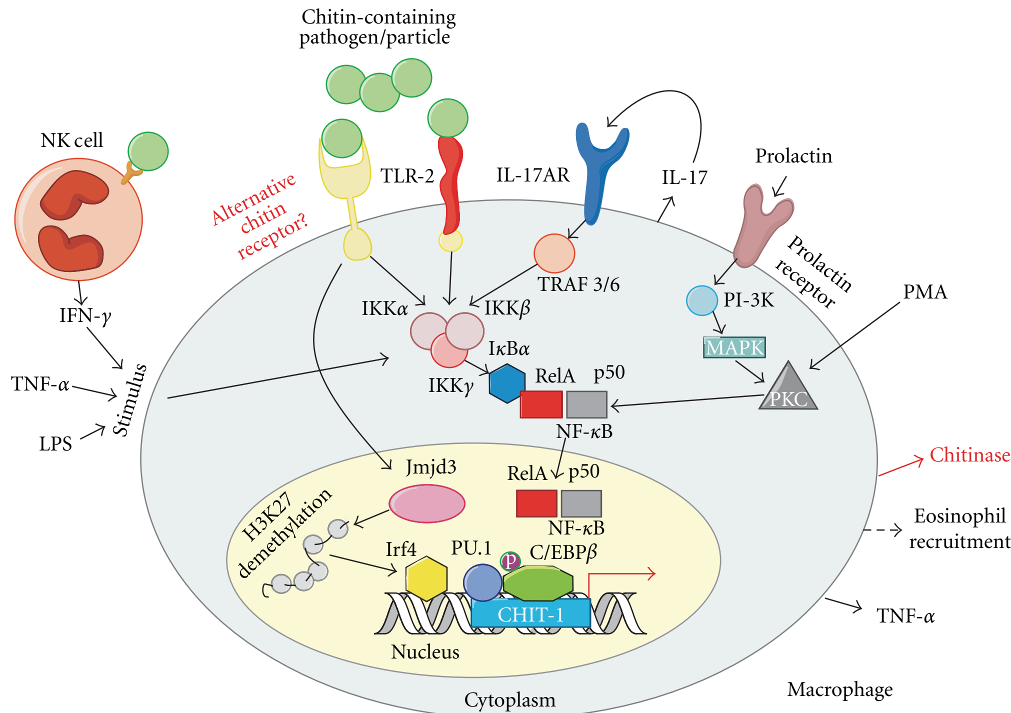

Figure 3: Hypothetical model for a molecular chitinase response to chitin-containing pathogens or particles. Chitin recognition leads to the expression of chitinase, TNF-α, IL-17, and eosinophil recruitment [83, 84]. NF-κB serves as the primary signaling pathway involved in the recognition of most fungal cell wall components (see Figure 2) [45]; therefore, its involvement in the expression of chitinase in response to a chitin-containing pathogen or particle is highly likely. Secretion of IFN-γ by NK cells induces macrophage priming caused by stimulation with a chitin-containing pathogen or particle [60–63]. IFN-γ, LPS, TNF-α, and PMA up-regulate chitinase activity [16–19], possibly through an NF-κB inflammatory-mediated pathway. IL-17 secretion upon macrophage stimulation with chitin increased levels of IL-17-AR and was TLR-2 dependent [84]. Either TLR-2 or an alternative chitin-specific receptor may recognize chitin-containing pathogens or particles and mediate chitinase activity. Prolactin stimulation of macrophages leads to chitinase expression via a PI-3K, MAPK, and NF-κB pathway [16–19]. Activation of Jmjd3, leads to demethylation of H3K27 and recruitment of the transcription factor Irf4, which is associated with M2 macrophage polarization in response to chitin stimulation [59]. Expression of the chitinase encoded by the CHIT-1 gene is regulated via PU.1 and C/EBPβ. The latter is phosphorylated (p) to induce CHIT-1 expression [85]. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; IFN-γ: interferon gamma; NK cells: natural killer cells; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor alpha; PMA: phorbol 12-myristate 13-acetate; IL-17: interleukin-17; IL-17-AR: interleukin-17A receptor; TLR-2: toll-like receptor-2; PI-3K: phosphatidylinositol 3-kinase; MAPK: Mitogen-activated protein kinase; Jmjd3: Jumonji domain containing-3; H3K27: histone 3 Lys27 demethylase; CHIT-1: chitotriosidase; C/EBPβ: CCAAT-enhancer-binding protein beta.

This mutant protein can bind chitin particles, but cannot degrade chitin [20, 86]. Macrophages from individuals with this 24-bp duplication in the *CHIT-1* gene produced *CHIT-1* RNA and small amounts of a 47 kDa protein, but no enzymatically active chitotriosidase [20]. Approximately 30–40% of the human population is heterozygous and 3–6% is homozygous for this duplication [20, 77, 86]. The use of chitinase activity as a disease biomarker may therefore be limited to patients with at least one wild-type *CHIT-1* allele.

The effect of environmental conditions on the occurrence of the most prominent chitinase polymorphism, the 24-bp duplication, was studied by Malaguarnera et al. DNA analysis was performed to compare the frequency of the exon 10 duplication allele in individuals from Mediterranean countries and sub-Saharan regions. This study found a higher frequency of individuals homozygous for the 24-bp duplication in Sicily and Sardinia, 3.73% and 5.45%, respectively, than in people from Benin and Burkina Faso (frequency of 0% homozygous for the duplication). The authors concluded that the presence of the *CHIT-1*-inactivating 24-bp duplication in Sicily and Sardinia was due to the improved, more sanitary environmental conditions as compared to Benin and Burkina Faso, which still face widespread parasitic diseases and the presence of multiple chitin-containing pathogens [77]. The lack of chitotriosidase activity in people with these polymorphisms may be compensated for by AMCase chitinase activity. However, there are also various polymorphisms that affect AMCase activity [22, 23, 87]. Therefore, a thorough immunogenetic haplotype analysis that involves *CHIT-1* and *AMCase* alleles, as well as chitin sensing and chitinase regulation pathways is needed to investigate the significance of human chitinase responses to fungal infections. It is possible that the dysregulation of chitin sensing or chitinase induction pathways could be associated with altered susceptibility for IFIs.

10. Concluding Remarks and Future Directions

An efficient method for early diagnosis and treatment of IFIs is needed [27, 30]. Exploiting host responses to IFIs could help to better understand fungal recognition by the immune system, and may reveal potential diagnostic markers of IFIs. A substantial increase in chitinase activity, in conjunction with other IFIs clinical signs and symptoms, or in conjunction with the β-1,3-glucan assay could be a biomarker indicative of a beginning fungal infection. Chitinase activity appears to play an important role in various diseases [13, 68], and therefore, a clear understanding of the processes of chitinase induction and regulation is desirable.

Chitinases can be induced by various stimuli including prolactin, TNF-α, IFN-γ, and PMA. And recombinant human CHIT-1 has demonstrated antifungal properties both *in vivo* and *in vitro* [25, 67]. Therefore, it is conceivable that artificial induction of chitinase production in patients that are at risk of fungal infections could increase their resistance to fungal pathogens. This strategy would be most effective in patients with genes encoding catalytically active chitinases. In summary, chitinase-based diagnostic assay or antifungal therapeutics may be developed in the near future.

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Review Article

Antifungal Resistance and New Strategies to Control Fungal Infections

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Despite improvement of antifungal therapies over the last 30 years, the phenomenon of antifungal resistance is still of major concern in clinical practice. In the last 10 years the molecular mechanisms underlying this phenomenon were extensively unraveled. In this paper, after a brief overview of currently available antifungals, molecular mechanisms of antifungal resistance will be detailed. It appears that major mechanisms of resistance are essential due to the deregulation of antifungal resistance effector genes. This deregulation is a consequence of point mutations occurring in transcriptional regulators of these effector genes. Resistance can also follow the emergence of point mutations directly in the genes coding antifungal targets. In addition we further describe new strategies currently undertaken to discover alternative therapy targets and antifungals. Identification of new antifungals is essentially achieved by the screening of natural or synthetic chemical compound collections. Discovery of new putative antifungal targets is performed through genome-wide approaches for a better understanding of the human pathogenic fungi biology.

1. Introduction

The fungal kingdom encompasses an enormous diversity of taxa with varied ecological niches, life-cycle strategies, and morphologies. However, little is known of the true biodiversity of Kingdom Fungi. Of the 1.5 million species estimated to belong to this kingdom, only about 5% were formally classified. Many fungi are parasites for plants, animals, human, and other fungi. Plant pathogenic fungi are able to cause extensive damage and losses to agriculture and forestry including the rice blast fungus, Dutch elm disease, and chestnut blight. Some other fungi can cause serious diseases in humans, several of which may be fatal if left untreated. These include aspergillosis, candidosis, coccidioidomycosis, cryptococcosis, histoplasmosis, mycetomas, mucormycosis, and paracoccidioidomycosis. The so-called dermatophytic and keratinophilic fungi can attack eyes, nails, hair, and especially skin and cause local infections such as ringworm and athlete's foot. Fungal spores are also a cause of allergies, and fungi from different taxonomic groups can provoke allergic reactions. In this paper, after a brief presentation of the medical impact of fungal infections at the global level and a summary of clinical treatments available today for clinicians, we will review the mechanisms underlying *in vitro* resistance to antifungals in fungal species of major importance in human medicine. Lastly, an overview of ongoing research undertaken to develop new therapeutic strategies to fight against fungal infections will be exposed.

2. Fungal Infections, Clinical Treatments, and Incidence of Antifungal Drug Resistance

2.1. Fungal Infections. At the beginning of the 20th century, bacterial epidemics were a global and important cause of mortality. In contrast, fungal infections were almost not taken into account. Since the late 1960s when antibiotic therapies were developed, a drastic rise in fungal infections was observed, and they currently represent a global health threat. This increasing incidence of infection is influenced by the growing number of immunodeficient cases related to AIDS, cancer, old age, diabetes, cystic fibrosis, and organ transplants and other invasive surgical procedures.

Table 1: Characteristics of main fungal infections worldwide.

Body location	Pathogen type	Organ	Most frequent genus	Estimated incidence of infection*
superficial	primary	Skin and hair	*Malassezia*	~140,000,000 cases/year
cutaneous	primary	Skin and nails	Trichophyton *Epidermophyton* *Microsporum*	~1,500,000,000 cases/year
mucosal	opportunistic	Vagina, digestive tract, urinary tract and	*Candida*	~75,000,000 cases/year ~9,500,000 cases/year
		eye	*Aspergillus, Fusarium*	~1,000,000 cases/year
systemic	opportunistic	any organ (lungs, brain, bloodstream etc.)	*Candida* *Aspergillus* *Cryptococcus* *Histoplasma* *Pneumocystis* *Coccidioidomyces* and so on	~300,000 cases/year ~350,000 cases/year ~1,000,000 cases/year ~500,000 cases/year >200,000 cases/year up to 300,000 cases/year

* adapted from "The Fungal Research Trust. How common are fungal diseases? Fungal Research Trust 20th Anniversary meeting. London June 18th 2011."

These infections are caused by two types of microorganisms: primary and opportunistic pathogens. Primary pathogens are naturally able to establish an infection in the healthy population. In contrast, opportunistic pathogens, among them commensal microorganisms of the healthy population, are able to develop infectious colonization of the human body when particular criteria, such as immunosuppression, are met. Fungal pathogens can be divided into two major groups: filamentous fungi and yeasts. Most of the primary pathogens are filamentous fungi, while most of the opportunistic pathogens are yeasts and some species of filamentous fungi are increasingly identified as opportunistic pathogens. It is also important to note that fungal infections can be classified in function of the tissue infected (see Table 1).

Superficial mycoses, such as *tinea versicolor*, are limited to the most external part of the skin and hair. These infections are most frequently caused by the species *Malassezia globosa* and *M. furfur*, which are estimated to be carried by 2% to 8% of the healthy population worldwide but could lead to *tinea versicolor* in some conditions that are still unclear [1].

Cutaneous and subcutaneous mycoses caused by dermatophytes fungi affect keratinized structures of the body. The most frequently involved dermatophyte genera are *Trichophyton*, *Epidermophyton*, and *Microsporum*. In most cases, cutaneous fungal infections require a challenge of immune system, and their incidence varies depending on the site of infection. For example, onychomycoses are very frequent in the global population, with an incidence varying from 5 to 25% [2].

Mucosal infections are mostly caused by opportunistic yeasts, and those belonging to the *Candida* genus are by far the most frequent. Vaginal, esophageal, oropharyngal, and urinary tract candidiasis are very frequent in immunocompromised patients. For example, esophageal candidiasis is associated with the entry into the clinical phase of AIDS and during the 1980s more than 80% of seropositive patients developed such an infection [3]. Fungal infections, of the eye are also classified as mucosal fungal infections, but are caused more frequently by *Fusarium* or *Aspergillus* species rather than *Candida* species.

Theoretically systemic mycoses may involve any part of the body, and a lot of species formerly considered as nonpathogenic are now recognized opportunistic pathogens responsible for deep-seated mycoses. These infections, with symptoms ranging from a simple fever to a severe and rapid septic shock, are very common in immunocompromised patients and are frequently associated with an elevated mortality rate. The most frequent pathogens involved in systemic fungal infections are *Candida*, *Pneumocystis*, *Histoplasma*, *Aspergillus*, *Cryptococcus, Mucor*, *Rhizopus*, and *Coccidioidomyces* [4–6].

2.2. Antifungal Agents. Despite extensive research dedicated to the development of new therapeutic strategies, there are only a limited number of available drugs to fight against invasive fungal infections. Indeed, only four molecular classes that target three distinct fungal metabolic pathways are currently used in clinical practice to treat essentially systemic fungal infections: fluoropyrimidine analogs, polyenes, azoles, and echinocandins. Several other classes, such as morpholines and allylamines are only used as topical agents due to either poor efficacy, or severe adverse effects when administered systemically.

2.2.1. Fluoropyrimidines. Fluoropyrimidines, of which only 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) are used in human medicine, are synthetic structural analogs of the DNA nucleotide cytosine (Figure 1).

5-FC was synthesized in 1957 by Duschinsky et al., initially as an antitumor therapy [7]. In 1963, Grunberg and coworkers discovered its antifungal potential by means of

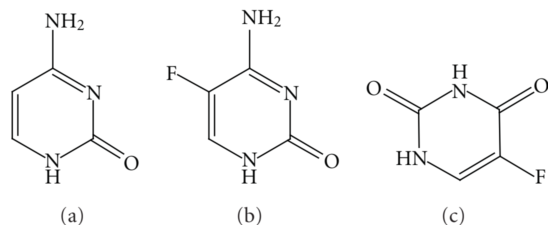

Figure 1: Chemical structures of cytosine (a) and of two fluoropyrimidines, 5-fluorocytosine (b), and 5-fluorouracil (c).

murine models of cryptococcosis and candidiasis [8]. Several years later 5-FC was successfully used for the treatment of systemic candidiasis and of cryptococcal meningitis [9].

5-FC possesses a broad range of activity. This drug is active against *Candida* and *Cryptococcus* genera. 5FC activity on *Phialophora*, *Cladosporium,* and *Aspergillus* genera is much less limited. 5-FC is also active against protozoa belonging to *Leishmania* and *Acanthamoeba* genus [10].

Due to its high hydrosolubility and small size, 5-FC possesses interesting pharmacokinetic properties, since it diffuses rapidly throughout body even when orally administered [12]. Generally, it produces negligible side effects, despite some severe adverse effects, such as hepatotoxicity or bone marrow lesions [11, 13–15], occurring in rare cases [16]. Surprisingly, these side effects are identical to those observed with 5-FU treatment, despite the fact that humans do not possess a cytosine deaminase enzyme that is responsible for the conversion from 5-FC into 5-FU in fungal cells [17, 18]. Some data suggest that the intestinal microbiome could be responsible for the 5-FU production and the observed side effects [19].

Despite its numerous pharmacological advantages, the use of 5-FC in clinical practice is decreasing because of the frequent occurrence of innate or acquired resistance to this drug in fungal pathogens. Thus, with few exceptions [20], 5-FC is never used as monotherapy but always in combination with another antifungal, such as amphotericin B [21, 22]. However, the elevated renal and liver toxicities of amphotericin B, that further increase 5-FC hepatotoxicity, has led to combination therapy of 5-FC more frequently with azole drugs.

5-FC itself has no antifungal activity, and its fungistatic properties are dependent upon the conversion into 5-FU [16, 20, 23]. The drug rapidly enters the fungal cell through specific transporters, such as cytosine permeases or pyrimidine transporters [24], before it is converted into 5-FU by the cytosine deaminase [16]. 5-FU itself is converted into 5-fluorouracil monophosphate (5-FUMP) by another enzyme, uridine phosphoribosyltransferase (UPRT). 5-FUMP can then be either converted into 5-fluorouracil triphosphate, which incorporates into RNA in place of UTP and inhibits protein synthesis, or converted into 5-fluorodeoxyuridine monophosphate, which inhibits a key enzyme of DNA synthesis, the thymidylate synthase, thus inhibiting cell replication (Figure 2) [16, 25, 26].

2.2.2. Polyenes. More than 200 molecules belonging to the chemical class of polyenes have an antifungal activity, most of them being produced by *Streptomyces* bacteria. However, only three possess a toxicity allowing their use in clinical practice: amphotericin B (AmB), nystatin, and natamycine.

Streptomyces bacteria synthesize polyenes through a gene cluster phylogenetically conserved within these species. This cluster contains genes coding for several polyketide synthases, ABC (ATP-binding cassette) transporters, cytochrome P450-dependent enzymes, and enzymes responsible for the synthesis and the binding of the mycosamine group [27]. Although it is possible to synthesize polyenes chemically, they are still produced from *Streptomyces* cultures for economic reasons.

Polyenes are cyclic amphiphilic organic molecules known as macrolides. Most of them consist of a 20 to 40 carbons macrolactone ring conjugated with a d-mycosamine group. Their amphiphilic properties are due to the presence of several conjugated doublebounds on the hydrophobic side of the macrolactone ring, and to the presence of several hydroxyl residues on the opposite, hydrophilic side (Figure 3) [28].

Polyene drugs target ergosterol, the main sterol component of fungal membranes. Their amphiphilic structure allows them to bind the lipid bilayer and form pores. Magnetic nuclear resonance data suggest that eight AmB molecules bind eight ergosterol molecules through their hydrophobic moieties, with their hydrophilic sides forming a central channel of 70–100 nm in diameter (Figure 4). Pore formation promotes plasma membrane destabilization, and channels allow leakage of intracellular components such as K^+ ions, responsible for cell lysis [28].

While structural data suggest that polyenes target ergosterol, and despite the fact that their binding to ergosterol was experimentally demonstrated [29–31], controversy remains over a possible intracellular mode of action. Some research has suggested that polyene drugs are able to induce an oxidative stress (particularly in *C. albicans* [32, 33]) as well as their activity seems to be reduced in hypoxic conditions [34].

Polyenes possess a lower but non-negligible affinity for cholesterol, the human counterpart of ergosterol. This slight affinity for cholesterol explains the high toxicity associated with antifungals and is responsible for several side effects [28]. For this reason, only AmB is given systemically, while nystatin and natamycin are only used locally or orally. These two last molecules fortunately possess a very limited systemic activity, since their absorption trough gastrointestinal mucosa is almost nonexistent [35, 36].

For these reasons, AmB is the most used polyene antifungal for systemic infections. Due to its high hydrophobicity and poor absorption through the gastrointestinal tract, it is necessary to administer AmB intravenously [28]. However, AmB administration is accompanied with adverse effects, mostly at the level of kidneys and liver. New AmB formulations, such as liposomal AmB or lipid AmB complexes, minimize such side effects [37].

For more than 40 years, AmB was one of the goldstandards for the treatment of systemic fungal infections due

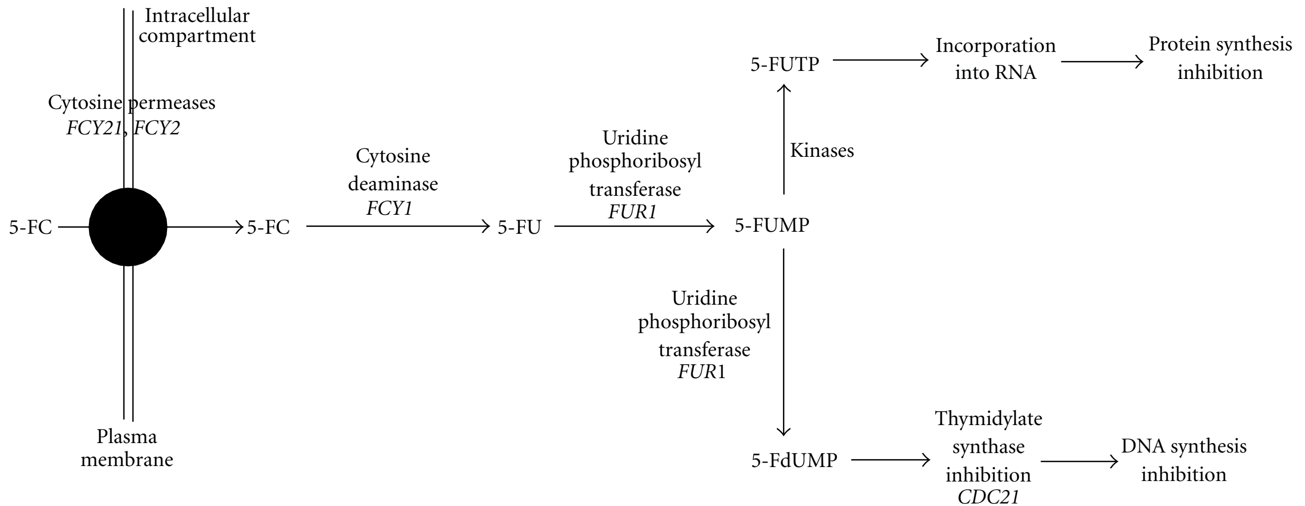

Figure 2: Intracellular metabolization and action mode of 5-FC in *S. cerevisiae*, adapted from [11]. In bold are indicated gene names of the respective enzymes. 5-FC: 5-fluorocytosine; 5-FU: 5-fluorouracil; 5-FUMP: 5-fluorouridine monophosphate; 5-FUTP: 5-fluorouridine triphosphate; 5-FdUMP: 5-fluoro deoxyribouridine monophosphate.

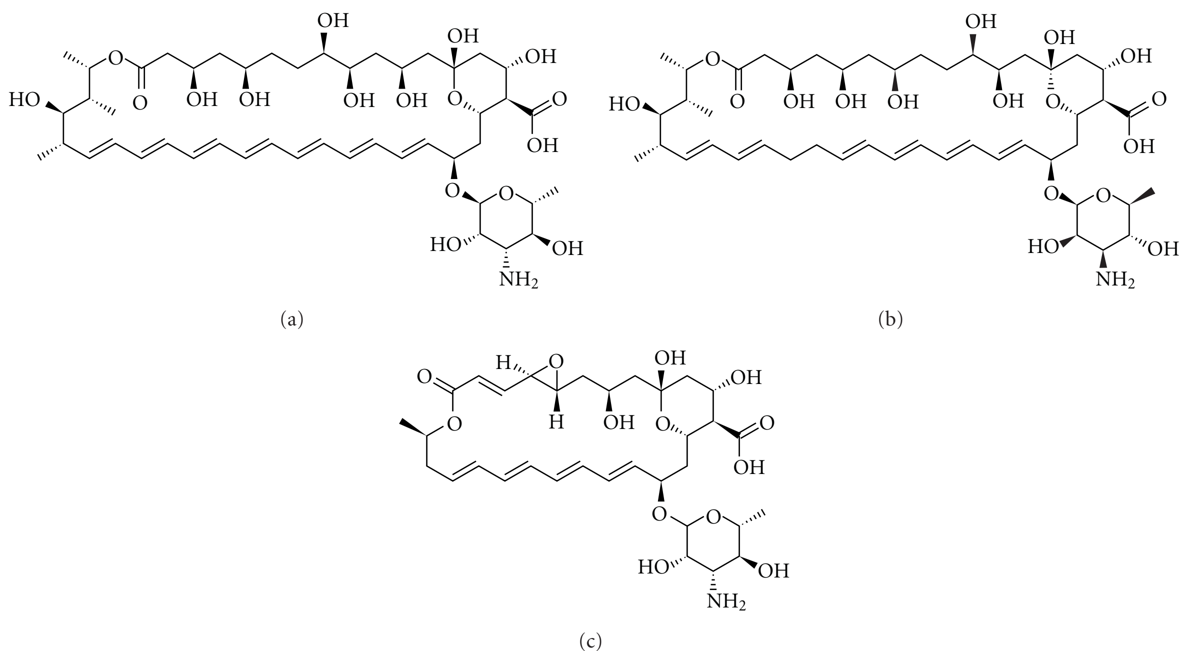

Figure 3: Chemical structures of amphotericin B (a), nystatin (b), and natamycin (c), three main polyene drugs.

to the low occurrence of acquired or innate resistance to this drug and also because of its broad range of activity [38]. Indeed, AmB is active against most yeasts and filamentous fungi. It is recommended for the treatment of infections caused by *Candida*, *Aspergillus*, *Fusarium*, *Mucor*, *Rhizopus*, *Scedosporium*, *Trichosporon*, *Cryptococcus*, and so on. AmB is also widely used to treat parasitic infections such as leishmaniasis and amibiasis [28]. Natamycin and nystatin are active against fungi belonging to the genera *Cryptococcus*, *Candida*, *Aspergillus,* and *Fusarium*. If nystatin is not used to treat molds infections, this drug is frequently used for the treatment of cutaneous, vaginal, and esophageal candidiasis, and natamycin can be used for the treatment of fungal keratosis or corneal infections [35].

2.2.3. Azoles. Azoles are by far the most commonly used antifungals in clinical practice, and consequently, they are also the mostly studied by the scientific community regarding their mode of action, their pharmacological properties, and the resistance mechanisms developed by microorganisms. Azole antifungals are also largely studied by pharmaceutical

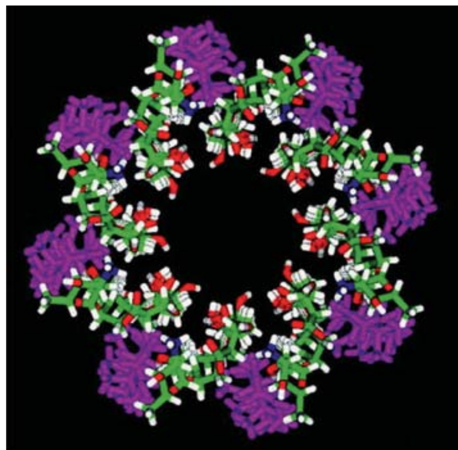

Figure 4: 3D model of pore formed by amphotericin B into lipid bilayer of the fungal plasma membrane, adapted from Baginski et al. [29]. Amphotericin B: white (H), green (C), red (O), and blue (N); ergosterol: pink.

companies, who seek to enhance their efficacy and to develop the *perfect antifungal*.

Azoles are cyclic organic molecules which can be divided into two groups on the basis of the number of nitrogen atoms in the azole ring: the imidazoles contain two nitrogen atoms, and the triazoles contain three nitrogen atoms (Figure 5) [39].

Azole drugs target the ergosterol biosynthetic pathway by inhibition of a key enzyme, the lanosterol 14alpha demethylase, encoded be the *ERG11* gene. This inhibition occurs through the binding of the free nitrogen atom of the azole ring to the iron atom of the heme group of the enzyme. The resulting accumulation and metabolism of 14alpha methylated sterol species leads to the synthesis of toxic compounds, which are unable to successfully replace ergosterol [40].

The first azole was synthesized in 1944 by Woolley [41], but it was not until 1958 that scientific community began to consider azoles as potential antifungal agents. In late 1960s, clotrimazole, econazole, and miconazole became available for treatment [42]. However, their use was restricted to external application due to their high toxicity when administered orally [43, 44]. In 1968, miconazole became the first antifungal available for parenteral injection, but due to its toxicity and relatively limited range among fungal species [45], its use decreased until it was no longer commercialized.

In 1981, the Food and Drug Administration (FDA) approved a new antifungal, ketoconazole, developed by Heeres and his coworkers [46]. This drug was the only antifungal available for treatment of systemic fungal infections caused by yeasts for the following ten years. However, there are several drawbacks to this drug. It is poorly absorbed when administered orally, and no ketoconazole form has ever been developed for intravenous injection. Moreover, it cannot cross the cerebrospinal barrier and is less active in immunosuppressed patients [42, 47–49]. It causes some severe side effects such as a decrease in testosterone or glucocorticoids production and liver and gastrointestinal complications [50–52]. Lastly, numerous interactions with other drugs were described. For these reasons, the triazoles were developed.

Fluconazole became available for use by clinicians in 1990 and provided many advantages over the use of imidazoles. Fluconazole is highly hydrosoluble and therefore can be easily injected intravenously. It is almost completely absorbed through the gastrointestinal tract, and it diffuses throughout the whole body, including cerebrospinal fluid [53, 54]. Fluconazole is suitable for the treatment of superficial candidiasis (oropharyngal, esophageal, or vaginal), disseminated candidiasis, cryptococcal meningitis, coccidioidomycosis, and cutaneous candidiasis. Due to its good pharmacokinetic properties as well as its broad spectrum of activity, fluconazole was the gold-standard treatment of fungal infections during the 1990s. Unfortunately, the overprescription of this drug by physicians for prophylaxis or treatment led to an increase in resistance to azole drugs. Moreover, fluconazole is almost ineffective against most molds.

Itraconazole was approved and made available by the FDA in 1992. This triazole possesses a broad spectrum of activity across fungal species comparable to this of ketoconazole and wider than fluconazole. Moreover, it is less toxic than ketoconazole and replaced it for treatment of histoplasmosis, blastomycosis, and paracoccidioidomycosis. Contrary to fluconazole, it is also used for the treatment of infections due to species belonging to the genera *Aspergillus* and *Sporothrix* [55]. However, itraconazole is hydrophobic and is thus more toxic than fluconazole. Itraconazole is only indicated for the treatment of onychomycosis, of superficial infections, and in some cases for systemic aspergillosis [56]. A new itraconazole formulation with an enhanced absorption and a decreased toxicity was approved by FDA in 1997 [57]. An injectable formulation of itraconazole was made available in 2001 [58].

Fluconazole and itraconazole are still not the perfect antifungals, since they have some nonnegligible drug interactions with such drugs that are used in chemotherapy or with AIDS treatment. These interactions can result in a decrease in azole concentration or even to an increase in toxicity [59]. Furthermore itraconazole and fluconazole are ineffective against some emerging pathogens like *Scedosporium*, *Fusarium*, and Mucorales, and resistance to azoles is increasingly reported [60].

So-called new generation triazoles have also been developed. Voriconazole and posaconazole were approved by FDA in 2002 and 2006, respectively. Ravuconazole is currently under clinical trial phase of drug development. They possess a wide range of activity, since they are active against *Candida*, *Aspergillus*, *Fusarium*, *Penicillium*, *Scedosporium*, *Acremonium*, and *Trichosporon*, and dimorphic fungi, dermatophytes, and *Cryptococcus neoformans* [61, 62]. While new generation triazoles were shown to be more effective against *Candida* and *Aspergillus* [62], compared to classical triazoles their side effects and drug interactions are similar to those observed with fluconazole and itraconazole [63]. Likewise, fungal isolates resistant to classical triazoles are also cross-resistant to new generation triazoles.

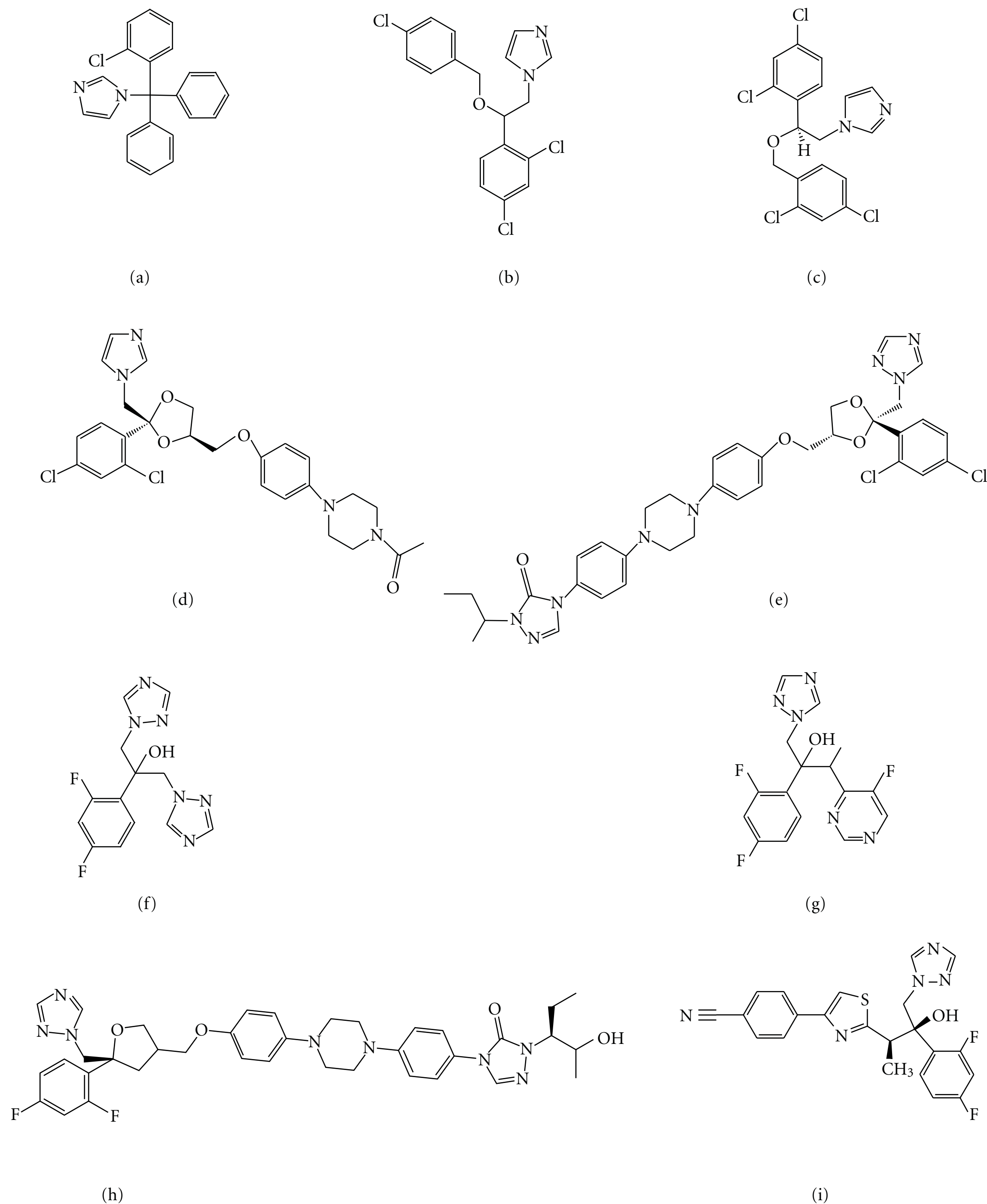

Figure 5: Chemical structures of the main azole antifungals, four imidazoles: clotrimazole (a), econazole (b), miconazole (c), and ketoconazole (d), two triazoles: itraconazole (e) and fluconazole (f), and three new generation triazoles: voriconazole (g), posaconazole (h), and ravuconazole (i).

2.2.4. Echinocandins. Echinocandins constitute the only new class of antifungals made available for clinicians to fight invasive fungal infections within the past 15 years [64]. Three echinocandins were currently approved for clinical use by the FDA in United States and later by the European Agency for the Evaluation of Medicinal Products (EMEA): caspofungin in 2001 by the FDA and in 2002 by the EMEA, micafungin in 2005, and lastly anidulafungin in 2006.

Echinocandins are synthetic derivatives of lipopeptides (Figure 6). These lipopeptides are naturally produced by several fungal species: *Aspergillus rugulovalvus* synthesizes caspofungin B, *Zalerion arboricola* synthesizes pneumocandin

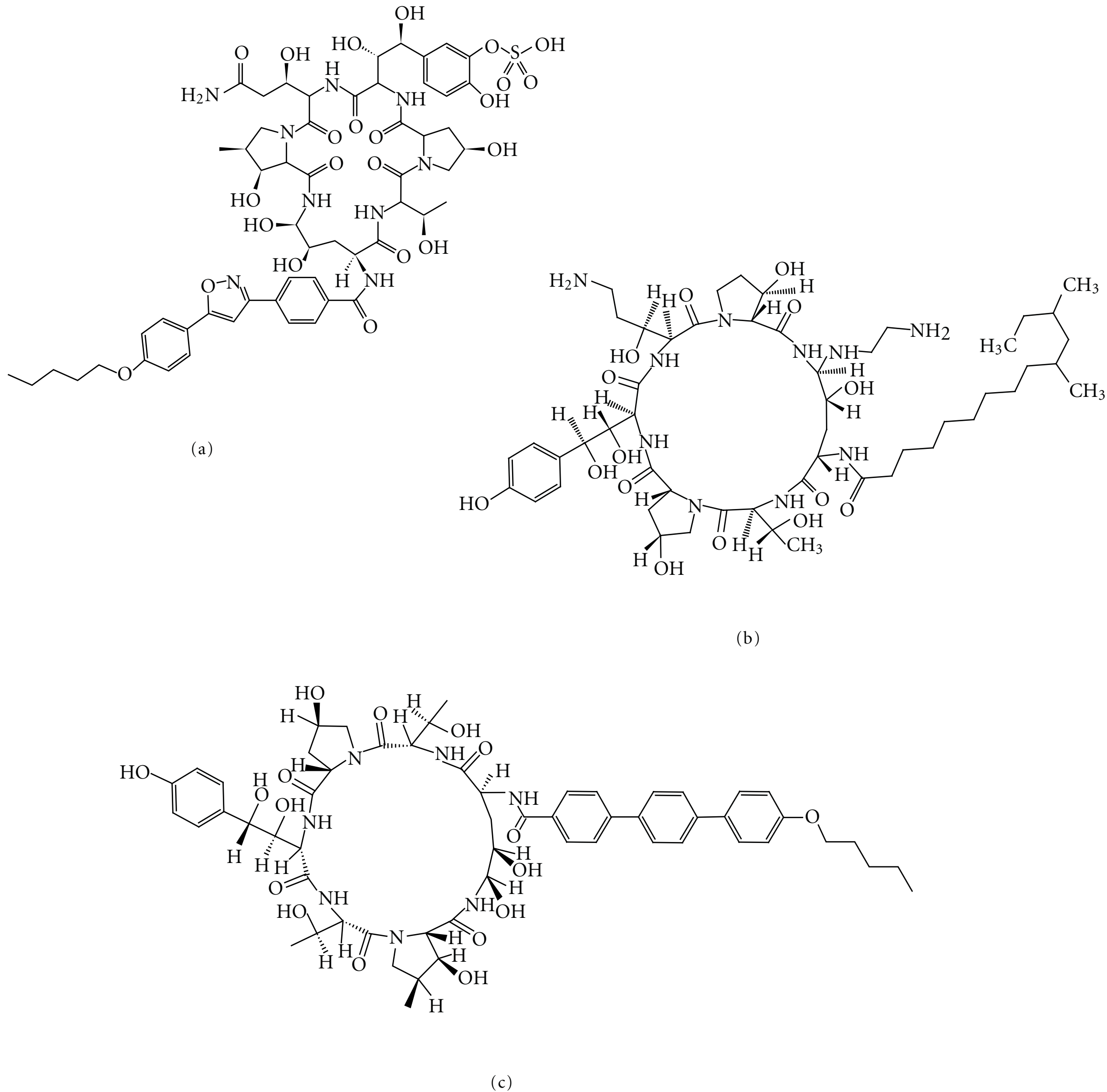

Figure 6: Chemical structure of the three echinocandins used in clinical practice: micafungin (a), caspofungin (b), and anidulafungin (c).

B, and *Papularia sphaerosperma* synthesizes papulacandin. Echinocandins are noncompetitive inhibitors of β(1-3)-glucan synthase, an enzyme that catalyzes the polymerization of uridine diphosphate-glucose into β(1-3) glucan, one of the structural components responsible for the maintenance of fungal cell-wall integrity and rigidity [65, 66]. β(1-3)-glucan synthase consists of an activating and a catalytic subunit encoded by *FKS* genes. In most fungi, two *FKS* genes are found within the genome. It has been shown in the model organism *Saccharomyces cerevisiae* that *FKS1* is expressed during the vegetative growth phase and *FKS2* during sporulation [67]. Echinocandins are able to inhibit both isoforms of the enzyme [68]. Inhibition of β(1-3)-glucan synthase leads to cell wall destabilization and to the leakage of intracellular components, resulting in fungal cell lysis [69].

These drugs are poorly absorbed in the gastrointestinal tract because of their high molecular weights and are therefore only used intravenously. Their pharmacologic properties are one of the reasons responsible for the approval of echinocandins by the FDA and the EMEA. These molecules possess a low toxicity (very rare side effects were reported) and are slowly degraded, and a daily injection is sufficient, and contrary to other antifungals, interactions between echinocandins and other drugs are rare [64]. Combined therapy between echinocandins and AmB or another azole often leads to a synergistic effect or at least to an additive effect [70, 71].

Another reason for which the echinocandins were approved is their activity spectrum. Indeed, echinocandins are active against most fungal species, including *Candida* and *Aspergillus*. For still unclear reasons, these molecules are

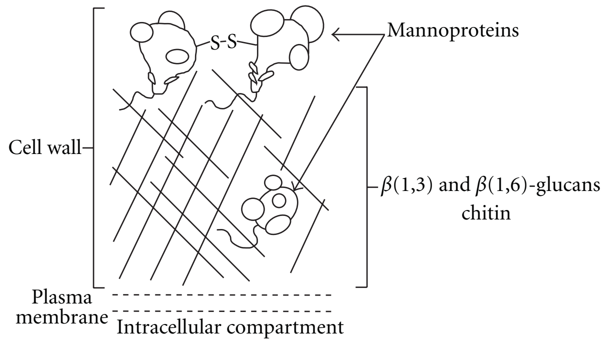

Figure 7: Schematic representation of *S. cerevisiae* cell wall, adapted from Stone et al. [69].

fungicidal in *Candida* but only fungistatic in *Aspergillus* [72, 73]. Moreover, fungicidal activity of echinocandins is species and isolate dependent within the *Candida* genus [74]. There exist several species within the fungal kingdom for which the echinocandins are ineffective. Such species include *Cryptococcus neoformans* [75] or species belonging to *Trichophyton* and *Fusarium* genera. Other species have an intermediate susceptibility to echinocandins, such as *Scedosporium apiospermum*, *S. prolificans*, and *Cladophialophora bantiana* [72]. However, echinocandins constitute a good alternative to fight against fungal infections and most of treatment of infections for which classical therapy with azoles or polyenes failed are successfully managed with echinocandins [64]. Therefore, caspofungin is indicated for the treatment of candidemia and invasive candidiasis, for fungal infection prophylaxis, and for the treatment of invasive aspergillosis for which itraconazole, voriconazole, or AmB is ineffective. Micafungin is used for treatment of candidemia and is particularly indicated for fungal infection prophylaxis in bone-marrow transplant patients. Anidulafungin has no particular indications, but its main advantage is its slow degradation in the body without liver or kidney involvement, thus it can be used in patients with liver and/or kidney insufficiencies [76].

What makes echinocandins unique is their fungal target. For many years, the fungal cell wall was considered to be a promising target for the development of new antifungal molecules [68]. The fungal cell wall contains elements that have no equivalents in human [77]. Its integrity is necessary for the fungal survival, since it provides a physical barrier against the host immune cells or against other microorganisms. Cell wall integrity is also responsible for osmolarity homeostasis and the maintenance of cell shape and size. Cell wall is also indispensable to essential enzymatic reactions and as an important role in cell-cell communication. The internal layer of the cell wall is composed of a β(1-3)-glucans and chitin web, in which are included some mannoproteins, while external layer is composed of mannoproteins (Figure 7) [77].

2.2.5. Other Antifungal Agents. Considering that the ergosterol biosynthetic pathway requires several enzymes that are unique to fungi, they constitute good targets for antifungal therapy, and three minor ergosterol biosynthesis inhibitors are used as topical antifungals. The allylamines and thiocarbamates, such as terbinafine and tolnaftate, both inhibit the *ERG1*-encoded enzyme, squalene epoxidase. The morpholines such as amorolfine act by inhibiting two different enzymes of the pathway, the Δ7,8-isomerase (encoded by *ERG24*) and the C14-reductase (encoded by *ERG2*). Despite their wide spectrum of activity, these antifungal agents are essentially used to treat dermatophyte infections such as *tinea capitis*, *tinea pedis*, and onychomycosis, because they do present numerous side effects.

Ciclopirox is also used as a topical antifungal agent, but its mode of action remains poorly understood in fungi [78, 79]. Another drug, griseofulvin, inhibits mitosis by interfering with microtubules function [80].

2.3. Incidence of In Vitro Resistance in Fungal Infection. The incidence of fungal infections has drastically increased over the past three decades and was simultaneously accompanied by increased acquired and innate resistance to antifungal drugs. However, antifungal resistance occurrence has to be considered independently for each antifungal class and for each fungal genus. Moreover, epidemiological data regarding incidence of resistance among fungal species is not identically distributed worldwide [81–83]. Lastly, clinical resistance, defined as the treatment failure in the patient, does not always correlate with *in vitro* resistance, measured as an increase in minimal inhibitory concentration of a drug. In this paper, only *in vitro* resistance incidence will be described.

2.3.1. 5-Fluorocytosine. 5FC resistance is a very common phenomenon [9, 16, 84]. The development of resistance can be intrinsic, as is the case for *C. tropicalis*, or acquired through the selection of resistant mutants after antifungal exposure. Within the *Candida* genus, 7% to 8% of clinical isolates are resistant to 5FC, and this frequency increases to 22% when only non*albicans Candida* species are considered. One to two percent of *Cryptococcus neoformans* clinical isolates are resistant to 5FC [85]. Filamentous fungi such as *Aspergillus* and dermatophytes are not susceptible to 5FC.

2.3.2. Polyenes. Despite the reported increase of polyene resistance, it remains a relatively rare event in clinical isolates of fungal pathogens [86], probably in relation with their mode of action, and the absence of systematic and standardized determination of susceptibility of clinical isolates [87]. The incidence of strains resistant to polyenes may thus be largely underestimated. Most fungal species are considered as susceptible to polyene drugs. However, some of them are intrinsically poorly susceptible to these antifungals, such as *C. glabrata*, *Scedosporium prolificans,* or *Aspergillus terreus* [38]. Some species are more prone to acquire polyene resistance. Among yeasts, one may cite *C. lusitaniae* [88, 89], *C. guilliermondii* [88], *C. krusei* [38], and *Trichosporon beigelii* and among filamentous fungi *Scedosporium apiospermum* and *Sporothrix schenckii* [90, 91].

Table 2: Nature, target, mode of action, and fungal resistance mechanisms of the major antifungal drugs used in human therapy.

Antifungal agent	Mode of action and cellular target	Mechanism of resistance
polyenes	binding to ergosterol	absence of ergosterol (loss of function mutation in *ERG3* or *ERG6*) decrease of ergosterol content in cells
azoles	inhibition of cytochrome p450 function: 14α-lanosterol demethylase (*ERG11*) sterol Δ^{22} desaturase (*ERG5*)	efflux mediated by multidrug transporters decrease of affinity in Erg11p by mutations upregulation of *ERG11* alterations in the ergosterol biosynthetic pathway
allylamines	inhibition of squalene epoxidase (*ERG1*)	unknown
morpholines	inhibition of sterol Δ^{14} reductase (*ERG24*) and the Δ^{7-8} isomerase (*ERG2*)	unknown
5-fluorocytosine	inhibition of nucleic acids synthesis	defect in cytosine permease deficiency or lack of enzymes implicated in the metabolism of 5-FC deregulation of the pyrimidine biosynthetic pathway
echinocandins	inhibition of β-1,3 glucan synthase (*FKS1&2*)	alteration of affinity of echinocandins for β(1,3)-glucan synthase

2.3.3. Azoles. The early 1990s was the start of a drastic increase in resistance among fungal clinical isolates. However, the improvement of antifungal therapeutic strategies throughout the last several years has helped to stabilize resistance frequencies. Increase in azoles use selected less susceptible species as well as those able to develop resistance. This led to a shift in the pathogenic fungal species encountered in clinic.

2.3.4. Echinocandins. Echinocandins resistance is a rare event [92]. For example, it is estimated that more than 97% of clinical isolates belonging to the *Candida* genus are susceptible to these drugs [93, 94]. Contrary to acquired resistance in other fungi, intrinsic echinocandin resistance in *Cryptococcus neoformans* is not linked with a *FKS1* or *FKS2* mutation. Indeed, *C. neoformans* β(1–3)-glucan synthase is inhibited by echinocandins, but this yeast is able to grow in the presence of high concentrations of these drugs. *C. neoformans* resistance to echinocandins seems to be due to a particular cell-wall polysaccharides composition in this species [95].

2.3.5. Incidence of In Vitro Resistance on Patient Care. As antifungal *in vitro* resistance poorly correlates with clinical outcome, better attention was needed to define parameters that produced reproducible and reliable intra- and interlaboratory results. For this purpose, two standardized methods for the testing of yeast and mould isolates (CLSI and Eucast) are recognized as the gold standards for drug susceptibility testing [96–98]. These standardized approaches produce susceptibility results comparable between laboratories, which may help to establish breakpoints for antifungal agents (see [96–98] for details). These breakpoints, defined as susceptibility ranges, together with pharmacokinetic and pharmacodynamic analyses and identification of resistance mechanisms, help to assess the *in vivo* activity of antifungal agents in invasive disease and therefore clinical outcome [99, 100].

3. Drug Resistance Molecular Mechanisms

Microorganisms develop mechanisms to counteract the fungicidal or fungistatic effects of all antifungals classes that are based on three major mechanisms, namely, (i) reducing the accumulation of the drug within the fungal cell, (ii) decreasing the affinity of the drug for its target, and (iii) modifications of metabolism to counterbalance the drug effect (Table 2). The molecular mechanisms leading to azole resistance have been most studied in yeast, and taking them as an example, such mechanisms are divided into four categories (Figure 8) [101]: (i) decrease in azole affinity for their target, (ii) increase in azole target copy number, (iii) alteration of ergosterol biosynthetic pathway after azoles action, and (iv) decrease in intracellular azole accumulation. In some highly resistant clinical isolates, sampled from long-term treated patients, several mechanisms of resistance are often combined [102, 103]. This increase in resistance along antifungal treatment is due to the sequential acquisition of different mechanisms [104–106]. In the following section, the molecular basis of the resistance mechanisms to antifungals will be described.

3.1. Increase of Drug Efflux

3.1.1. ABC Transporters. *CDR1* and *CDR2* (*Candida* drug resistance 1 and 2) from *C. albicans* are the two major ABC transporters involved in azole resistance in this species. *CDR1* and *CDR2* can be coordinately upregulated in some azole-resistant strains or by exposure to a wide variety of chemically unrelated inducers such as terbinafine, amorolfine, fluphenazine, or steroid hormones. Several *cis*-acting regulatory elements responsible for the regulation of

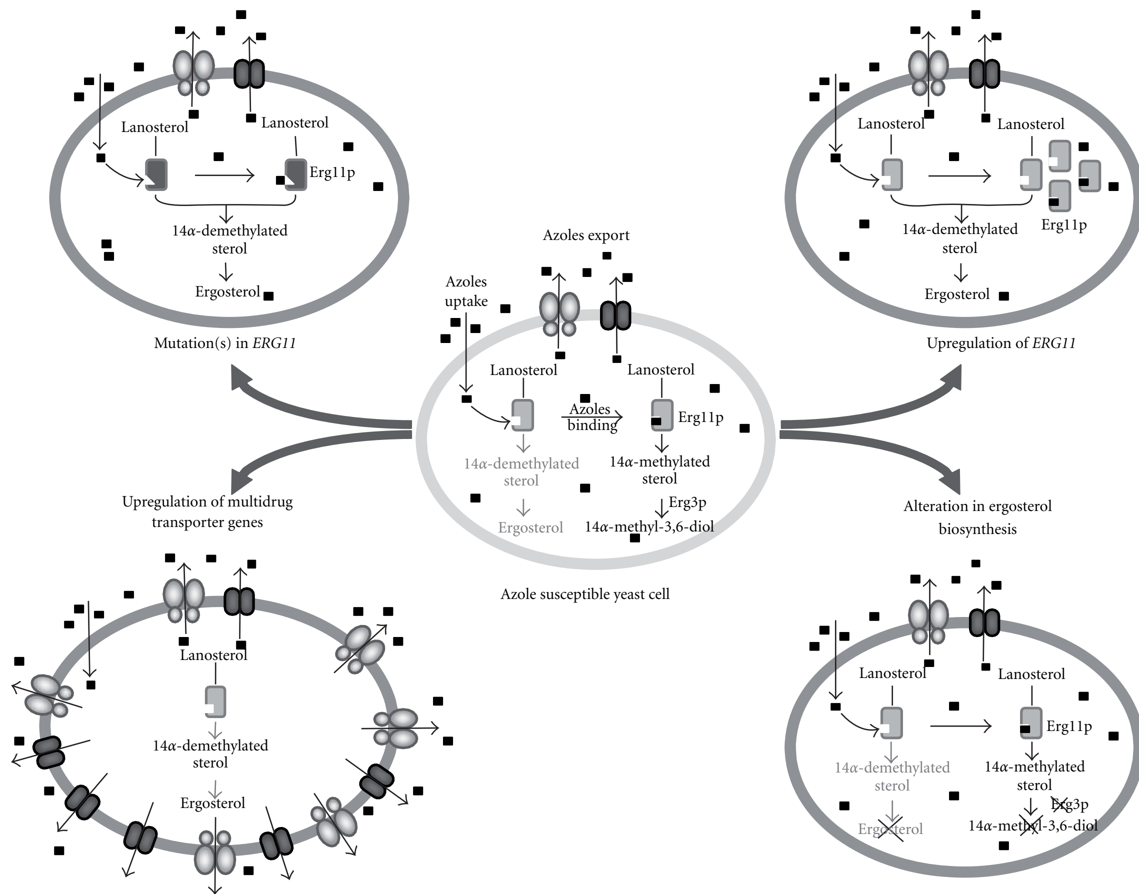

Figure 8: Mechanisms of resistance to azole compounds in *C. albicans*.

these two genes were identified by several investigators [107–111]. Promoter deletion studies have revealed 5 different regulatory elements in the *CDR1* promoter including a BEE (basal expression element), a DRE (drug responsive element), two SREs (steroid responsive element), and a NRE (negative regulatory element) (see Table 3 for details). Internal deletions of the BEE and DRE motifs in the *CDR1* promoter affect basal *CDR1* expression and drug-induced expression, respectively [107]. SRE1 and SRE2 were reported to be involved in the response to steroid hormones: with SRE1 responding only to progesterone and SRE2 to both progesterone and β-oestradiol [108]. Finally, the deletion of the NRE motif leads to an increase in the basal expression of *CDR1* [110, 111]. In contrast to *CDR1*, the *CDR2* promoter contains only a DRE motif (Table 3) [107]. Among these different *cis*-acting elements, DRE was the only element involved in constitutive high expression and in transient upregulation of both *CDR1* and *CDR2*. This DRE sequence was functionally analyzed by systematic mutation each base of the initially described DRE sequence [107, 112]. The data obtained from systematic mutational studies are in agreement with ChIP-Chip assays performed with the *trans*-acting factor binding to the DRE [113]. In other *Candida* species, functional homologues to *CDR1* and *CDR2* were described as involved in drug resistance. In *C. glabrata*, *CgCDR1* and *CgCDR2* (formerly denoted *PDH1*) as well as *SNQ2* (another ABC transporter coregulated with *CgCDR1* and *CgCDR2*) are upregulated in azole-resistant clinical isolates and participate in azole resistance [114–118]. All the three genes contain *cis*-acting elements in their promoters, so-called PDRE. These elements are similar to those described in *S. cerevisiae* for *PDR5*, an ABC transporter involved in drug resistance of *S. cerevisiae* [119, 120]. Disruptions of *CgCDR1* and *CgCDR2* lead to hypersusceptibility to fluconazole, cycloheximide, and chloramphenicol [115, 117]. In both *C. albicans* and *C. glabrata*, *CDR1* was shown to be the main contributor in azole-resistance among the ABC-transporters [121–123]. Other ABC-transporters from *C. dubliniensis* (*CdCDR1* and *CdCDR2*) [124, 125], *C. krusei* (*ABC1* and *2*) [126, 127], *C. tropicalis* (*CDR1*-homologue), and *C. neoformans* (*CnAFR1*, AntiFungal Resistance 1) were reportedly upregulated in azole-resistant isolates. In *A. fumigatus*, *atrF,* and *AfuMDR4* are upregulated in itraconazole-resistant strains [128–130]. The *cis*-acting regulatory elements of these genes are still awaiting in-depth dissection analysis. The overexpression of ABC-transporters have also been identified as a resistance mechanism to azole in *Aspergillus nidulans* [131, 132].

The identification of *trans*-acting factors regulating ABC-transporters in pathogenic fungi relied first on the well-described *S. cerevisiae* PDR network as a model [138–142].

Table 3: *Cis*-acting elements involved in drug resistance.

	Organism	Gene	Regulatory element: Name	Regulatory element: Sequence	Position respectively to the ATG	*Trans*-acting factor
ABC transporters	*C. albicans*	*CDR1*	BEE	—	−960 to −710	?
			DRE	ACGGATATCGGATATTTTTTT	−460 to −439	Tac1
			NRE	CTGATTGA	−335 to −328	?
			SRE1	GGAGTAGCAAGTGTGTCAAGAACCTGAATTC	−740 to −711	?
			SRE2	TTATCCGAAACGCTTTACTCCTCTATTATT	−691 to −661	?
		CDR2	DRE	ACGGAAATCGGATATTTTTTT	−221 to −201	Tac1
	C. glabrata	*CgCDR1*	PDRE	TTCCGTGGAA	−1201 to −1192	CgPdr1
		CgCDR2	PDRE	TTCCGTGGAA	−560 to −551	CgPdr1
MFS transporters	*C. albicans*	*CaMDR1*	HRE/YRE	—	−561 to −520	Cap1/?
			BRE/ MDRE	ACGGTAAAATCCTAATTGGGAAAAATACCGAGAATGA	−296 to −260	Mcm1/Mrr1
			AR1	—	−397 to –301	?
			AR2	—	−588 to –500	?
			AR3	—	−287 to −209	?
	C. glabrata	*CgFLR1*	YRE3	TTAGTAA	−372 to −366	CgAp1
ERG11	*C. albicans*	*ERG11*	ARE	AATATCGTACCCGATTATGTCGTATATT	−224 to −251	Upc2
	C. glabrata	*ERG11*	SRE			Upc2A

Since the Zn2-Cys6 transcription factors *PDR1/PDR3* are master regulators of this network in *S. cerevisiae*, an *in silico* search for *PDR1/PDR3* homologues in fungal genomes was performed. Data so far available found only one functional homologue in *C. glabrata* [120]. CgPdr1p has 40% and 35% identity with Pdr1p and Pdr3p, respectively [143], and was able to complement a *pdr1*Δ *S. cerevisiae* mutant strain. Likewise, *PDR1* deletion in *C. glabrata* leads to a loss of *CgCDR1* and *CgCDR2* regulation and to a sharp decrease in azole MICs. [144]. Three studies have identified separate gain-of-function mutations in *CgPDR1* alleles of azole-resistant strains which are responsible for constitutive high expression of *CgCDR1*, *CgCDR2*, *SNQ2*, and *CgPDR1* itself (Figure 9) [120, 145, 146].

Attempts to identify *C. albicans PDR1/3* functional homologues were undertaken to complement the absence of *PDR1/PDR3* in *S. cerevisiae* by genetic screens. Several genes were identified including *FCR1* and *FCR3* (FluConazol Resistance) [147–149] and *SHY1*-3 (Suppressor of Hypersusceptibility) [150] (formerly, resp., named *CTA4, ASG1* and *ATF1*). *FCR1*, *CTA4, ASG1*, and *ATF1* encode Zn2-Cys6 transcription factors, while *FCR3* encodes a bZip transcription factor. Even though *FCR1* was able to restore *PDR5* expression in a *pdr1*Δ/*pdr3*Δ *S. cerevisiae* mutant strain, its disruption in *C. albicans* resulted in decreased susceptibility to fluconazole, suggesting that *FCR1* acts as a negative regulator of fluconazole susceptibility [147]. Nevertheless, the target genes of *FCR1* in *C. albicans* are not yet known. Up to now, the relevance of *FCR3* in azole resistance has not been addressed in *C. albicans*. *CTA4, ASG1*, and *ATF1* expression in *S. cerevisiae* could restore *PDR1/PDR3* functions in *S. cerevisiae*; however, their disruption in *C. albicans* did not affect azole susceptibility and expression of *CDR1* and *CDR2* [150]. An additional regulator of *CDR1* was identified by a genetic screen in *S. cerevisiae* with a *LacZ* reporter system under the control of the *CDR1* promoter. A *C. albicans* gene was subsequently identified that encodes for a protein CaNdt80p similar to the *S. cerevisiae* meiosis specific transcription factor Ndt80p. Disruption of *CaNDT80* in *C. albicans* was shown to affect basal expression levels of *CDR1* in *C. albicans* and reduce the ability of this gene to be upregulated in the presence of miconazole [151, 152]. More recently, Ndt80p was shown to have a global effect on azole-resistance through is regulon which includes many genes involved in ergosterol metabolism [153].

The release of the entire data from the *C. albicans* genome sequence has encouraged other approaches for identifying *trans*-regulators of *CDR1* and *CDR2*. Since the DRE motifs present in the promoter of *CDR* genes contains two CGG triplets that are potentially recognized by Zn2-Cys6 transcription factors (TF) [154–157], it was likely that one of the 78 ORFs encoding proteins with Zn2-Cys6 signatures could be involved in the regulation of *CDR1* and *CDR2*. Interestingly, genome data revealed that three of these ORF (the so-called "zinc cluster") were located in tandem close to the mating type locus (*MTL*) at a distance of 14 kb [158]. Homozygosity at the *MTL* locus is associated with the development of azole resistance in *C. albicans* [159], thus indicating that one the genes of the zinc cluster could control *CDR1* and *CDR2* expression. As a matter of fact, deletion of one of these Zn2-Cys6 TF-encoding genes in an azole-susceptible strain led to increased drug susceptibility and loss of transient *CDR1* and *CDR2* upregulation in the presence of inducers. This gene was named *TAC1* for transcriptional

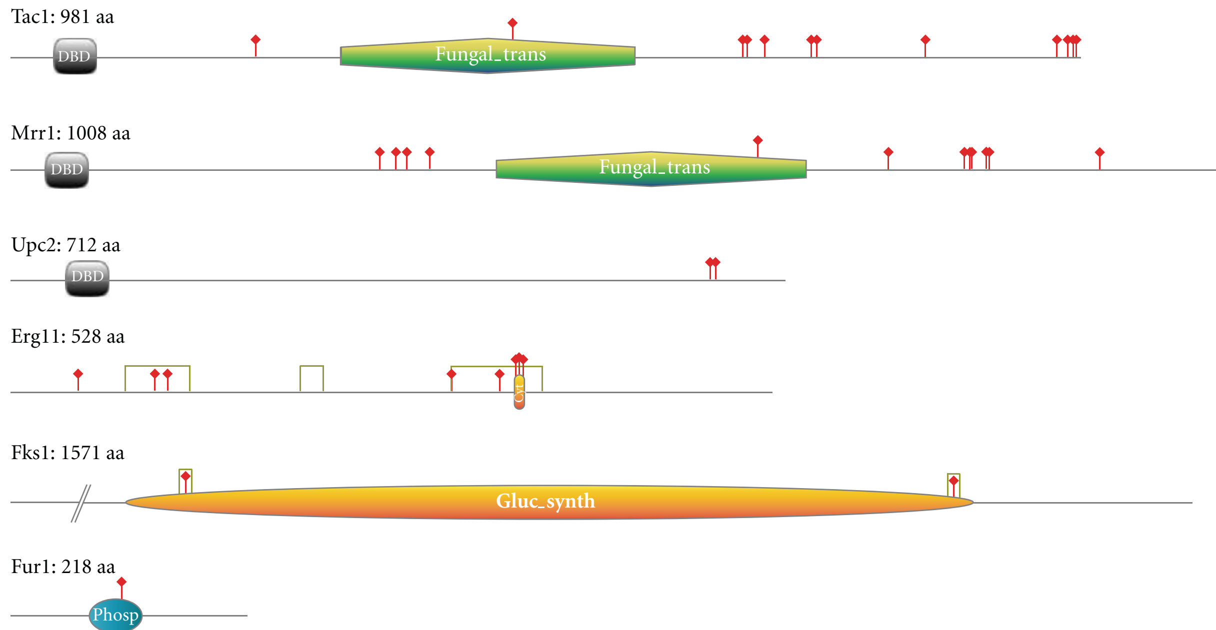

Figure 9: Point mutations affecting antifungal susceptibility in clinical isolates of *C. albicans*. Indicated functional domains were determined using either Prosite or Pfam tools. Only mutations which involvement in antifungal resistance was experimentally demonstrated are indicated by a red stick. Hot spot mutations in Erg11 and Fks1 are delimitated by gray boxes (Point mutation localization references: Tac1 [112], Mrr1 [133], Upc2 [134], Erg11 [135], Fks1 [136], Fur1 [137]). Drawings of the proteins were made with Prosite My Domain-Image Creator tool.

activator of *CDR* genes [158]. Consistent with the mutant phenotype, Tac1p can bind *in vitro* and *in vivo* to the DRE [112, 158]. However, *TAC1* is not involved is the basal expression of *CDR1* controlled at least by the BEE [158]. Hyperactive alleles that confer constitutive high *CDR1* and *CDR2* expression, and therefore drug resistance to a *tac1*Δ/Δ mutant strain of *TAC1*, were isolated from azole-resistant strains. Wild-type and hyperactive alleles differed by point mutations defined as gain-of-function mutations (GOF). Up to now, at least 15 GOF were described in *TAC1* at 12 different positions [112, 158, 160, 161] (Figure 9). Wild-type and hyperactive alleles are co-dominant for the expression of their phenotypes [112, 158, 160, 161], and because of this property, high drug resistance levels correlate with homozygosity of hyperactive alleles. Interestingly, the *TAC1* locus and the associated *MTL* are rendered homozygous in the development of azole resistance. Such events are accomplished by rearrangements on chromosome 5 including mitotic recombinations on one chromosome 5 arm or the loss of one chromosome 5 homologue followed by duplication [160]. Increase of resistance can still be obtained by isochromosome formation with the left arm of the chromosome 5. This allows for the increase of drug resistance genes present on this chromosome (among which *TAC1* and *ERG11*) and thus can contribute to drug resistance increase [160–164]. Up to this date, regulation of Tac1p activity remains unknown.

3.1.2. Major Facilitator Superfamily (MFS) Transporters. In *C. albicans*, *MDR1* (MultiDrug Resistance 1, previously named *BEN*^{r} for Benomyl resistance) is a transporter currently shown to be the only MFS transporter involved in azole resistance of clinical isolates [165, 166]. *MDR1* is not usually expressed at detectable levels in fluconazole-susceptible isolates, but is constitutively upregulated in some fluconazole-resistant isolates. As for *CDR1* and *CDR2*, *MDR1* can be specifically transiently upregulated by drugs such as benomyl, cycloheximide, methotrexate, and several oxidizing agents [165]. MFS transporters are known to be involved in azole resistance of other fungal species. Homologues of *MDR1* in *C. dubliniensis* and *C. tropicalis*, named *CdMDR1* and *CtMDR1*, respectively, are upregulated in azole-resistant strains [167–170]. In *A. fumigatus*, *in vitro*-generated itraconazole-resistant isolates show constitutive high expression level of the MFS transporter, AfuMDR3 [128]. The role of *cis*-acting regulatory elements in resistance was investigated in the *C. albicans MDR1* gene by separate studies. Two of the studies undertaken by Rognon et al. and Riggle and Kumamoto identified a similar region, called BRE (benomyl response element) or MDRE (*MDR1* drug resistance element) respectively. This region is responsible for the constitutive high expression of *MDR1* in fluconazole-resistant isolates [171, 172] and was also shown to be responsible for the response to benomyl [172]. A second regulatory element involved in the response of *MDR1* to oxidative stress is designated HRE (H_2O_2 response Element). This region contains two YRE (*YAP1* response element) motifs [173], one perfectly conserved (-532 TTAGTAA-526) and the other with two mismatches (-549 TAACTAT-543). Interestingly, the HRE is not required for constitutive upregulation of *MDR1* in azole-resistant isolates. A separate study undertaken by Hiller et al. described three distinct

cis-activating regions (1, 2, and 3) in *MDR1*. Region 2, which overlaps with encompassing the HRE, was implicated in benomyl-dependent *MDR1* response [174]. Region 1 and 3, close to the BRE/MDRE region, were required for a constitutive high expression of *MDR1* in an azole-resistant isolate.

MDR1 expression was shown to be regulated by at least four *trans*-acting factors: Cap1p [175–177], Mrr1p [178, 179], Upc2p [180, 181], and Mcm1p [182]. Nucleotide sequence data of *cis*-acting elements has provided some clues to their identification. When comparing the *MDR1 cis*-acting elements with existing transcription factor binding site databases, several putative *trans*-acting elements were identified. As mentioned above, the HRE of *MDR1* contains Yap1p binding sites. The bZip transcription factor Cap1 was shown to directly interact with the *cis*-acting domains [177] and to be involved in drug resistance [175]. The BRE/MDRE motif contains a perfect match for the Mads-box transcription factor Mcm1p in its sequence. Mogavero et al. showed that Mcm1p acts as a coregulator for Cap1 and Mrr1p and is not required for *MDR1* upregulation by H_2O_2 but is required for full *MDR1* induction by benomyl [182]. Genome-wide transcriptional analyses of clinical isolates that exhibit *MDR1*upregulation permitted the identification of a Zn2-Cys6 transcription factor that is coregulated with *MDR1* [179]. Deletion of *MRR1* in azole-resistant strains abolishes the constitutive overexpression of *MDR1*, therefore identifying Mrr1p as a central regulator of *MDR1*. Like for *TAC1*, two types of alleles were distinguished for *MRR1*. Wild-type alleles are needed for a transient upregulation of *MDR1* by drug exposure. In contrast, hyperactive alleles confer constitutive overexpression of *MDR1* and therefore also confer increased resistance to fluconazole [179]. Wild-type and hyperactive alleles differ by GOF mutations and until now, 14 GOF mutations at 13 positions were described in hyperactive Mrr1 [180] (Figure 9). Interestingly, hyperactive Mrr1 proteins were also shown to be able to confer Mdr1p-independent drug resistance probably through the regulation of oxydoreductases implicated in the detoxification of yeast cells after fluconazole exposure [183]. A blast analysis in *C. dubliniensis* allowed for the identification of a gene encoding for a protein that shares 91% of identity with Mrr1 of *C. albicans* [133] and able to complement a *CaMrr1*Δ mutant strain. The properties of CdMrr1 are similar to those of CaMrr1 and two types of alleles can also be distinguished. Until now 5 GOF mutations were identified and analyzed in hyperactive CdMrr1 proteins [133].

3.2. Target Alteration

3.2.1. Target Mutation. Another mechanism by which fungal pathogens are able to develop resistance is a decrease in antifungal affinity for their respective targets, without a major decrease in target activity. Such is the case for azole drugs, in which a decreased affinity between azole and a mutated lanosterol 14α-demethylase, can lead to resistance. A point mutation in the *ERG11* gene that codes for lanosterol 14α-demethylase leads to the complete inhibition of the binding capacity of the azole drug to its target [184, 185]. Numerous of these point mutations identified in *ERG11* were previously described, and their involvement in azole resistance was experimentally demonstrated for fungi such as *Cryptococcus neoformans* [186], *C. albicans* [187], (see also Figure 9), and *C. tropicalis* [167]. In *Aspergillus fumigates, CYP51A* and *CYP51B* encode two distinct forms of 14α-demethylase and mutations in the first of these two genes seem to be the most frequent mechanism responsible for azole resistance in clinical isolates. In this species, it was demonstrated that the nature of the nucleotide mutation, and therefore, the nature of the amino acid substitution, influences the development of resistance to different azole agents [188–192]. Interestingly, it was demonstrated that some clinical isolates share common mutations in Cyp51A with environmental azole-resistant strains, suggesting that some clinical azole resistant isolates might originate from the environment [193–195].

While target site alteration is far from being the most significant mechanism of resistance to azole drugs, it is the only known mechanism by which fungal pathogens are able to develop resistance to echinocandin drugs. This was demonstrated for *S. cerevisiae* and *C. albicans*. Echinocandin resistance is systematically associated with point mutations in either *FKS1* or *FKS2* [196, 197]. Analysis of the location of these mutations within the *FKS* genes led to the characterization of two regions, the so-called "hot spots", integrity of which seems to be essential for enzyme activity [136]. In contrast to azoles and *ERG11*, mutations in *FKS1* did not alter the β-glucane synthase affinity for its target but decreased only the enzyme processivity [198]. Hot-spot mutations have also been identified in other species, such as *C. glabrata* [196, 199], *C. krusei* [200], *Scedosporium apiospermum* [201], and *A. fumigatus* [202, 203] (Figure 9).

Numerous enzymes of the pyrimidine salvage pathway are involved in 5FC mode of action and thus numerous molecular mechanisms could lead to resistance to this drug [16, 204]. The most frequently found mechanism in clinical isolates of pathogenic fungi is a point mutation in the *FUR1* gene that encodes the enzyme responsible for the conversion of 5FU into metabolites able to enter the cytosine metabolism (Figure 9) [14]. *FUR1* mutation leads to complete resistance to both 5FC and 5FU in fungi. A second, frequently reported mutation leads only to resistance of 5FC. This second mutation is a point mutation in the *FCY1* gene that codes for cytosine deaminase, the enzyme responsible for the conversion from 5FC into 5FU. Several such point mutations that lead to decreased activity of the cytosine deaminase were identified, essentially in *Candida* yeast species such as *C. glabrata* [205, 206] and in *S. cerevisiae* [207].

3.2.2. Target Expression Deregulation. A third mechanism of drug resistance is the deregulation of the drug target expression. For drugs targeting, the biosynthesis of ergosterol, such as azoles, terbinafine, or fenpropimorph, even relative short exposures of two to three hours lead to the transient upregulation of the *ERG* gene family in *C. albicans, glabrata, tropicalis*, and *krusei* [208]. These data suggest a common regulation of the ergosterol biosynthetic pathway in the

presence of inhibitors. Longer azoles *in vitro* exposures (minimum 24 h) lead to constitutive upregulation of *ERG* genes, including *ERG11* [209], and decrease drug susceptibility. In clinical isolates of *C. albicans* and *C. dubliniensis* resistant to azoles isolated from HIV patients, upregulation of *ERG11* was described as a minor mechanism often combined with other more major mechanisms of resistance such as pump overexpression or *ERG11* mutations [103, 169, 210]. The overexpression of *ERG11* originates either by gene dosage effect through duplication of the gene or by upregulation of the gene by a *trans*-acting factors, both hypotheses were verified. In *C. albicans* and *C. glabrata*, it was shown that increased azole resistance due to *ERG11* upregulation was in fact due to genome rearrangement via formation of an isochromosome in *C. albicans* and duplication of a chromosome in *C. glabrata*, and therefore amplification of *ERG11* [163, 211]. In *Cryptococcus neoformans,* the well-known *SRE1* gene was shown to regulate the ergosterol biosynthesis pathway and also to be involved with virulence of the fungus [212]. In *S. cerevisiae*, the *ERG* gene family was shown to be regulated by two zinc cluster transcription factors encoded by *ScUPC2* and *ScECM22*. Two homologues of *ScUPC2* were found in the genome of *C. glabrata*: *CgUPC2A* and *CgUPC2B*. It appears that while both transcription factors regulate sterol biosynthesis and exogenous uptake, only CgUpc2A is responsible for the regulation of the transcription of the *ERG* gene family in response to sterol inhibitors [213]. In *C. albicans*, only one gene homologue of *ScUPC2/ScECM22* was found and named *CaUPC2* [214, 215]. It was shown that CaUpc2 is necessary for the upregulation of *ERG* genes in the presence of ergosterol synthesis inhibitors. Moreover, the *upc2* Δ/Δ mutant shows increased susceptibility to most drugs and a decrease in sterol uptake as compared to the wild-type strain [214, 215]. Further studies demonstrated the ability of CaUpc2 to bind to the ARE motif in the promoter of *C. albicans ERG11* (Table 3) [215, 216]. Genome-wide location analysis of CaUpc2 confirmed the SRE motif as the DNA binding site, and also confirmed the *ERG* gene family as a CaUpc2 target as well as *CDR1*, *MDR1*, and *UPC2* itself as new target genes. Analysis of clinical strains resistant to fluconazole with upregulated *ERG11* expression, demonstrated the existence of a hyperactive allele of *CaUPC2* that confers intrinsic upregulation of *ERG* genes. Currently, two GOF mutations were described for CaUpc2 (Figure 9) [134, 180].

3.3. Metabolism Modification

3.3.1. Echinocandins Paradoxical Effect. Some yeasts and filamentous fungi are able to grow in elevated echinocandin concentrations much higher than the MICs [136, 217]. This phenomenon, called paradoxical effect, is due to the metabolic adaptation of microorganism and is mediated by the cell wall integrity signalization pathway. This response is the direct consequence of the β(1-3)-glucans synthesis inhibition and the subsequent cell wall composition modifications, upon echinocandin administration [218, 219]. Several studies suggest that the magnitude of the paradoxical effect is variable depending on the microorganism itself as well as on the echinocandin nature. Therefore, the paradoxical effect would be more pronounced in the presence of caspofungin as compared to anidulafungin or micafungin [220]. However, the clinical significance of paradoxical effect has never been studied nor has it ever been observed in echinocandin-treated patients [86].

3.3.2. De Novo Synthesis of Pyrimidines. It is possible that 5FC resistance could be the consequence of an overall induction of the *de novo* pyrimidine biosynthetic pathway. In this case, the antifungal drug competes with the regular pyrimidine intermediate metabolites for incorporation into nucleic acids [16]. This increase in activity of the *de novo* pyrimidine biosynthetic pathway is reflected by an increased expression of the *CDC21* gene, whose product is inhibited by 5FC [205]. *FUR1* mutations could lead to 5FC resistance. However, its downregulation has also been demonstrated to be involved in 5FC decreased susceptibility. A 4-fold decreased expression of this gene of high importance in 5FC mode of action is sufficient to lead to a total resistance to this pyrimidine fluorinated analog in *C. glabrata* [84].

3.3.3. Ergosterol Biosynthesis Pathway Alteration. Modifications of main metabolic pathways could also lead to azole drugs resistance. For example, alteration of the late steps of the ergosterol biosynthetic pathway through inactivation of the *ERG3* gene gives rise to cross-resistance to all azole drugs [101]. Indeed, the antifungal activity of azole drugs relies on the synthesis of toxic 14α methylated sterols by the late enzymes of this pathway. A point mutation that occurs in the *ERG3* gene can lead to the total inactivation of C5 sterol desaturase. In this case, toxic 14α methylated sterols are no longer synthesized and even in the presence of azole drugs sterols species able to replace ergosterol are generated. While very uncommon, this mechanism was identified in several clinical isolates of *C. albicans* [221–223].

3.3.4. Plasma Membrane Composition Variation. Polyene drugs do not require internalization into fungal cells in order to exert their antifungal activity, since they incorporate into the plasma membrane from the external side. Thus, they escape metabolizing enzymes and efflux systems, and the only possibility for fungi to develop resistance to polyene is to modify their target, ergosterol. However, ergosterol is responsible for the integrity and fluidity of the plasma membrane, and therefore, possibilities to compensate for its absence are very limited. Although rarely described, resistance mechanisms responsible for acquired or innate resistance to polyene drugs were studied in several fungal species. In each case, resistance to polyenes results from a decrease or total absence of ergosterol in the plasma membrane through mutations in nonessential genes of the ergosterol biosynthetic pathway [224]. Molecular polyene resistance mechanisms were described in laboratory mutants of yeasts belonging to the *Candida* genus and in *S. cerevisiae*. Thus, both *ERG11* deletion in *C. albicans* [225] and *ERG3* deletion in *S. cerevisiae* [226] lead to mutants with cross-resistance to azole and polyene drugs. Likewise, *ERG6*

inactivation in *C. lusitaniae* [227] and *S. cerevisiae* results in polyene resistance [228]. Regarding clinical isolates, very few data is available. Only a few studies have associated polyene resistance to an *ERG3* mutation in clinical isolates of *C. albicans* [221, 229] and to an *ERG6* mutation in *C. glabrata* [230, 231].

3.3.5. Biofilms. "United we stand, divided we fall". This statement is certainly true concerning the fight between fungi and antifungals. It is well characterized that microbial communities engulfed in a polysaccharides-rich extracellular matrix, also known as biofilm, are by far more resistant to antifungal drugs than isolated cells. Fortunately, few pathogenic species within the fungal kingdom are able to form biofilms. The mostly known and widely studied of those species able to form biofilms are the species of the *Candida* genus [232]. Another yeast frequently responsible for biofilm-associated infections is *Cryptococcus neoformans* [233, 234]. Some clinical cases have also reported the involvement of other yeast species, such as *Pichia fabianii* [235] or *Trichosporon asahii* [236]. Additionally, it is now accepted that filamentous fungi, and particularly those of the *Aspergillus* genus, can grow as biofilms in humans [237–239]. Fungal biofilms are frequently polymicrobial biofilms, meaning that bacterial species frequently associate with one or several fungi [240, 241]. In medical mycology, biofilms constitute a real concern in the fields of invasive and dental medicine. They constitute a nonnegligible source of nosocomial fungal infections, essentially through the use medical devices. Moreover fungal biofilms are resistant to almost all the currently used antifungals, with the exceptions of echinocandins and lipid formulations of AmB [242]. The molecular mechanisms underlying the persistence of the fungal biofilms despite antifungal treatment remain unclear. It is likely that biofilm resistance is the result of a combination a multiple factors, among them an increased expression of efflux pumps, a modification of plasma membrane composition, and the biofilm-produced extracellular matrix itself [232, 243].

4. Development of New Antifungal Strategies

Current antifungal treatments are limited in their capacity to treat infections, especially systemic infections and no considerable advancements in antifungal therapies were developed recently. New therapies are therefore needed against pathogenic fungi. Several approaches were developed during the last several years in order to find new solutions. Researchers aim to discover new antifungal drugs either by testing already existing medical compounds, compounds from natural sources such as plants, sea, microorganisms or by systematic screens of chemical compound libraries. Researchers also strive to elucidate the underlying biology of fungal microorganism both *in vitro* and *in vivo*. Host-fungal interactions play a critical role for all fungal pathogens. Targeting this interaction provides novel therapies, which could be used alone or in combination with existing antifungal drugs. Such a combination may also determine the development of antifungal drug resistance.

4.1. Development of New Antifungal Active Compounds. Much effort has gone towards analyzing the antifungal properties of what is called natural compounds (NP) or natural bioactive compounds isolated from plants, other microorganisms, or marine organisms [244–246]. Some such compounds are investigated because their known triggering mechanisms important for fungi, while other compounds are tested blindly for their antifungal properties. Currently, none of these studies have produced a compound suitable for the clinical trial stage although interesting results were obtained.

Other studies focused on *in vitro* screens of several drugs currently used in clinical practice for their potentiation of the antifungal effect of the fungistatic agent fluconazole (FLC) on *Candida albicans*. This facilitated the discovery of several compounds, such as inhibitors of the calcineurin [247, 248] or Tor pathways [249–251], efflux pump inhibitors (derived compounds of milbemycin) [252–254], and more recently, antibodies against heat-shock 90 protein (HSP90) [255]. In particular, inhibitors of the calcineurin pathway were shown to be fully active *in vivo* in the potentiation of fluconazole, and they also led to a dramatic decrease in fungi virulence [256–260].

Systematic screening of chemical compounds libraries was also undertaken, essentially by industrial laboratories as an attempt to discover new antifungal compounds. High throughput screening of the legacy Schering-Plough compound collection has recently lead to the discovery of a new glucan synthase inhibitor effective again *C. albicans* and *C. glabrata* [261–263].

Some analysis used reverse genetic assay in which, *C. albicans* heterozygous deletion or transposon disruption mutants collection were screened for growth under treatment with collections of chemical compounds [264, 265]. This approach allowed identification of both antifungal drugs and the genes related to the mechanism of action of the related compounds.

Another type of high-throughput screens of chemical libraries was achieved measuring the viability of drug-treated *Caenorhabditis elegans* infected with *C. albicans* [266]. Compounds can be simultaneously screened for antifungal efficacy and host toxicity, which overcomes one of the main obstacles in current antimicrobial discovery. A pilot screen for antifungal compounds using this novel *C. elegans* system identified 15 compounds that prolonged survival of nematodes infected with the medically important human pathogen *C. albicans*. One of these compounds, caffeic acid phenethyl ester (CAPE), had effective antifungal activity in a murine model of systemic candidiasis and had *in vitro* activity against several other fungal species [266]. In addition, this whole-animal system may enable the identification of compounds that modulate immune responses and/or affect fungal virulence factors that are only expressed during infection.

4.2. Genome-Wide Studies to Detect Potential New Antifungal Targets. The improvement of already existing antifungal drugs and the limitation of drugs resistance apparition has helped to elucidate the basic biology of the fungal pathogen. For this purpose, several groups made efforts to develop

collection of systematic mutants essentially for *C. albicans*. An important difficulty for antifungal therapy is to develop drugs that exploit factors unique to fungi, which can be challenging considering that organism are eukaryotic and share many conserved biological pathways. Genes that are essential to fungal survival are possible targets for drug development.

Using the GRACE (gene replacement and conditional expression) or CPR (conditional promoter replacement) technologies, some research groups have assessed the essentiality of *C. albicans* and *Aspergillus fumigatus* genes [267, 268]. One study identified 567 essential genes in *C. albicans* [267]. And another study screened 54 genes of *A. fumigatus* based on ortholog functions and essentiality in *C. albicans* and *S. cerevisiae* [268], of which 35 were defined as essential in *A. fumigatus*. Authors were able to show that while the ERG11 gene family (CYP51A and ERG11B) is essential in *A. fumigatus*, the individual genes themselves are not. These analyses provide interesting and fully informative data for antifungal drug design and improve upon previous *in silico* analyses that when using *S. cerevisiae* data were only able to identify 61% of homologous genes reported in the genes found in the Roemer et al. analysis [267].

The diploid state of the genome presents a major problem to the development of a mutant collection. Therefore, some collections consist of heterozygous deletion [265] or transposon disruption mutants [264, 265]. Other collections contain homozygous transposon disruption mutants based on the random insertion thanks to the Tn7 transposon to a UAU cassette [269, 270]. These collection were first restricted to the transcription factors of *C. albicans* [269, 271, 272] but continue to be enlarged for the entire genome [270, 273]. Other collections consist of deletion mutants constructed with PCR generated deletion cassettes, with two different markers for each allele in the case of *C. albicans* [274, 275]. Such collections are now being constructed for *C. glabrata*.

Three kinds of analyses detailed below were performed with these collections. They aimed a better understanding of the modifications occurring in the fungi submitted to antifungal treatments or of the relationship developed between the fungus and its host all along the infectious process. Such knowledge might improve the actual therapy to avoid resistance development or might allow playing on the host-fungus equilibrium to improve recovery of patients.

First of all, treating strains with already known antifungal drugs and analyzing for example, growth modification and later transcriptional rewiring, some authors try to better understand drugs mechanisms of action and/or to find synergistic effect between them [272, 274]. Gene encoding the transcription factor Cas5 was found to be involved in the response to caspofungin [272]. Other studies showed that *AGE3*, which encodes an ADP-ribosylation factor GTPase activating effector protein, if deleted, abrogates fluconazole tolerance in *C. albicans*. Interestingly, Brefeldin A, an inhibitor of ADP-ribosylation factor, resulted in a synergistic effect with other drugs for *C. albicans* as well as for *Aspergillus* [273]. Finally, Homann et al. screened a collection of 143 transcription factor mutants under 55 distinct conditions among which exposition to fluconazole and 5FC, and they conclude in their analysis that nearly a quarter of the knockout strains affected sensitivity to commonly used antifungal drugs [274].

Other studies were geared better understand the biology of fungal species. For this purpose, mutant collections were subjected to a wide range of environmental conditions, modifying elements such as pH, salt concentration, carbon sources, oxidative conditions, temperature, and availability of essential elements such as metals (iron, copper, zinc, etc.) [274, 276].

Understanding the relationship between fungus and host during infection may provide further information useful for the improvement of antifungal treatment. In order to analyze the cross-talk occurring between fungus and host during the infectious process, researchers screened the colonization properties of mutants directly in hosts. One study that was performed with 1201 gene knockout mutants of *Cryptococcus neoformans* analyzed their *in vivo* proliferation profile in the murine lung, and they were able to identify 40 infectivity mutants [277]. Gene deletions in these mutants were previously uncharacterized and did not show any defect in traits known to be linked to virulence (polysaccharide capsule formation, melanization, and growth at body temperature). At least, four other similar studies were performed with *C. albicans* mutants. Two of them were done in invertebrate host models such as *C. elegans* or *D. melanogaster* [266, 278]. Interestingly, the *Cas5* Δ/Δ mutant, which has already been shown to be important for caspofungin response, was shown to be less virulent in both invertebrate models of infection [278, 279]. Finally, this transcription factor was demonstrated as crucial for cell wall integrity, and its importance in virulence was confirmed in the mice intravenous model of infection [278]. Two other studies screened collections of *C. albicans* mutants directly in mice by pools of mutants that were previously tagged [280, 281]. One collection was restricted to Zn2-Cys6 transcription factors (TF) mutants and the other was composed of mutants affecting about 11% of the entire *C. albicans* genome with no respect to a gene class. In both cases, mutants were also screened for traits known to be linked to virulence, such as the ability to filament and proliferate as well as the ability to grow at 42°C, at high and low pH, and in oxidative conditions. Noble et al. identified 115 mutants among the 674 screened with attenuated infectivity, but normal morphological switching and proliferation [281]. More precisely, they identified glycolipid and glucosylceramide as the first small molecules synthesized by *C. albicans* that are specifically required for virulence. Vandeputte et al. identified two Zn2-Cys6 TF mutants within 77 tested. These mutants displayed attenuated infectivity in their pool test, which was also confirmed in independent single strains infection of mice *ZCF13* and *ZCF18* [280]. Both genes were previously uncharacterized. *ZCF18* showed a slight growth defect in contrast to *ZCF13* which grew normally at body temperature, but slightly less at 42°C. *ZCF13* mutant displayed an abnormal morphology, producing strongly filamentous colonies on YPD medium at 35°C and displaying high invasion ability. *ZCF18* deletion also led to a slight enhancement of

colony wrinkling. Both genes are currently under further analysis.

Unfortunately, whenever promising, up to now, no new compound and/or new target have been selected for further development from these approaches.

5. Conclusion

These last years were very rich in better knowledge of molecular basis of antifungal resistance and more generally of the metabolism of pathogenic fungi. Antifungal drug resistance appears to essentially be due to point mutations in either drug targets or transcription factors regulating actors of the resistance. In the near future, high throughput diagnostic tools could be used in the course of treatment of fungal infections in order to detect resistance and adjust therapeutic strategies accordingly before any clinical evidence and therefore allow a rapid adjustment of the antifungal treatment.

One of the challenges of finding new antifungal targets in *C. albicans* was the lack of sophisticated screening technologies often employed with other fungal species such as *Saccharomyces cerevisiae*. The recent application of genome-wide studies to pathogenic fungi for both host-pathogen interactions and the biological study will hopefully encourage and facilitate the development of new effective therapeutic strategies. Such improvements in antifungal treatment may lead to a better clinical outcome.

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Review Article

Candida Biofilms and the Host: Models and New Concepts for Eradication

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Biofilms define mono- or multispecies communities embedded in a self-produced protective matrix, which is strongly attached to surfaces. They often are considered a general threat not only in industry but also in medicine. They constitute a permanent source of contamination, and they can disturb the proper usage of the material onto which they develop. This paper relates to some of the most recent approaches that have been elaborated to eradicate *Candida* biofilms, based on the vast effort put in ever-improving models of biofilm formation *in vitro* and *in vivo*, including novel flow systems, high-throughput techniques and mucosal models. Mixed biofilms, sustaining antagonist or beneficial cooperation between species, and their interplay with the host immune system are also prevalent topics. Alternative strategies against biofilms include the lock therapy and immunotherapy approaches, and material coating and improvements. The host-biofilm interactions are also discussed, together with their potential applications in *Candida* biofilm elimination.

1. Introduction

Biofilms, adherent microbial communities embedded in a polymer matrix, are common in nature. However, they are also a persistent cause of hygiene problems in the food industry and in the medical field [1]. Biofilms result from a natural tendency of microbes to attach to biotic or abiotic surfaces, which can vary from mineral surfaces and mammalian tissues to synthetic polymers and indwelling medical devices, and to further grow on these substrates [2–4]. Candidiasis, caused most frequently by *Candida albicans*, and to a lesser extent by *C. glabrata*, *C. tropicalis,* or *C. parapsilosis*, is often associated with the formation of biofilms on the surface of medical devices and tissues [5]. *Candida albicans* is a dimorphic fungus and is part of the commensal human micoflora. It is also an opportunistic pathogen of the human body when its proliferation is not controlled by the host immune system. It is one of the most often identified agents in nosocomial infections and is capable of invading virtually any site of the human host, from deep tissues and organs, to superficial sites such as skin and nails, to medical implants and catheters [6]. *C. albicans* biofilm development has been characterized in various model systems both *in vitro* and *in vivo* [7–9] and consists of distinct phases. The initial step consists of the adhesion of fungal cells of the yeast form to the substrate. It is followed by a phase of cell filamentation and proliferation, which results in the formation of multiple layers of sessile cells of different morphologies, including pseudohyphal and hyphal cells. The next step of maturation results in a complex network of cells embedded in extracellular polymeric material, composed of carbohydrates, proteins, hexosamine, phosphorus and uronic acid, as well as host constituents in natural settings [10]. There is indeed evidence that host glycoproteins, nucleic acids, and cells, such as neutrophils, may participate in the maturity of the matrix, in particular on mucosal sites [11–13]. The establishment of the biofilm extracellular matrix (ECM) represents a unique characteristic of biofilms. Quantity and composition of the matrix vary from one species to another and in different sites of infection depending on environmental cues,

Table 1: Examples of *Candida* biofilm models *in vitro*.

Models *in vitro*	Device	Used for
Closed systems (discontinuous growth conditions over time (nutrient depletion, accumulation of secondary metabolites))	(i) 96-well polystyrene microtiter plate (ii) Discs/pieces of catheter in 6- to 24-well plate (discs made of silicone, polyurethane, polycarbonate, polystyrene, stainless steel, Teflon, polyvinyl chloride, hydroxyapatite, and porcelain)	Easy and widespread use: comparative analyses between strains and species [33–39] to antifungal susceptibility tests [40]
	(iii) Calgary biofilm device (80 pegs immersed into a standard 96-well plate)	Biofilm formation studies by different *Candida* species [41]
	(iv) *Candida* biofilm chip (several hundreds nanobiofilms encapsulated in collagen and formed on a glass slide treated to obtained a monolayer of hydrophobic coating)	High-throughput biofilm studies [42]
Flow systems (Continuous growth conditions)	(i) CDC biofilm reactor (24 biofilms can be formed simultaneously)	Comparative analysis of biofilm quantification methods [43]
	(ii) Microfermentors (biofilms formed on a Thermanox slide glued to a glass spatula)	Gene expression analyses [44]
	(iii) Modified Robbins device (adapted to hold several individual discs)	Study of the effects of shear forces and nutrient supplies on *C. albicans* biofilm formation [45]
	(iv) Flow biofilm model (silicone elastomer strip placed into a polypropylene conical tube)	Study of *C. albicans* biofilm development, architecture, and drug resistance [46]
Shear stress conditions	Rotating disc system (silicone catheter devices placed under a shear force of 350 revolutions per minute)	*C. albicans* biofilm architecture and development [47]

such as nutrient availability and mechanical stimuli [14–17]. Matrix synthesis by *Candida* biofilm cells has been shown to be minimal in static conditions in comparison to dynamic environments [10], aggravating biofilm formation on mucosal and abiotic sites where there is a fluid flow, such as on the oral mucosa, the urethra, or central venous catheters. The last step, dispersion of cells from a biofilm, plays a key part in the biofilm developmental cycle as it is associated with candidemia and disseminated invasive disease [18].

Pathogenic microbes that build biofilms are potential causes of constant infections that defy the immune system and resist antimicrobial treatment, partly due to the matrix-inherent limited exposure of the cells within a biofilm to these types of immunological and medical arsenals [19–22]. Other mechanisms of biofilm resistance have been suggested, such as slow growth, differential regulation of the cell metabolic activity caused by nutrient limitation and stress conditions, and cell density [23–25]. In addition, the ability to adhere, as a unique prerequisite to form a biofilm, is a fast process, which makes the prevention of biofilm development difficult with the current antimicrobial tools and strategies.

Biofilms are diverse communities and therefore vary depending on the microbe, the surface, and the colonization niche [5, 26–30]. This paper gives an update on the recent efforts made in establishing alternative means of eradication and also prevention of *Candida* spp. biofilms, by developing new models of biofilm formation in flow conditions, as well as high-throughput rapid screening analyses *in vitro*. Newly developed *in vivo* models anticipate a shift of interest towards mixed fungal-bacterial biofilms and their role in pathogenesis in mucosal infections in particular. Keeping in mind that there is no unique model representative of all biofilms, it remains quite a challenge to tackle biofilm inhibition. One of the most attractive perspectives is the development of antimicrobe materials, and the latest findings are presented here.

2. *Candida* Biofilm Models

2.1. Models In Vitro. Biofilm formation is a multistep growth behaviour that results from complex physical, chemical, and biological processes [31, 32]. Because of the versatility of the milieu in which *Candida* biofilms can develop in the human host, from the oral cavity contributing to dental plaque formation to the blood stream in intravenous catheters and the urinary tract, it seemed necessary to reproduce *in vitro* as many conditions as possible to establish common and specific characteristics of *Candida* biofilm formation. In that respect, a multitude of *in vitro* studies has been described that relates to the impact of different types of substrate, nutritional supplies, in flow or static conditions, on adhesion and biofilm properties of several *Candida* species, and recent findings are presented next. An overview of the *in vitro* models available to study *Candida* biofilms is provided in Table 1.

2.1.1. Candida Species and Substrates Specificities. While biofilm formation is a general characteristic of many microbes, biofilm features such as architecture, matrix composition, and resistance to antifungal drugs are species and substrate dependent. And examples that demonstrate variation in biofilm ability and structure are numerous. Some studies are discussed below, and in particular studies related to *Candida* biofilms formed on dental materials. Interest has indeed grown in investigating the role of *Candida* species and the effect of the type of material in the development of denture stomatitis [48]. For example, in a comparative study, cell counts analyses showed that saliva-coated discs harboured less *C. glabrata* cells than untreated discs, while the number of *C. albicans* cells was not affected by the saliva coating [33]. However, both species adhered better on hydroxyapatite (HA) surface than on two other types of dental material, polymethylmetacrylate and soft denture liner. Surprisingly, dual species experiments showed that *C. glabrata* displayed higher cell counts when grown in the presence of *C. albicans* than when grown alone. In contrast, hyphal development by *C. albicans* seemed to be reduced in the presence of *C. glabrata* in most of the conditions tested. These data may help understand the impact that *Candida* species may have on each other, as mixed species communities are being identified in clinical samples [49]. In another case study, using discs as support for biofilm formation *in vitro*, HA substrate appeared to be less prone to *Candida* adherence than acrylic denture, porcelain, or polystyrene when not coated with saliva [34]. In addition, the effect of serum and similar materials on biofilm development of *C. albicans* clinical isolates was also evaluated *in vitro* [35]. Disc coupons made of polycarbonate, polystyrene, stainless steel, polytetrafluoroethylene (also known as Teflon), polyvinyl chloride (PVC), or HA were used in a high throughput assay. For all surfaces tested, the presence of serum increased biofilm formation. However, in absence of serum, Teflon supported higher biofilm production than any other material, likely due to its high roughness and hydrophobicity properties.

The differential ability to form biofilm of 84 strains from several *Candida* species, including *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis,* was assessed on clinical materials, such as Teflon and PVC. All species, with the exception of *Candida glabrata,* favoured Teflon [50]. In this study, *C. glabrata* together with *C. krusei* strains were not highly proficient in forming dense biofilms, as quantified by colony-forming units. Moreover, *C. parapsilosis* strains showed the least uniformity in the ability to form biofilm, followed by *C. tropicalis* and *C. albicans*. While some variability in the ability to form biofilms between strains of *C. albicans* has been documented *in vitro*, a study by MacCallum et al. [51] revealed that biofilm formation *in vitro* did not significantly vary between strains of the four major clades of *C. albicans*, classified according to single-nucleotide polymorphisms determinations and analysis of DNA repeat sequences [52]. However, high variation in the ability to form biofilm among strains of *C. parapsilosis* and less extensive biofilm formation by *C. glabrata* specimens has been illustrated in a few studies by crystal violet staining and confocal laser scanning microscopy [36–38]. Strain-dependent variation in biofilm formation was also observed among isolates of two genetically nonidentical classes of *C. parapsilosis*, namely, *C. orthopsilosis* and *C. metapsilosis* [39, 53]. All three species could form biofilms, but metabolic activity of biofilm cells differed between strains of the same species. However, conflicting data with different isolates reported the inability of *C. orthopsilosis* and *C. metapsilosis* to form biofilm in polystyrene 96-well plate assay *in vitro* [54, 55]. Biofilm formation among *C. parapsilosis sensu strictu* strains was also found to vary according to the geographical regions and the body sites from which the isolates came from [56]. Isolates from blood and cerebrospinal fluid seemed more prone to form biofilms than isolates from nails, catheters, and mucosa. Overall, these data suggest a high variability in biofilm ability of strains of *C. parapsilosis* and related species, perhaps due to inadequate models or to an intrinsic poor ability to establish the biofilm growth by these species.

In a Calgary biofilm model adapted to *Candida* spp., *C. krusei* developed the largest biofilm mass in comparison to *C. albicans*, *C. glabrata*, *C. dubliensis,* and *C. tropicalis* [41]. This model, allowing 80 biofilms to be formed at once, seemed to be very favourable to *C. krusei* biofilm development as biofilms of that species constituted of thick multilayered structures composed of pseudohyphal cells, while the other species formed sparse biofilms.

In a last example of novel *in vitro* models of biofilm formation on various soft contact lenses, analyses revealed differences in hyphal content and architecture of the fungal keratitis causative agents *Fusarium* and *C. albicans* [57]. Polymers such as balafilcon A and galyfilcon A were favourable to filamentous growth of *C. albicans*, while others such as etafilcon A and lotrafilcon A sustained biofilms formed mainly of yeast cells. In addition, differences in biofilm formation were also observed between peripheral and central regions of the lenses, with dense biofilms formed preferentially in the centres of the lenses. Although a direct relationship between the lens ionic charge and water content and the ability of fungi to form biofilm could not be established, these data confirm previous findings that irregular surface texture of materials affect both cellular morphology and biofilm mass [58].

2.1.2. Synthetic Media and Flow Systems Mimicking In Vivo Conditions. The physiological specificity of infection sites is also an important factor, and efforts have been made to reproduce some major environmental cues *in vitro*, such as mimicking the blood flow or the urine. Biofilms grown in synthetic urine medium were comparable to those grown in the commonly used cell culture RPMI medium [59]. And time course studies revealed that the development of both types of biofilm followed a similar pattern, with an initial adherence phase, followed by growth, proliferation, and maturation. The biofilms differed slightly in their architecture, as biofilms grown in synthetic urine medium seemed to be less complex and less dense, with a larger proportion of yeast cells rather than elongated cells. Increased nutritional supply promoted biofilm formation in

another model of artificial urine medium, highlighting once again the importance of reproducing as closely as possible the physiological conditions to gain relevant information [60]. *C. tropicalis* biofilms were also characterized in artificial urine medium, on urinary catheters in a flow model [61]. Cells were able to colonize the catheters in the presence of the artificial urine medium and to detach from these silicone catheters, illustrating their capacity to colonize distal sites.

Biofilms grown in static conditions have been predominantly studied, in comparison to flow-based systems, due to a low cost, a rapid processing of large number of samples, and limited technical requirements. However, in order to maintain their niches in dynamic environments, biofilms *in vivo* endure shear forces generated by the constant flow of physiological fluids [62]. Gene expression analyses revealed only a marginal difference between biofilms grown in static conditions, such as microtiter plates or serum-treated catheters, and those grown in a flow system in microfermentors [44]. Interestingly, the biofilm transcriptomes were not strongly affected by factors such as nutrient flow and aerobiosis, in contrast to the gene expression of free-living cells. However, a few studies indicated that biofilms grown under flow conditions, in CDC reactors or modified Robbins devices, contain more extracellular matrix and more biomass [10, 43, 45]. Mature biofilms formed in a flow of replenishing nutrients consist of a dense network of yeast cells, pseudohyphae, and hyphal cells. In a simple flow model, using a silicone strip placed in a conical tube, *C. albicans* biofilms grew thicker than biofilms grown in static conditions, and grew faster as an 8-hour-grown flow biofilm had similar biomass as a 24-hour-grown static biofilm [46]. The authors speculated that uninterrupted food supply prohibited adverse conditions, such as nutrient starvation and toxic accumulation, and hence promoted rapid cell proliferation. A parallel study, using a rotating disc system (RDS) to impose shear forces at physiological levels to biofilms developed on catheter pieces, illustrated similar results as biofilms under shear stress grew thinner but denser than those in no-flow conditions [47]. In the RDS model, less cells adhered at first, but by 24 h biofilms displayed similar metabolic activity and dry weight as those obtained in the static model. Suggestions that explained the increased growth rate in shear conditions included an increased rate of maturation in these conditions and a natural selection of more robust cells capable of withstanding the fluid friction by growing faster.

2.1.3. High-Throughput Biofilm Models. Another important aspect of *in vitro* biofilm modelling is the development of high-throughput systems of particular interest in the large-scale screening of antibiofilm molecules. Most studies so far have made use of the 96-well microtiter plate assay [40]. In this model, biofilms are formed directly on the bottom of the wells, and the quantification method is based on the ability of sessile living cells to reduce tetrazolium salt (XTT) to water-soluble orange formazan compounds. In an effort to upscale biofilm production, a *C. albicans* biofilm chip system (*Ca*BChip) has recently been developed by Srinivasan et al. [42]. The high-density microarray platform is composed of more than 700 independent and uniform nanobiofilms encapsulated in a collagen matrix and provides the first miniature biofilm model for *C. albicans*. Despite the several-thousand-fold miniaturization, the biofilms formed on the chip displayed phenotypic characteristics, such as a multilayer of yeast, pseudohyphae and hyphal cells, and a high level of antifungal drug resistance, consistent with those of biofilms formed by standard methods. However, echinocandins were not proficient to eradicate biofilm in this system, potentially due to their binding to the collagen matrix. In a second generation of the biofilm chip, other nonprotein matrices will be investigated. While this system steps-up the number of biofilms that can be produced at once in static conditions, the next step may be to develop high-throughput flow biofilm systems adapted to *Candida* spp. Such a tool has been described based on a device comprised of microfluidic channels that provide fluid flow to 96 individual bacterial biofilms [63]. The effects of antimicrobial agents on the biofilms were rapidly screened, and viability was quantified by fluorescence measurements. These high-throughput techniques will certainly contribute greatly to the discovery of novel antibiofilm molecules.

2.2. In Vivo Models of Candida Biofilms

2.2.1. Biofilm Models on Inert Substrates. *In vivo* models are undisputedly required to appreciate the hostile environment that conditions biofilm formation (Table 2). A few *Candida* biofilm models, mostly associated to catheter infections, have been developed in several rodents, giving insights on the *in vivo* biofilm structure and the efficacy of various antifungal agents [70]. The catheter-related *in vivo* biofilm models resulted in biofilm formation within 24 h and consisted of complex structures of yeast and elongated cells embedded in extracellular matrix, similar to those observed in *in vitro* model systems [8]. While susceptibility to azoles was reduced in these models, liposomal amphotericin B lock therapy and treatment with caspofungin or chitosan proved to be efficient against *in vivo* biofilms [64, 65, 71]. Central venous catheter models (CVCs) are also useful for the investigation of the kinetics and occurrence of dissemination of the microorganisms to other organs, demonstrated by colonisation by *C. albicans* of the kidneys in the rat model [8]. In addition, the development of a CVC model in mice will allow comparison to other modes of infection, in particular to the commonly used disseminated candidiasis by tail vein infection. A murine model for catheter-associated candiduria was recently developed and illustrated the role of *Candida* biofilms in the persistence of the urinary tract infection [66]. It also outlined differences between murine and human catheter-related candiduria in terms of bladder inflammation and fungal burden in the urine. In another catheter-related *Candida*-associated infection model, we developed a subcutaneous foreign body system suitable for *C. albicans* [9]. This model, of nondisseminated nature, allowed the study of biofilm development for long periods of time (Figure 1) but required the use of immunosuppression treatment of the animals due to the high inflammatory response associated with implant of foreign devices. However, efficacy of the

Table 2: *Candida* biofilms *in vivo* models.

Models *in vivo*	Device	Developed in
Catheter-associated models	(i) Central venous system	Rat [8], rabbit [64], mouse [65]
	(ii) Candiduria model	Mouse [66]
	(iii) Subcutaneous foreign body system (biofilms developed after 2 to 6 days in infected implated catheter fragments)	Rat (immunosuppressed before and during biofilm development) [9]
Candida-associated denture stomatitis models	(i) Acrylic denture material attached to the hard palate (biofilms developed between the hard palate and the device)	Rat (immunosuppressed on day of infection) [67]
	(ii) Custom fitted denture system (cast fabrication of a fixed part that is attached to the posterior palate and a removable part fitted to the anterior palate)	Rat [68]
Mucosal model of oropharyngeal candidiasis	Biofilms developed on the tongue after infection by swabbing and drinking water contaminated with *Candida* cells	Mouse (immunosuppressed on day of infection) [12]
Vaginitis model	*In vivo* and *ex vivo* models	Mouse (treated with estradiol prior infection) [69]

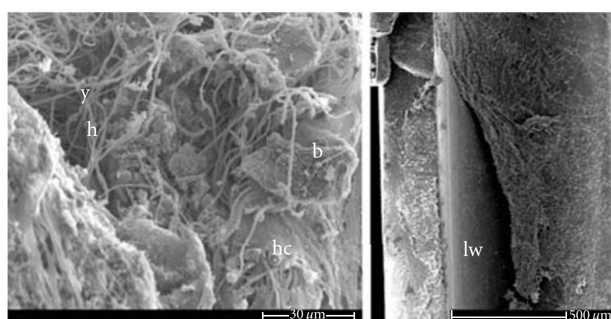

Figure 1: Scanning electron microscopy images of wild type *Candida albicans* biofilms developed *in vivo* in the denture model (left panel) and in the subcutaneous model (right panel). Elements such as hyphal cells (h), yeast cells (y), bacterial cells (b), host cells (hc) and catheter lumen wall (lw) are highlighted. Images were adapted from the work of Nett et al. [67], and S. Kucharíková and P. Van Dijck (MCB Laboratory, VIB, K.U. Leuven, unpublished data), respectively.

echinocandin anidulafungin, by intraperitoneal injections, was demonstrated against *C. albicans* biofilm in this *in vivo* system [72]. These *in vivo* models are all suited for further study of novel antifungal therapies and for the use of novel material technologies, including less adherent surfaces and material coating with fixed or releasing antifungal agents (see the next section).

A relatively cost- and time-effective *Candida* biofilm model on acrylic denture material, which does not require the *ex vivo* mold process, was illustrated recently [67]. In this rat model, biofilms developed between the hard palate and the denture material, following *Candida* inoculation in that 1 mm space (Figure 1). Fungal invasion of the palate and the tongue and neutrophils infiltration also occurred, indicating that the model was consistent with that of acute human denture stomatitis. Interestingly, the denture model offers the possibility to study mixed biofilm structure and behaviour in response to antimicrobial treatments, as the biofilms were composed of both bacterial and fungal cells. Finally, biofilms developed on the denture model were inherently resistant to fluconazole, in accordance with previous findings [8, 72], but also to the echinocandin micafungin, in contrast with previous investigations performed in a different model [73]. A plausible explanation suggested by the authors is that the mixed biofilm nature combined with the specific site of infection, the oral cavity, is the cause of that antifungal resistance. An alternative rat model of *Candida*-associated denture stomatitis recently described differs by the use of animal-fitted devices [68]. In this system, a removable part of the device makes the replacement of the infected device a relatively easy step. These models promise to deliver an alternative mean of testing novel antibiofilm molecules.

2.2.2. Biofilm Models on Biotic Surfaces. Tools and models to study biofilm formation developed on implanted materials are numerous and indicative of the increased medicinal use of such implants. Biofilms formed on live surfaces are much less characterized, yet they are recognized as causing or aggravating numerous chronic diseases [74]. Besides dental plaques, few reports have investigated biofilm development in clinical samples. Biotic biofilms are poorly understood as tissue samples are sparse and not easily accessible. The oral cavity is an accessible *in vivo* model for studying protein-surface interactions and has been well characterized for bacterial biofilm [75]. A mucosal model of oropharyngeal candidiasis was recently proposed to characterise *C. albicans* mucosal biofilms *in situ* in mice [12]. Keratin, originating from desquamating epithelial cells, constituted a large proportion of the biofilm matrix. First evidence was given that epithelial cells, neutrophils, and commensal oral bacteria co-exist within the fungal mucosal biofilm developed on mouse tongue. Bacteria were mostly found on the apical part of the biofilm, and very few were seen to

invade the tongue epithelium layer. This model highlights the complexity of mucosal biofilms, as host elements and commensal organisms contribute in an active or passive manner to the structure of the biofilms.

C. albicans can also form biofilms on the vaginal mucosa, illustrated by two *in vivo* and *ex vivo* models in immunocompetent estradiol-treated mice [69]. *C. albicans* vaginal biofilms consisted of yeast and hyphal cells embedded in extracellular material, illustrated by ConA staining of the interspersed matrix. In the *ex vivo* model using vaginal explants, no exogenous nutrients were provided, yet biofilms were formed most likely by scavenging host nutrients.

Host-pathogen interactions in biofilm settings have not yet been elucidated, but comparison between these models promises to identify model-specific fungal and host elements.

2.3. Mixed Species Candida Biofilms. The relative contribution and the role of bacteria-*Candida* interactions in the pathogenesis of mucosal infections are yet to be established. However, there is clear evidence that multimicrobial interactions have a central role in the context of human disease [76]. For example, microbial diversity was illustrated in a biofilm-related infection of the urinary tract [77]. Out of 535 clinical samples of urinary catheters, *Candida* spp. were identified among the 39 different microbial taxa isolated. Single-species samples represented 12.5% only. *C. albicans* was isolated in 141 samples, and other *Candida* species were present in other 82 samples. Biofilm formation ability of each isolated strain was quantified *in vitro*, yet not in an artificial urine medium, and cut-off values were used to define no, weak, intermediate, and strong biofilm producers. *C. tropicalis* isolates were the strongest biofilm producers among the *Candida* species. Certain species of bacteria did not show biofilm formation ability in this study. These data illustrates the fact that, in multispecies biofilms, some have a great potential to cause biofilm-based infections, while others may be more passive members of the structured community. Commensalism, mutual cooperation, and antagonism make the interactions within mixed biofilms complex [78, 79]. A summary of bacteria-*Candida* interactions and their effect on fungal development is provided in Table 3. Bacteria can interact with *C. albicans* cells within mixed biofilms, and in particular with hyphal cells. The methicillin-resistant Gram-positive *Staphylococcus aureus* had the highest hyphal association, in comparison to *S. epidermidis*, *Strepococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis,* and *Escherichia coli* in decreasing order, respectively [80]. However, interaction between *S. aureus* and *C. albicans* did not result in reduced or altered biofilm viability. In another study, addition of bacteria to preformed *Candida* biofilms *in vitro* had an antagonistic effect on biofilm cell mass, often in a cell-density-dependent manner [81]. With all inoculums tested, *P. aeruginosa* reduced significantly the fungal biofilm mass when added during the first few hours of biofilm development. In a different experimental assay, preformed bacterial biofilms significantly reduced adhesion and biofilm growth of *C. albicans* [82]. Moreover, simultaneous addition of bacteria and *C. albicans* cells showed that in all cases fungal adhesion was decreased, whereas bacterial biomasses were not affected.

Hypotheses of synergistic relationships between microbes have been suggested, and in particular within mixed biofilm communities [83]. For example, bacterial adhesion was observed on the tongue mucosa of *C. albicans*-infected animals but not of noninfected animals, in a mucosal model of oropharyngeal candidiasis [12]. Synergistic cooperation can also perturb susceptibility to antimicrobial treatment. For example, *S. aureus* resistance to vancomycin was enhanced in mixed biofilms with viable *C. albicans* cells, whereas susceptibility of the fungal cells to the antifungal amphotericin B was not altered [84]. Binding of the fungus to the bacterial cells occurs via the *Candida*-specific adhesin proteins, including Als3, Eap1, and Hwp1, as demonstrated by heterologous expression of these cell wall proteins in the model yeast *Saccharomyces cerevisiae* [85]. The role of adhesins in single- and multispecies biofilm formation is not discussed here but can be found in previous reports [86–88].

3. Antibiofilm Strategies: Research and Development

The current therapies against fungal diseases [96], employing one of the five classes of antifungals (polyenes, pyrimidine analogues, allylamines, azoles, and echinocandins) administrated orally or intravenously, are not discussed in this paper. Each antifungal compound has advantages and limitations related to its spectrum of activity and mode of action. The susceptibility of *Candida* biofilms to the current therapeutic agents remains low, with the exception of the echinocandins [97, 98]. However, these compounds have been employed in different approaches, such as lock therapy or material coating as releasing agent. These alternative methods and their perspective of usage are discussed below.

3.1. Lock Therapy Approach and Prevention against Catheter-Related Blood Stream Infections. Nosocomial infections associated with medical devices represent a large proportion of all cases of hospital-acquired infections [99]. In particular, insertion of any vascular catheter can result in a catheter-related infection, as microorganisms can colonise catheter external and internal surfaces. Some of the favourite niches of colonisation of *Candida* spp. include indeed vascular and urinary catheters and ventricular assist devices, which can be accompanied with high mortality rates [100]. Adherence to the catheter surface, facilitated by host proteins such as fibronectin and fibrinogen, can then lead to biofilm formation [101]. The antimycotic lock therapy approach is currently recommended and employed in treating catheter-related bloodstream infections (CRBSI), in particular for long-term catheters, according to the Infectious Diseases Society of America guidelines [102]. However, lock therapeutic treatment is pathogen– specific as catheter removal is recommended for CRBSI caused by *Candida* species and *Staphylococcus aureus*. The lock therapy involves the instillation of high doses of an antimicrobial agent (from 100- to 1000-fold the minimal inhibitory concentration,

Table 3: Interspecies relationship with *Candida* spp. growth and biofilm development.

Bacterial species	Effect on *C. albicans* hyphal growth	Effect on *Candida* biofilm
Staphylococcus aureus (+)	Associates to hyphal cells (56%) [80]	No antagonistic effect in dual biofilms with *C. albicans* (BacLight LIVE/DEAD assay) [80]
Staphylococcus epidermidis (+)	Associates to hyphal cells (25%) [80]	Reduced adhesion and biofilm formation by a glycocalyx producer strain (CFU counts) [82]
Streptococcus pyogenes (+)	Associates to hyphal cells (25%) [80]	
Streptococcus mutans and Streptococcus intermedius	*S. mutans* inhibits hyphal formation [89, 90]	No significant effect on biofilm viability at densities ranging from $6.25 \cdot 10^5$ to $1 \cdot 10^7$ cells/mL (bacteria added to preformed 3-hour-old biofilms; polystyrene *in vitro* model; CFUs analyses) [81]
Streptococcus gordonii (+)	Stimulates hyphal growth [91]	Promotes mixed biofilms with *C. albicans* [91]
Pseudomonas aeruginosa (−)	(i) Associates to hyphal cells (17%) [80] (ii) Reduced hyphal growth in *C. albicans-P. aeruginosa* dual biofilms [81] (iii) Binds hyphae and kill *C. albicans* [92]	(i) Reduced adhesion and biofilm formation by a nonglycocalyx producer strain (CFU counts) [82] (ii) Reduction of biofilm mass ranging from 40% to 80% in a density-dependent manner [90] (iii) Mutual biofilm inhibition between *Pa* and *C. albicans*, *C. krusei* and *C. glabrata*; decreased biofilm formation of *C. parapsilosis* and *C. tropicalis* in presence of *Pa*; increased CFUs of *Pa* in presence of *C. tropicalis* [93]
Escherichia coli (−)	Associates to hyphal cells (5.7%) [80]	(i) Reduction of biofilm mass ranging from 50% to 80% [81] (ii) Mutual decrease in biofilm cell mass between *Ec* and *C. albicans*; inhibition of biofilm development by *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. dubliniensis*; increased *Ec* cell numbers within *C. tropicalis* and *C. dubliniensis* biofilms [94]
Lactobacillus acidophilus		Inhibition of viable biofilm cell mass by 40% [81]
Bacillus subtilis	Associates to hyphal cells (2.5%) [80]	
Actinomyces israelii (+)		Some inhibition of biofilm at high densities [81]
Prevotella nigrescens and Porphyromonas gingivalis	Inhibition of *C. albicans* hyphal development [95]	Reduction of *C. albicans* biofilms, only at high densities [81]
Klebsiella pneumoniae, Serratia marcescens, and Enterobacter cloacae		Decreased biofilm formation (CFU counts) [82]

(MIC)) directly into the catheter in order to “lock” it for a certain period of time (from hours to days) [103].

Few reports are currently available on the usage of antifungal lock solutions in clinical practice, but they seem to indicate the curative effect of this kind of treatment [104, 105]. *In vitro* studies are more prevalent at the moment and seem to also favour the use of antifungal lock therapy to eliminate *Candida* spp. biofilms, and in particular with the usage of echinocandins [106]. For example, biofilm metabolic activity formed on silicone by *C. albicans* and *C. glabrata* could be effectively reduced by a 12 h lock treatment with micafungin (at 100–500x MIC), which was shown to persist for up to 3 days [107]. Caspofungin had an intermediate effectiveness in the same study, as its activity did not persist as long against *C. glabrata* biofilms. While these results are promising for potential use of the lock technique to treat infected catheters, 100% biofilm inhibition could not be achieved. Sterilization of catheters was obtained *in vivo* by lock treatment with amphotericin B lipid complex (ABLC) in a rabbit model of catheter-associated *C. albicans* biofilm [108]. However, in this study, the lock solution was administrated a few hours a day for a prolonged period of time (7 days). Synergistic antibiofilm combinations, used as lock solutions, between classical antimicrobial agents and other compounds such as the mucolytic agent N-acetylcysteine, ethanol, or the chelating agent EDTA, are also effective against *S. epidermidis* and *C. albicans* individual and mixed biofilms [109]. In a similar approach, recent results suggest that the combination of antibacterial agents with Gram-positive activity, including doxycycline and tigecycline, with known antifungals, such as AMB, caspofungin, and fluconazole, can be useful for the treatment of *C. albicans* biofilms [110, 111].

The prevention of CRBSI has also been the focus of research and randomized controlled trials [112]. In a systematic assessment, Hockenhull et al. [113] showed the clinical effectiveness of CVCs treated with anti-infective agents (AI-CVC) in preventing CRBSI. While trials are still required to determine the most cost and clinical-effective anti-infective product, the routine usage of AI-CVC

will often be limited if appropriate use of other practical care behaviour is not employed in intensive care units. Antifungal impregnated CVCs have been tested in animal models. The echinocandin caspofungin was employed to prevent *C. albicans* biofilm formation in a biofilm model in mice. *C. albicans* biofilm formation was greatly reduced in CVCs that had been pretreated for 24 h with high doses of caspofungin. The dissemination to the kidneys was also reduced by such therapy [65]. Similarly, the use of chitosan, a polymer isolated from crustacean exoskeletons, as a pretreatment of catheters to prevent *C. albicans* biofilm formation was validated in a CVC biofilm *in vivo* model [71]. The use of lock technique or preventive impregnation of antifungals in combating catheter-associated infection seems promising, but not yet convincing on a cost effective point of view as huge doses are still needed to eradicate fungal growth.

3.2. Material Coating and Novel Antibiofilm Surfaces. A developing field of research focuses on the usage of modified materials or coated surfaces to prevent adherence and biofilm development. Implant materials are prone to biofilm formation affecting health in general and duration of the implant in particular. Surface characteristics, such as surface roughness, surface free energy, and chemistry, can influence the type and the feature of the biofilms [114, 115]. For example, *C. albicans* adhesion is enhanced if the roughness of the denture materials is increased [116]. It is nowadays conceivable that coatings may be engineered to promote selective adhesion, with possible attachment to cell tissue (for implant in bone contact) but not to microbes. They may also address the second phase of biofilm development involving quorum sensing, by inhibiting cell-cell communication signals [117, 118]. Biomaterial modifications as a way to prevent biofilm development have been the focus of intense research, in particular in the field of bacterial biofilms [119], but the latest findings on their impact on *Candida* biofilms are discussed next.

3.2.1. Surface Modifications. Surface properties of medical devices constitute a major factor contributing not only to the stability in the body but also to their performance and lifetime *in vivo* and their colonization by microorganisms. In that matter, albumin adhesion is beneficial since it has been shown to prevent binding of microorganisms, while fibrinogen has the opposite effect [120]. Chemical grafting of polyethylene and polypropylene surfaces, functionalized with cyclodextrins, yielded a change in protein adsorption profile of these polymers, by promoting adsorption of albumin and reducing adhesion of fibrinogen to the material surface [121]. In addition, these modified substrates incorporated well the antifungal agent miconazole, leading to reduced biofilm formation by *C. albicans in vitro*. Modified polyethylene and silicone rubbers proved to be very efficient in inhibiting *C. albicans* biofilm formation *in vitro* [122]. These cytocompatible materials were also capable of releasing for several hours considerable amount of an anionic antimicrobial drug, nalidixic acid, suggesting their use as drug-eluting systems.

Modifications of polyurethanes dental biomaterials by addition of surface-modifying end groups were successfully employed to manage *C. albicans* biofilm formation [123]. In addition, correlation between contact angle and biofilm formation was surface dependent. Increased hydrophobicity resulted in increased metabolic activity of the biofilms grown on polyetherurethane, while they inversely correlated for biofilms formed on polycarbonate surfaces. Addition of 6% polyethylene oxide to Elastane 80A showed to be the best combination as no biofilm could be observed on that surface. Biofilms on voice prostheses consist of mixed populations that can include *C. albicans*. Modification of the silicone surface of the prostheses has been employed to limit *C. albicans* colonization, as opposed to incorporation of antimicrobial agents in order to avoid the occurrence of resistance [124]. Silicone disks grafted with C1 and C8 alkyl side chains reduced adherence and biofilm formation of *C. albicans* by up to 92%. Longer side chains did not show as good results, and combinations of quaternizing agents did not work synergistically either. Similarly, grafting of cationic peptides, such as the salivary peptide Hst5 and synthetic variants, onto silicone rubber, inhibited biofilm formation by up to 93%, in a peptide-dependent manner [125].

3.2.2. Surface Coatings. Fungicidal or fungistatic materials have been employed to fabricate or coat the surfaces of medical devices and have a great potential in reducing or eliminating the incidence of biofilm-related infections. Dental resin material coated with thin-film polymer formulations containing the polyene antifungals nystatin, amphotericin B, or the antiseptic agent chlorhexidine, were used in *C. albicans* biofilm assays [126]. Biofilm reduction was the greatest on chlorhexidine containing polymers, while the other formulations were much less efficient. Similarly, multilayered polyelectrolyte thin films containing an antifungal β-peptide incorporated within the layers of the films inhibited the growth (and hyphal formation) of *C. albicans* by 74% after 2 h of contact [127].

The polysaccharide dextran is widely used in medicine and is also one of the main components of dental plaque. Cross-linked dextran disks soaked with amphotericin B solutions, described as amphogel, kills fungi within 2 hours of contact and can be reused for almost 2 months without losing its efficacy against *C. albicans* [128]. This antifungal material is biocompatible and could be used to coat medical devices to prevent microbial attachment. It was recently used for local antifungal therapy in the form of injectable cross-linking hydrogels [129]. Nitric oxide can antagonise cell proliferation by signalling rather than by toxic effect. It regulates bacterial biofilm dispersal and has also been employed in releasing xerogel to attenuate *C. albicans* adherence and biofilm formation [130]. The nitric-oxide-based method is still at the experimental level, due to poor water solubility and stability.

Coating of medical material surfaces has been employed and tested with several types of coating molecules, including the naturally occurring polymer chitosan and antimicrobial peptides such as Histatin 5 (Hst5). Surfaces coated with the polymer reduced the viable cell number in biofilms by

more than 95%, in the case of *C. albicans* and also for many bacteria such as *Staphylococcus aureus* [131]. Chitosan, which is proficient against a wide range of pathogenic microbes, disrupts cell membranes as cells settle on the surface. The use of such polymer offers a biocompatible tool for further coating design of medical devices. Acrylic disks precoated with Hst5 prove to be efficient in inhibiting biofilm formation of *C. albicans*, especially in the later stage of development, while biofilm sensitivity to the antimicrobial peptide was the same as the one of free-living cells [132]. The utility and potential of selected peptides, as therapeutic molecules, including the β-glucan synthesis inhibitors, the histidine-rich peptides, and the LL-37 cathelicidin family are being determined and could be used as coating compounds against adherence and biofilm formation [133, 134].

The possible applications of biomaterial modification remain to be clearly established and approved. Shift from a commensal bacterial biofilm to a more pathogenic biofilm involving *Candida* spp. in the oral cavity for instance is believed to be more influenced by mucosal inflammation and the general well-being of the host than on the nature and surface properties of the material itself [135]. However, development of materials that can fully abolish microbial adherence is a promising perspective against biofilm formation. The discrepancy between antimicrobial coatings killing the biofilm-proficient organisms and antimicrobial releasing coatings to prevent biofilm formation is a current issue.

3.3. Quorum Sensing Molecules and Natural Byproducts. Adhesion and biofilm formation by *C. albicans* cells can be modulated by physical and chemical signals from the oral bacterium *Streptococcus gordonii* [91]. Indeed, most *Streptococcus* species possess the antigen I/II, a cell-wall-anchored protein receptor that mediates binding to *C. albicans*. Moreover, *C. albicans* hyphal and biofilm development are greatly enhanced by *S. gordonii*, which also relieved the fungal cells from the repressing effect of the quorum sensing molecule farnesol [91]. Farnesol, a sesquiterpene and signalling molecule produced by *C. albicans*, represses biofilm formation *in vitro* [136]. Conversely, tyrosol, a 2-(4-hydroxyphenyl) ethanol derivative of tyrosine, accelerates hypha production in the early stages of biofilm development and is secreted at least 50% more by biofilm cells than by planktonic cells [137]. Several studies demonstrated that farnesol actually increases fungal pathogenicity in animal models, potentially by interfering with normal progression of cytokine induction [138–140]. Analogs of farnesol have been identified that fail to induce pathogenicity and yet retain farnesol ability to block hyphal development [141]. While these analogs did not protect mice from candidiasis, they may be of interest in biofilm inhibition. Indeed, a number of molecules with farnesol-like activity, that can induce the shift to the yeast form of growth, have been identified in Gram-negative bacteria. For instance, the signalling molecule, homoserine lactone, produced by *Pseudomonas aeruginosa* represses *C. albicans* filamentation [142]. *P. aeruginosa* also produces several phenazines that exhibit antifungal activity against *C. albicans* [143]. Uptake of the phenazines generated reactive oxygen species production and led to fungal cell death. In mixed biofilms, binding of the toxins to the fungal cells has a negative influence on *C. albicans* growth.

In a different approach, Valle et al. [144] demonstrated that the use of nonantibiotic molecules, such as polysaccharides, produced by competitive commensal organisms can antagonize biofilm formation. A better knowledge of the microbial community behaviour and in particular of the interaction between commensal and pathogen organisms would help to combat predominance of the infectious or disease causative agents. In this scheme, natural products produced by cells within a biofilm contribute to the dynamic of the community and may play an antiadhesion role for nonwanted other microorganisms [145]. Bacterial lipopolysaccharides also modulate adhesion and biofilm ability of several *Candida* species, in an interspecies-dependent manner [146]. It is not known how mixed populations affect the host immune response in response to infection. The overall population behaviour results from a potential selective advantage to either or both species. While communication is the key, interpretation is the code. Identification and alterations of the communication signals would certainly result in a better understanding of how species coexist and permit a better control of biofilm formation [147]. Targeting quorum sensing molecules or associated signalling mechanisms is an open field of research at present, but the use of quorum quenching enzymes or quorum sensing inhibitors naturally produced by other species could help in the finding of novel antibiofilm agents [148, 149].

3.4. Host Responses to Biofilms: Perspective of Immunotherapy. With the number of people considered at high risk for microbial infections constantly increasing, immunotherapy seems to offer a great potential despite the complexity of the interaction between the host defence system and the pathogen [150]. The ability of human pathogens, such as *Candida* spp., to cause infections depends on a constant and sometimes discontinuous battle between the pathogen and the host immune system [151]. Recognition of *Candida*-specific pathogen-associated molecular patterns (PAMPs) by dedicated pattern recognition receptors (PRRs) such as Toll-like receptors and lectins activates the innate effector cells (macrophages, dendritic cells, and neutrophils), which in turn produce a variety of soluble factors, including cytokines and chemokines [152]. However, little is yet known about the interactions between human phagocytes and *Candida* spp. biofilms, while immunotherapeutic treatment against candidiasis has been undertaken [153, 154]. Chandra et al. [155] demonstrated that adherent peripheral blood mononuclear cells (PBMCs) enhanced the ability of *C. albicans* to form biofilm. They also observed that phagocytosis of the fungal cells within a biofilm did not occur while their free-living counterparts were phagocytosed. These data defined the novel concept that *Candida* biofilms seem to have an immunosuppressive effect. Inactivated PBMCs on the other hand did not induce this enhanced growth behaviour, nor did lipopolysaccharide-activated PBMCs, suggesting that the stimulated biofilm formation resulted from (a) *Candida*-biofilm-induced secretory factor(s). Indeed, the cytokine profile of PBMCs following coculture with planktonic or

biofilm cells of *C. albicans* differed greatly, with IL-1β as the cytokine most highly overexpressed by contact with biofilms. Supporting these data, a recent study showed that phagocytes alone induced much less damage to biofilms than they did to free-living cells or to resuspended biofilm cells, which lacked the overall structure of biofilms and most of the matrix [156]. Using confocal laser scanning microscopy, Katragkou and coworkers deducted that human phagocytes looked like unstimulated cells, presenting a rounded shape when in presence of biofilms. This was also confirmed by a reduced cytokine production in a biofilm-phagocyte coculture, compared to a planktonic cells-phagocytes mix. Phagocytes appeared entrapped within the structured network of cells and matrix and were unable to internalize cells within biofilms. Moreover, *C. albicans* and *C. parapsilosis* biofilms were more susceptible to the additive effects between phagocytic host defence and the echinocandin anidulafungin than to each separately and to the combination of the azole voriconazole with phagocytes [156, 157]. These data validate the findings that echinocandins can influence host cell interactions with biofilm [158].

Pathogens have evolved many mechanisms of defence to avoid being recognized by the host environment [159–161]. *C. albicans* can evade immune attack by masking its cell wall β-glucan component, a potent pro-inflammatory signature carbohydrate, under a thick layer of mannoproteins. Clear evidence showed that exposing the β-glucans by treatment with the antifungal drug caspofungin elicited a stronger immune response [158]. These data suggest that echinocandin treatment may enhance immunity [162]. Masking of β-glucans depends on a complex network of cell wall remodelling, and targeting these regulatory processes may identify novel antifungal possibilities. For example, disruption of the MAPK pathway regulated by the extracellular signal-induced Cek1 kinase triggered a greater β-glucan exposure, which resulted in an enhanced immune response compared to the wild-type strain [163]. There are conflicting data regarding the role of the β-glucan receptor Dectin-1, expressed widely on phagocytes, in antifungal immunity [164]. However, studies suggested that Dectin-1 is required for fungal killing and induction of early inflammatory responses. These findings are of interest for biofilm recognition by the immune system, as β-1,3-glucans are found in high amounts in the extracellular matrix of *Candida* biofilms *in vitro* and *in vivo* [10, 12, 165]. Biofilms developed on soft tissue are associated with infiltration of the infected sites by neutrophils, which can then confer innate immune protection [166]. In *C. albicans*, Hyr1, encoding a GPI-anchored cell wall protein, has been shown to confer resistance to neutrophil killing *in vitro* and in the oral mucosal tissue biofilm model [12, 167]. In addition, vaccination with a recombinant Hyr1p protected mice against hematogenously disseminated candidiasis. Immunotherapeutic strategies, such as vaccination, anti-*Candida* antibodies, and cytokine therapy, are under investigation to treat *Candida* infections [168]. However, their applicability in treating biofilm-related infections is still in a preliminary state. In that framework, recent data showed that pretreatment of *C. albicans* cells with antibodies targeting the complement receptor 3-related protein led to reduced adhesion and biofilm formation *in vitro* [169]. In another study, anti-*C. albicans* antibodies from chicken egg yolk were employed as antiadherent molecules [170]. While the adherence of *C. albicans* was reduced, biofilm inhibition was only observed in absence of serum, as the activity of the antibody was very much reduced against germ tubes, of which the formation is induced in the presence of serum. *In vivo* studies of the antibody-based approach remain to be investigated in the context of biofilms.

4. Concluding Remarks

The large panel of biofilm models suitable for *Candida* research highlights the diversity of niches in which the fungus can develop ranging from biotic to abiotic surfaces. However, the role and nature of host-pathogen interactions during biofilm formation are only starting to get unveiled. The search for an antibiofilm treatment is a complex subject which requires improved knowledge of the pathogen itself, and also of the host response to adhesion and biofilm formation, the properties of the substrates onto which biofilm develop, and the interactions within microbial communities. The field of chemoinformatics may assist the development of novel antibiofilm compounds, based on already identified good candidate molecules [171]. This approach may also reveal better coating agents for material surfaces that would persist long periods of time *in vivo*. The use of natural compounds, from dietary plants or probiotics, may also be considered as they are better tolerated by humans.

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Review Article

Hyphal Growth in Human Fungal Pathogens and Its Role in Virulence

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Most of the fungal species that infect humans can grow in more than one morphological form but only a subset of pathogens produce filamentous hyphae during the infection process. This subset is phylogenetically unrelated and includes the commonly carried yeasts, *Candida albicans*, *C. dubliniensis*, and *Malassezia* spp., and the acquired pathogens, *Aspergillus fumigatus* and dermatophytes such as *Trichophyton rubrum* and *T. mentagrophytes*. The primary function of hypha formation in these opportunistic pathogens is to invade the substrate they are adhered to, whether biotic or abiotic, but other functions include the directional translocation between host environments, consolidation of the colony, nutrient acquisition and the formation of 3-dimensional matrices. To support these functions, polarised hyphal growth is co-regulated with other factors that are essential for normal hypha function *in vivo*.

1. Introduction

The commonly carried yeasts, *Candida albicans*, *C. dubliniensis*, and *Malassezia* spp., and the acquired pathogens, *Aspergillus fumigatus* and the dermatophytes are opportunistic pathogens and afflict individuals whose immune systems are compromised or dysfunctional, often in combination with other predisposing factors. *Candida* spp. are regarded as part of the commensal flora, colonising multiple mucosal sites, including the GI tract. They can cause irritating superficial oral and vaginal infections "thrush", invasion of the skin and nails (onychomycosis), and, if released into the bloodstream of immunocompromised patients, fatal systemic infections due to the formation of inflamed lesions in internal organs. *Malassezia* spp. are also carried by the majority of the population. All but one species lacks fatty acid synthase activity, so *Malassezia* localise to epidermal surfaces that are rich in secreted sebum, such as the scalp, chest, and back [1–3]. *Malassezia* cause a variety of superficial fungal infections, including seborrhoeic dermatitis, dandruff, *Tinea versicolor*, atopic dermatitis, and psoriasis, and often exacerbate other skin conditions. *Aspergillus* spp. are obligately filamentous and grow as mycelia in the soil. They produce microscopic airborne conidia which are inhaled into the lung, from where they are normally cleared by the activity of airway cilia and alveolar macrophages. Failure to do so can lead to several diseases, including invasive aspergillosis, where the organism is carried in the blood to other organs, and aspergilloma, the formation of fungal foci in cavities formed in a prediseased lung. The obligate hyphal growth of *Aspergillus*, where little or no cell shedding occurs, is a contributing factor to morbidity due to the potential delay in diagnosing bloodstream infection [4]. The attributable mortality rate for *Aspergillus* spp. is 50–80%, the highest rate for fungal infections in humans [5]. Dermatophytes are acquired from the environment through passage from mammalian reservoirs and between humans. Approximately one-third of the European population is estimated to suffer from acute dermatophyte infections such as *Tinea pedis* "athlete's foot" through their ability, shared with *Candida* spp., to degrade heavily keratinised skin and thrive in warm, moist conditions [6]. A further threat posed to health by these filament-forming pathogens is their propensity to develop pseudomembranous biofilms. *C. albicans* biofilms appear as thick, creamy-white plaques on mucosal surfaces, the typical manifestation of thrush. Aspergilloma foci in the lung display many of the characteristics associated with biofilms. *C. albicans*, *Malassezia*, and *A. fumigatus* are also found in biofilms on abiotic medical plastics. The biofilm environment poses significant medical problems since it promotes resistance to

antifungal drugs, acts as a reservoir for seeding further infection, and compromises the mechanical function of devices such as voice prostheses and contact lenses [7, 8].

Many of the fungi that cause disease in humans grow as free-living mycelia in the environment but convert to a yeast morphology once inside the human body in response to the raise in temperature. Collectively known as "the dimorphs," this group is able to cause severe and sometimes fatal infection whilst existing solely in the yeast form, illustrating that hypha formation *per se* is not essential for fungal virulence. Microscopy of diseased tissue show that *C. albicans*, *Malassezia*, and dermatophytes reversibly switch between yeast and hyphal morphologies *in vivo*. When reverse genetics became available in *C. albicans* nearly two decades ago, it was shown that loss of the ability to make this transition resulted in avirulence [9]. The fungi most associated with morbidity and mortality in humans, therefore, require a morphogenesis programme for full virulence, underlining the need to understand the temporal and spatial role of morphogenesis during disease progression. The challenge has been partially met by the development of genetic tools and infection models for *C. albicans*, *C. dubliniensis*, and *A. fumigatus*, where there is inherent difficulty in studying the development of infection *in vivo*. Less emphasis has been placed on the study of the *Malassezia* and dermatophytes because they cause only superficial infections and are more amenable to topical treatment. Nevertheless, such infections are extremely common and can cause considerable distress to chronic sufferers. Reverse genetics approaches and genome sequencing data are now becoming available for these organisms and highlight the contrasts and commonalities with the more well-studied pathogens [2, 10–12].

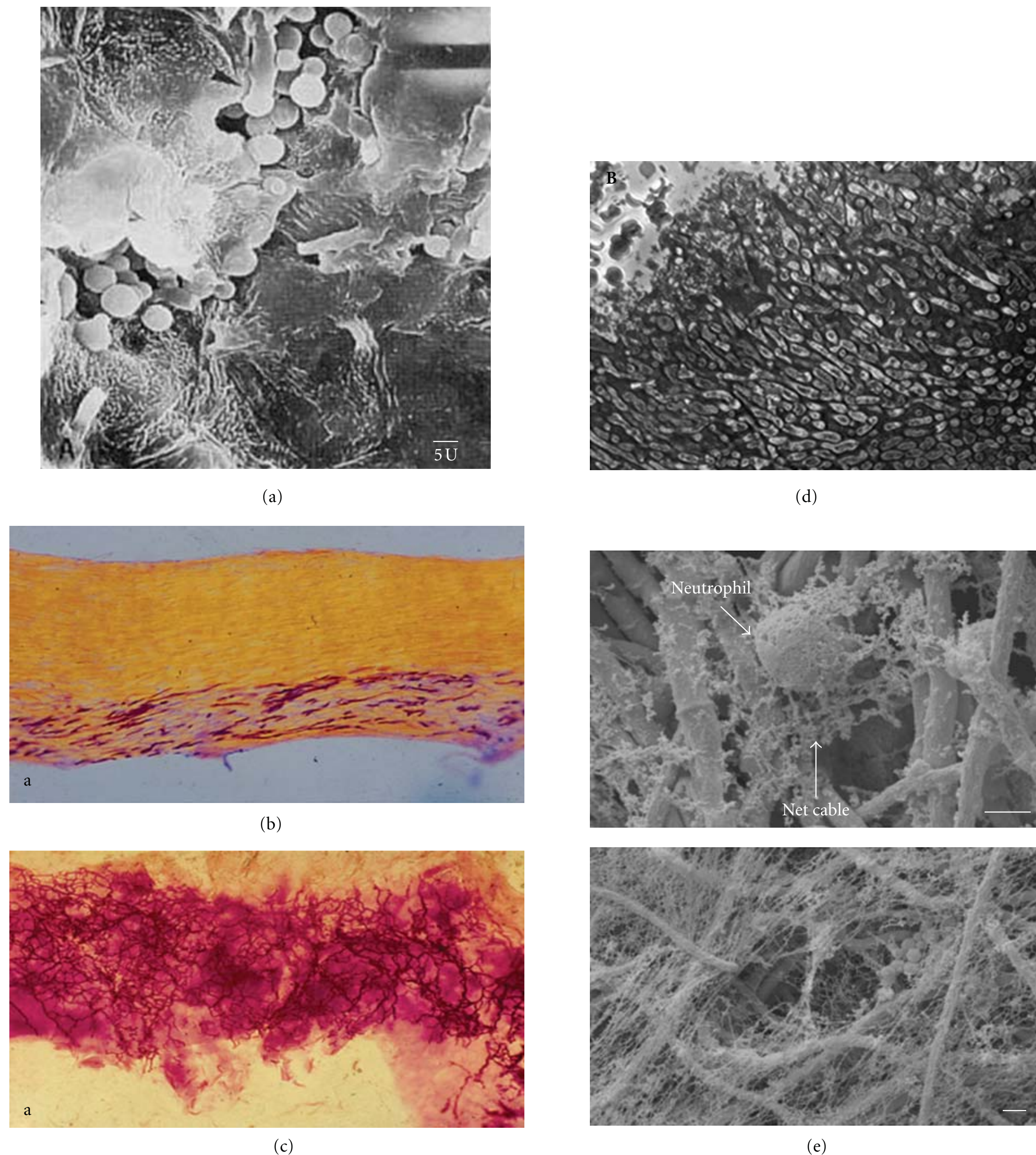
We are gaining a better understanding of the role of hyphae in disease and appreciate that hyphae come as a complex package of morphological features, supported by hypha-specific, site-specific, and time-dependent gene regulation. The bottom line, perhaps not surprisingly, is that hypha formation confers the ability to actively penetrate host tissue. However, there is still much to learn about how hyphae are deployed effectively during tissue translocation, adherence and nutrient acquisition *in vivo*. The regulation of morphogenesis in *C. albicans* and models of hyphal growth are covered in excellent reviews elsewhere [18–24]. This paper focuses on presenting what is known about hyphal growth during infection by the major hypha-forming human pathogens and considers the specific functions and mechanical and structural properties that hyphae confer during virulent fungal growth.

2. Hyphal Growth in Disease

Fungi infect a diverse range of host body sites. *Malassezia* and the dermatophytes are limited to specific environments partially due to nutrient availability, but *C. albicans* and *A. fumigatus* can tolerate high internal body temperatures and a wide range of ambient pH values, oxygen availability, and nutrients. Each environment presents challenges that select for fungi with specific adaptations.

2.1. Keratinised Cell Layers. The epidermis is made up of dense, heavily keratinised cell layers that are constantly undergoing a process of renewal. Three major groups of filament-forming fungi commonly infect the keratinised and cornified layers of skin, nails, and hair—*Candida albicans*, the dermatophytes and the lipophilic *Malassezia* spp. Conidia and hyphae are observed skin scrapings and in lesions caused by these fungi, which face the challenge of retaining a foothold on a surface that is being constantly shed. *Candida* spp. and *Malassezia* spp. are part of the commensal flora and growth in the yeast form is generally asymptomatic and tolerated by the immune system. Although phylogenetically distant, these fungi have evolved a similar arsenal of lipases and proteases for life on humans, some of which are specifically expressed during growth as hyphae [2, 25]. *Malassezia* yeasts are thought to be taken up by keratinocytes and exist as facultative intracellular parasites by actively suppressing the inflammatory response [26]. *Malassezia* hyphae are not well studied because they are slow growing *in vitro* and require specialised growth media that contains a source of lipid. *In vivo*, hyphae are observed only in individuals with hyperactive sebaceous-gland activity, where the presence of excess sebum appears to be the inducer of morphogenesis [27]. *M. globosa* grows as yeast and hyphae in localised cavities formed within the rough landscape of the skin. The hyphae are short but penetrate keratinised skin cells to gain access to deeper cavities below, where growth reverts to yeast, and new colonies are formed (Figure 1(a)) [13, 14]. One of the roles of *M. globosa* hyphae, therefore, appears to be the rerooting of infection in nutrient-rich, deep cornified layers to replace the older fungal colonies that are passively brought to the epidermal surface and sloughed off. In lipophilic *Malassezia* spp., morphogenesis is thought to be a response to the presence of excess sebum [28]. In *M. globosa*, fungal lipases break down sebaceous triglycerides, producing abundant unsaturated fatty acids as unwanted byproducts, which act as immunostimulatory molecules in individuals with poor epidermal integrity [3, 29, 30]. Eleven protein allergens have been identified from *Malassezia* species, including a conserved Heat Shock Protein (HSP70) [31]. Interestingly, the *C. albicans* surface invasin, Ssa1, is also an HSP70-like protein [32, 33]. The specific involvement of hyphae in the generation of immunostimulatory molecules by *Malassezia* has yet to be addressed.

The keratinolytic dermatophytes, *T. mentagrophytes* and *Arthroderma benhamiae*, are acquired from other mammalian hosts and thrive in warm, damp skin [11]. Dermatophytes are visible as arthroconidia and hyphae within the epidermal layers and surrounding hair shafts, although the *in situ* signals that induce morphogenesis are not known. In addition to hyphae, *T. mentagrophytes* cells produce thin fibrils, proposed to be adhesin molecules, which help attach the fungus to the skin surface [28]. The deeper layers of the epidermis are penetrated by hyphae that meander and produce branches that extend parallel to the predominant cell layers (Figure 1(b)). It is thought that further penetration is inhibited by limited iron availability due to the activity of host ferritin in the underlying layer of the dermis [34]. No direct penetration of the keratinised cells by dermatophytes



(a) (d) (b) (c) (e)

Figure 1: *In vivo* growth of filamentous fungal pathogens. (a) Hyphae of *Malassezia globosa* translocate deeper within the keratinised epidermal layer where they establish new colonies and revert to growth as yeast [13]. (b) Dermatophyte hyphae follow the keratinised layers that run parallel to the nail surface [14]. (c) Hyphae of *Candida albicans* growing in a multidirectional manner within the nail, often forming helical twists [14]. (d) Histological section of aspergilloma in the lung showing the tightly packed *Aspergillus fumigatus* hyphae surrounded by a matrix material and with no immune-cell infiltrate [15]. (e) Production of neutrophil extracellular traps (NETs) against *A. fumigatus* hyphae where, unlike the safe haven of a fungus-derived biofilm matrix, hyphae are instead imprisoned in a host-derived matrix of neutrophil DNA and calprotectin, a protein which chelates the divalent cations that are required for fungal growth [16, 17].

has been reported, but fungal cells flatten themselves against the substratum, a further possible aid to adhesion [28]. In contrast, *C. albicans* growth in the nail showed a more chaotic pattern of colonisation. Hyphae were entangled and less likely to stratify within the epidermal layers than dermatophytes or *Malassezia* species [14]. Additionally, many hyphae grew in a helical or spiral growth trajectory, a contact-dependent and low-nutrient response in this fungus

(Figure 1(c)) [35, 36]. Detection of an epidermal infection by the host leads to the proliferation of epidermal cells to increase shedding of the stratum corneum and the microbes contained within it. The physical penetration by hyphae and their accompanying enzymatic activity, therefore, contribute to the thickened and chaotic appearance of the skin and nail bed that is a marked feature of epidermal infection.

2.2. Biofilms on Mucosal and Abiotic Surfaces. The formation of hyphae is a key feature in the development of the 3-dimensional structure of fungal biofilms, which present specific clinical problems due to their relative resistance to treatment with antifungal drugs and their potential to release infective cells. Biofilms formed by *C. albicans* are the primary cause of mucosal infection in susceptible humans, where the plaques formed by overproliferation of adhered cells to vaginal or oral epithelia are easily visible [38, 43]. Surprisingly little is known about the activation of morphogenesis by *C. albicans* during mucosal infection, although predisposing factors such as gender, age, poor oral hygiene, and underlying chronic conditions are well-documented. In *in vitro* models of epithelial infection, the germination of hyphae commences as soon as yeast cells are introduced into the system [44]. This begs the question as to whether this process is actively suppressed during commensalism, or whether hyphae are constantly germinating *in vivo* but immediately being cleared by the immune system. Contaminated medical plastics, such as catheters and prostheses, also offer stable substrates for biofilm formation (Figure 2(a)(i)), and recent studies of aspergilloma suggest that these enclosed foci of matrix-bound *A. fumigatus* hyphae found in the diseased lung also display the characteristic properties of biofilms (Figure 1(d)) [15, 45]. *In vitro* studies of the temporal process of biofilm formation by *C. albicans* on inert, abiotic substrates show that yeast cells provide an early-stage, adhesive base layer, from which hyphae germinate to generate a thick, loosely structured matrix (Figure 2(a)(iii)). As the biofilm matures, the matrix becomes embedded through the production of fungal exopolysaccharides (β-glucan), protein, hexosamine and extracellular DNA [46, 47]. Hypha formation is not essential for biofilm establishment or maintenance, but biofilms formed by yeast alone are thin and more easily removed from surfaces by mechanical disruption, suggesting that the tangle of hyphal filaments serves to strengthen the structure [48]. Studies of *C. albicans* biofilms formed on mucosal tissue showed that this structure is retained *in vivo*, but the matrix is more complex, comprising yeast, hyphae, extracellular β-glucan, bacteria, keratinised squamous cells, and foci of host neutrophils [38].

Hyphae in mature biofilms show a strong propensity to invade the underlying substrate, even when there is little nutrient value (Figure 2(a)). In the mucosa, intercalation of hyphae disrupts the epithelial layer, which activates a localised inflammatory response. Remarkably, the hyphae of *C. albicans* and a variety of other filamentous pathogens are able to penetrate the soft medical silicones which are used in the manufacture of prosthetic devices and contact lenses. Hyphal infiltration causes the silicone to expand and stiffen, thus, compromising the function of the device (Figure 2(a)(ii)) [7, 8]. Mucosal biofilms form at sites occupied by multiple species of microbes [49]. The presence of bacteria within *C. albicans* biofilms is, therefore, not surprising, and studies suggest that the growth of hyphae within biofilms is likely to be modulated *in vivo* by species such as *Streptococcus mutans* and *Streptococcus gordonii*. These oral bacteria can attenuate or promote hyphal growth, respectively, through physical interactions and chemical signalling via the production of quorum-sensing molecules [50, 51]. *C. albicans* produces its own quorum-sensing molecules, farnesol, and tyresol, which are negative and positive regulators of morphogenesis, respectively, [52–54]. The role of hyphae in mucosal infection by *C. albicans* is, therefore, modulated by a three-way interaction between the fungus, resident bacteria and the ambient host environment.

2.3. Systemic Dissemination. A. fumigatus and *C. albicans* both cause bloodstream infections in humans (invasive aspergillosis and disseminated candiasis) and employ two primary methods by which to translocate from one environment to another within the body. The first method is through the passive uptake of fungal particles by host cells, and the second is by a method that is unique to filamentous fungi-active penetration of host cell membranes by the hyphal tip [55]. Passive uptake occurs either during receptor-mediated engulfment of the fungus by phagocytic cells with the aim of killing the microbe or by nonphagocytic endothelial or epithelial cells where molecules on the fungal surface stimulate their own endocytosis (Figure 2(b)) [40, 56, 57]. *C. albicans* and *A. fumigatus* both appear to use this method early in the infection process, translocating by induced uptake into superficial epithelial cells or the endothelial cells that line blood vessels. It has been observed that germinating *C. albicans* hyphae are more efficiently endocytosed than yeast cells [58]. This could be the result of evolutionary pressure on the fungus because the immune system is activated by the presence of hyphae, making escape into a safer environment a matter of some urgency for the fungus. This is of particular importance in the bloodstream because exposure to serum is a strong inducer of morphogenesis in *C. albicans*. The molecular interactions that stimulate the uptake of *C. albicans* by nonphagocytic cells are being elucidated and involve the expression of fungal surface invasins, Als3 and Ssa1. These proteins have alternative cellular functions (Als3 is an amyloid-like, hypha-specific adhesion, and Ssa1 is an intracellular heat-shock protein), but Ssa1 interacts with N-cadherin on endothelial cells and both interact with E-cadherin on epithelial cells [33, 57, 59]. *In vitro* assays show that *A. fumigatus* conidia may translocate from the lung alveoli by induced uptake into Type II alveolar epithelial cells, which display immune cell activity [4, 60, 61]. Escape from the intracellular host environment requires morphogenesis followed by sustained polarised growth. *C. albicans* mutant cells with deletion of Eed1, which is involved in polarity maintenance, could penetrate epithelial cells during the onset of hyphal growth (Figure 2(b)(i)) but became trapped intracellularly when polarity could not be maintained [39]. As growth reverted to yeast, the mutant was unable to punch its way out of the host cell, and hence, dissemination into

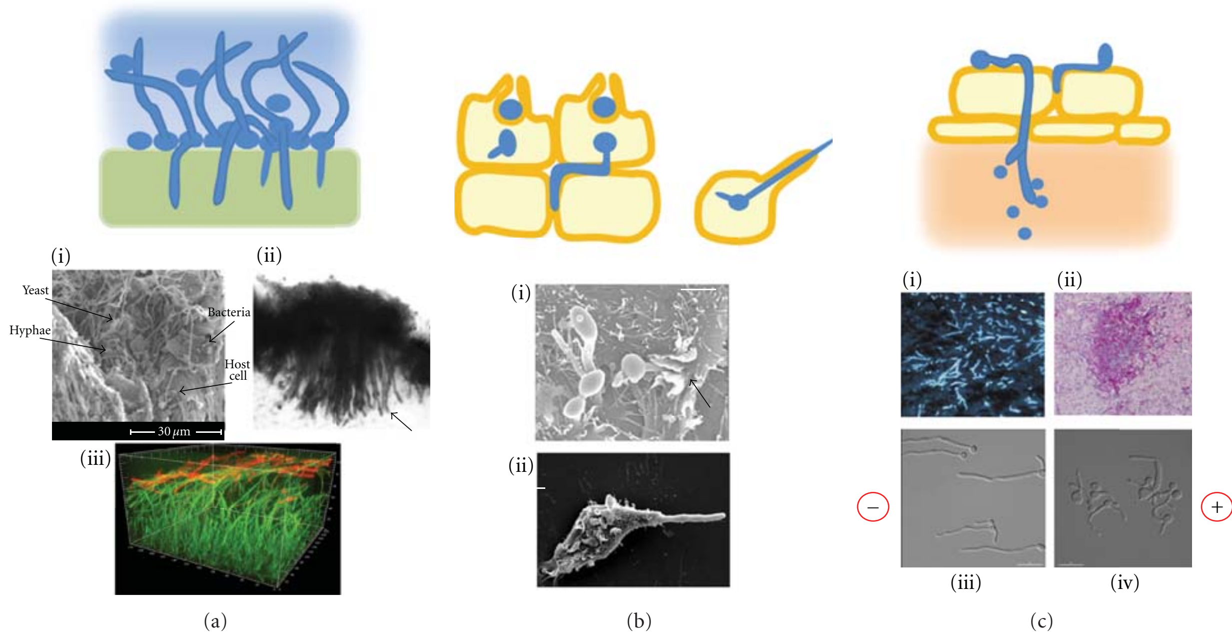


Figure 2: Models and examples of hyphal invasion *in vivo*. (a) Biofilms form on mucosal and abiotic surfaces by initial adhesion of yeast cells, followed by hypha germination and the deposition of extracellular matrix polysaccharides (blue). On mucosa or soft silicones, hyphae penetrate the underlying layers. (i) Rat denture biofilm formation after 48 h [37]. (ii) Hyphae penetrate the silicone of a voice prosthesis, causing it to expand and stiffen [7]. (iii) *In silico* construction of a biofilm showing the thick hyphal matrix (green) and leading edge of β-glucan deposition at the hyphal tips (red) [38]. (b) Induced uptake of yeast and newly germinating hyphae by epithelial cells or phagocytosis by macrophages is followed by sustained polarised growth, which breaches the host cell plasma membrane and permits the escape of the fungus. (i) *C. albicans* hyphae are engulfed by epithelial cells during induced uptake [39]. (ii) *C. albicans* avoids being killed by a macrophage by undergoing morphogenesis and breaching the macrophage membrane [40]. (c) Active penetration of endothelial cells and reversion to yeast growth in the tissue below. (i) Biopsy of murine lung with invasive aspergillosis, showing septate hyphae stained with blancophor [41]. (ii) Histological section of murine kidney showing *C. albicans* lesion containing yeast, hyphae, and infiltrate of neutrophils (courtesy D. MacCallum). (iii) Galvanotropism: *C. albicans* wild-type hyphae orient towards the cathode when grown in an applied electric field (10 V/cm) [42]. (iv) All tropic responses were abolished in the *rsr1*Δ mutant, which was attenuated in virulence. Cell polarity was maintained, but hyphal tip directionality was erratic [42].

the underlying cell layers did not occur. Sustained hyphal growth is, therefore, important for the endocytic route of tissue invasion in *C. albicans*, and Eed1 may also be required for escape from host macrophages after phagocytosis (Figure 2(b)(ii)).

This finding underscores a fundamental difference between the mechanisms used by pathogens that undergo morphogenesis and those that infect solely as yeast, such as *Cryptococcus neoformans*. The dissemination of *C. neoformans* from the lung also occurs via induced uptake, but the organism has an alternative way of escaping from the host cell. Unlike *C. albicans*, it is able to modify the intracellular environment of the host cell and proliferate until it finally causes host-cell lysis or is expelled from it by nonlytic exocytosis [62, 63]. Induced uptake appears to be a common strategy for the first step of translocation within the host, but pathogenic fungi have alternative mechanisms for breaking free from the host vehicle. In hypha-generating fungi, the second stage of invasion is one of morphogenesis followed by the active penetration of host cell membranes by hyphal tips (Figure 2(c)). This enables the fungus to establish fungal masses in the underlying matrix of solid organ tissue where, compared to the bloodstream, infiltrates of host immune cells find it relatively difficult to access the invading fungus (Figures 2(c)(i) and 2(c)(ii)).

3. The Physical Properties of Hyphae

3.1. Morphogenesis and Morphology. Reversible morphogenesis offers fungi a choice between two lifestyles within the host. When cells divide as yeast, a mother cell and her daughters remain confined at a single site and must compete with each other for nutrients. If nutrients are scarce, the formation of hyphae allows new cells to be produced sequentially by expansion at the tip. The bulk of the mother-cell cytosol, which contains most of the elements required to generate new cells, is pushed forward by turgor pressure coupled with expansion of the vacuoles positioned sub-apically [64]. Thus, the tip cell actively extends while the sub-apical cells lie dormant until new nutrients are assimilated. In *C. albicans*, each cell compartment is approximately 20 μm long, so, after 5 cell divisions, the fungus can potentially cover a distance of 100 μm. This is more than enough to escape from a phagocyte, anchor within a cell layer, or penetrate endothelia and reach the solid organs below. Once a more favourable

environment is reached, the fungus can choose to revert once again to growth as yeast.

Morphogenesis from yeast or conidia is stimulated by a perceived change in the environment. For *A. fumigatus*, the signal for germination might be the presence of moisture in the lung. For *Malassezia*, it is thought to be the sensing of lipids. In *C. albicans*, morphogenesis has been studied extensively *in vitro*. It requires the integration of multiple signalling pathways involved in the sensing of ambient conditions, such as pH, temperature, and nitrogen availability (see reviews by [18, 19]). The complexity of these inputs reflects the unusually wide variety of host environments this fungus is capable of colonising. Signals act on the master regulators of morphogenesis, activators Cph1 and Efg1, and suppressors, Tup1 and Nrg1. The *efg1*Δ/*cph1*Δ double mutant has been widely used to study the role of morphogenesis in virulence and host responses because it does not produce hyphae neither does it express hypha-specific genes (HSGs) that are co-regulated with morphogenesis, making it difficult to pinpoint the key features of morphogenesis that are required for virulence. Early comparative expression studies revealed that several HSGs encode surface proteins that are involved in adhesion or host interactions and are essential for full virulence: Als3 is an adhesin and invasin, Hyr1 is involved in interaction with neutrophils, and Hwp1 delivers strong adhesion properties because it is a substrate for cross-linking to extracellular matrix by host transglutaminase [65, 66]. Other transcription factors that lie downstream of the Efg1/Cph1 master regulators have been identified as regulators of subsets of HSGs under defined *in vivo* conditions. These include Ume6, a master regulator of hypha-specific genes, Czf1 (embedded growth), Bcr1 (biofilm maturation), Eed1 (escape after endocytosis), and Hgc1, which suppresses cell separation and is expressed at the hyphal tip only [39, 67–72]. Analyses of temporal and spatial gene expression during infection, coupled with studies of physical changes induced by the environment in other fungi, suggest that a combination of site-specific and hypha-specific gene expression is likely to produce hyphae with subtly different properties [73, 74]. It is possible that some of the HSGs of unknown function are involved in modulating the structural status of hyphae. For example, *C. albicans* doubles the chitin content of the cell wall during hyphal growth, suggesting that increased rigidity is required, and other changes such as turgor pressure or the degree of cell wall crosslinking, could be important for the fungus to meet niche-specific challenges.

Morphogenesis has a significant consequence for the fungus because it exposes surface molecules that alert the immune system to its presence. The primary mechanism is through the detection of pathogen-associated molecular patterns (PAMPs), microbe-derived molecules that are recognised as nonhost by phagocytes. PAMPs that are derived from fungi include cell wall polysaccharides (galactomannan and galactofuranose in *A. fumigatus*; β-glucans, chitin phosphomannan in *C. albicans*), fungal surface proteins, secreted fungal enzymes and their breakdown products, and ATP released during host-cell lysis [31, 40, 75–77]. During epithelial colonisation by *C. albicans* and *Malassezia* yeasts, the fungal PAMPs that would otherwise induce an inflammatory response are masked from the immune system or simply not generated by the yeast form. *A. fumigatus* conidia are contained within a hydrophobic coat of RodA fibrils. Although taken up and cleared by macrophages, the inflammatory response is not activated unless conidia swell and germinate, when RodA is degraded and the cell wall polysaccharides are exposed [78, 79]. Mucosal defence at most body sites is mediated by epithelial cells and macrophages, which specifically recognise hyphae [80–82]. On detection of hyphal PAMPs via Dectin-1 and other receptors, cytokine signalling activates a group of proteins called the inflammasome, which is expressed within mucosal macrophages and dendritic cells [83]. The inflammasome processes and releases IL-1β, which recruits T cells and neutrophils, the key line of defence against *C. albicans* and *A. fumigatus* via phagocytosis and the deployment of neutrophil extracellular traps (NETs) (Figure 1(e)) [16, 84–86]. Thus, morphogenesis allows the fungus to be "seen" by the innate immune system so is only a virulence factor if host immunity is somehow defective.

3.2. Directionality. The hyphal tip is of particular importance because it controls the direction of new growth in response to the environment, steering the hypha around obstacles or towards nutrients [87]. In plants, the direction of polarised cell growth is determined by specific cues from the environment, such as light or gravity, which elicit pre-programmed directional responses, or tropisms. In fungi, contact-dependent growth behaviour, or thigmotropism, has been studied in some detail in plant pathogens. Growing hyphal tips are able to detect defined topographical features on the host leaf, enabling them to locate host penetration sites. For example, the hyphae of *Cymodothea trifolii* follow depressions at the cell boundaries of its host, white clover, because the stomatal pores are located at cell junctions [88]. A different growth strategy has evolved in *Uromyces appendiculatus*, a rust fungus whose host bean plant arranges its stomata transversely across the leaf. Here the hyphae grow perpendicularly to leaf depressions to maximise the chance of finding a penetration site. These responses can be induced on inert surfaces that topographically mimic the host, demonstrating that hyphae can sense surface topography through thigmotropism [89]. Are fungal tropic responses important for disease progression in humans? *A. fumigatus* has been termed "angiotropic" because its hyphae readily find their way into pulmonary blood vessels [90]. However, it is not clear whether they are actively responding to chemical or topographical clues and how this tropism is relieved when the fungus exits the bloodstream. The growth behaviour of *T. mentagrophytes* and *M. furfur* in the stratum corneum has been described as "meandering," but this does not mean that the growth direction is random as it could be defined by local signals such as adhesion molecules. Other topical fungi, such as *T. violaceum*, *T. glabrum*, and *Microsporum gypseum*, specifically invade hair follicles, so are potentially following chemotropic gradients that define this site [91, 92]. Studies of tropisms in *C. albicans* and *C. dubliniensis* have shown that growth around small obstacles (thigmotropism) can be elicited in a calcium-dependent manner [87, 93–95]. However, no chemotropic growth has been observed in response

to cAMP or retinoic acid, which can mediate growth behaviour in mammalian cells, although polarised mating projections do respond chemotropically to mating pheromone (Brand, unpublished data) [96]. Instead, pathogenic fungi seem to be hard-wired to penetrate any substrate they are in contact with, since hyphae in biofilms formed on inert silicones invade the abiotic material despite the apparent lack of extrinisic biological signals [7]. *C. albicans* responds to other external stimuli *in vitro*, displaying aerotropism under hypoxic conditions and galvanotropism, where hyphae germinate and orient towards the cathode in an applied electric field (Figure 2(c)(iii)) [94, 97, 98]. Galvanotropism is also calcium dependent in *C. albicans* and is a common feature of many types of polarised and migratory mammalian cells [99, 100]. These tropisms are potentially relevant to hyphal guidance within the host but are difficult to isolate in the complex *in vivo* environment. An exception is contact-dependent sinusoidal and helical growth, which occurs *in vivo* during onychomycosis when hyphae are embedded in the keratinised nail, and can be replicated *in vitro* in nutrient-poor, high-strength agar (4–6%) or on cellophane [36]. Helical growth is also observed in *Aspergillus* spp. that invade soft contact lenses and, therefore, could be a general hyphal response to embedding in a dense, dehydrated matrix [8]. While further tropic responses to *in vivo* stimuli have yet to be identified, normal regulation of hyphal tip directionality does seem to be involved in tissue penetration. In the *C. albicans rsr1*Δ mutant, where polarised growth is maintained but the direction of tip growth is erratic, hyphae are insensitive to all *in vitro* tropic stimuli (Figure 2(c)(iv)). Their ability to penetrate oral epithelial cells was reduced by 50%, and they were not able to cause cell damage compared to the control strain [42]. A refinement of this study confirmed that Rsr1 is required for damage to an epithelial monolayer but is not required for damage in deeper cell layers [44]. This suggests that a hyphal tip must orient correctly against the outer host surface to achieve initial cell penetration, but, once embedded, directional control is not required within a tissue matrix. This and other studies demonstrate that hyphal steering can be uncoupled from polarised growth, but there is no straightforward correlation between the loss of response to tropic stimuli that generate responses *in vitro* and avirulence *in vivo*.

3.3. Force and Adhesion. Directional growth has to be coupled with mechanical force if hyphae are to push the surrounding matrix out of the way or to penetrate physical barriers. The hyphae of *A. fumigatus* and *C. albicans* are clearly capable of both feats within the host, yet little work on the biomechanics of tissue penetration in mammalian hosts has been carried out. More is known about the penetration of leaf cuticles by plant fungal pathogens. The waxy coating of the leaf is sufficiently tough to require the formation of specialised structures called appressoria, from which a penetration peg emerges to pierce the host leaf below. Two conditions must be in place for this to be successful. First, the appressorium must generate a high internal turgor pressure, which can be up to 5.85 MPa (58 atm) [101]. Secondly, the appressorium must become sufficiently anchored to the host leaf so that the penetration peg enters the plant rather than pushing the appressorium away from it. Thus, adequate adhesion to the host is required before the necessary pressure can be exerted by the fungus. This observation may explain why hyphae need to be so much stickier than yeast and why many of the genes upregulated during morphogenesis in *C. albicans* are related to adhesion. Although yeast cells adhere to the host perfectly well, their complement of adhesins may simply not be able to deliver the anchorage required during the application of hyphal tip pressure to an obstacle. The generation of sufficient adhesion by emerging hyphae may be a time-dependent process, where the number of crosslinks formed with the host increases with hypha length. This in turn could be influenced by whether the hypha is growing on a two-dimensional surface (low surface area of contact) or in a three-dimensional matrix (hypha surrounded by contact points). The need to establish multiple adhesion sites may be why active penetration of the host is not seen in the early stages of tissue invasion [44].

A further aid to applying force is turgor pressure, which must be accompanied by wall loosening at the tip so that the turgor pressure generated can be used maximally to apply tip pressure against an object [73]. The turgor pressure of hyphae has been determined in very few fungal species. In *Achlya bisexualis* and *Armillaria gallica* hyphae, it was found to be 0.6–0.8 MPa (6–8 atm), and the latter was able to apply a tip pressure of 17% of the turgor pressure against an object [73, 102]. In a rare study of force applied by a human pathogen, the oomycete *Pythium insidiosum*, it was calculated that the pressure generated by the hyphal tip was 0.3 MPa (3 atm). This was sufficient to drive a fungal tip through a stiffened 8% agar matrix (0.1 MPa or 1 atm) but was one hundred-fold less than that required to penetrate intact human skin [103]. Similar calculations have been undertaken for plant pathogens, and together the evidence indicates that host tissue penetration is likely to involve a combination of hyphal tip pressure and the hydrolytic activity of fungal exoenzymes [103, 104]. Turgor pressure and the secretion of wall tensioning and degradative enzymes must be coordinated at the site of growth to promote tissue penetration. Given the large number of hydrolytic enzymes that are secreted by hyphae, any study into which of them are involved in aiding tissue penetration, or whether their importance varies by body site, would require the isolation and analysis of apical-cell gene expression, which is no trivial task.

4. Future Perspectives

Although there are alternative routes to fungal virulence, many chronic and acute fungal infections involve the formation of hyphae during all or part of the infection process. The inbuilt propensity of hyphae to drill down into a substrate seems straightforward, but we are finding that many aspects of morphogenesis and hyphal growth behaviour are subtle and have yet to be explained. How are hyphal tips guided and controlled? What are the tropic mechanisms by which *A. fumigatus* enters and exits the bloodstream? How can tissue invasion by hyphae be separate from tissue damage?

Are the structural properties of hyphae regulated differentially depending on body site? To understand how hyphae behave in the host environment, we will need to combine genetic approaches with cell physics. As the genome sequences and molecular tools become available in *Malassezia* and dermatophytes, about which we know relatively little, we should be able to generate a more global understanding of how hyphal growth is deployed against the host.

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Review Article

Invasive Mold Infections: Virulence and Pathogenesis of *Mucorales*

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Mucorales have been increasingly reported as cause of invasive fungal infections in immunocompromised subjects, particularly in patients with haematological malignancies or uncontrolled diabetes mellitus and in those under deferoxamine treatment or undergoing dialysis. The disease often leads to a fatal outcome, but the pathogenesis of the infection is still poorly understood as well as the role of specific virulence determinants and the interaction with the host immune system. Members of the order *Mucorales* are responsible of almost all cases of invasive mucormycoses, the majority of the etiological agents belonging to the *Mucoraceae* family. *Mucorales* are able to produce various proteins and metabolic products toxic to animals and humans, but the pathogenic role of these potential virulence factors is unknown. The availability of free iron in plasma and tissues is believed to be crucial for the pathogenesis of these mycoses. Vascular invasion and neurotropism are considered common pathogenic features of invasive mucormycoses.

1. Introduction

The *Mucorales*, which is the core group of the traditional *Zygomycota* [1–3], have been recently reclassified into the subphylum *Mucoromycotina* of the *Glomeromycota* phylum of the kingdom Fungi [4]. This new classification does not include *Zygomycota,* because the authors consider the phylum polyphyletic, indeed the name zygomycosis, which encompassed infections caused by members of *Mucorales* and *Entomophthorales*, has become obsolete [4]. The *Mucorales* are characterized by aseptate (coenocytic) hyaline hyphae, sexual reproduction with the formation of zygospores, and asexual reproduction with nonmotile sporangiospores. They are ubiquitous in nature, being found in food, vegetation, and soil [1–3]. The majority of the invasive diseases are caused by genera of the *Mucoraceae* family, and the resulted disease is called mucormycosis [1–3, 5–7]. Transmission occurs by inhalation of aerosolized spores, ingestion of contaminated foodstuffs, or through cutaneous exposure, the latter being the most important mode of acquisition of mucormycosis in immunocompetent hosts [6, 8]. Risk factors for invasive diseases include uncontrolled diabetes mellitus, haematological malignancies, bone marrow and solid organ transplantation, deferoxamine therapy, corticosteroid therapy, or other underlying conditions impairing the immune system [9]. Limited activity of some principal classes of antifungal drugs (i.e., echinocandins and azole derivatives) as well as vascular invasion and neurotropic activity could explain the high mortality seen in mucormycosis [9, 10]. This paper, together with others published in this special issue, reviews the clinical spectrum of and risk factors for mucormycosis with particular emphasis on the role of fungal traits interacting with human host defences.

2. Epidemiology

A few members of the *Mucorales* (Table 1) are able to grow in human tissues causing a wide spectrum of clinical diseases. The entity and severity of the disease depends on the interaction between the fungus and the host immune

Table 1: Agents[a] of mucormycosis belonging to *Mucorales* order of the *Glomeromycota* phylum.

Order	Family	Genus	Species	Maximum growth temp (°C)
Mucorales	*Mucoraceae*	*Rhizopus*	*oryzae*	>37°C
			microsporus	>37°C
			azygosporus	>37°C
			schipperae	>37°C
		Mucor	*circinelloides*	>37°C
			indicus	>37°C
			racemosus	32°C
			ramosissimus	36°C
		Rhizomucor	*pusillus*	>37°C
		Lichteimia (*Absidia*)	*corymbifera*	>37°C
		Apophysomyces	*elegans*	>37°C
	Cunninghamellaceae	*Cunninghamella*	*bertholletiae*	>37°C
	Saksenaeaceae	*Saksenaea*	*vasiformis*	>37°C
	Syncephalastraceae	*Syncephalastrum*	*racemosum*	>37°C

defences [5, 7, 11]. In their exhaustive review, Roden and coworkers analysed 929 cases of documented infections caused by members of the former *Zygomycota* since 1885 [9]. They found that 19% of patients did not have any underlying disease at time of infection, while diabetes (36% of cases) was the main risk factor for developing the infection among patients with underlying conditions [9]. More recently, 230 cases of infections were collected in 13 European countries between 2005 and 2007 [12]. The majority of patients (53%) had haematological malignancies (44%) and haematopoietic stem-cell transplantation (9%) as underlying conditions, while only 9% of patients presented diabetes mellitus as the main risk factor [12]. *Rhizopus* spp. are the most common causative agents of invasive mucormycosis, *Mucor* spp. and *Lichteimia* (formerly *Absidia*) spp. rank as second and/or third cause [6–9]. Although mucormycosis remains a highly fatal disease, its burden is still low, as well documented by Pagano and coworkers [13]. They were able to demonstrate that mucormycosis affected about 0.1% of 11,802 patients with hematologic malignancies. Among the 346 cases of proven and probable mold infections, only 14 (4%) were caused by members of *Mucorales* [13]. In immune-competent subjects, mucormycosis generally develops as a consequence of traumatic injuries, and the disease commonly involves skin even if possible dissemination from skin to contiguous organs can occur [9, 11].

3. The Infection

Mucormycosis can be classified in rhinocerebral, pulmonary and disseminated, abdominal-pelvic and gastric, and cutaneous or chronic subcutaneous diseases. Common features of rhinocerebral, pulmonary, and disseminated diseases include blood vessel invasion, hemorrhagic necrosis, thrombosis, and a rapid fatal outcome.

Rhinocerebral mucormycosis is more often associated with uncontrolled diabetes mellitus and ketoacidosis than malignancies or deferoxamine therapy. Inhaled spores colonize at first the upper turbinates and paranasal sinuses and cause sinusitis. Depending on the underlying disease, the fungus can rapidly invade the central nervous system, causing symptoms like an altered mental state, progression to coma, and death within a few days [1–3, 5–11].

Pulmonary mucormycosis is commonly seen in patients with leukemia, lymphoma, solid organ or bone marrow transplantation, and diabetes but is occasionally reported also in apparently healthy subjects. Disease manifestations vary from a localized nodular lesion to cavitary lesions and dissemination; in the latter case, massive hemoptysis generally occurs. Crude mortality is lower (60%) in cases of isolated lesions than in severe pulmonary (87%) and disseminated (95%) diseases [9].

Gastrointestinal disease is a rare manifestation of mucormycosis, and it is mainly associated with malnutrition in presence of predisposing factors, especially in children with amoebic colitis, typhoid, and pellagra [11]. In the most severe cases, the disease can be characterized by ulceration of the mucosa and invasion of blood vessels with subsequent production of necrotic ulcers, this form of the disease is fatal [3, 11].

Cutaneous mucormycosis may be a primary disease following skin barrier break or may occur as a consequence of hematogenous dissemination from other sites, and the outcome of the disease is strictly dependent on the patients' conditions. Primary cutaneous mucormycosis can involve the subcutaneous tissue as well as the fat, muscle and fascial layers [3].

4. Treatment

Treatment of mucormycosis combines surgical intervention and antifungal therapy. Liposomal amphotericin B is the drug of choice for the therapy of mucormycosis. The *in vitro* susceptibility testing for amphotericin B gives a broad

range of values according to the genus and the species. With the exception of posaconazole, the azole derivatives show a limited *in vitro* activity against *Mucorales*, and the echinocandins have a limited activity against these fungi [14]. Studies of *in vitro* combination of posaconazole with amphotericin B showed synergistic effects against hyphae of some species [15]. In addition, combination therapy with liposomal amphotericin B plus caspofungin or posaconazole and posaconazole with colony-stimulating factor has been successfully used in experimental infections [10, 16–18]. In humans, combination therapy (liposomal amphotericin B plus echinocandins or posaconazole with or without iron chelation) has been used as aggressive antifungal treatment following surgical resection of the damaged tissue [19–23]. Patients treated with combination of antifungal drugs had a better survival outcome than those treated with amphotericin B alone [20, 21]. A promising therapeutic approach consists of the use of iron chelation. Although deferoxamine therapy is associated with a high risk to develop mucormycosis [2, 3, 5–7, 9–11, 24], newer iron chelators (deferiprone and deferasirox) have not been associated with increased risk of mucormycosis and have been used as therapeutic agents in cases of experimental mucormycosis [24].

5. Virulence Traits and Pathogenesis

According to Casadevall and Pirofski [25]: "*Quantitative and qualitative measures of virulence vary as a function of host factors, microbial factors, environmental factors, social factors and interactions amongst them*". This concept is especially true if we consider opportunistic microorganisms such as fungi. Macrophages and neutrophils play the major role in immune defence against agents of mucormycosis. Prolonged neutropenia is thus the main risk factor for developing the disease. Moreover, therapeutic interventions (i.e., corticosteroid therapy), that cause functional defects in macrophages and neutrophils, represent additional risk factors for mucormycosis. Diabetes itself can impair the function of neutrophils contributing to the severity of the mucormycosis in patients with ketoacidosis [26]. An important protective factor against mucormycosis is the low concentration of free iron in plasma and tissues. Many of the underlying diseases listed above as predisposing factors for developing mucormycosis share an iron overload as a consequence of iron tissue burden, elevated serum transferring, or increased nontransferrin-bound iron [24]. Iron is essential for *Mucorales* either enhancing their growth and hyphal development *in vitro* or increasing their pathogenicity *in vivo* [27]. Hemodialysis patients under treatment with deferoxamine (DFO), an iron chelator, are particularly at risk for mucormycosis, and Boelaert and coworkers [28] reported a high mortality (89%) in 46 patients who developed severe mucormycosis during DFO treatment. The same group [27, 29] was able to demonstrate that *Mucorales* use DFO as a xeno-siderophore, being capable to detach iron from DFO in a very efficient manner. More recently, other investigators confirmed the importance of iron in the pathogenicity of *Mucorales* by studying the expression of the FTR1 (high-affinity iron permease of *R. oryzae*) gene and its product [30]. The authors were able to demonstrate the effect of gene disruption and gene silencing on *R. oryzae*, which was unable to acquire iron *in vitro* and showed a reduced virulence in mice. Consistently, anti-Ftr1p antibodies protected mice from *R. oryzae* infection [30]. Angioinvasion with subsequent infarction of the surrounding tissue is uniformly present in all cases of severe disseminated mucormycosis [31]. Specific adhesion to endothelial cells and internalization of the fungus by the endothelial cells are important for the pathogenic strategy of *Mucorales* [32]. More recently, Liu and coworkers [33] demonstrated that a novel host receptor (the glucose-regulated protein 78 [GPR78]) facilitates the invasion of human endothelial cells by *Rhizopus oryzae*. This study demonstrated that in the presence of high iron and glucose concentrations, such as in diabetic subjects, there is a direct relationship between an increased expression of GPR78 and an increased damage to endothelial cells in diabetic mice [33]. Involvement of the CNS is common in invasive mucormycosis, *Mucorales* are capable to gain access to the central nervous system (CNS) by local vessels invasion or direct extension from paranasal sinuses [1–3, 5–11]. Another possible mechanism, involving a retrograde extension of the fungi into CNS by means of the nerves, was hypothesized by Frater and coworkers [31]. By evaluating the histologic features of 20 patients with invasive disease, they found a high percentage of perineural invasion. A further fascinating hypothesis concerning the virulence of *Mucorales*, in particular of *Rhizopus* species—the most common etiological agents of disseminated mucormycosis—is a possible involvement of endosymbiotic bacteria in the pathogenesis of the disease [34]. The authors formulate their hypothesis on the basis of the ability of *Rhizopus* species to live with endosymbiotic toxin-producing bacteria [35] and of the existing link between emergence of mucormycosis and the increased drug resistance of Gram-negative bacteria seen in the recent decades. Later on, both the groups of researchers demonstrated that endosymbiotic toxin-producing bacteria were not essential for the pathogenesis of mucormycosis [36, 37]. Other potential virulence factors of *Mucorales* could be proteolytic, lipolytic, and glycosidic enzymes as well as metabolites like alkaloids or mycotoxins as agroclavine. However, their direct involvement in human cases of mucormycosis has been still to be documented [3].

6. Diagnosis

Histology and culture are still the most important diagnostic approaches for mucormycosis because of the lacking of molecular diagnosis methods standardized or commercially available. Moreover the β-1–3 glucan detection is not useful in this kind of infection due to the extremely low content of this molecule in the *Mucorales* [38, 39]. Timely diagnosis of invasive mucormycosis is essential due to the rapid progression of the disease, and because signs and symptoms of the infection could mimic other invasive fungal infections. Tissue biopsies are the clinical specimens of choice and

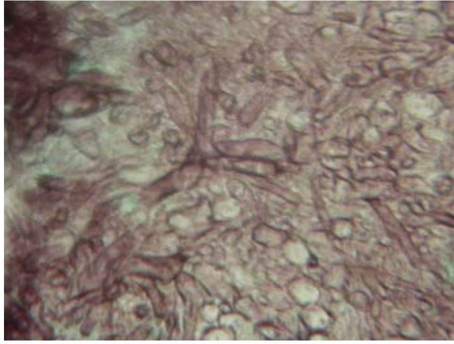

Figure 1: Aseptate hyphae with wide branching angles and large diameter from a lung fungus ball suggestive of mucormycosis (GMS stain 400×).

should be submitted to histopathological and microbiological examination. When cultures are performed, it should be remembered that slicing rather than grinding of the samples should be adopted, because grinding could result in the loss of viability due to the coenocytic characteristics of the mycelium. Microscopic detection of aseptate or pauciseptate hyphae with a large diameter and wide branching angles is suggestive of mucormycosis (Figure 1). Histopathological examination of the infected tissues reveals inflammatory response, often entirely filled with neutrophils, invasion of arterial and venous walls (angioinvasion) with subsequent infarction, and perineural invasion [31].

7. Conclusion

Invasive mucormycosis is an important cause of morbidity and mortality in patients with impaired immune defence and severe underlying diseases. In immunocompromised or debilitated patients, the disease is rapidly progressive, refractory to antifungal therapy, and often cause of death. Several characteristics of *Mucorales* have been involved in the pathogenesis of the infection as potential virulence factors, but a trait that can be considered a specific determinant of virulence has not been defined yet. Angioinvasion, neurotropism, and iron uptake are common characteristics of *Mucorales* that trigger diseases in humans. Many open issues remain to be clarified on the interaction between members of the *Mucorales* order and the host immune response. Different therapeutic approaches, especially the combination therapy, seem to have a promising impact on the clinical outcome of this infection. However, the development of the most severe forms of mucormycosis and the subsequent outcome is strictly dependent on the efficiency of the host immune system.

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Review Article

Application of Bioluminescence Imaging for *In Vivo* Monitoring of Fungal Infections

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Fungi can cause severe invasive infections especially in the immunocompromised host. Patient populations at risk are increasing due to ongoing developments in cancer treatment and transplantation medicine. Only limited diagnostic tools and few antifungals are available, rendering a significant number of invasive fungal infections life threatening. To reduce mortality rates, a better understanding of the infection processes is urgently required. Bioluminescence imaging (BLI) is a powerful tool for such purposes, since it allows visualisation of temporal and spatial progression of infections in real time. BLI has been successfully used to monitor infections caused by various microorganisms, in particular bacteria. However, first studies have also been performed on the fungi *Candida albicans* and *Aspergillus fumigatus*. Although BLI was, in principle, suitable to study the infection process, some limitations remained. Here, different luciferase systems are introduced, and current approaches are summarised. Finally, suggestions for further improvements of BLI to monitor fungal infections are provided.

1. Introduction

Bioluminescence imaging (BLI) is a noninvasive technique that can be used to track microorganisms in living animals. To use this technique cells are generated that emit light from enzyme-catalysed oxidation reactions. Light emission is detected by highly sensitive charged coupled device (CCD) cameras that allow the collection of single photons [1]. The light-generating enzymes are generally called luciferases, although several different luciferases from a wide variety of organisms and without structural relatedness are known [2]. Nevertheless, all characterised luciferases have in common that they need oxygen for the light-emitting reaction but use different substrates and cofactors and emit light at different wavelengths. A short summary of key features of some frequently used luciferases that will be described in more detail is shown in Table 1.

BLI has been used to monitor gene expression and disease progression caused by pathogenic bacteria [3]. Furthermore, the system has also been applied to monitor growth of implanted tumour cells for investigating the effectiveness of therapeutic approaches [4, 5]. In terms of eukaryotic microorganisms such as fungi, bioluminescence imaging has mainly been used for highly sensitive monitoring of gene expression [6, 7], whereas infection studies on fungi are basically limited to the dimorphic yeast *Candida albicans* [8, 9] and the filamentous ascomycete *Aspergillus fumigatus* [10, 11]. These investigations have shown that BLI can be used as a powerful tool to study the infection process, but a fine-tuning of the system is required to increase its suitability. Thus, this paper summarises the key features of different luciferase systems, reports on the results obtained for fungal infections, and provides suggestions for future applications and improvements.

2. Bacterial Luciferases

For the generation of bioluminescent bacteria generally a *lux* operon from a prokaryotic origin [12] is used. This operon not only harbours genes coding for the heterodimeric luciferase (*luxA* and *B*) with a native mass of approximately 77 kDa [13] but also genes required for the production of the luciferase substrate (*luxC*, *D*, *E*). The latter genes are

Table 1: Key features of selected luciferases from different phylogenetic origins.

Luciferase origin	Organism (family)	Substrate	Cosubstrate	Composition (mass)	Localisation (native)	Peak emission (nm)
Vibrio spec.	Bacteria (*Vibrionaceae*)	Long-chain aliphatic aldehyde	O_2; $FMNH_2$	heterodimer (77 kDa)	Cytoplasm	490
Photorhabdus spec.	Bacteria (*Enterobacteriaceae*)	Long-chain aliphatic aldehyde	O_2; $FMNH_2$	heterodimer (77 kDa)	Cytoplasm	490
Photinus pyralis	Firefly (*Lampyridae*)	Benzothiazoyl-thiazole	O_2; ATP	monomer (62 kDa)	Peroxisome	561–578*
Pyrophorus plagiophthalamus	Click beetle (*Elateridae*)	Benzothiazoyl-thiazole	O_2; ATP	monomer (62 kDa)	Peroxisome	548–594
Renilla reniformis	Sea pansy (*Renillidae*)	Benzylimidazo-pyrazinone coelenterazine	O_2	monomer (35 kDa)	Cytoplasm	480
Gaussia princeps	Copepod (*Metridinidae*)	Benzylimidazo-pyrazinone coelenterazine	O_2	monomer (19 kDa)	Secreted	480

* Peak emission is temperature sensitive and gradually shifts to 612 nm at 37°C [22].

responsible for production of a long-chain aldehyde that is oxidised together with a reduced riboflavin phosphate ($FMNH_2$) for the light-generating reaction [14]. To enable a heterologous expression of the *lux* operon in various bacteria, several optimisation strategies have been applied involving the use of different promoters and codon adaptations as required by the respective host [15]. Such adaptations allowed studying the infection process of several bacteria such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus anthracis, Mycobacterium tuberculosis*, and others [3, 16–19].

Besides using the *lux* operon for virulence studies, it is also used as a reporter system to study promoter activity in bacteria under various environmental conditions. Here, although the reporter system is functional when all genes are located in a single operon, some substrate limitations can occur that limit the linearity of the reporter system. Recent studies have shown that this limitation is circumvented by independent and constitutive expression of the substrate-producing genes *luxC*, *D*, *E* and by only controlling expression of the luciferase-encoding genes by the promoter of interest [20].

Attempts have also been made to use the bacterial system for expression in eukaryotes since the bacterial *lux* operon provides the only system for which the genes for substrate synthesis are known. This allows the production of autobioluminescent cells without the need for external addition of luciferase substrates. However, since eukaryotes need a promoter controlling the expression of each single gene and, additionally, require a codon adaptation of the *lux* genes, studies using *lux* genes in eukaryotes are rather limited. Recently, it was possible to transfect mammalian HEK293 cells with a *lux* cassette harbouring codon-optimised genes linked by internal ribosomal entry sites to limit the number of promoter elements due to bicistronic gene expressions [21]. By this method it was possible to detect a minimum of 20,000 HEK cells *in vitro*. Additionally, after subcutaneous injection it was possible to redetect these *lux* cassette-expressing cells from living mice [21]. However, although this system allows the continuous long-term observation of a cell population without the need for external substrate addition, the system still possesses several drawbacks. Despite the constitutive expression of a flavin reductase gene, the amount of reduced $FMNH_2$ appeared limited in the eukaryotic system since the addition of $FMNH_2$ to the culture medium strongly increased luminescence signal intensity. Thus, although the substrate for luciferase can be autonomously produced by the cell, one of the essential cosubstrates remains limited. Furthermore, the use of these expression cassettes in other eukaryotes such as fungi will need species-specific adaptations involving time-consuming cloning procedures. Additionally it should be noted that, despite synthetic optimisation of *lux* genes for increased expression and production rates in HEK cells, luminescence signal intensity appeared rather low. Thus, combined with the short wave length of light emission (peak emission at 490 nm), this system may have limitations in monitoring cell proliferation from deeper tissues.

3. Beetle Luciferases

Studies using luciferases in research on eukaryotes frequently use enzymes that derive from luminous beetles (*Lampyridae*) or click beetles (*Elateridae*). The most prominent examples are the firefly luciferase from *Photinus pyralis* and the luciferase from the click beetle *Pyrophorus plagiophtalamus* [23]. Luciferases from these beetles are monomeric enzymes with a molecular mass of approximately 62 kDa and a protein sequence identity of around 50%. Additionally, the native protein sequences from both species display a C-terminal peroxisomal import sequence (SKL) for targeting to specialised peroxisomes of photocytes in the beetle lantern organ. Due to this targeting sequence, luciferases are also transported to peroxisomes of other eukaryotes as shown for mammalian cells, yeast, and plants [24, 25]. Although fireflies and click beetles are only distantly related, luciferases from both families use the same substrate, which is a heterocyclic water soluble acid (benzothiazoyl-thiazole) generally called luciferin. Luciferin is oxidised in an ATP-dependent manner leading to oxyluciferin, AMP, CO_2, and

light. The quantum yield of this reaction was determined as 0.88, which is the highest yield for any luciferase-catalysed reaction characterised so far [23]. Click beetle luciferases differ in the wavelength of emitted light ranging from green (548 nm) to orange (594 nm), and this range has been observed from individual beetles implying that different luciferases may be produced within the same insect [26]. In contrast, the firefly *P. pyralis* only produces a single luciferase with a peak intensity of light emission at 561–578 nm [22, 23]. However, it should be noted that a spectral shift to >610 nm (612–617 nm) occurs at pH below 7.5, temperatures above 35°C, addition of denaturants or several heavy metals [22, 27]. Thus, it has been shown that light produced by a firefly luciferase within a mammalian system (body temperature ≈37°C) is red shifted [22]. Although this shift is accompanied by a reduced quantum yield [23], it is compensated by an increase in luciferase activity [22] and, thus, allows a better detection of microorganisms from deep tissues due to reduced light absorption from haemoglobin [28].

Genes for synthesis of luciferin are still unknown, and the external addition of this luciferase substrate is required to obtain bioluminescent signals. To investigate whether this requirement for luciferin supplementation limits the suitability of the system *in vivo*, the distribution of luciferin was studied in a murine system. In a qualitative analysis it was shown that 20 min after intraperitoneal injection luciferin was detectable in tissue homogenates from brain, skin, and liver. Furthermore, the repeated injection of 126 mg/kg of luciferin appeared to have no adverse toxic effect on animals [29]. However, quantitative analyses using either radioiodine labelled [30] or ^{14}C-labelled luciferin [31] showed that distribution velocity and tissue accumulation vary depending on the route of luciferin application (intravenous versus intraperitoneal) and time of measurement after application. Both studies independently showed that luciferin availability especially in brain, muscles, and bones remains at low rates regardless of the application route and may, thus, act as a limiting factor for luciferase activity detection. Nevertheless, although substrate concentrations may be too low to allow substrate saturation for the light-emitting reaction, at least a qualitative assessment of bioluminescence can be envisaged from all targeted organs. Thus, although the number of injections and amount of luciferin applied to individual animals should be kept to a minimum, tracking of an infection at various time points and from different body sites should be possible despite the uneven substrate distribution in living animals.

As mentioned above, beetle luciferases require ATP for substrate oxidation, and this feature can be used to determine minute amounts of ATP released from cell lysates [32]. However, this feature of beetle luciferases may also act as a drawback during *in vivo* measurements. Due to the ATP dependence of these luciferases, not only the substrate luciferin may be limited, but also the ATP concentration, which is dependent on the physiological state of the cell. Thus, co-substrate requirements, ATP in the beetle luciferase, and $FMNH_2$ in the bacterial *lux* system are major disadvantages of these bioluminescence-based reporter systems.

4. Coelenterazine-Dependent Luciferases: Rluc and Gluc

Another type of luciferases frequently used as a reporter in eukaryotic cells oxidises the substrate benzylimidazopyrazinone coelenterazine (short coelenterazine) independent of ATP or $FMNH_2$. Thus, these luciferases do not require a special physiological state of the host cell (despite the presence of oxygen) to act as a reporter and can be measured in cell-free extracts by coelenterazine addition. Two coelenterazine-dependent luciferases with different characteristics are mainly used as reporters: (i) the luciferase from the sea pansy *Renilla reniformis* (Rluc) and (ii) from the copepod *Gaussia princeps* (Gluc).

Renilla luciferase has been purified to homogeneity and biochemically characterized approximately 35 years ago. The enzyme is a monomer of 35 kDa that emits light at 480 nm. Although it is rather stable at elevated temperatures, it tends to irreversibly form inactive dimers and multimers when stored at concentrations exceeding 0.5 mg/mL [33]. Compared to the quantum yield of firefly luciferases, the quantum yield of Rluc with coelenterazine is approximately 10 times lower with only about 6-7%. Furthermore, also the substrate turnover rate appears rather low [33], which is a drawback in terms of sensitivity of the system. In addition, although the light emission at 480 nm is ideally suited to excite the *R. reniformis* green fluorescent protein, light at this wavelength is strongly absorbed by haemoglobin. Nevertheless, since bioluminescence background signals from animals are generally very low, Rluc has been successfully used to generate reporter cells for *in vivo* monitoring of cancer development in murine systems [34–36]. However, it has been reported that the multidrug resistance MDR1 P-glycoprotein (Pgp) efficiently removes coelenterazine from the cell and may, thus, lead to an underestimation of bioluminescence by the Rluc system [37]. In contrast, such a substrate export has not been described for the luciferin of firefly luciferase. It has also been shown that the distribution of coelenterazine is strongly dependent on the route of application. Studies on Rluc-expressing *Trypanosoma brucei* cells showed that the parasites were detected at different body sites depending on coelenterazine injection either via the intravenous or the intraperitoneal route [38]. Thus, although firefly luciferin is also not evenly distributed between different body sites bioavailability appears superior compared to coelenterazine. In addition, coelenterazine tends to autooxidation in the presence of serum albumin which enhances background signals, a phenomenon that is not observed with firefly luciferin [39].

The luciferase from *Gaussia princeps* has several different features compared to Rluc. Gluc is naturally secreted [40] and assumed to keep predators' attention while the copepod escapes. The secretion signal of this luciferase is also active in other eukaryotic cells as exemplified by expression studies in CHO and HepG2 cells [41]. Although, as described for Rluc, native Gluc consists of a monomeric structure, its molecular mass of 19 kDa is significantly smaller than that of Rluc [42]. Furthermore, although coelenterazine is also used as a substrate and light at a wavelength of 480 nm is emitted in

an ATP-independent manner, light intensity is much higher than that of Rluc [43]. The enzyme additionally possesses a very high thermostability retaining more than 50% activity after 30 min incubation at 95°C [44].

The secretion of Gluc has several advantages that may also turn into disadvantages of the system. Due to its high stability, Gluc can be easily identified from culture supernatants, thus enabling to detect intracellular events from the environment [43]. Additionally, the extracellular localisation of Gluc provides an easier access to the substrate coelenterazine that can be limited in Pgp-positive cells by using the intracellular Rluc system. However, the secretion of Gluc also leads to an uncontrolled distribution of Gluc via the blood stream causing elevated background levels and less distinct signals at the site where luciferase-producing cells are located. To minimise the drawbacks of secretion attempts have been made to produce membrane-bound Gluc by fusing the enzyme with the CD8 transmembrane domain and expressing the construct in T cells. This procedure resulted in increased signal intensities of transfected cells and allowed *in vivo* monitoring of T-cell recruitment to implanted cancer cells [45]. Thus, future experiments may favour specific Gluc versions rather than Rluc for *in vivo* imaging.

5. Luciferases as Reporter Systems in Fungi

First successful approaches to produce firefly luciferase in the yeast *Saccharomyces cerevisiae* were published in 1988 [46]. The native cDNA was cloned under control of the alcohol dehydrogenase promoter and allowed a maximum luciferase yield of 10 ng/mL of culture. However, this amount was assumed to be too low for use as a suitable *in vivo* reporter. Thus, different promoters were selected to increase luciferase production rates. Accompanied with optimised culture and assay conditions it was possible to detect luciferase activity from a minimum of 1×10^6 intact yeast cells [47]. However, this sensitivity still appeared too low for using the firefly luciferase system as a suitable reporter for gene expression analyses from intact cells. A search for the problems causing this low sensitivity led to the speculation that the peroxisomal localisation of native firefly luciferase mediated by the C-terminal SKL sequence might limit the availability of substrates and thus causing low light emission. Indeed, removal of the peroxisomal-targeting sequence enhanced light emission of transformed cells by two to three orders of magnitude. In addition, growth speed of cells harbouring the modified luciferase gene was significantly enhanced compared to cells expressing the native gene sequence, indicating that the peroxisomal localisation affected the natural physiology of yeast cells [6]. Subsequently, luciferase assay systems were developed for yeast cells that expressed both, firefly and *Renilla* luciferase at the same time allowing two study two independent cellular responses by BLI [48, 49].

Similar to *S. cerevisiae*, first approaches to use the firefly luciferase as a reporter in the dimorphic yeast *Candida albicans* were only partially successful [50]. Expressions of the firefly luciferase under control of the phase-specific *WH11* promoter yielded no detectable bioluminescence. However, this negative result was attributed to the presence of several leucine residues encoded by CUG triplets, which are translated into serine in *C. albicans* and might have led to a nonfunctional protein [51, 52]. Thus, to generate a bioluminescent reporter system suitable to study gene expression in *C. albicans* the *Renilla* luciferase was used, because its gene does not contain in frame CUG codons. Indeed, use of Rluc led to well-detectable bioluminescence and was suitable to study gene expression of various genes in both cell-free extracts and intact cells [51]. Subsequently, the Rluc system was also used to investigate the effectiveness of a tetracycline-regulated expression system in *C. albicans* [53]. Later, the firefly luciferase was revisited. By exchanging all CUG codons from a vector carrying the firefly luciferase gene designed for expression in mammalian cells it was possible to generate bioluminescent *C. albicans* cells. This firefly luciferase system was suitable for use as a selection marker in the transformation of clinical wild-type isolates without the need for other growth-disturbing markers that could affect cellular physiology [54]. However, although this system worked well for *in vitro* analyses, the system displayed some problems in infection studies as will be outlined below.

Bioluminescent reporters have also been generated for use in filamentous fungi. In a trial for studying the expression of a xylanase gene from *Aspergillus oryzae* in the close relative *Aspergillus nidulans* a luciferase reporter system was used. Unfortunately, no details on the source of luciferase had been provided, and it can only be speculated that a firefly luciferase was used. However, the observed bioluminescence correlated with the expected expression pattern of the xylanase gene and indicated that BLI should be functional also in filamentous fungi [55]. Subsequently, BLI was discovered for investigating circadian rhythms in the ascomycete *Neurospora crassa* by using a firefly luciferase gene with improved codons for expression in mammalian cells [56]. However, initial studies revealed that light emission from intact cells was rather low and efforts were made to increase signal intensity. Thus, a fully codon-optimised synthetic version of the luciferase gene was constructed that emitted high bioluminescence signals when fungal transformants were grown on media supplemented with luciferin. By this method it was possible to follow the circadian expression of clock-controlled genes over several days [7].

In summary, although studies using luciferase systems in fungi are still rather limited, this technique seems to provide a powerful tool to study gene expression from intact cells. Additionally, the use of dual reporter systems, such as a combination of Rluc and firefly luciferase, allows the independent monitoring of gene expression by BLI, because both luciferases are highly specific for their respective substrate.

6. Bioluminescence Imaging in Fungal Virulence: *Candida albicans*

Investigations using BLI for monitoring fungal infections *in vivo* are basically limited to *C. albicans* and *Aspergillus fumigatus*. Despite the small number of investigations,

the detailed investigations on system limitations may help to design reporter systems that could improve the BLI technique also for other fungi.

As mentioned above, Doyle et al. constructed a firefly luciferase for use in *C. albicans* in which all CUG codons of a luciferase gene adapted for expression in mammalian cells were replaced by CUU codons allowing correct translation of the gene into a functional protein [54]. This system was, in principle, suitable to follow the induction of gene expression as shown by the induced expression of the hyphal-specific gene *HWP1* under hyphae-inducing conditions. However, when light emission was studied from constitutively active promoters, it was noted that bioluminescence was significantly lower in hyphae than in yeast cells although cell-free extracts from both cell types revealed similar bioluminescence levels. Thus, it was concluded that the uptake of luciferin might be hampered by the reorganised cell wall in *C. albicans* hyphae [54]. However, the same group also investigated the suitability of the system for studying pathogenesis in mice by BLI [8]. In a vulvo-vaginal infection model the system was suitable to visualise the persistence of *C. albicans* within the vaginal lumen. To allow substrate saturation in these experiments luciferin was directly applied to the vaginal lumen and light emission correlated with the number of *C. albicans* cells. Furthermore, in vulvo-vaginal infected mice a topical treatment with miconazole revealed clearance of the infection as visualised by BLI. However, imaging of systemic candidiasis was hampered by bioluminescence intensities that were too dim to follow pathogenesis, and this was attributed to the formation of hyphae during tissue penetration of *C. albicans* cells.

Although the explanations provided by the author referring to a limited substrate uptake by hyphae sound valid, other reasons could also explain why monitoring of systemic infections failed. First, a much stronger bioluminescence is required when studying infections from deep tissues compared to more superficial infections. This is due to strong light absorption from haemoglobin, and, as a general rule, it can be assumed that light emission intensity is reduced by a factor of 10 by each cm of tissue depth [16]. Although the firefly luciferase gene was codon adapted in terms of the in frame CUG codons, an inspection of the codon adaptation index (CAI) against highly expressed *C. albicans* genes by using the dCAIoptimizer reveals a CAI of only 27%. Thus, translation of the luciferase-coding RNA might have been limited by a shortage of the respective tRNAs. Synthesis of a fully codon-adapted luciferase gene might, therefore, significantly increase protein production rates and the accompanied bioluminescence signal intensity.

Another limitation of the gene sequence utilised could have derived from the peroxisomal-targeting sequence of the luciferase used for constructing the bioluminescent *C. albicans* cells. As outlined above, studies on *S. cerevisiae* showed that light emission is strongly reduced when luciferase is targeted to yeast peroxisomes [6]. Thus, not only the structure of the cell wall, but also the amount of peroxisomes and the available ATP content within these compartments may vary in *C. albicans* hyphae and may limit substrate availability for the luciferase reaction. Thus, the simple removal of the peroxisomal-targeting sequence might have enhanced the suitability of the system for studying systemic infections. At the moment, studies are under way to confirm both hypotheses.

However, due to the described limitations of studying systemic *C. albicans* infection by use of the firefly luciferase, Enjalbert et al. investigated the suitability of a *Gaussia* luciferase for monitoring *C. albicans* infections [9]. Since Gluc is naturally secreted, it was assumed that cellular barriers would not hamper substrate availability. However, to avoid a systemic distribution of Gluc, the gene was fused with the sequence coding for the glycophosphoinositol-linked cell wall protein PGA59. This strategy was similar to that described for trapping Gluc on the surface of T cells [45]. *C. albicans* cells producing the cell-wall-anchored Gluc were compared to *C. albicans* cells expressing Rluc, and the highly superior luminescence intensity of the Gluc system was confirmed. Light emission was in a range that allowed visualisation from intact cells even by the naked eye. Additionally, it was shown that, when using as constitutive expression system, no significant differences were observed between yeast cells and hyphae [9]. This system was suitable to study the progression of infection after subcutaneous, cutaneous, and vaginal infections. In cutaneous infections, coelenterazine was directly added to the abraded skin area, whereas in subcutaneous infections coelenterazine was supplied subcutaneously and in vaginal infections by applying the substrate to the vaginal lumen. By these methods it was ensured that coelenterazine reached the site of infection in sufficient amounts and light intensities correlated well with the fungal burden.

When the cell-wall-bound Gluc system was used to study progression of systemic infections, no satisfying results were obtained. Under *in vivo* conditions the detected luminescence intensities did not exceed background levels although homogenized kidneys incubated with coelenterazine revealed luminescence signals [9]. Several reasons may account for this failure to monitor systemic infections. One major problem, as discussed by the authors, might have derived from the limited distribution of coelenterazine after intraperitoneal injection. This seems reasonable, since a similar observation was made for experiments performed with *T. brucei* in which intraperitoneal or intravenous injection of coelenterazine revealed different results for the localisation of parasites [38]. However, only the intraperitoneal coelenterazine injection had been utilised to study systemic *C. albicans* infections, and it cannot be excluded that an intravenous substrate injection might have been able to track the infection. However, in terms of investigating the dissemination to yet unknown body sites, the problems of substrate availability cannot be neglected. Another problem might have been derived from the light emission wavelength of the selected luciferase. Gluc emits light at a peak wavelength of 480 nm with no emission above 600 nm [43]. Since haemoglobin and tissue absorbance of light are much less pronounced at wavelengths above 600 nm [1], the strong absorption at 480 nm accompanied by an autooxidation of coelenterazine might have hampered the detection of *C. albicans* from deep tissues. Thus, coelenterazine-based

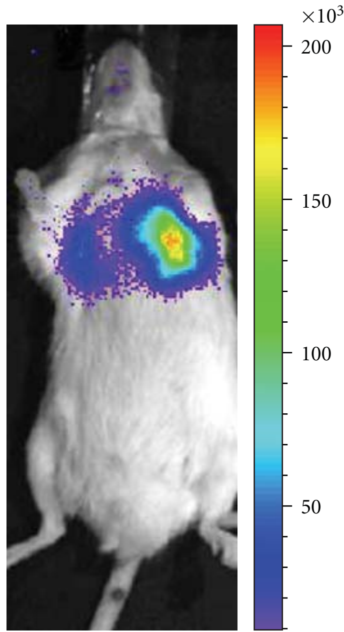

Figure 1: Bioluminescence imaging of invasive aspergillosis. The depicted mouse was immunosuppressed with cortisone acetate and infected intranasally with the bioluminescent *A. fumigatus* strain C3. Bioluminescence was acquired 28 h after infection. Light emission is detected from both lung lobes indicating the establishment of bronchopulmonary aspergillosis. (Figure kindly provided by O. Ibrahim-Granet).

systems might only become suitable reporters for systemic *C. albicans* infections when substrates are developed that allow enhanced tissue distributions and a red shift of light emission.

7. Bioluminescence Imaging in Fungal Virulence: *Aspergillus fumigatus*

BLI has also been investigated for its suitability to monitor progression of *A. fumigatus* infections from living mice. For this purpose, the firefly luciferase codon optimised for expression in mammalian cells but without a peroxisomal-targeting sequence had been used [10]. To obtain strains with a constitutive luciferase production expression of the firefly luciferase was controlled by the glyceraldehyde-3-phosphate dehydrogenase promoter. Indeed, light emission was easily detectable from intact cells by either using a microplate reader or other bioluminescence imaging devices. Since *A. fumigatus* is a filamentous fungus, this observation indicates that the cell wall of hyphae does not necessarily cause problems for intracellular luciferin availability. The system was also suitable to study the antimycotic efficiency of several drugs under *in vitro* conditions.

When tested in a murine model for invasive bronchopulmonary aspergillosis (IBPA) by infecting mice immunocompromised by cortisone acetate, strong bioluminescence signals were obtained from the infected lung already 24 h after infection (Figure 1). Thus, this model provides the first example in which a deep tissue infection of a fungus was successfully monitored by BLI [10]. However, signal intensities strongly declined at later days, and histopathologic analyses showed a severe infiltration of immune effector cells consisting of mainly neutrophils that attacked fungal hyphae. Although subsequent analyses by qPCR showed that indeed the amount of living fungal cells did not increase three days after infection compared to a 24 h time point [11] the decline of the bioluminescence signal was surprising. Additionally, when an antibody-mediated neutrophil depletion strategy was used to render mice susceptible for IBPA, luminescence signals remained rather low and histopathology revealed a rapid and severe infiltration of monocytes to the site of infection.

To explain this phenomenon of decreasing luminescence signal intensity, it needs to be mentioned that all luciferases require dissolved oxygen for the oxidation of the respective substrate. Thus, it was speculated that the severe inflammatory process might cause anaerobic niches within the lung tissue [10]. In addition, within necrotic tissues the distribution of luciferin from the intraperitoneal site may be low causing substrate limitation at the site of infection. These speculations were supported by the fact that lungs removed at necropsy and injected with oxygen-saturated luciferin revealed luminescence signal intensities that correlated well with the expected fungal burden [10, 11].

The limitations of fungal quantification were less pronounced when mice were rendered leukopenic by using the cytostatic drug cyclophosphamide [11]. Under this regimen the amount of fungal biomass is heavily increasing, and no immune effector cells can be recruited to attack hyphae. Quantification of the bioluminescence signals showed that under this immunosuppression regimen signal intensity steadily increased until mice succumbed to infection. Thus, the firefly luciferase system, in principle, appears suitable to study the temporal and spatial progression of infection.

Preliminary analyses have also shown that the bioluminescent *A. fumigatus* strain seems suitable to monitor progression of disseminated disease and signals were easily detected from all infected organs (unpublished results). However, to increase the sensitivity of the system, we are currently constructing and testing different *Aspergillus* species that carry a synthetic firefly luciferase gene adapted to the *A. fumigatus* codon usage (Figure 2). It is expected that the sensitivity from this construct will increase by at least a factor of 10. This increase would be sufficient to track even minute amounts of hyphae from infected tissues and should allow following the efficiency of antifungal therapy under *in vivo* conditions.

8. Conclusion

Different luciferase systems have been established for use in infection studies. The bacterial *lux* operon seems well applicable for studying bacterial infection, but appears less suitable for use in eukaryotic cells. Here, mainly coelenterazine-dependent and beetle luciferases are used, but both systems have limitations.

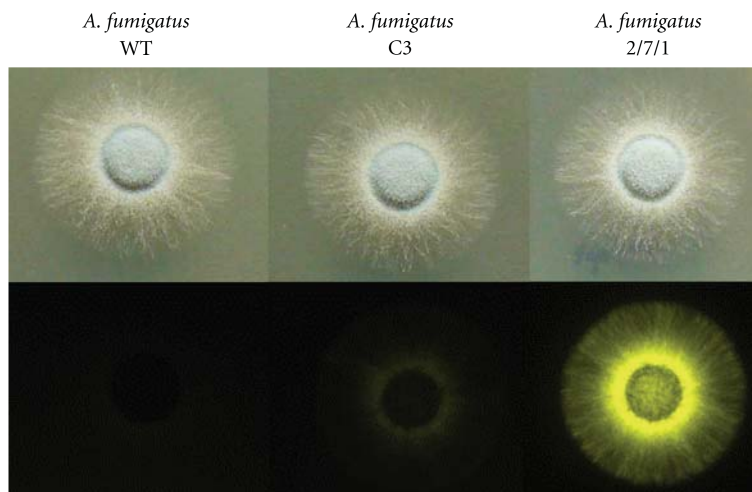

Figure 2: Visualisation of bioluminescence from *A. fumigatus* colonies. The *A. fumigatus* wild-type strain CBS144.89 and the bioluminescent strains C3 and 2/7/1 are shown. Strains were grown for 48 h in the presence of 1 mM D-luciferin on glucose minimal medium. The upper lane shows a daylight photograph, the bottom line bioluminescence images of the colonies acquired by a medium sensitive Versa Doc luminescence imaging system. Strain C3 carries four ectopic integrations of the *P. pyralis* luciferase gene codon adapted for expression in mammalian cells. Only a moderate light emission is detected. Strain 2/7/1 carries two integrations of the *P. pyralis* gene codon optimised for expression in *A. fumigatus*. Light emission is strongly enhanced.

To overcome all problems the optimal bioluminescence reporter system should fulfil the following characteristics: (i) high expression rates, (ii) strong signal intensity with high quantum yield of the reaction, (iii) able to track deep infections with light emission far above 600 nm, (iv) independent from the host response (oxygen independent), (v) produce its own substrate intracellular, and (vi) should not affect physiology of the cell.

Unfortunately, at the moment such an optimal reporter system does not exist. Thus, the best suited system needs to be selected in regard to the scientific question. While superficial infections may be studied by coelenterazine-dependent luciferases, the limited substrate distribution and the short wavelength do not favour its selection for systemic infections. However, especially for gene expression studies in culture media, the Gluc system seems to provide an excellent candidate. Furthermore, besides coelenterazine and dissolved oxygen these luciferases are independent of additional cofactors that need to be provided by the host cell. Beetle luciferases are much larger proteins (three times the size of Gluc) and have been shown, under some circumstances, to affect fungal growth rate. Furthermore, they require cellular ATP as an essential cofactor that needs to be used from the cellular pool. However, light emission of these luciferases is above 600 nm under *in vivo* conditions, and luciferin distribution appears more homogenous. Thus, firefly luciferases may provide a better suited system for *in vivo* monitoring of fungal infections. Nevertheless, subcellular localisation and codon adaptations may play important roles for successfully applying this system to research.

In the future, investigations using BLI with other fungal pathogens will be performed. These studies will help to optimise luciferase reporter systems and will contribute to the understanding of pathogenicity mechanisms. A special feature will derive from the *in vivo* monitoring of the efficiency of antifungals in individual animals. Thereby, BLI will not only reduce the required number of animals but also allow identifying optimised treatment strategies to combat life-threatening fungal infections.

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Review Article

Experimental Models of Cryptococcosis

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Cryptococcosis is a life-threatening fungal disease that infects around one million people each year. Establishment and progression of disease involves a complex interplay between the fungus and a diverse range of host cell types. Over recent years, numerous cellular, tissue, and animal models have been exploited to probe this host-pathogen interaction. Here we review the range of experimental models that are available for cryptococcosis research and compare the relative advantages and limitations of the different systems.

1. Introduction

Cryptococcosis presents in three forms—cutaneous, pulmonary, and meningococcal—and is a life-threatening fungal disease. Although the genus *Cryptococcus* contains more than 50 species of free-living basidiomycete fungi, only two, *C. neoformans* and *C. gattii,* are significant pathogens of humans [1, 2]. Disease is typically caused in the immunocompromised, such as HIV/AIDS or organ transplant patients, and is usually attributable to *C. neoformans*. However, incidences of cryptococcosis in otherwise healthy individuals have been rising, and *C. gattii* is primarily responsible for these cases [2, 3]. Cryptococcal infection is acquired through inhalation of basidiospores or desiccated yeast cells into the lungs, from where cryptococci can potentially disseminate to all organs but with a predilection to the brain [1, 4–8]. Cryptococcal meningitis is estimated to kill 600,000 people annually worldwide, with more than 80% of deaths occurring in Sub-Saharan Africa [9].

In the course of infection through airways to lungs and from lungs to brain, *Cryptococcus* must overcome two major barriers: the innate and adaptive immune mechanisms of the host. The former consists of anatomical or physical barriers such as the mucosal or lung epithelium, the blood-brain barrier of the CNS, and phagocytic cells such as neutrophils, monocytes/macrophages, and dendritic cells. Successful evasion of the host defences results in cryptococcal colonization of host tissues and hence cryptococcosis. To better understand the pathogenesis of this disease, numerous *in vivo* and *in vitro* models have been developed to investigate features of cryptococcosis and address questions such as the course of cryptococcal infection, invasion of cellular barriers, and interactions with—and evasion of—the immune response.

The aim of this review is to present all reported experimental models of Cryptococcosis and summarise recent and/or prolific discoveries using these. This will hopefully provide an evaluation of how different models can aid Cryptococcal research and give food for thought on how current and new models could be utilised in novel ways.

2. *In Vitro* Cellular Models

2.1. Monocytes and Macrophages. The role of monocytes and macrophages in cryptococcosis has been widely studied. Macrophages detect, phagocytose and kill extracellular microorganisms, and present antigen to T cells [10, 11]. These disease factors have been explored using *in vitro* models.

Macrophage phagocytosis of nonopsonised cryptococcal cells is very poor, but dramatically enhanced by complement or immunoglobulin-based opsonins [12]. Dysfunctional phagocytic apparatus cripples the immune response; for example monocytes from HIV/AIDS patients that were unable to phagocytose cryptococci failed to induce lymphoproliferative response in a macrophage—lymphocyte coculture system [13]. Once *Cryptococcus* cells have been engulfed,

macrophages can present Cryptococcal antigen and induce IL-1 expression and T-cell proliferation *in vitro* [14, 15]. However, cryptococci show a remarkable ability to survive and proliferate within macrophages, an adaptation that has been explored using both live imaging (Figure 1) [16–19] and gene expression [20, 21] approaches in macrophage cell lines (including J774 and RAW) and primary cells (including bone-marrow-derived murine cells and peripheral blood monocyte-derived human cells) [16, 17, 20, 22–24]. A noteworthy consideration when using *in vitro* models is that the *Cryptococcus*-cell interaction can differ depending on the type of macrophage used. For example, cryptococcal expulsion rates were found to be significantly higher in human primary macrophages compared to the J774 cell line [17]. However, despite the fact that different macrophage types are known to vary significantly in their behaviour *in vivo* [25], this aspect has yet to be extensively investigated in the context of cryptococcosis.

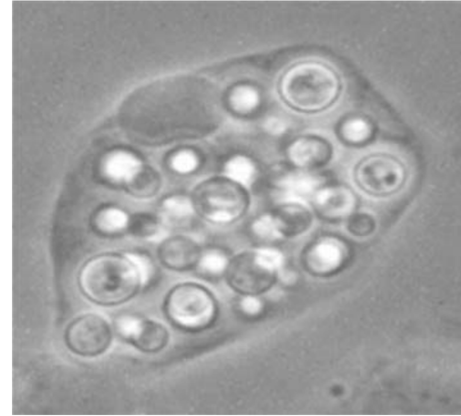

Figure 1: *Cryptococcus neoformans* can proliferate to a density of hundreds of yeast cells within macrophages. Here a J774 macrophage has been partially lysed to reveal intracellular *C. neoformans* after 18 hr of incubation. Image: W. Sabiiti.

2.2. Dendritic Cells. Dendritic cells (DCs) constitute vital mediators of the initiation of adaptive immune response [26] and are regarded as professional antigen presenters. Although less well studied than macrophages, several aspects of DC function have been documented *in vitro*. Phagocytosis of live and heat-killed cryptococci and antigen presentation have been demonstrated in both human peripheral blood mononuclear cells (PBMCs), derived DCs and mouse bone-marrow-derived dendritic cells (BMDCs) [27, 28]. Internalization of live *C. neoformans* induced minimal TNF-alpha production by human monocyte-derived DCs and none in mouse-derived BMDCs, whereas human DCs incubated with acapsular cryptococci produced significantly higher amounts of TNF-alpha. This suggests that presence of capsule inhibits protective cytokine production. While it is clear from these studies that DCs can internalize and process both dead and live cryptococci, it is not known whether both dead and live antigen induce the same type and intensity of cytokine response. In addition, it is as yet undetermined whether intracellular cryptococci are eradicated by DCs or whether they survive, proliferate, and escape as seen in macrophages.

2.3. Neutrophils. Neutrophils make up the largest population of phagocytes and are the first to be recruited to areas of infection. However their short lifespan means that they are a challenging cell type used in *in vitro* experimentation. Despite this limitation, investigations have shown that neutrophils are able to kill cryptococci *in vitro* [29, 30]. Although neutrophil cryptococci killing is typically considered to be mediated by the oxidative burst, neutrophils retain partial anticryptococcal function even when treated with respiratory burst inhibitors [31]. Unlike macrophages, neutrophils are also able to control extracellular cryptococci [32], although cryptococci produce mannitol, superoxide dismutase, and other ROS scavengers which help protect against extracellular killing by the respiratory burst [32, 33]. To date, however, it is not known whether cryptococci can persist to any degree within neutrophils, or how this interaction is modulated by the presence of macrophages at the infection site.

2.4. Eosonophils. Eosonophils, mostly associated with inflammatory response to helminthic parasites, have also been shown to be capable of phagocytosing and killing *C. neoformans* [34, 35]. *Cryptococcus*—eosonophil interactions have been studied using primary rat peritoneal eosinophils, in which it has been demonstrated that uptake of cryptococcal cells is strongly enhanced by antibody opsonization and is mediated by FcγRII and CD18 [34, 35]. Garro and colleagues further showed that coincubation of *C. neoformans*-stimulated eosinophils with CD4+ and CD8+ T cells from infected mice resulted in proliferation of T cells in an MHC 1- and 2-dependent manner, and hence eosinophils can act as antigen presenters and induce adaptive immune response to cryptococcal infection [35].

2.5. Lymphocytes. Lymphocytes, particularly CD4+ T cells, are important in the cell-mediated immune response to cryptococcosis [36]. Lymphocyte—*Cryptococcus* interaction models have used either primary mouse-derived or human PBMC-derived T lymphocytes and, to a lesser extent, immortalized T-cell lines. Studies using human lymphocytes have demonstrated a direct interaction between *C. neoformans* and lymphocytes. Both T cells and NK cells attach to *Cryptococcus* and directly inhibit its growth *in vitro* [36, 37]. Lymphocyte—*Cryptococcus* conjugate formation was enhanced when lymphocytes from mice immunized with heat killed *C. neoformans* were coincubated with cryptococcal yeast cells [38], suggesting that prior exposure activates this process, presumably through antigen processing and presentation by phagocytes to lymphocytes [39]. Intriguingly, both CD4+ and CD8+ T lymphocytes can kill extracellular cryptococci via granulysin, NK cells utilise perforin to achieve the same ends [40–43].

3. *In Vitro* Physical Barrier Models

3.1. Lung Epithelium. The inhalation route of infection makes the lung the first internal organ to be colonized

by *Cryptococcus*. The interaction between *Cryptococcus* and epithelial cells lining the alveolar spaces is thus critical in regulating cryptococcal entry into the circulation system. The mechanisms that allow *Cryptococcus* to penetrate human alveolar cells remain largely uncharacterised. Initial investigation showed that glucuronoxylomannan is an important factor permitting the attachment of yeast to host cell and subsequent infiltration of, and damage to, the host intracellular environment [44]. *In vitro* experiments have also provided insight into how the capsule virulence factor promotes the ability of *Cryptococcus* to cause infection at the lung barrier. Human lung epithelial cells can internalize both encapsulated and acapsular strains of *C. neoformans* [45]. The lung surfactant protein-D (SP-D) was shown to bind to and promote phagocytosis of both encapsulated and acapsular *C. neoformans* by macrophages *in vitro* [46]. However, encapsulated cryptococci are not efficiently opsonized by SP-D and those that were phagocytosed resisted intracellular killing by macrophages [47]. In addition, the lung is the site of granuloma formation during pulmonary cryptococcosis (and potentially during latent infections). To date, however, no models have been established to address the role of lung epithelial cells in granuloma formation.

3.2. Blood-Brain Barrier. Cryptococcal meningoencephalitis is the most devastating and fatal form of cryptococcosis. Modelling the blood-brain barrier (BBB) with the appropriate neurovascular properties has been a prominent challenge in cryptococcosis research. Isolation of human, rat and mouse brain microvascular endothelial cells (BMECs), which can be propagated *in vitro* to monolayers with BBB-like properties, has greatly improved the study of *Cryptococcus*—BBB interactions (Figure 2). Both human and mouse immortalized BMEC cell lines are commercially available and have been used in several studies, although freshly isolated BMECs are not so readily accessible.

Cryptococcus must penetrate the blood-brain barrier in order to cause meningococcal infection. Several studies using either primary or immortalized human, rat and mouse BMEC as BBB models have shown that BMEC can associate with and internalize yeast cells [39, 48–50]. Transwell chamber systems with BMEC monolayers have been used in some of the studies to demonstrate the occurrence of transcytosis across this cell layer [39, 50]. However *in vivo* the BBB is a complex tissue in which BMECs are supported by pericytes and astrocytes, a feature which cannot at present be recreated *in vitro*. In this context, the recent application of intravital imaging to examine cryptococcal traversal across the BBB *in vivo* represents a major advance for the field [51].

4. *In Vivo* Models

In vivo models can complement results of *in vitro* studies and also stand alone as valuable tools with which to make new discoveries and test out hypotheses. In this context, it is important to make the distinction between models that are used because they are vectors or carriers of human disease, and those that are used purely for their amenability in an experimental context. Those that are studied because they naturally harbour *Cryptococcus*, for example the koala [52], will not be discussed here. Instead we concentrate below on the range of model organisms exploited to undertake a whole-organism approach to studying *Cryptococcus*. In addition, the use of invertebrate models is an increasingly popular approach in many areas of biomedical research, including that of human fungal pathogens. We place a relatively large focus on these novel, emerging models over the more established and well-used vertebrate systems in order to highlight potential avenues for exploiting new model systems for cryptococcal research.

4.1. Invertebrate Models. Invertebrates can be excellent models for disease. Advantages over their mammalian counterparts include reduced maintenance costs, fewer ethical restrictions, relatively short reproduction times, and large brood sizes. A key argument for the use of invertebrate models is that features of their immune systems allow rigorous investigation into human disease. Whereas vertebrates have evolved innate and adaptive immunity, invertebrates possess only the innate system, the most ancient form of pathogen defence. The basic underlying mechanisms of immune response can therefore be studied without potential confusion from adaptive immunity, which can be very species- or even individual-specific.

A well-rehearsed argument that doubts the degree to which invertebrate models can be of value in biomedical research centres on the concept that the human immune system is vastly different from the invertebrate system. Indeed, adaptive immunity allows hosts to recognise molecular details of different invading pathogens, construct specific proteins to fight them, and maintain these proteins to protect against subsequent infections. However despite its lack of immune memory, the innate system of invertebrates is highly sophisticated and intricate and does not simply involve the same stereotypical response to every pathogen it encounters [53, 54]. Studies from organisms that use only innate immunity have demonstrated the complexity of this immune response [55, 56], and how it can vary according to a range of biotic and abiotic factors.

In addition, many human pathogens probably evolved their virulence mechanisms in more primitive organisms [57–60]. In the case of *Cryptococcus sp.*, these primitive hosts could have included free-living nematodes and amoebae. Adaptations that allowed the pathogens to escape natural invertebrate predators could have been translated into virulence factors for infecting mammalian hosts, and therefore studying interactions between the invertebrates and microbes could elucidate valuable information about disease.

Invertebrates are also simple and efficient models in which to study fungal virulence factors, such as capsule growth and melanin production [59, 61]; morphological dimorphism [62]; phospholipase production [63]. Antifungal activity can also be tested rapidly and economically using invertebrate models, due to their amenability to high-throughput screens of chemical libraries that can measure key factors in drug development including host immune response, efficacy, and toxicity [64, 65].

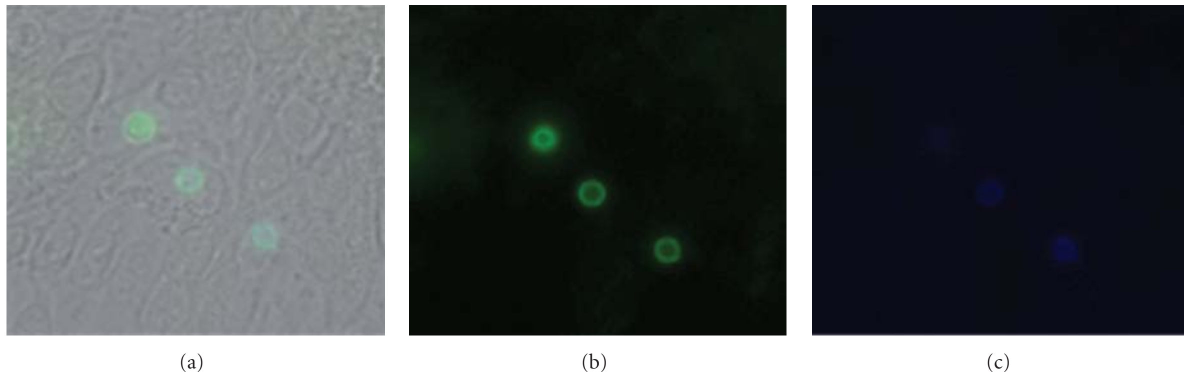

Figure 2: Fluorescence microscopy to determine adherent and internalized *Cryptococcus neoformans* on brain microvascular endothelial cells. *C. neoformans* serotype A strain H99 cells were prestained with FITC (green) before 4 hr incubation with brain endothelial cells and then counterlabelled with Calcofluor White (blue) to label extracellular adherent yeasts. (a) Merged image to reveal both endothelial cells and associated yeast cells, (b) and (c) Fluorescence images revealing the FITC and Calcofluor stained yeast cells, respectively.

4.1.1. Caenorhabditis elegans. *Caenorhabditis elegans* has been employed for immunological study of many pathogens, and the past decade has seen it established as a model for *Cryptococcus neoformans* infection [61]. In this system a culture of *C. neoformans* in YPD liquid media is spread onto agar plates, usually with the addition of antibiotics, and incubated overnight. When *C. elegans* are transferred to these plates they acquire the pathogen orally, leading to fatal disease [66]. Critical similarities exist between pathogenicity in *C. elegans* and mammalian hosts, for example strains of *Cryptococcus* with reduced virulence in mammalian hosts generally show similar attenuation in *C. elegans* [61]. These parallels support the case for *C. elegans* constituting a sound model for investigating basic mechanisms underlying *Cryptococcus* infection, disease, and treatment.

The molecular basis of infection and disease can be rigorously investigated in *C. elegans*, due to its amenability as a genetic model, and consequently a wealth of knowledge has been accumulated concerning immunity at the gene level. The potential to screen large numbers of mutant pathogens in *C. elegans* and identify genes important for virulence demonstrates a particular strength for the nematode model. A recent investigation identified mutations in *C. neoformans* that caused reduced survival in *ex vivo* cerebral spinal fluid and commensurate attenuation in *C. elegans* and the rabbit [67]. In addition, genes important for *C. neoformans* virulence in mammalian systems have been shown to be instrumental in causing death in *C. elegans* killing assays, highlighting a parallel between the immunity and pathogenesis of the *Cryptococcus*-human and *Cryptococcus*-worm model systems. For example, *C. neoformans* genes associated with virulence in both mammalian and nematode models include those associated with signal transduction pathways (GPA1, PKA1, PKR1, RAS1), laccase production (LAC1), and the {alpha} mating type [61].

C. elegans can use nonpathogenic forms of *Cryptococcus*, for example *C. laurentii* and *C. kuetzingii*, as a food source in the laboratory [23]. This lends support to the theory that the nematode is a natural predator of *Cryptococcus* and could therefore be an ideal model in which to investigate the evolution of virulence factors that have enabled certain strains of *Cryptococcus* to become pathogenic to worms and other hosts.

However, a profound limitation of *C. elegans* as a model for investigating pathogenesis of mammalian disease is that the mode of infection is completely different to that in mammals. The nematode ingests the pathogen, and cryptococcal growth is restricted to the intestine, in marked contrast to the lung inhalation and subsequent dissemination that is characteristic of human infections. Along with the limitations associated with this entirely different mode of infection, it is not possible to administer an exact pathogen dose in this model, which could severely constrain the scope of certain experiments. In addition, phagocytes are not present in *C. elegans*, so in terms of studying phagocytosis in a whole organism model a more favourable invertebrate model may be an insect or amoeba.

4.1.2. Amoeboid Models. Soil amoebae are environmental reservoirs of human pathogens [68] and therefore show promise as model hosts in the laboratory setting. Amoebae feed by phagocytosis of microorganisms, in a process that is essentially similar to the phagocytosis of microbes by human macrophages [69]. Amoebae therefore provide a simple model in which to investigate aspects of this fundamental immune response. Additionally, there are a number of characteristics that make these organisms valuable experimental models, primarily with respect to genetic tools.

Acantamoeba castellanii. In terms of using invertebrate hosts to study evolution of human pathogen virulence, the interaction between *Cryptococcus sp.* and *A. castellanii* provides interesting and unique opportunity for investigation. *Cryptococcus* can cause amoebae to lyse following phagocytosis [70], and a popular theory postulates that this adaptation to overcome predation could have been a precursor to the escape from mammalian phagocytes that cryptococci demonstrate *in vitro* [71]. In support of this, characteristics that promoted *Cryptococcus* survival in *A. castellanii* were also identified as virulence factors that enhanced *Cryptococcus* parasitism

in macrophages [71]. Although *A. castellanii* may not be as genetically well characterised as the amoeboid model *D. discoideum*, the former has the advantage of remaining viable in laboratory conditions above 25°C, therefore more accurately simulating conditions of human infection, which occur in the critical 32–37°C window [72].

An important feature of the *A. castellanii* model is that this organism is killed by *Cryptococcus*. On a basic level this suggests that *Cryptococcus* readily acts as a pathogen in amoebae, which could confer an advantage over another invertebrate, such as *D. melanogaster*, which is not killed by injection of *Cryptococcus* and therefore may not be such a valuable a model of pathogenesis.

Dictyostelium discoideum. *Dictyostelium discoideum* has proved a useful tool for studying intracellular proliferation of human pathogens including *Cryptococcus.* The small haploid genome of *D. discoideum* has been sequenced [73], and is particularly amenable to genetic manipulation [68, 74].

Important parallels in terms of cryptococcal virulence are present between *D. discoideum* and human hosts. For example the fungal capsule is important for *C. neoformans* infection of *D. discoideum*, as acapsular mutants did not replicate in the amoebae [59]. Another important finding in *D. discoideum* showed that *C. neoformans* caused disease with increased efficiency following growth in the amoeboid model, indicating that adaptations for survival in the host translated into virulence factors [59].

4.1.3. Insect Models. After pathogen recognition, the insect immune response follows a characteristic sequence of events involving two major classes of effector systems—cellular and humoral [75, 76]. The humoral immune response concerns the production of antimicrobial peptides and induction of enzyme cascades which function to minimise harm caused by the pathogen. Circulating haemocytes within the insect haemocoel produce the cellular response and rapidly fight the pathogen using three key cellular defences: phagocytosis, nodule formation, and encapsulation [77, 78]. Assays to measure insect immune response can be applied to a variety of species and employed in a high-throughput manner. For example, *in vivo* phagocytosis can be assessed by nondestructive extraction of haemolymph containing phagocytosed pathogens from insects and then analysed using *in vitro* assays. Given the importance of phagocytosis in cryptococcosis research, insects may therefore prove a valuable experimental tool.

Despite the many advantages of insect models for human disease research, there are a number of important limitations. Insects lack most organs found in humans, such as lungs, kidneys, and hearts. Given that organs such as the lungs are particularly important in *Cryptococcus* infection and disease, this highlights an obvious limitation of insects rather than mammalian models for this fungal pathogen. In a similar vein, the blood-brain barrier is a particularly important area of investigation for *Cryptococcus* research, but the lack of blood capillaries in the insect body means that this aspect of the disease cannot be studied in insect hosts.

Galleria mellonella. The larvae of the greater wax moth, *Galleria mellonella*, have been used as whole-organism virulence models for various species of pathogenic fungi. This organism can live at mammalian body temperature, thereby allowing temperature-sensitive investigation of pathogenesis. The larva also provides a good infection model because it is easy to inoculate with specific doses of fungal pathogen, owing largely to its size (approximately 1.5–2.5 cm in length), which contrasts with the smaller size of insects such as *Drosophila*, in which controlling dose per insect is challenging [79]. To date, the *G. mellonella* model has primarily been used to assess virulence and/or antifungal activity. Inoculation of microbes into the haemocoel is minimally invasive because piercing of the haemocoel is not required. Instead applying gentle pressure can open up the base of the proleg, and a needle is inserted. The aperture reseals on the release of pressure [79]. This nonpiercing method means that immune response directed at wound repair does not affect the results of experiments, which would be the case if insects were injected through the cuticle.

G. mellonella has been advocated as a particularly good model for diseases that disseminate through the body via the bloodstream, such as *Cryptococcus neoformans* and *Candida albicans* [79]. The development of *G. mellonella* as a model for *Cryptococcus neoformans* was first achieved by Mylonakis and colleagues in 2005 [80]. All *C. neoformans* strains tested caused death of the insect host, despite effective phagocytosis by insect haemocytes [80]. This suggests that *Cryptococcus* virulence may rely on the same mechanisms in *G. mellonella* as it does in *in vitro* assays, during which the phagocytic immune response is evaded and even exploited by the pathogen.

Recently the *G. mellonella* system was employed to investigate whether the antifungal activity of Fluconazole could be enhanced with the addition of other drugs. Fluconazole proved to have greater beneficial effect for *G. mellonella* survival when administered in combination with the antihistamine Astemizole and a closely related analog (A2) [81]. The results of this *in vivo* study were supported and enhanced by an *in vitro* experiment, in which the usually fungistatic Fluconazole became fungicidal when combined with Astemizole and A2 [81]. This investigation demonstrates how survival data from an insect model that is relatively easy to infect and monitor in a high-throughput manner can support *in vitro* mechanistic discovery.

Drosophila melanogaster. Infection of *D. melanogaster* with *C. neoformans* can be achieved by a variety of methods, and both dissemination of disease and local infection can be investigated. Injection into the haemocoel is done using a small needle inserted into the thorax or abdomen (e.g., [23]). This represents a systemic infection because the pathogen is administered directly into the haemolymph. A limitation of this method is that it is far removed from the natural pathway of infection, as microorganisms such as *Cryptococcus* would not be able to cross the insect cuticle unless an opening was

already present. An alternative method is to administer the pathogen by incorporating it into the fly food.

Interest in *D. melanogaster* as a model of human disease has grown in recent years, with the continuing realisation that immune signalling pathways are highly conserved between fly and human [82, 83]. Of particular relevance to fungal disease is the finding that Toll receptor activation downstream of fungal infection leads to the production of antifungal peptides by *D. melanogaster* [84].

Wild-type *D. melanogaster* shows resistance to fungal pathogens administered by injection into the haemocoel including *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. Experiments have shown that the Toll pathway is crucial in this resistance [84, 85]. In contrast, the Toll pathway is not involved in pathogen defence when the method of *Cryptococcus neoformans* infection is ingestion [85]. This research using *Drosophila* has suggested that *Cryptococcus neoformans* may elicit different responses in the systemic- and digestive-related immunity, in this host at least [85].

4.2. Vertebrate Models. For certain elements of fungal pathogen research, the benefit of the adaptive immune system of vertebrates will always be a feature that renders them more valuable as models than invertebrates. Vertebrate models have been extensively covered in other reviews (e.g., [86, 87]) but are discussed briefly below.

4.2.1. Mouse (Mus musculus). The majority of *Cryptococcus*-related work utilising mammalian systems has been carried out using mice. Due to the fact that murine models are well established as valuable study systems in many research areas including fungal pathogenesis, an in-depth discussion of mice in *Cryptococcus* research is not within the scope of the present review. Briefly, however, mouse models are a popular choice because they are well established and characterised in medical research, and a variety of genetic backgrounds are available. Infection with *Cryptococcus* can be achieved by a variety of methods, including intranasally, intraperitoneally, intracerebrally, intravenously, intratracheally, and via inhalation [88, 89], which opens up a range of experimental opportunities. In addition, the vast array of information available on the mouse immune system means that parallels and pitfalls can be readily identified, allowing for the design of highly refined experiments.

4.2.2. Rat (Rattus rattus). The rat model is comparable to mouse, but with a few advantages. The slightly larger body size allows a variety of experimental manipulations to be achieved with relative ease, including endotracheal intubation, bronchoalveolar lavage, serial venepuncture, CSF collection, radiography, computed tomography (CT), and magnetic resonance imaging (MRI) [88]. The rat has also been reported to develop chronic pulmonary cryptococcosis in the wild [90], suggesting this organism as a potentially valuable disease model. A model mimicking pulmonary cryptococcosis establishment, induced by *C. gattii* intratracheal inoculation in immunocompetent rat hosts, was recently established [88]. This opens up the opportunity to use this model to study more complex elements of cryptococcal etiology, such as long-term latency or the emergence of *C. gattii* as a primary pathogen. The rat model has been very useful in research into mammalian host responses to *Cryptococcus*. For example expression of acidic mammalian chitinase (AMCase) in response to infection by *Cryptococcus*, which contains chitin in its cell wall, could be a potential mediator of asthma [91].

4.2.3. Guinea Pig (Cavia porcellus). The Guinea pig model was first established for cryptococcal disease relatively recently by Kirkpatrick et al. [92]. The larger size of Guinea pigs compared to mice means they are ideal for more sensitive experimental manipulation. For example, intravenous inoculation can be relatively easily achieved in Guinea pigs. Another benefit of the Guinea pig model for fungal research is that oral doses of antifungals sufficient to control fungal infections are similar to the doses in humans [93]. An example of the utilisation of the Guinea pig model is the investigation into the effectiveness of an intravenous delivery of the antifungal itraconazole in fighting disseminated fungal infections including cryptococcosis [93].

4.2.4. Rabbit (Oryctolagus cuniculus). The rabbit has been advocated as a model for cryptococcal meningitis mainly due to its size in comparison to other mammalian study systems. For example, Steen et al. [94] chose the rabbit model because its relatively large body size enables the study of yeast at the site of infection, which is more difficult in smaller mammals such as mice and Guinea pigs. Historically the rabbit model was not an appealing choice for cryptococcal disease research because the organism appeared to be naturally resistant to this pathogen [95]. However the potential for this model increased when pretreatment with corticosteroids and subsequent *Cryptococcus* inoculation successfully resulted in the development of chronic cryptococcal meningitis [95]. A recent utilisation of this model involved measuring survival of *Cryptococcus* mutants in rabbits to establish whether mutations causing reduced attenuation in cerebral spinal fluid showed corresponding reduced virulence *in vivo* [67]. This is an example of the use of a whole-organism model to validate hypotheses of disease attenuation developed from *in vitro* experiments.

4.2.5. Zebrafish (Danio rerio). The zebrafish is emerging as an attractive model system for a variety of human diseases. Mutagenesis and screening can be done on a large scale with the zebrafish, which is a fairly unique feature in vertebrate models. Another benefit to this model is that it can be maintained at relatively low cost, with few ethical restraints. Thus the zebrafish in many ways has the economical advantages of an invertebrate model whilst also possessing all of the vertebrate immune system features that researchers in fungal pathogenesis require.

The zebrafish has not yet been used to investigate infection and disease in relation to *Cryptococcus*—however this application is under development (Figure 3). This model has

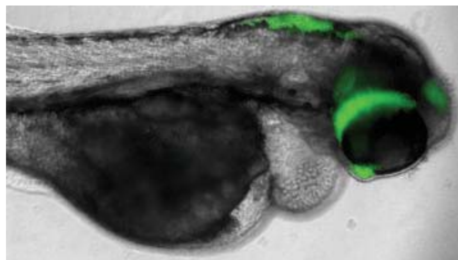

Figure 3: Zebrafish embryo 48 hours after infection with *Cryptococcus neoformans* strain H99 expressing GFP. Image Courtesy of S. A. Johnston, University of Birmingham, UK. GFP-expressing yeast was developed by Voelz et al. [99].

been used for other human fungal pathogens, lending hope to the idea that a disease model for *Cryptococcus* could soon be developed. For example, a comprehensive infection model of the zebrafish with *Candida albicans* has been established, in which pathogen morphogenesis and gene expression, and host immune response, were monitored [96]. Fish were killed by *C. albicans* in a dose-dependent manner, and infection was established at a number of different sites, indicating that this organism could be a valuable tool in fungal research.

5. *Ex Vivo* Models

Ex vivo organ culture could be considered a way of achieving a balance between the advantages of *in vitro* and *in vivo* experimental models. The use of perfused organs provides a relatively authentic physiological environment and permits the study of pathogens crossing membranes, disseminating through host tissue, and migrating to the bloodstream—processes not captured by *in vitro* experimentation. Organs from higher animals, which would otherwise carry enormous ethical and economic implications, can be studied when perfused organs are chosen over whole animal models. For example, haemoperfused liver from pigs was recently established as a model for *C. albicans* investigation [97]. This model could be adapted for the study of other human fungal pathogens including *Cryptococcus*. An *ex vivo* model has also been established for swine trachea [98], and since *Cryptococcus* infects the lungs, this model could be of particular interest to adapt for investigation of this pathogen.

6. Closing Remarks

Cryptococcal research utilises a diverse range of experimental model systems, spanning from individual cells to unicellular whole organisms to mammalian models. Progress from hereon may benefit from different research disciplines exchanging knowledge and skills so that models already established in one field, such as drug development, can be adapted for another area of research such as pathogen evolution.

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Review Article

Histoplasma Virulence and Host Responses

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Histoplasma capsulatum is the most prevalent cause of fungal respiratory disease. The disease extent and outcomes are the result of the complex interaction between the pathogen and a host's immune system. The focus of our paper consists in presenting the current knowledge regarding the multiple facets of the dynamic host-pathogen relationship in the context of the virulence arsenal displayed by the fungus and the innate and adaptive immune responses of the host.

1. Introduction

Histoplasmosis was first described in 1906 by Darling among the workers of the Panama Canal [1], and it is currently the most common cause of fungal respiratory disease with almost 500,000 individuals acquiring the fungus each year [2]. The etiologic agent responsible for histoplasmosis is *Histoplasma capsulatum*, a thermally dimorphic fungus with worldwide distribution. The fungus is primarily found in soil, where it exists in a mycelia form. In the United States, highly endemic areas include regions along the Mississippi and Ohio River valleys, where seroprevalence studies have shown that up to 80% of individuals are skin test positive for histoplasmin [3].

The entry portal of *H. capsulatum* is through inhalation of aerosolized of 2–4 μm diameter microconidia [4]. Morphogenesis is initiated after infection with the conidia developing into a 2–4 μm oval yeast form. The fungus is rapidly ingested by macrophages and neutrophils, but manages to avoid intracellular destruction. Intracellular yeast can be transported diffusely via the lymphatics and into the bloodstream. Nevertheless, initial infection is typically contained by innate and adaptive host responses. In immunocompetent individuals, the pulmonary disease is usually subclinical to limited, typically with flu-like symptoms, including fever, cough, headaches, and myalgias. However, lethal disease can occur in otherwise healthy individuals who acquire a large inoculum infection. Additionally, severe primary infection is more common and reactivation of latent infection occurs in immunocompromised persons, particularly in HIV-infected population and transplant recipients. Disseminated disease occurs in a small fraction of infected individuals, but this form of histoplasmosis continues to carry a high fatality rate even in patients receiving appropriate medical treatment [5]. More recently, treatments with inhibitors of tumor necrosis factor-α have been shown to place patients at high risk for developing histoplasmosis [6].

2. *H. capsulatum* Virulence Factors

The characterized virulence determinants of *H. capsulatum* are mainly surface expressed molecules that mediate the interaction between the fungus and the host's immune cells allowing the pathogen to evade destruction by innate immune response and facilitate the replication of the yeast in its new environment.

Heat shock protein 60 (HSP60), which has important roles in chaperoning intracellular proteins and supervising adequate protein folding, has also recently been described as an essential surface molecule, mediating the recognition and phagocytosis of the yeast by macrophages [7]. It acts as a ligand for CD11/CD18 macrophage receptor and, despite low number of antigenic sites, the coupling with the CR3

receptor is followed by rapid ingestion of the yeast. The interaction between *Histoplasma* and macrophage through HSP60 binding to CR3 results only in a mild host immune reaction, as it does not lead to a significant activation of phagocytes in the absence of other costimulatory signals [8]. This process allows the microorganism to survive and replicate inside the host cells [9, 10].

Heat shock protein 82 (HSP82) is another important molecule in normal development of *H. capsulatum* that also participates in the response to cellular stresses; it binds to a variety of cellular proteins, keeping them inactive until they have reached their proper intracellular location or have received the proper activation signal [11]. The role of HSP82 is further complicated by the thermal dimorphism displayed by *Histoplasma*, since changes in temperature represent both a stress inducer and a signal for yeast phase transformation. Recently, Edwards et al. demonstrated that a reduction in HSP82 decreases *Histoplasma* virulence in macrophages and severely impairs the fungus' ability to infect lungs in a murine infection model [12]. This suggests that a low basal level of HSP82 expression is sufficient only to preserve cellular functions at mammalian body temperature, but not to withstand other stresses encountered during infection. The temperature values during febrile episodes in the host, for example, represent one stress that requires HSP82 function, and defective mutant strains show a decreased ability to recover from transient in vitro incubation at 40°C. However, even at 37°C these defective mutants showed decreased virulence within macrophages, despite having identical growth in culture at similar temperature; this implies that HSP82 extends its role to enduring additional, nonthermal stresses during host infection. One example is the ability to survive oxidative stress, as shown by peroxide challenge studies [12].

YPS3 is a yeast phase-specific gene encountered in a subset of *H. capsulatum* strains. The encoded protein is found both as a fungal cell wall constituent and as a secreted molecule [13]. The exact function of this gene remains to be determined, but its importance in virulence is a certainty, since YPS3 mutants are attenuated in vivo [14].

The production of cell wall melanin is associated with virulence for diverse fungi; *H. capsulatum* conidia and yeast produce melanin or melanin-like pigments in vitro and yeast cells are melanized during mammalian infections [15]. The melanization process decreases the susceptibility of the fungus to amphotericin B and caspofungin and melanin can abrogate the potency of certain host defense mechanisms, such as free radicals and microbicidal peptides [16, 17].

Calcium-binding protein (CBP) represents another important factor in *Histoplasma* pathogenicity. CBP is secreted by the fungal cells during the yeast-phase of intracellular growth within the macrophage [18], and its importance for the virulence of the fungus was demonstrated both in vitro and in vivo. For example, CBP1 gene deletion yeast cells were rapidly cleared from the lungs of infected mice. Additionally, *H. capsulatum* growth is inhibited in limiting calcium conditions [19]. One of the hypotheses is that calcium acquisition represents an important factor for intracellular survival of the microorganism; another hypothesis targets the modulating effect of CBP in binding calcium to facilitate optimal phagolysosomal conditions for yeast growth.

Many *H. capsulatum* strains express α-(1,3)-glucan on their yeast cell surface. This polysaccharide forms a layer that conceals cell surface β glucans, which have antigenic properties, eluding the identification by the host phagocytic cells. The β glucan found in the cell wall of *Histoplasma* and other fungi is recognized by the Dectin-1 receptor on macrophages resulting in the triggered formation of reactive oxygen species and secretion of proinflammatory cytokines [20]. Confirmatory evidence for the role of the α-glucan was obtained using *Ags-1*-deficient *Histoplasma* mutant strains, where yeasts lacking the cell wall α-(1,3)-glucan were attenuated for virulence [21].

Histone 2B (H2B) has also been found to play a role in pathogenesis [22]. Histones are mainly intracellular components, but a study investigating passive immunity through administration of monoclonal antibodies from mice immunized with *Histoplasma* revealed antibody recognition of H2B present on the cell wall. The means by which historically intracellular-based molecules, as HSP60 or H2B, reach the yeast cell wall where they can interact with host cells was unclear until recently when macromolecular transport to the extracellular space was demonstrated to occur via vesicular secretion and active vesicular transport [23, 24].

Hydroxamate siderophores production by *Histoplasma* is another newly characterized virulence factor. Strains defective for the gene coding for siderophore production display impaired intracellular growth in both human and murine macrophages, which can be reversed by either exogenous iron addition or restoration of SID1 expression [25, 26].

3. Host Defense Mechanisms

After being exposed to *Histoplasma*, the host relies on both innate and adaptive immune response mechanisms to neutralize the pathogen and withstand infection. Macrophages and dendritic cells have major roles in the activation of cellular pathways, and the numerous cytokines, especially IFN-γ and TNF-α, significantly impact host responses.

Macrophages have a central role in the interaction between the fungus and the host, although their contribution has a dual nature. They represent the first line of defense during infection with *H. capsulatum*, as they rapidly phagocytose the inhaled conidia and transforming yeast cells, and the infected macrophage subsequently activate effector T cells and enhance the release of Th1-associated proinflammatory cytokines (IL-12, IFN-γ, and TNF-α) [27, 28]. Deprivation of zinc and iron is amongst the means used by macrophages to neutralize the intruding pathogen [29], along with production of superoxide, nitric oxide, and lysosomal hydrolysis. However, the fungus displays various mechanisms to elude destruction after phagocytosis. For example, *H. capsulatum* yeast cells are able to regulate the pH of the phagolysosomes at a neutral pH (approximately pH 6.5), where lysosomal hydrolases have decreased activity. Hence, the *H. capsulatum* yeast cells manage to survive and even replicate inside macrophages [30].

Dendritic cells are also an important effector of the innate immunity. They are able to phagocytose and degrade the fungal cells with higher efficacy than macrophages, which might be due to recognition of the pathogen via a different type of receptor (fibronectin receptor on dendritic cells versus CD18 on macrophages) [31]. Dendritic cells also are extremely efficient at processing and presenting antigens to specific CD8 T cells, either following ingestion of the yeast, or through "cross presentation" of fungal antigens engulfed from infected apoptotic macrophages [32]. In a recent study, the addition of antigen-presenting dendritic cells was found to suppress excessive production of IL-4 by CD4 T cells in lungs of CCR2-deficient mice infected with *H. capsulatum*, demonstrating the importance of these cells in the regulation of immune responses [33].

Cellular immunity is crucial in the host defense against intracellular pathogens; therefore, T cells, as the central effectors of the cellular immunity, have a substantial role in neutralizing *H. capsulatum* yeast cells. Mice depleted of both CD4 and CD8 T cells have accelerated time to death after challenge with *H. capsulatum* yeast cells, especially in a primary histoplasmosis model, which underlines the importance of the interaction between the two cell subsets in withstanding *Histoplasma* infection by eliciting a Th1 response [34]. CD4 cell depletion is associated with survival during primary infection, as a result of impaired IFN-γ production. The elimination of CD8 T cells results in decreased clearance of yeast cells in primary but not secondary infection. One particular subpopulation of T cells, $V\beta4^{+}$ T cells, is preferentially expended during infection with *H. capsulatum,* and elimination of these cells from mice impairs their ability to resolve infection [35]. Th17 T cells and their interaction with regulatory T cells have recently been linked via the chemoattractant mediator CCR5 to the host's ability to effectively combat *H. capsulatum* infection; increases in Th17 cytokines and reductions in the number of regulatory T cells were associated with accelerated fungal clearance in CCR5-deficient animals [36].

Although cytokine responses are complex in histoplasmosis and alter over the course of disease, the main cytokines involved in *Histoplasma* clearance from the host are IL-12, IFN-γ, and TNF-α [34]. IL-12 through its ability to regulate IFN-γ production is critical in inducing a protective immune response in primary infection with the pathogen. IFN-γ is pivotal for the host's innate resistance to systemic infection with *H. capsulatum.* Survival of mice is significantly reduced in IFN-γ-deficient mice as well as in wild-type mice treated with neutralizing antibody to IFN-γ [37]. Patients with impaired IFN-γ signaling due to genetic defects are at increased risk for severe disease forms and administration of the cytokine can be therapeutic. For example, a report of recurrent disseminated *H. capsulatum* osteomyelitis in a patient with genetic IFN-γ receptor 1 deficiency describes progressive clearing of all bone lesions and normalization of inflammatory markers following subcutaneous therapy with IFN-γ [38]. Although IFN-γ is critical in primary infection, survival in secondary infection can be achieved in the absence of IFN-γ, as immunization of IFN-γ-deficient mice with an initial sublethal inoculum can prolong the survival of these mice when subsequently challenged with a high concentration of *H. capsulatum* yeast cells [39]. The major mechanism by which these mice were able to control secondary infection was through increased production of TNF-α.

TNF-α is a key modulator of disease in both primary and secondary histoplasmosis, though different protective mechanisms are involved in these conditions [34, 40]. In primary infection, decreased survival of TNF-α-deficient mice has been attributed to an impaired ability to generate reactive nitrogen intermediates in the alveolar macrophages, although inducible nitric oxide synthase expression in lung tissue is preserved. During secondary infection, the increased mortality is largely due to a biased host reaction to a Th2-type response that is associated with elevated levels of both IL-4 and IL-10. These findings parallel the clinical data that clearly demonstrates that therapy with TNF-α inhibitors poses a significant increased risk for reactivation of latent histoplasmosis with a greater likelihood of severe, disseminated disease [8].

Humoral immune responses generally have a limited role in the clearance of intracellular pathogens; however, the protective role of antibodies against surface molecules of *H. capsulatum* has been described. Administration of monoclonal antibodies to *Histoplasma* H2B reduces fungal burden, decreases pulmonary inflammation, and prolongs survival in murine infection models [22]. The protective response was associated with increased levels of IL-4, IL-6, and IFN-γ. Similarly, antibodies to *H. capsulatum* HSP60 prolong the survival of the lethally infected animals [41, 42].

4. Discussion

Histoplasmosis is the most common endemic dimorphic fungal pathogen of man. The continuously expanding population of immunocompromised patients, secondary to the ongoing HIV epidemic, the increasing use of immunosuppressant therapies and rising number of transplant recipients, represents a high risk cohort for histoplasmosis. The mortality rate associated with invasive histoplasmosis is still unacceptably high, despite the use of broad spectrum antifungal agents, which emphasizes the need for developing novel therapies and effective preventive strategies.

As outlined in this paper, targeting virulence determinants of *H. capsulatum* and attempts to modify the capacity of the host to respond to the fungal invader are actively being pursued by researchers. Recent studies investigating the capacity of *H. capsulatum* to release a large number of proteins and other immunologically active compounds [23, 24, 43, 44] demonstrate the breadth of the fungus' ability to modify host responses. The high frequency of disease in the endemic areas and the increasing prevalence of the disseminated disease forms justify the development of adequate immunization strategies. Most recently, data has shown that vaccine-induced fungus-specific Th17 cells can confer protection against pulmonary histoplasmosis by recruiting and activating neutrophils and macrophages to the alveolar space [45]. Harnessing the host's existing

armamentarium or supplementing the host's capacity, such as with the administration of cytokines or antibody to *H. capsulatum*, will be rich areas of study for the future.

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Review Article

Dermatophyte Virulence Factors: Identifying and Analyzing Genes That May Contribute to Chronic or Acute Skin Infections

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Dermatophytes are prevalent causes of cutaneous mycoses and, unlike many other fungal pathogens, are able to cause disease in immunocompetent individuals. They infect keratinized tissue such as skin, hair, and nails, resulting in tinea infections, including ringworm. Little is known about the molecular mechanisms that underlie the ability of these organisms to establish and maintain infection. The recent availability of genome sequence information and improved genetic manipulation have enabled researchers to begin to identify and study the role of virulence factors of dermatophytes. This paper will summarize our current understanding of dermatophyte virulence factors and discuss future directions for identifying and testing virulence factors.

1. Introduction

Dermatophytes are the most common cause of fungal infections worldwide, resulting in treatment costs of close to half a billion dollars annually in the USA [1, 2]. The World Health Organization estimates global prevalence of dermatomycoses to be approaching 20% [3]. Despite this, researchers lack a sophisticated understanding of dermatophyte pathogenesis [4].

Dermatophytes are the group of filamentous fungi that are the most common cause of cutaneous mycoses. The diseases caused by these organisms are generally named after the part of the body that is infected rather than the infecting organism. For example, tinea pedis refers to athlete's foot and tinea unguium refers to a nail infection. Dermatophyte infections are generally superficial, but immunocompromised patients can experience severe, disseminated disease [5]. Although dermatophyte infections are treatable, there is a high rate of reinfection; it remains to be determined whether this is due to relapse (the fungus not being completely eradicated during treatment) or a new infection [6].

The dermatophytes include three genera of molds in the class Euascomycetes: *Trichophyton*, *Microsporum*, and *Epidermophyton*. Dermatophytes are grouped according to their habitat as being either anthropophilic (human associated), zoophilic (animal associated), or geophilic (soil dwelling). Anthropophilic species are responsible for the majority of human infections; however, species from all three groups of dermatophytes have been associated with clinical disease. Human infections caused by anthropophiles tend to be chronic, with little inflammation, whereas infections caused by geophiles and zoophiles are often associated with acute inflammation and are self-healing [7].

The recent sequencing and annotation of several dermatophyte genomes, as well as advances in techniques for genetic manipulation of dermatophytes, provide resources that will aid in elucidating the mechanisms of virulence of these ubiquitous organisms. An understanding of the specific virulence factors that contribute to dermatophyte pathogenesis would aid in the design of effective therapeutics. This paper will summarize the current state of understanding of dermatophyte virulence factors and comment on future directions for their studies.

2. Virulence Factor Identification

2.1. Virulence Factor Identification Using Bioinformatics Approaches. The sequencing of seven dermatophyte genomes has

recently been completed, and the sequences have been made publically available via the Broad Institute database [8]. The Broad Institute sequenced and annotated the genomes of five dermatophyte species. *Trichophyton rubrum* (*Tr*) is anthropophilic and the most common cause of dermatophyte infections in humans worldwide. *Trichophyton tonsurans* (*Tt*) is also anthropophilic and is a major cause of tinea capitis (scalp ringworm). *Tt* was recently found to be present in more than 30% of students in some grade levels at US schools [9]. *Trichophyton equinum* (*Te*) is closely related to *Tt* but is zoophilic and primarily associated with disease in horses. *Microsporum canis* (*Mc*) is also zoophilic and is the most common cause of tinea capitis in Europe [10]. *Microsporum gypseum* (*Mg*) is a geophile that is associated with gardener's ringworm. The strains selected for sequencing are all clinically relevant (associated with human disease) [4] and have been characterized with respect to growth rate, conidiation, and drug susceptibility [11]. Genome sequences of the remaining two species, the phylogenetically related zoophiles *Arthroderma benhamiae* (*Ab*, a teleomorph of *Trichophyton mentagrophytes*) and *Trichophyton verrucosum* (*Tv*), were recently completed by the Hans Knoell Institute (Jena, Germany) and published [12]. These organisms cause a highly inflammatory infection in humans. As expected, comparison of the seven dermatophyte genomes indicates that these species are closely related phylogenetically and each is more closely related to the others than to *Coccidioides immitis* or the dimorphic fungi (unpublished data). This is in agreement with a comparative study of five dermatophyte mitochondrial genomes, which suggested a recent divergence of the dermatophyte clade [13].

All seven genomes were found to encode high numbers of protease-encoding genes compared to related, nondermatophytic fungi ([12] and unpublished data). In particular, dermatophytes appear to have expanded sets of endopeptidases, exopeptidases, and secreted proteases. In contrast, there is little difference in abundance of carbohydrate enzymes of the CAZy family designation [14, 15] between dermatophytes and dimorphic fungi (unpublished data). This highlights the important role of protein degradation in the lifestyle of dermatophytes.

Secretome analysis of *Ab* during growth on keratin confirmed that proteases made up the largest group of identified secreted proteins [12]. The *Ab* and *Tv* genome sequences also revealed a relatively high number of secondary metabolite gene clusters, and expression of some of these genes were confirmed to be up- or downregulated during keratinocyte infection by *Ab* [12].

As described above, disease caused by human-adapted organisms *Tr* and *Tt* tends to be chronic with low inflammation, whereas zoophiles (*Te*, *Mc*, *Ab*) and geophiles (*Mg*) generally cause an inflammatory infection. The availability of sequence information now allows researchers to use a bioinformatics approach to make predictions about which genes are involved in virulence, as well as differences between species that have adapted to different ecological niches. Preliminary unpublished analysis indicates that among the five genomes sequenced by the Broad Institute, most genes are conserved in all five species, the majority of which are annotated. This is not surprising and confirms the genetic relatedness of the dermatophytes. Of the annotated genes that are unique to a particular species, there does not appear to be any trend. However, there are a number of hypothetical genes unique to each species that potentially play a role in niche adaptation and pathogenicity (unpublished data).

In many pathogenic eukaryotes, including protozoan parasites and fungi, there is a trend for clinical isolates to have cryptic, modified sexual cycles. Although originally thought to be asexual, *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* are all examples of fungi that cause systemic disease and were shown to have sexual cycles [16, 17]. It is therefore possible that other pathogenic fungi, including anthropophilic dermatophytes, have sexual cycles that have not yet been identified under laboratory conditions. Bioinformatic analysis identified the mating locus in each of the sequenced dermatophyte species, and PCR analysis with additional strains of the species has identified the other mating type, except for *Tr*, where the second mating type has not yet been identified [18]. Recent work has shown normal mating with progeny in the geophilic species *Mg* [19] and the ability of *Tr* to initiate but not complete a mating cycle with a related species of the opposite mating type [20]. Whether *Tr* is able to mate during growth on human skin remains to be determined, and the potential contributions of mating to virulence represent an area of active research.

Knowledge of the mechanisms of pathogenesis of other fungi also leads to predictions of virulence factors in dermatophytes. For example, the dipeptidyl peptidase DppIV was identified in *Mc* based on sequence similarity [21]. Additionally, dermatophytes have recently been shown to produce melanin or melanin-like compounds, which are predicted to play a role in virulence based on the known role of melanins in other pathogenic fungi [22]. Similarly, *Tr* has been shown to produce xanthomegnin, a toxin previously known to be produced by *Aspergillus*, in culture and during human infection [23]. The complete annotated sequences of dermatophyte genomes will aid in identifying additional putative virulence factors based on sequence similarity, which will then need to be tested experimentally to confirm expression and role during infection.

2.2. Virulence Factor Identification Using High-Throughput Screens. The *T. rubrum* Expression Database [24, 25] provided an important starting point for transcriptional analysis by collating expressed sequence tags (ESTs) and transcriptional profiles from microarrays, resulting in the identification of numerous genes involved in growth during a variety of conditions. Complete genome sequencing will enhance our ability to identify open reading frames (ORFs) in future studies.

Screens have historically been used to identify gene products likely to play a role in virulence. Dermatophytes are known to infect keratinized structures such as skin, hair, and nails, and therefore the ability of dermatophytes to degrade keratin is considered a major virulence attribute. In support of this, a correlation between keratinase activity and pathogenesis has been observed for *Mc* [26] and dermatophytes have been shown to secrete more than 20 proteases

in vitro when grown in medium containing protein as the sole nitrogen source (reviewed in [27]). As discussed above, genome analysis confirmed expansion of protease genes in the seven dermatophyte genomes ([12] and unpublished data).

Given the importance of keratin to the pathogenic lifestyle of dermatophytes, studies that aimed to identify virulence factors have often examined the response of dermatophytes to growth on keratin. For example, subtractive suppression hybridization (SSH) approaches have been used to compare *Tr* during growth on keratin as compared to glucose [28] or minimal medium [29]. Select genes identified in this manner were confirmed to be upregulated during interaction with keratinocytes [29]. These included a homeobox transcription factor and a zinc-finger protein, which are candidates for acting as transcriptional regulators during infection.

Kaufman et al. found that thioredoxin, cellobiohydrolase, and the protease-encoding gene *Tri m 4* had increased transcription during growth of *T. mentagrophytes* (*Tm*) with keratin as compared to glucose alone [30]. Zaugg et al. constructed a cDNA microarray for *Tr* and examined gene expression during growth on soy and soy + keratin as compared to rich medium (Sabouraud) to find factors induced by one or both proteins [31]. They found that growth in soy or soy + keratin activated a large set of genes encoding secreted endo- and exoproteases, as well as other proteins potentially implicated in protein degradation, some of which appeared to be keratin specific. Interestingly, the authors noted that upregulation of enzymes in the glyoxylate cycle was also observed during growth on soy or soy + keratin, as compared to Sabouraud. The glyoxylate cycle has been implicated in virulence of other microorganisms [32], and its upregulation during dermatophyte growth on keratin was confirmed for *Ab* by Staib et al., who examined gene expression of *Ab* under the same conditions [33]. They found induction of similar sets of orthologous protease-encoding genes as compared to the *Tr* data.

However, a growing body of evidence suggests that not all keratin-induced proteases play a role during infection. For example, although *Tri m 4* was induced by the presence of keratin, it was not significantly upregulated when homogenized skin was provided as the sole nutrient source [30]. Furthermore, some of the prominent keratin-induced genes of *Ab* were not found to be strongly upregulated *in vivo* during guinea pig infection and the protease-encoding gene *SUB6* was strongly upregulated *in vivo* but not detectably activated during growth on keratin [33]. Instead, Staib et al. found just one protease-encoding gene, *MCPA*, which was strongly induced during both infection and growth on keratin [33]. Their study identified nonprotease genes, such as those encoding a putative opsin-related protein and enzymes of the glyoxylate cycle, which were upregulated *in vivo*. Likewise, Burmester et al. found that only some of the keratin-induced proteases were strongly expressed during fungus-keratinocyte interaction [12]. Interestingly, they found that secondary metabolites were induced during interaction with keratinocytes. Previous work has shown that antibiotic substances are produced by dermatophytes that may help the fungi compete against bacteria also present on the skin [34, 35]. It is possible that differentially regulated secondary metabolites, perhaps including antibiotics or pigment production, play a role in dermatophyte infection by providing the fungi with an advantage over other microorganisms present on the skin.

Expression of specific secreted subtilisins and metalloproteases was monitored by RT-PCR during *Tr* growth *in vitro* in the presence of keratin, elastin, collagen, or human skin sections [36]. *SUB3*, *SUB4*, and *MEP4* had increased expression under all four conditions (compared to growth in glucose medium). The increased expression of *SUB3* is consistent with *in vivo* findings in *Ab*, although *SUB3* of *Ab* was upregulated only at a low level [12]. Furthermore, *SUB4* was not found to be upregulated during guinea pig infection by *Ab*.

Together, these results indicate that secreted proteases are not the only virulence factors of dermatophytes and indeed that not all proteases play an overlapping role during infection. Some proteases may be used only during specific stages of infection or might have a more general role in growth that is not specific to virulence. Furthermore, it is possible that each of the dermatophyte species will have a unique program of expression for proteases and other putative virulence factors during infection.

In order to identify additional factors that play a role during infection, investigators have examined the transcriptional response of dermatophytes exposed to environmental factors such as growth on lipids [37, 38], changes in pH [38, 39], and the presence of antifungal drugs [38, 40–45]. They have also assessed transcript abundance during different stages of growth [46–49]. The relationship of these environmental factors to the pathogenesis of dermatophytes is a continuing area of research.

3. Testing the Role of Putative Virulence Factors

3.1. Models for Testing Virulence. Although dermatophytes were initially studied in experimental human infections [50], the current most common animal model for studying virulence factors of dermatophytes is the guinea pig [51]. This model has been useful for zoophiles [33, 52–54] but does not provide an accurate infection model for most anthropophilic species [4]. A murine model has been useful for studying the immune response to dermatophytes [55, 56], but again the mice only develop disease in response to infection by zoophiles. An alternative to mammalian models has been to rely on growth of the dermatophyte on keratinized surfaces such as sterilized nail fragments as an indication of pathogenicity [57, 58]. Despite its relative ease, this is a nonquantitative model based solely on the observed (qualitative) ability of the dermatophyte to grow. Its continued use in virulence studies highlights the need for development of a more appropriate model of anthropophilic dermatophyte infection.

Galleria mellonella (wax moth) larvae are an established virulence model for several fungal pathogens, including *Candida*, *Cryptococcus*, and the mold *Aspergillus* [59–65].

The *Galleria* model has several advantages as a virulence model, including an immune system with some similarities to the human innate immune system [61, 66]. However, *Galleria* does not appear to be a useful model to study the pathogenic mechanisms of dermatophytes [11].

Recently, researchers have used skin explants as a model for dermatophyte adherence and invasion [67–71]. *Tm* and *Tr* have been tested in this model. Conidial suspensions or pieces of mycelium are applied to a skin explant, and germination and hyphal invasion are monitored microscopically. Dermatophytes can adhere to and invade *ex vivo* skin explants [67–69], and dermatophyte growth is inhibited by antifungal drugs [70]. A reconstructed feline epidermal model [72] and a feline *ex vivo* epidermal model [52] have also been reported for the study of *Mc*, a zoophile whose natural host is cats.

Human epidermal tissues are commercially available, which abrogates the need for researchers to have access to clinical samples and provides a greater degree of standardization between labs that wish to use skin explants as a virulence model. Dermatophyte microconidia are able to germinate and cause damage to these tissues (our own unpublished data). Skin explants therefore represent a possible virulence model to study the initial stages of dermatophyte infection.

3.2. Virulence Factor Genes That Have Been Tested. There are a few cases in which a gene hypothesized to play a role in virulence has been deleted or knocked down. Due to the historical difficulties of genetic manipulation of dermatophytes, gene deletions are often not complemented. However, recent genetic advances have provided a foundation for genetic manipulation of dermatophytes [53, 73–77]. Ideally, future studies of dermatophytes should include both a deletion and a complementation of the mutation to definitively prove the role of a gene product in virulence.

A study on the gene encoding malate synthase (AcuE, a key enzyme of the glyoxylate cycle) provided an excellent step towards this goal. *ACUE* was identified as being upregulated during infection of guinea pigs by *Ab* [33]. Grumbt et al. constructed a deletion, Δ*acuE*, and compared its growth to the parental strain on different carbon sources as well as during guinea pig infection [53]. Although they did not see a difference in pathogenicity between the mutant and the wild type, they did see differences in growth when provided with 0.5% olive oil as the sole carbon source, with Δ*acuE* being unable to grow. Complementation with the wild-type allele restored growth.

TruMDR2 is a gene identified in *Tr* that is predicted by sequence similarity to encode an ATP-binding cassette (ABC) transporter protein [78]. Deletion of this gene results in increased susceptibility to some antifungal compounds [78]. *TruMDR2* was found to be upregulated during growth in the presence of keratin as compared to glucose [28, 58], suggesting a role for the transporter protein during infection. To confirm this, the wild-type and deletion mutants were compared for growth on nail fragments. As predicted, the deletion mutant showed a reduced ability to grow [58].

Tr also encodes a protein with sequence similarity to *pacC*, a pH-regulated transcription factor in *Aspergillus nidulans* [57]. Disruption of *pacC* results in reduction of keratinolytic activity and a reduction in the ability to grow on nail fragments [57].

As discussed above, *SUB3* has been identified in screens as a putative virulence factor in *Tr* and *Ab*. *SUB3* expression and activity has also been monitored in *Mc*, where *SUB3* was identified as a 31.5 kDa secreted protein with *in vitro* keratinolytic activity [79]. It was found to be expressed during natural *Mc* infection of cats, although presence of *SUB3* did not correlate to disease state as *SUB3* was found in both symptomatic and asymptomatic infections [79, 80]. Expression of *SUB3* was experimentally reduced using RNA-mediated silencing [21], and the resulting strain was tested in a feline *ex vivo* adherence model [52]. Arthroconidia from the *SUB3* RNA-silenced *M. canis* strain had reduced adherence to feline epidermis compared to the control strain. Although the control-strain arthroconidia did not adhere equally well to epidermis from each of three different cats, for each cat the *SUB3* RNA-silenced strain had a reduction in adherence that was statistically significant. The strains were also tested for their ability to cause lesions in the guinea pig model, but no difference in virulence was observed [52]. The authors conclude that *SUB3* is required for adherence to, but not invasion of, the epidermis. It is also possible that the function of other secreted subtilisins masked the loss of *SUB3* or that the guinea pig model does not completely mimic feline infection.

In *Mc*, the *dnr1* gene, which has sequence similarity to nitrogen regulatory genes of other filamentous fungi, is able to complement a loss-of-function nitrogen regulatory mutation (*areA*) in *Aspergillus nidulans*. Disruption of *dnr1* in *Mc* caused a reduction in the ability of the fungus to grow on medium containing keratin as the sole nitrogen source [81]. A similar result was seen for *Tm* (teleomorph: *A. vanbreuseghemii*) when *tnr1*, a gene with sequence similarity to *areA* and *dnr1*, was disrupted [77]. Neither of these mutants have been studied in a virulence model. Virulence studies of these and other factors identified, for example in screens or through bioinformatics approaches, will be essential to determining the contribution of each factor to disease.

4. The Role of the Immune System

Fungal virulence is the result of interplay between the infecting organism and the host. During dermatophyte infection, cell-mediated immunity is widely considered to be responsible for modulating dermatophyte disease [7, 82–87] and fungal antigens activate T-suppressor and T-helper cells [56]. Differences specific to the host are thought to be important in determining the relative susceptibility of individuals, with factors such as age, gender, and genetics all likely to play a role [85, 87]. These clinical factors will not be reviewed here.

A recent review of host-dermatophyte interactions is available [83]. We will briefly describe these as they relate to dermatophyte virulence factors. The most numerous cells in the epidermis are keratinocytes, indicating that dermatophytes must primarily interact with these cells. Interestingly, keratinocytes seem to exhibit a differential response following exposure to different dermatophyte species.

Tani et al. measured cytokine production by epidermal keratinocytes following coculture with *Tm*, *Tt*, and *Tr* [88]. Of these, *Tm* causes an acute inflammatory response, whereas *Tt* and *Tr* are anthropophiles that cause minimal inflammation. Although all three species induced Interleukin- (IL-) 8 secretion, coculture of keratinocytes with *Tm* resulted in higher levels of IL-8 production than coculture with *Tt* or *Tr*. Additionally, *Tm* but not *Tt* or *Tr* was able to induce secretion of TNFα. Similar results were found when cytokine secretion profiles of human keratinocytes were compared during dermatophyte infection with *Ab*, a zoophile and causes a severe inflammatory response, and *Tt* [89]. They found that both species caused an increase in expression of IL-8, which was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). However, *Ab* also induced secretion of a broad spectrum of cytokines, whereas *Tt* did not. These studies support the hypothesis that different dermatophyte species induce different immune responses in the host, contributing to the relative severity of the infection.

Keratinocytes are not the only cells that will interact with the dermatophytes. Phagocytic cells are attracted to the site of infection (reviewed in [87]), and an ability of dermatophytes to survive those interactions would contribute to pathogenesis. Campos et al. determined that *Tr* conidia could germinate in a macrophage, resulting in macrophage death [90].

Certainly, it would be advantageous for anthropophiles to downregulate the immune response so as to facilitate chronic infection, and fungal factors that modulate the host immune response represent potential virulence factors. Indeed, mannan extracted from the cell wall of *Tr* inhibited lymphoproliferation of human mononuclear leukocytes *in vitro* [91]. Addition of filtrate solution from *Tm* or *Tr* was shown to induce secretion of IL-1α and basic fibroblast growth factor in keratinocytes [85], with more IL-1α being secreted in cells exposed to the *Tm* filtrate. This suggests that at least some of the putative virulence factors involved in modulating the immune response might be secreted by the fungi. It is likely that cell-associated as well as secreted factors contribute to the dermatophyte's ability to exaggerate or suppress an inflammatory response.

Temporal expression of proteases has also been postulated to contribute to the relative intensity of the inflammatory response [84]. Recent data comparing secreted proteolytic and lipolytic enzymes in *Tt* and *Te* support this idea [92]. *Tt* and *Te* are adapted to different host species (humans and horses, resp.), and *Tt* causes a mild chronic disease whereas *Te* causes an inflammatory disease in humans. Of the 31 genes studied, each had ≥99.5% sequence identity between the two species; however, transcriptional analysis identified differences in expression during growth on keratin [92]. For example, of the subtilisin-like proteases examined, *Sub6* and *Sub7* had significantly higher expression in *Tt* compared to *Te*, whereas *Sub1* and *Sub5* had significantly higher expression in *Te* compared to *Tt* [92].

Arthroconidia are produced during some infections and might aid survival in the nail and transmission of the infection to a new host. Arthroconidia can be formed by a majority of *Tr* clinical isolates during growth on nail powder under specific laboratory conditions [93] and have decreased susceptibility to some antifungals compared to microconidia [94]. The precise contribution of arthroconidia to infection and the mechanisms by which arthroconidia interact with host cells remains an area of investigation.

Few studies have examined dermatophyte gene expression in response to human cells [12, 30], and those that have serve to highlight the fact that growth in the presence of keratin does not necessarily reflect conditions during infection. The precise mechanisms by which dermatophyte species interact with host cells at the molecular level remain unknown. There is a clear need to identify the dermatophyte factors involved in pathogenesis, and a logical starting point is to identify dermatophyte genes and proteins that are upregulated during interactions with epithelial cells. To this end, transcriptional and proteomic profiling of dermatophytes during infection of human epidermal tissue, in addition to a bioinformatic approach, may identify additional potential virulence factors. These studies must go further, though. It is imperative that we test the expression and role that these factors play during infection. Only then can we expand our list of true virulence factors of dermatophytes and use the knowledge to inform directions for therapy and preventative measures.

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Review Article

Protein Glycosylation in *Aspergillus fumigatus* Is Essential for Cell Wall Synthesis and Serves as a Promising Model of Multicellular Eukaryotic Development

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Glycosylation is a conserved posttranslational modification that is found in all eukaryotes, which helps generate proteins with multiple functions. Our knowledge of glycosylation mainly comes from the investigation of the yeast *Saccharomyces cerevisiae* and mammalian cells. However, during the last decade, glycosylation in the human pathogenic mold *Aspergillus fumigatus* has drawn significant attention. It has been revealed that glycosylation in *A. fumigatus* is crucial for its growth, cell wall synthesis, and development and that the process is more complicated than that found in the budding yeast *S. cerevisiae*. The present paper implies that the investigation of glycosylation in *A. fumigatus* is not only vital for elucidating the mechanism of fungal cell wall synthesis, which will benefit the design of new antifungal therapies, but also helps to understand the role of protein glycosylation in the development of multicellular eukaryotes. This paper describes the advances in functional analysis of protein glycosylation in *A. fumigatus*.

1. Introduction

The *Aspergilli* are filamentous fungi, which are multicellular eukaryotes with a relatively simple life cycle. Over 200 species have been classified in the genus *Aspergillus*. Many of them have long been used in food production, industrial fermentation, and agriculture. On the other hand, a few, such as *A. fumigatus*, *A. flavus*, *A. niger*, *A. nidulans,* and *A. terreus*, are opportunistic fungal pathogens, causing life-threatening invasive aspergillosis (IA) in immunosuppressed patients [1, 2], in which *A. fumigatus* is the predominant pathogen [3–5]. The crude mortality for IA is 60–90% and remains around 29–42% even when treatment is given [6]. The main reasons for patient death are late diagnosis and the low efficiency of the drug therapies available to treat IA.

The fungal cell wall is a protective physical barrier against adverse environmental conditions. The fungal cell wall is a unique organ not found in mammalian cells. It maintains cell shape and provides osmotic protection [7, 8] and has therefore been recognized for a long time as an ideal drug target. Indeed, several cell-wall-targeted drugs, such as echinocandins, caspofungin, micafungin, and anidulafungin, have been introduced as therapies. For example, echinocandins, which inhibit synthesis of β-1,3-glucan, a crucial component of the cell wall are effective in the treatment of invasive fungal infections including IA [9]. Unfortunately, the echinocandins also trigger an increase of chitin [9, 10], which partially compensates for the loss of β-1,3-glucan and reduces the efficiency of treatment due to the complicated mechanism of cell wall biogenesis in *A. fumigatus*. Therefore, a more profound understanding of the mechanisms of cell wall biosynthesis in *A. fumigatus* would help to improve the efficiency of drug therapies, especially for drugs which target the cell wall.

The cell wall of *A. fumigatus* is composed of a unique β-1,3/1,4-glucan skeleton with chitin and galactomannan covalently linked to the nonreducing ends of β-1,3-glucan. The cell wall is mainly coated with GPI proteins, which contain N- and O-glycans [11, 12]. While there is no doubt that glycosylation is involved in cell wall organization, the functional importance of protein glycosylation in cell wall

organization has, until recently, remained poorly understood. However, during the past few years, it has become increasingly evident that glycosylation is vital for cell wall synthesis and thus vital for growth and morphology of *A. fumigatus*.

Basically, all eukaryotes possess three types of protein glycosylation, N-glycosylation of asparagine residues, O-glycosylation of threonine and serine residues, and glycosylphosphatidylinositol-anchoring (GPI-anchoring) of the C-terminus of some proteins. Humans lacking individual glycosyltransferases suffer from severe congenital diseases, known as carbohydrate-deficient glycoprotein syndromes (CDGs) [12–14]. Clearly, the sugar components of proteins play a major role in embryonic and postembryonic development of humans as well as of all higher eukaryotes. However, the molecular details leading to CDGs are only vaguely understood. During the past 20 years, the combination of carbohydrate chemistry and biology has developed rapidly. It is now known that carbohydrates play increasingly important roles in regulating the development of higher organisms [15]. However, the mechanism by which carbohydrates play a role in development and disease is still unclear. Our knowledge of protein glycosylation comes mainly from investigation of the model yeast *S. cerevisiae* and of mammalian cells [15]. Although investigation of the model yeast has been very useful in elucidating the biochemical features of protein glycosylation at the cellular level, they cannot reveal the complicated functions of glycosylation in the development of multicellular eukaryotes. Therefore, investigation of glycosylation in the multicellular fungus *A. fumigatus* not only helps understand the mechanism of cell wall synthesis in this species but also provides insights into the role of glycosylation in the development of multicellular eukaryotes. This paper concentrates on protein glycosylation in *A. fumigatus*, which will be discussed with respect to the enzymatic pathways involved and their functional importance. Furthermore, the utility of *A. fumigatus* as a model for glycosylation during development of multicellular eukaryotes will be outlined.

2. Cell Wall Organization and Its Compensatory Mechanism in *A. fumigatus*

2.1. Cell Wall Organization. The cell wall of *A. fumigatus* is mainly composed of β-1,3-glucans that are highly branched with β-1,6 linkages. Together they constitute a three-dimensional network with a large number of nonreducing ends, to which chitin, galactomannan, and β-1,3/1,4-glucan are covalently anchored [16]. The cell wall is mainly coated with GPI proteins, which contain N- and O-glycans derived primarily from the process of glycosylation [11, 17]. Glycoproteins such as Gel2p and Ecm33p are also involved in cell wall polysaccharide synthesis. Gel2p is a member of a new family of GPI-anchored β1,3-glucanosyltransferases. Deletion of *gel2* leads to slower growth, abnormal conidiogenesis, an altered cell wall composition, and reduced virulence [18, 19]. It has been proposed that Gel2p is responsible for the elongation of β-1,3-glucan side chains of β-1,3/1,6 branched glucan to provide new nonreducing ends. Ecm33p is also involved in maintaining proper cell wall architecture though its function is unknown. Disruption of *ECM33* results in morphogenetic aberrations such as defects in conidial separation, increase of chitin in conidial cell walls, rapid conidial germination, and increased virulence [20, 21]. It is clear that glycoproteins are directly, as structural components of the cell wall, and indirectly, as enzymes required for cell wall synthesis, involved in maintaining proper cell wall architecture in *A. fumigatus*. However, it remains unclear how glycosylation affects the function of these proteins.

2.2. Cell Wall Integrity (CWI) Signaling Pathway. Increased chitin synthesis has been known as an important compensatory response to cell wall stress both in *S. cerevisiae* and filamentous fungi [22–26]. In *S. cerevisiae*, the cell wall is required to maintain cell shape, which is essential for the formation of a bud and hence cell division. The yeast cell remodels its rigid structure to accommodate cell expansion during vegetative proliferation, mating pheromone-induced morphogenesis, and nutrient-driven filamentation through the cell wall integrity (CWI) signaling pathway. Cell wall defects or damage induces the cells to activate the CWI pathway to survive, and the compensatory mechanism characterized by an increased chitin content is triggered [27].

The CWI signaling pathway in *S. cerevisiae* is activated in response to low osmolarity, thermal stress, or mating pheromone and polarized growth. It is comprised of a family of cell surface sensors coupled to the small G-protein Rho1p, which activates the CWI MAPK cascade via protein kinase C (Pkc1p). This signaling cascade activates the expression of genes encoding for cell wall proteins that stabilize the cell wall. Meanwhile, activated Rho1p also activates a set of additional effectors such as Bni1p and Bnr1p formin proteins, Skn7p transcription factor, and the Sec3p exocyst component, which regulate a diverse set of processes including β-glucan synthesis at the site of cell wall remodeling, gene expression related to cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site [28].

A family of cell surface sensors is responsible for detecting and transmitting the status of the cell wall to Rho1p [28]. These sensor molecules include Wsc1p (Hcs77p/Slg1p) [29–31], Wsc2p and Wsc3p [31], and Mid2p and Mtl1p [32, 33]. Among these cell wall stress sensors, Wsc1p and Mid2p appear to be the most important and serve a partially overlapping role in CWI signaling. The extensive O-mannosylation of Mid2p and Wsc1p is important to their stability [34]. Reduced O-mannosylation leads to incorrect proteolytic processing of these proteins, which in turn results in impaired activation of the PKC1 pathway and finally causes cell death in the absence of osmotic stabilization [35]. More recently, N-glycan is found to be directly involved in Mid2p sensing. It has been shown that both the extent of the N-linked glycan and its distance from the plasma membrane affect Mid2p function. Non-N-glycosylated Mid2p fails to perceive cell wall challenges [36]. These observations demonstrate that N- and O-glycosylation are important for CWI sensing and thus important for cell wall biogenesis and polarized growth in yeast.

The presence of *A. fumigatus* genes encoding for proteins homologous to the yeast Rho1p, Rho3p, and Cdc42p suggests a similar mechanism for the CWI pathway. Indeed, it has been recently shown that Af*cdc42*/*CDC42*, Af*rho1*/*RHO1*, and Af*rho3*/*RHO3* are highly expressed in the mutant devoid of Cwh41p (glucosidase I), which suggests an activation of these genes induced by cell wall damage [37]. Also, increased expression and activation of the *A. fumigatus* MpkAp, an ortholog of the *S. cerevisiae* Mpk1p, is also induced by cell wall damage [38, 39]. It is becoming clear that, as in yeast, defects in cell wall integrity also trigger the CWI MAPK cascade in *A. fumigatus*.

On the other hand, in contrast to yeast, little is known about the cell wall stress sensors in *A. fumigatus*. In the last release of the *A. fumigatus* genomic database (http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/index.php~main), only one protein (AFUA_5G09020) is annotated as a homologue of the Wsc4p, which does not appear to contribute to CWI signaling in yeast. Therefore, the *A. fumigatus* cell wall stress sensor molecule remains to be identified and investigated.

3. Importance of Glycosylation in *A. fumigatus*

The precursor of all mannose residues found in galactomannan, glycoprotein, and GPI anchor in *A. fumigatus* is GDP-mannose. Therefore, its biosynthesis has drawn special attention. In all eukaryotes, the activation of mannose initiates from formation of mannose 6-phosphate (Man-6-P), which occurs by one of two routes: direct phosphorylation of mannose by hexokinase or interconversion from fructose 6-phosphate (Fru-6-P) via phosphomannose isomerase (PMI), and the latter pathway requires three enzymes: PMI, phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GMPP). Fru-6-P is converted to Man-6-P by PMI, and then Man-6-P is converted to mannose 1-phosphate (Man-1-P) by PMM. Subsequently, Man-1-P is ligated with the guanosine 5-triphosphate molecule (GTP) to form GDP-mannose by Man-1-P guanylyltransferase [40–63].

The interconversion of Man-6-P and Fru-6-P catalysed by PMI is the first committed step in the synthesis of Man-containing sugar chains and provides a link between glucose metabolism and mannosylation. PMI deficiency is the cause of carbohydrate-deficient glycoprotein syndrome type Ib (CDG-Ib, OMIM 602579) in humans, but the clinical symptoms and aberrant glycosylation can be corrected with dietary mannose supplements [40].

Genes encoding for PMIs have been investigated in several fungal species, such as *S. cerevisiae*, *Candida albicans*, *A. nidulans*, and *Cryptococcus neoformans* [48–51]. The *S. cerevisiae* PMI is encoded by the *PMI40* gene [51]. The *pmi* $^{-}$ mutant shows a significantly reduced growth rate at high concentrations of mannose. Biochemical and genome-wide analysis reveals that excess mannose leads to an accumulation of Man-6-P, which mainly inhibits the activity of phosphoglucose isomerase (PGI) and thus represses glycolysis, protein biosynthesis, and cell wall biogenesis [52].

Table 1: Properties of PMIs from different species.

Species	MW (kD)	K_m for Man-6-P (mM)	V_{max} (μmol/min/mg)	Reference
Aspergillus fumigates	55	1	753	[54]
Saccharomyces cerevisiae	45	0.65	980	[51]
Escherichia coli	42	ND	ND	[41]
Candida albicans	49	1.24	1200	[42]
human	46.7	0.25	110	[42]
Porcine	ND	0.17	140	[42]
Salmonella typhimurium	42.6	1.34	833.3	[43, 44]
Xanthomonas campestris	58	2	33.5	[45]
Pseudomonas aeruginosa	54	3.03	0.83	[46]
Burkholderia cepacia	55	9.01	21	[47]

ND, not detected.

The *A. nidulans manA1* mutant exhibits abnormal ballooning of hyphal tips and eventually ceases to grow [53]. Disrupted *MAN1* mutant of *C. neoformans* leads to poor capsule formation, reduced polysaccharide secretion, morphological abnormalities, and attenuated virulence [50]. In *A. fumigatus*, PMI activity is essential for viability and plays a central regulatory role in both glycosylation and energy production. Deletion of the *A. fumigatus pmi1* gene leads to uncoupling of the link between energy production and glycosylation and accumulation of Man-6-P, which then results in defects in cell wall integrity, conidiation, and morphology [54]. Although extracellular mannose can rescue the growth of PMI deficient mutants in *A. fumigatus*, both lower and higher concentrations of mannose lead to a reduction in the levels of α-glucan in the cell wall and an accumulation of Man-6-P [54]. The phenotypes associated with the mutant under mannose starvation are mainly due to an insufficient supply of GDP-Man required for cell wall synthesis. The abnormal morphology associated with the Δ*pmi1* mutant under mannose-replete conditions is mainly ascribed to an accumulation of Man-6-P, which cannot efficiently enter glycolysis, instead becoming trapped in a cycle of dephosphorylation and rephosphorylation resulting in depletion of intracellular ATP. It should be pointed out that the PMI in *A. fumigatus* mainly catalyzes the conversion of Fru-6-P to Man-6-P, and its binding affinity for Man-6-P is similar to that of yeasts but different from the ones from bacteria or animals (Table 1). This suggests that it may be possible to design a specific inhibitor for fungal PMIs [54].

GMPP is the final enzyme in the pathway generating GDP-mannose. Several GMPPs have been identified and characterized in different species [55–59]. In *S. cerevisiae* and *C. albicans*, GMPP is essential [60, 61], while in *Leishmania*

mexicana GMPP is not required for viability [62]. Repression of GMPP in yeast leads to pleiotropic phenotypes including cell lysis, failure of cell separation, impaired budding and hyphal switching, clumping and flocculation, and cell wall defects [61]. Repression of expression of *A. fumigatus* GMPP *srb1*, a homologue of yeast *SRB1*/*PSA1*/*VIG9*, leads to hyphal lysis, a defective cell wall, impaired polarity maintenance, and branching site selection, as well as rapid germination and reduced conidiation. In contrast to yeast, inducible repression of *srb1* expression in *A. fumigatus* does affect the ability to direct polarity establishment and septation [63].

These reports imply that mannose activation is specifically crucial for the synthesis and organization of the cell wall and thus essential for survival of fungal species. This further suggests that glycosylation is essential for the viability of pathogenic fungal species such as *A. fumigates,* and inhibitors that specifically block mannose activation in fungi may be potential drugs to treat fungal infections.

4. Biosynthesis and Function of N-Glycosylation in *A. fumigatus*

N-glycosylated proteins contain oligosaccharides that are N-glycosidically linked to the γ-amido group of asparagine. This type of glycoprotein has been intensively studied in many model systems from yeast to human cells with respect to their structure, biosynthesis, and function [15]. It has been shown that the formation of the highly variable N-linked oligosaccharides is initiated by the assembly of a lipid-linked oligosaccharide $Glc_3Man_9GlcNAc_2$-PP-Dol by a series of glycosyltransferases located on the cytoplasmic and luminal faces of the ER membrane. The most complete understanding of biosynthesis of the lipid bound precursor has been obtained from *S. cerevisiae* and from mammals. As far as it is known, the corresponding reactions proceed almost identically in other eukaryotes [15].

Subsequently, the Dol-PP-linked $Glc_3Man_9GlcNAc_2$ is transferred as a whole to an asparagine residue within an N-X-T/S consensus sequence of a nascent peptide, which is catalyzed by the oligosaccharyltransferase (OST), and then the N-glycosylated proteins are modified in a species-specific manner and transferred through the secretory pathway to the cell surface where they either get exported or anchored to the plasma membrane, to the extracellular matrix, or to the cell wall (Figure 1).

4.1. Initiation of N-Glycosylation. OST is a membrane complex consisting of several subunits. In *S. cerevisiae*, the OST complex consists of at least eight different subunits, including Ost1p, Ost2p, Wbp1, Stt3p, Swp1p, Ost4p, Ost5p, and Ost3p/Ost6p [64–67]. Although the function of each subunit is still unclear, Stt3p is believed to be the catalytic subunit [68–70], and its homologues are found in almost all eukaryotes [71]. The *S. cerevisiae STT3* is an essential gene [72, 73]. It appears that the *A. fumigatus stt3* is also essential as no viable knockout mutant has been recovered [39]. Repression of the *stt3* gene in *A. fumigatus* leads to a severe retardation of growth and a slight defect in cell wall integrity [39]. Further analysis shows that repression of *stt3* upregulates expression of the genes responsible for glucan and chitin synthesis, especially *gel1*, *gel2*, *fskA*, *chsE,* and *chsG*. Indeed, an increase of cell wall mannoprotein and chitin was observed following repression of the *stt3* gene. However, this upregulation of chitin is not accompanied by an activation of the MpkA kinase. Indeed, only the unfolded protein response (UPR) is induced. As the UPR has been shown to be involved in CWI signaling in *A. fumigatus* [74], it is likely that UPR, instead of the MpkA-dependent CWI signaling pathway, is the major compensatory mechanism induced by repression of the N-glycosylation in *A. fumigatus* [39].

4.2. N-Glycan Processing and Protein Folding Quality Control in ER. Once $Glc_3Man_9GlcNAc_2$ is transferred to proteins, the N-glycan is processed sequentially in the ER and Golgi. N-Glycan processing is initiated by the removal of the glucose residues catalyzed by ER glucosidase I and glucosidase II.

In mammalian cells, N-linked glycan plays a decisive role as a quality control (QC) of the folding of secretory proteins, which is composed of calnexin, calreticulin, UDP-glucose:glycoprotein glucosyltransferase (GT) and glucosidase II (GII) and is essential for cellular survival [75–77]. N-glycans initially serve to increase the hydrophilicity of the as-yet-unstructured nascent polypeptides. Subsequently, the two outermost glucose residues of the N-glycan are removed by the sequential action of glucosidase I (GI) and GII to the monoglucosylated form, which is recognized and bound by calnexin, a type I ER membrane lectin, and calreticulin, its soluble relative. For many glycoproteins, the interaction with calnexin or calreticulin slows down the rate of folding but increases efficiency. GII-catalyzed removal of the third glucose residue follows the dissociation of folding substrates from calnexin and is required for release of native polypeptides from the ER and transport to their final destination. The folding sensor GT adds back a terminal glucose to promote reassociation of nonnative polypeptides released from calnexin, thus prolonging their retention in the ER folding environment. Cycles of de-/reglucosylation might be protracted until the polypeptide released from calnexin fulfills quality control requirements. When correct folding is not achieved, an ER-specific N-glycan-dependent pathway of degradation removes the misfolded proteins. When N-glycosylation is inhibited, the most commonly observed effect is the generation of misfolded, aggregated proteins that fail to reach a functional state [75, 76].

Before entering the QC system, the outermost glucose residue of the N-glycan is trimmed by ER glucosidase I [78]. A human inherited glucosidase I deficiency has been reported to result in neonatal birth with severe generalized hypotonia and dysmorphic features [79].

Unlike mammalian cells, *S. cerevisiae* lacks a calnexin cycle and GT and only has an effective mannosidase I-dependent ERAD system [80, 81]. The yeast glucosidase I (Cwh1p) is encoded by the *CWH41* gene [82]. Mutational defects in the *CWH41* gene cause severe and selective instability of glycoprotein Kre6p, a putative Golgi glucan synthase required for β-l,6-glucan synthesis [23, 83, 84].

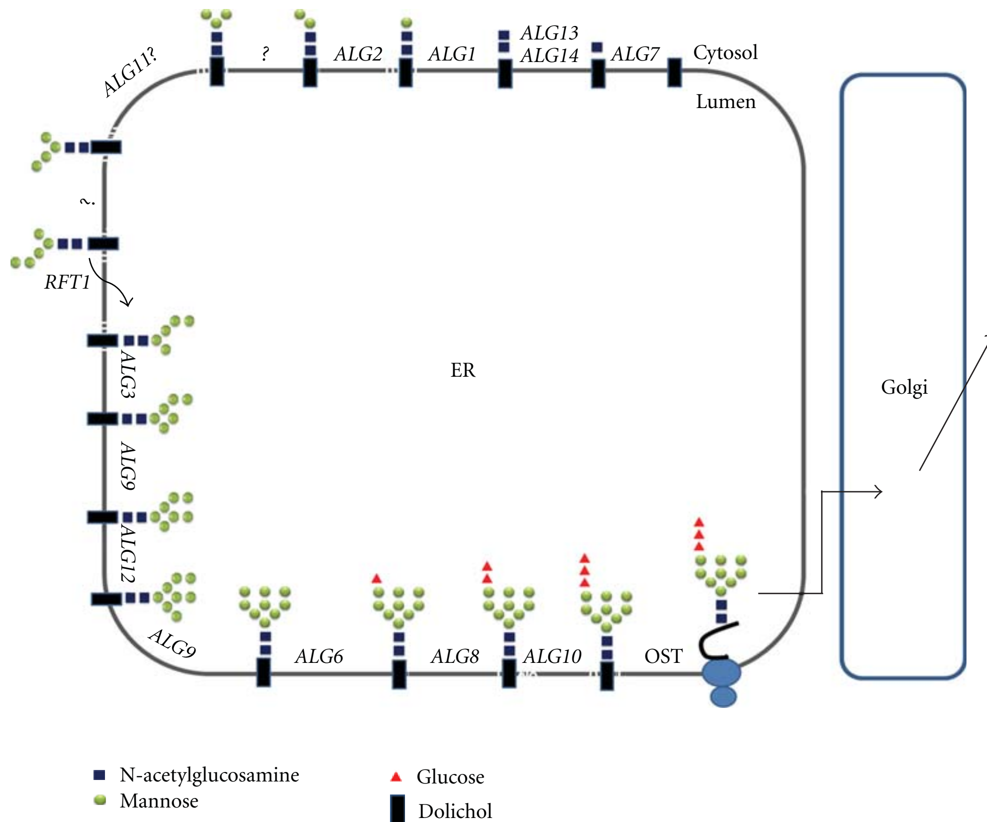

Figure 1: Biosynthesis of the lipid-bound oligosaccharide and transfer of the oligosaccharide to the nascent polypeptide in the endoplasmic reticulum of *S. cerevisiae*. The identified *ALG* genes for the respective glycosylation reactions are indicated. Synthesis starts at the cytoplasmic face with UDP-GlcNAc and GDP-Man as donors. The $Man_5GlcNAc_2$-PP-Dol is then transferred to the luminal side with the help of Rft1 and elongated to the full-length lipid-linked oligosaccharide $Glc_3Man_9GlcNAc_2$-PP-Dol by using Dol-P-Man and Dol-P-Glc as donors. The oligosaccharide is subsequently transferred to the γ-amido group of the asparagine residues within the consensus sequence Asn-X-Ser/Thr of nascent secretory proteins. This reaction is catalyzed by the oligosaccharyltransferase (OST) complex.

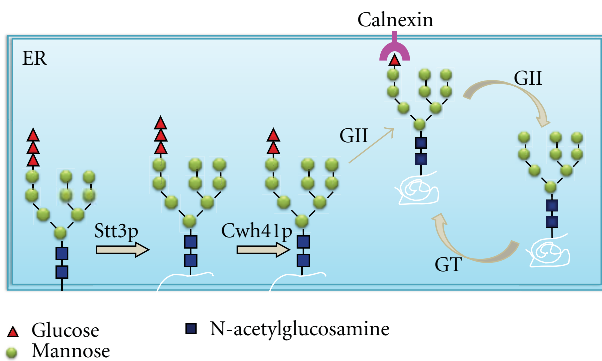

Figure 2: N-glycan-dependent quality control of protein folding in *A. fumigatus*. In *A. fumigatus*, the N-glycan-dependent QC system is composed of calnexin, UDP-glucose: glycoprotein glucosyltransferase (GT) and glucosidase II (GII).

Some filamentous fungi have been proposed to possess N-glycan-dependent QC of glycoprotein folding based on fungal genome sequence data [85]. Recently, evidence that filamentous fungi possess an N-glycan-dependent QC system has been reported in *A. fumigatus* [37, 86]. Indeed, calnexin (AAS68033), glucosidase II, and GT have been annotated in the last release of the *A. fumigatus* genomic database (Figure 2) [87]. Zhang et al. [37] reported that deletion of the *cwh41* gene in *A. fumigatus* results in defective N-glycan processing of the proteins secreted by *A. fumigatus*. Although Af*cwh41* is not essential for hyphal growth and virulence, a severe reduction in conidial formation, abnormalities of polar growth and septation, and a temperature-sensitive deficiency of cell wall integrity were documented. Also, the genes encoding Rho-type GTPases (Rho-type GTPase/CDC42) were upregulated, which suggests that the CWI pathway was activated in the mutant [37].

4.3. N-Glycan Proces sing in the Golgi. After processing by two ER α-glucosidases, the N-glycan is further processed by the action of various α1,2-mannosidases, which can remove one or more of the four α1,2-linked mannose residues. In mammalian cells, $Man_9GlcNAc_2$ is converted to $Man_5GlcNAc_2$ by the action of ER and Golgi α-mannosidases, which is the precursor for complex, hybrid, and high-mannose N-glycans [88]. In *S. cerevisiae*, a specific ER α1,2-mannosidase converts

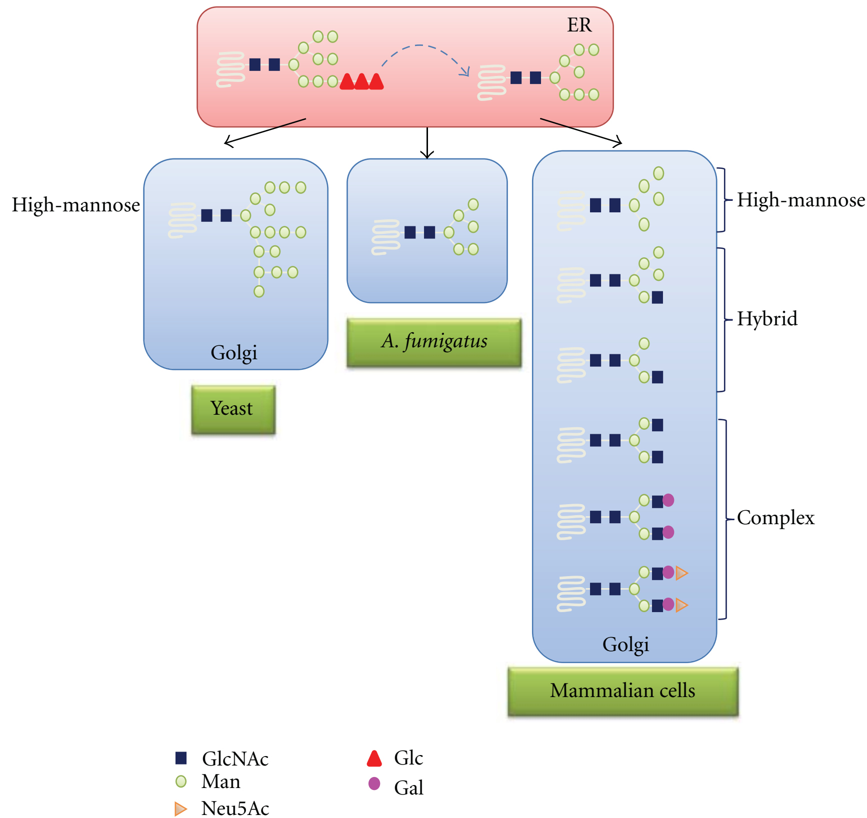

FIGURE 3: Comparison of N-glycans in yeast, *A. fumigates,* and mammalian cells. After processing by two ER α-glucosidases, the N-glycan is further processed by the action of various α1,2-mannosidases. In *S. cerevisiae*, a specific ER α1,2-mannosidase converts $Man_9GlcNAc_2$ into $Man_8GlcNAc_2$, which is elongated in the Golgi to form an outer chain containing up to 200 residues of mannose. In *A. fumigatus,* the N-glycans on mature glycoprotein are $Man_6GlcNAc_2$. In mammalian cells, $Man_9GlcNAc_2$ is converted to $Man_5GlcNAc_2$ by the action of ER and Golgi α-mannosidases, which is the precursor for complex, hybrid, and high-mannose N-glycans.

$Man_9GlcNAc_2$ into $Man_8GlcNAc_2$, which is elongated in the Golgi to form an outer chain containing up to 200 residues of mannose [89, 90]. *A. saitoi* and *Trichoderma reesei* have been found to produce N-glycan structures containing five mannose units ($Man_5GlcNAc_2$), suggesting further processing of the $Man_9GlcNAc_2$ precursor [91, 92]. The N-glycans on mature secreted glycoprotein produced by *A. fumigatus* are $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_8GlcNAc_2$, in which $Man_6GlcNAc_2$ is the major glycoform [37]. These observations demonstrate that N-glycan synthesis in filamentous fungi seems to differ from that in yeast and is similar to that in higher eukaryotes (Figure 3). Although small N-glycans are commonly found on glycoproteins of *A. fumigatus*, $Hex_{5-13}HexNAc_2$ glycans on the galactomannoproteins, and $Man_{5-9}GlcNAc_2$ as well as $Gal_{f1}Man_{5-7}GlcNAc_2$ structures on other secreted glycoproteins have been identified in *A. fumigatus* [93, 94]. The enzyme (UDP-Gal_p mutase) required to synthesize the requisite UDP-Gal_f donor has been shown to be an important factor in biosynthesis of the cell wall in *A. fumigatus* [94], while the gene/enzyme responsible for the transfer of Gal_f has not been identified. Recently, the *A. fumigatus* Och1, a key mannosyltransferase for synthesis of elaborated protein N-glycans in yeast, has been identified. Deletion of the *och1* gene results in a reduction of sporulation in the presence of high calcium concentrations. This evidence suggests that polymannosylated N-glycans exist in *A. fumigatus* and certain proteins engaged in sporulation require N-glycan outer chains to be fully functional [95].

The α-mannosidases have been classified into two groups: Class I and Class II [102, 120, 121]. Class I α-mannosidases include ER Man_9-mannosidase, endomannosidase, and Golgi mannosidase I. Class II α-mannosidases include the lysosomal mannosidases, Golgi mannosidase II, yeast vascular mannosidase [98, 99], and ER α-mannosidase II [122, 123]. Several Golgi α-mannosidases have been cloned and characterized from *Penicillium citrinum* [124, 125], *A. saitoi* [126], *A. oryzae* [127], *T. reesei* [128], and *A. nidulans* [102, 129]. These enzymes are all monomeric with a molecular weight of 50–60 kDa and show the maximal activity in the semiacidic condition (pH 4–6).

Class I α-mannosidase is known to play an important role in the processing of mannose-containing glycans. In *Drosophila melanogaster,* deletion of the Golgi mannosidase I (*MAS-1*) results in viable progeny, and the null organisms synthesize the same range of oligosaccharides as the wild-type ones, albeit at different ratios [130]. In *S. cerevisiae,* disruption of the ER α-mannosidase gene does not prevent outer chain synthesis [96]. In the last release of the

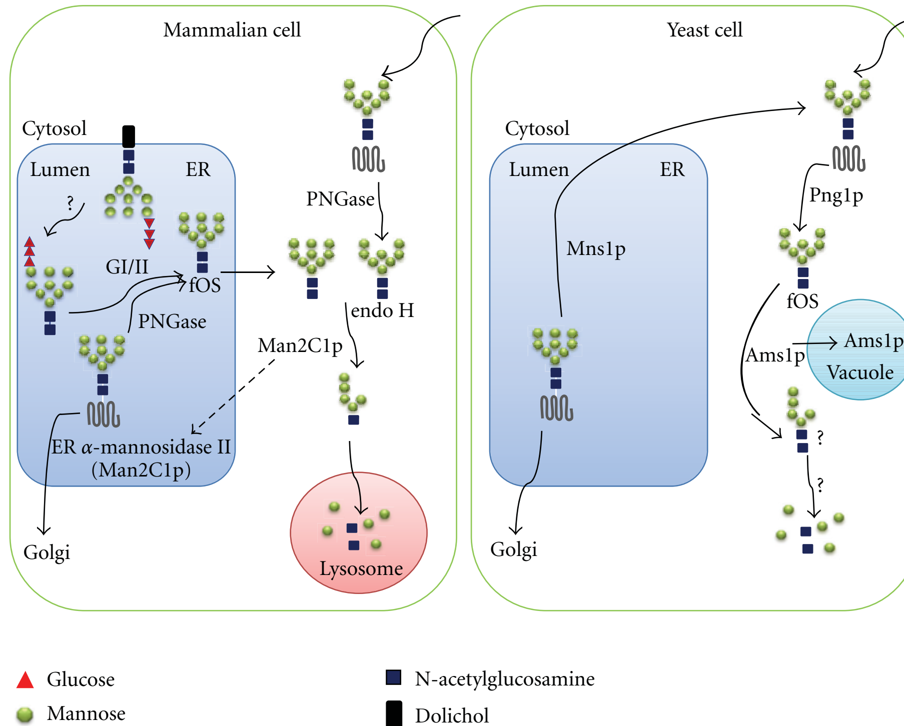

Figure 4: Comparison of free oligosaccharide catabolism in mammalian and yeast cells. Glycoprotein biosynthesis in mammalian cells is accompanied by the generation of free oligosaccharides (fOS) from both OST-mediated hydrolysis of $Glc_3Man_9GlcNAc_2$-PP-dolichol in the lumen of the ER and peptide N-glycanase (PNGase-) mediated de-N-glycosylation of newly synthesized glycoproteins, which undergo ER associated protein degradation (ERAD), either in the ER or the cytosol. fOS that are liberated in ER can be transported into the cytosol. In the cytosol, fOS are trimmed by an endo-β-D-*N*-acetylglucosamine H (endo H-) like enzyme and the α-mannosidase Man2C1p to yield $Man_5GlcNAc$, which can be imported directly into lysosomes to be degraded. In *S. cerevisiae*, fOS are released from glycoproteins in the cytosol by Png1p, a counterpart of mammalian PNGase. Then the Png1p-generated fOS may be processed in the cytosol by Ams1p, the yeast cytosolic α-mannosidase. It should also be noted that no structural studies have been performed on the products that can be generated from $Man_8GlcNAc_2$ by Ams1p, and the ultimate fate of such products remains obscure. On the other hand, two Png1p-independent fOS pools, $Man_3GlcNAc_2$ and $Man_8GlcNAc_2$, are also seen in *S. cerevisiae*. The pool comprising small fOS ($Man_3GlcNAc_2$) appears to be disposed of by unknown enzymes in the vacuole. The pool containing mainly $Man_8GlcNAc_2$ may be generated and disposed of along the secretory pathway.

TIGR database (http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/index.php~main) [87], nine *A. fumigatus* genes are annotated to encode α-mannosidases, including XP_749038.1, XP_754794.1, XP_751252.1, XP_751819.1, XP_752444.1, XP_752825.1, XP_753592.1, XP_751114.1, and XP_750572.1. Among them, MsdSp (XP_752825.1) has been identified to encode a Class I α1, 2-mannosidase and acts on $Man_8GlcNAc_2$ to produce $Man_6GlcNAc_2$. Deletion of the *msdS* gene leads to a defect in N-glycan processing, as well as a reduction of cell wall components (including α-glucan, β-glucan, mannoprotein, and chitin) and reduced conidiation. Morphological analysis reveals abnormal polarity and septation. However, deletion of the *msdS* has no effect on fungal growth and virulence [97].

4.4. Degradation of N-Glycan. In mammalian cells, free oligosaccharides (fOS) are generated by OST-mediated hydrolysis of $Glc_3Man_9GlcNAc_2$-PP-dolichol in the lumen of the ER or peptide N-glycanase (PNGase)-mediated de-N-glycosylation of newly synthesized glycoproteins either in the ER or the cytosol. fOS that are liberated in the lumen of the ER can be transported into the cytosol, where they are trimmed by an endo-β-D-*N*-acetylglucosamine H (endo H)-like enzyme and the α-mannosidase Man2C1p in order to yield an oligosaccharide, $Man_5GlcNAc$, that can be imported directly into lysosomes to be degraded (Figure 4) [122, 123, 131–135]. In humans [136] and cattle [137–139], a deficiency in α-mannosidase results in the lethal disease mannosidosis, a rare lysosomal storage disease with a collection of clinical symptoms including progressive mental retardation, impaired hearing, dysostosis multiplex, immune defects, elevation of serum and urinary oligosaccharide levels, and an enlargement of lysosomes in most cell types resulting from the accumulation of undegraded oligosaccharides.

The rat Man2C1p is involved in oligosaccharide catabolism of misfolded glycoproteins in the lumen of the ER which have been retrotranslocated into the cytoplasm for proteolytic disposal [131–133]. A proteolytically cleaved version of the rat Man2C1p has been found in the lumen of the ER where it is believed to be involved in the early stages

of glycoprotein maturation (also called ER α-mannosidase II) (Figure 4) [122, 123].

The yeast cytosolic α-mannosidase Ams1p, a counterpart of Man2C1p, is also involved in the processing of fOS. Since the yeast Png1p is mainly localized to the cytosol, it is proposed that the Png1p-generated fOS may both be generated and processed in the cytosol [100]. The role of the yeast Ams1p is to aid in recycling macromolecular components of the cell under nutrient deprivation [101]. Interestingly, after its synthesis in the cytosol, the Ams1p is translocated into the vacuole by the cytosol-to-vacuole targeting pathway [101], which suggests a common feature shared by the *S. cerevisiae* Ams1p and its mammalian counterparts. However, the yeast Ams1p only participates in recycling or utilizing of oligosaccharide but not in processing of N-glycan (Figure 4) [100]. It should also be noted that no structural studies have been performed on the products that can be generated from $Man_8GlcNAc_2$ by Ams1p, and the ultimate fate of such products remains obscure [135]. On the other hand, two Png1p-independent fOS pools, $Man_3GlcNAc_2$ and $Man_8GlcNAc_2$, are also seen in *S. cerevisiae*. The pool comprising small fOS ($Man_3GlcNAc_2$) appears to be disposed of by unknown enzymes in the vacuole. The pool containing mainly $Man_8GlcNAc_2$ may be generated and disposed of along the secretory pathway [135].

Similarly, *A. nidulans* α-mannosidase IIC is also proposed to be involved in oligosaccharide catabolism [102]. Both *A. nidulans* α-mannosidase IIC and *S. cerevisiae* Ams1p are not essential for normal cellular function since disruption of these genes has no visible effect on growth or morphology [98, 99, 102].

In contrast to its counterpart in yeast or *A. nidulans*, the *A. fumigatus* Ams1p is required for normal cellular function. Deletion of the *A. fumigatus ams1* leads to a severe defect in conidial formation, especially at a higher temperature. In addition, abnormalities of polarity and septation are associated with the ΔAf*ams1* mutant. These results show that the Af*ams1* gene is required for morphogenesis and cellular function in *A. fumigatus* [103]. The involvement of the Af*ams1* gene in polarized growth demonstrates that the processes involved in fOS regulation are important for *A. fumigatus*. It is likely that the Ams1p is involved in cell wall synthesis and thus polarity through the CWI pathway. Therefore, probably the ΔAf*ams1* mutant could serve as a simple model to investigate the mechanism of α-mannosidosis.

4.5. Functions of N-Glycosylation in Cell Wall Synthesis, Morphology, and Polarity. Functional analyses of the genes required for N-glycosylation reveal that protein N-glycosylation is important for cell wall synthesis, morphogenesis, and polarized growth in *A. fumigatus*. 2-D gel analysis reveals that deletion of the *cwh41* gene encoding glucosidase I in *A. fumigatus* leads to ER stress, which induces overexpression of HSP70 and calnexin chaperone and activates the ERAD. Meanwhile, the proteins required for actin rearrangement are found to be underexpressed or missing, which is consistent with the observation of random localization of actin fibers in the mutant [86]. These observations, for the first time, clearly suggest that N-glycosylation contributes to proper folding and trafficking in *A. fumigatus*. It appears that proteins involved in cell wall biosynthesis in *A. fumigatus* are more dependent on the N-glycan-dependent folding system. As in yeast, cell wall defects also trigger the CWI signaling pathway in *A. fumigatus*, which activates downstream effectors that regulate cell wall biogenesis and polarized growth. Zhang et al. [37, 86] have proposed that the proteins required for cell wall synthesis or cell wall stress sensing are substrates of *A. fumigatus* Cwh41p and require glucose trimming for their proper localization and function. Misfolding of these proteins would cause cell wall defects, which then leads to activation of the ERAD and Rho-type GTPases-mediated CWI pathway. Moreover, activation of CDC42 in the CWI pathway also activates SepA, an upstream organizer of actin ring formation at septation sites, and thus causes abnormal polarized growth associated with the Δ*afcwh41* mutant (Figure 5). Although the phenotypes associated with different N-glycosylation mutants vary, the finding that all of these mutants exhibit phenotypes associated with cell wall defects, abnormal polarization, and morphological changes can all be explained by this proposed model.

Obviously, more investigations are needed to identify and characterize all of the proteins affected by N-glycosylation in *A. fumigatus*. This information would be key to understanding the complex compensatory mechanisms participating in cell wall biosynthesis in *A. fumigatus*, which would serve as a basis to develop new antifungal therapies, as well as help to elucidate the molecular mechanism of human diseases associated with defects in glycosylation.

5. Biosynthesis and Function of O-Glycosylation

O-mannose glycosylated proteins were first discovered in yeast and filamentous fungi, and recently this type of glycoproteins has also been described in mammals. The O-mannosylation most likely occurs in all animals, with the exception of nematodes (e.g., *Caenorhabditis elegans*); it is also not detected in plants (*Arabidopsis thaliana*, *Oryza sativa*). However, it has also been discovered in one bacterial species (*Mycobacterium tuberculosis*) [15]. In mammalian cells, the inner O-linked mannose is elongated with the first addition of a N-acetylglucosamine and then various sugars [140]. In the case of yeast, the O-mannose type carbohydrate chain starts with a serine/threonine-linked mannose, which is extended to an oligomannose chain. In *A. fumigatus*, the O-linked glycans on cell wall mannoproteins are found to be Glcα1, 6Man, Gal*f*β1, 6Manα1, 6Man, Gal*f*β1, 5Gal*f*β1, 6Manα1, 6Man and Gal*f*β1, 5[Gal*f*β1,5]$_3$ Gal*f*β1, 6Man [141], while only a single mannose residue was detected on secreted proteins [113]. A further type of protein O-glycosylation, in which a single β-O-linked GlcNAc residue is linked to serine and threonine occurs in animals, plants, and filamentous fungi, but not in *S. cerevisiae*. For this type of protein modification, a considerable number of reviews are available [142–144]. Therefore, this type of O-glycosylation is not discussed in this paper.

Protein O-mannosylation is initiated by a family of protein O-mannosyltransferases (PMTs) that are evolutionarily conserved from yeast to human [145, 146]. In *S. cerevisiae*

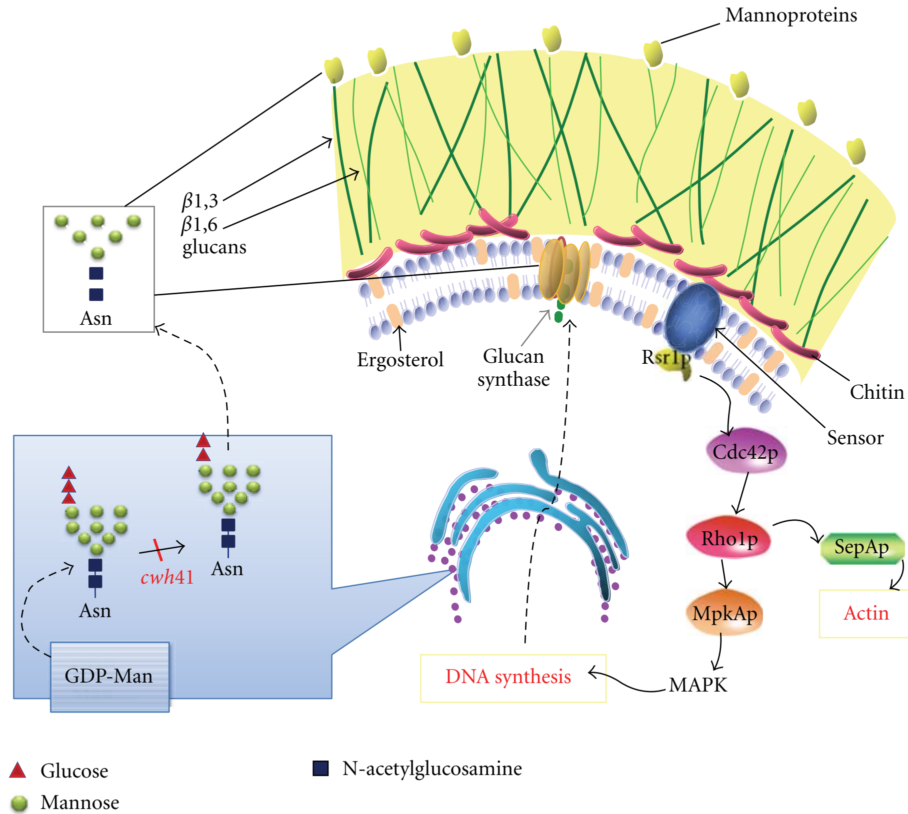

FIGURE 5: Proposed model of functions of N-glycosylation in *A. fumigatus*. Proteins that are required for cell wall synthesis or cell wall stress sensing require N-glycosylation for their proper folding, localization, and function. Disruption of N-glycosylation in *A. fumigatus* results in misfolding and degradation of these proteins, which thus causes cell wall defects and then leads to the activation of the Rho-type GTPases (Rsr1p/Bud1p-Cdc42p-Rho1p-) mediated CWI pathway to compensate for cell wall defects. Meanwhile activation of the CDC42 in the CWI pathway also activates SepA, an upstream component of actin rearrangement, leading to abnormalities in polarized growth.

a total of seven PMT family members (ScPmt1–7p) are present [104, 147], which fall into three major groups of homology: (i) Pmt1/5/7, (ii) Pmt2/3/6, and (iii) Pmt4. Genes with significant homology to *PMT*s have been cloned in humans, mice, and *Drosophila* [148–150]. Specific protein substrates that are O-mannosylated by ScPmt1p, ScPmt2p, or ScPmt4p have been described in *S. cerevisiae* [151–153].

In comparison with *S. cerevisiae*, the PMT family is less redundant in higher eukaryotes. In *Drosophila* only two PMT family members are present (*rotated abdomen* and *twisted*) [149, 150]. The same is true for mice and humans (POMT1 and POMT2) [148, 150]. Mutations in human POMT1, a homologue of the yeast Pmt4, cause Walker-Warburg Syndrome (WWS), which is characterized by severe congenital muscular dystrophy, neuronal migration defects, and structural abnormalities of the eye [13]. Targeted deletion of *Pomt1* in mice results in embryonic lethality due to defects in the formation of the Reichert's membrane, the first basement membrane to form in the embryo [154]. Mutations of the *Drosophila* PMT homologues alter muscle structures and the alignment of adult cuticle [155].

The *PMT* family is crucial for viability, cell wall integrity, and morphogenesis in several fungal species, such as *S. cerevisiae*, *S. pombe*, *C. albicans* and *C. neoformans*, *A. nidulans*, and *A. fumigatus* [108, 109, 111–115, 145, 156]. In *S. cerevisiae*, single *pmt1* mutants fail to grow in anaerobic conditions on some media [105]. The *pmt1,2,3*-triple mutants grow only in osmotically stabilized medium, whereas the *pmt1,2,4*- and *pmt2,3,4*-triple mutants are not viable in any conditions, indicating that PMT protein activity is essential in *S. cerevisiae*, although individual genes are dispensable [104].

C. albicans contains five *PMT* genes. The *pmt1* mutants are viable, but they are defective in undergoing cellular differentiation from yeast to a true hyphal growth form under some conditions [106]. The virulence of the *pmt1* null mutant is significantly attenuated, which is likely due to reduced O-glycosylation of the *C. albicans* adhesin Als1p [106]. The *pmt1,4*-double mutants are not viable. The *pmt* phenotypes are closely linked to alterations in cell wall components, including cell wall mannoproteins and polysaccharides [107].

In *S. pombe* only one member of each PMT subfamily is present, namely, *oma1*$^+$, *oma2*$^+$, and *oma4*$^+$. Deletion of *oma2*$^+$, as well as simultaneous deletion of *oma1*$^+$ and *oma4*$^+$ is lethal. Characterization of the viable *S. pombe oma1*D and *oma4*D single mutants shows that reduced O-mannosylation results in abnormal cell wall and septum formation, therefore

severely affecting cell morphology and cell-cell separation [108]. In *C. neoformans*, three *PMT* genes are present. Pmt4p is essential for morphogenesis and virulence [109]. Recently, Willger et al. showed that *PMT2* is an essential gene, and the double *pmt1pmt4* deletion is lethal [110].

Filamentous fungi, such as *A. fumigatus*, *A. nidulans*, *Neurospora crassa,* and *Fusarium gramineum*, contain only three *pmt* genes that belong to the PMT1, PMT2, and PMT4 subfamilies, respectively [107, 108, 113]. Each of the PMTs appears to function independently. All single *pmt* mutants in *A. nidulans* are viable but showed reduced growth at elevated temperatures and defects in morphogenesis [111, 112]. The double deletion *pmtA/pmtC* (orthologues of the *PMT2* and *PMT4*) and *pmtB/pmtC* (orthologues of *PMT1* and *PMT4*) are synthetically lethal.

Previously, Zhuo et al. have shown that a single deletion of *A. fumigatus pmt1* results in temperature-sensitive phenotypes [113]. When the *A. fumigatus* Δ*pmt1* mutant was grown on solid complete medium at 37°C, no difference was found between the mutant and the wild type. A strongly retarded growth, however, was observed when this mutant was grown at 42°C and 50°C. This temperature-sensitive phenotype could be complemented by the addition of 1 M sucrose in the media. Further analysis shows that the mannoprotein, α-glucan, and chitin in the cell wall of the mutant grown at 37°C are increased, while β-glucan is reduced. When the *A. fumigatus* Δ*pmt1* mutant was cultured at 42°C, the α-glucan was increased, while the β-glucan was decreased, and the mannoprotein and chitin content remained unchanged. Moreover, deficient conidiation and reduced germination have been documented at 42°C [113]. As compared with the *S. cerevisiae pmt1* mutants, the *A. fumigatus* Δ*pmt1* mutant, as well as the *C. albicans* and *S. pombe pmt1* mutants, shows more severe defects in cell wall integrity. This significant phenotype could be explained by the fewer members of *PMT* family presented in *A. fumigatus, C. albicans,* and *S. pombe*. However, in a recent study by Mouyna et al., the *A. fumigatus pmt1* mutant does not show any visible phenotype [115]. In the report by Zhuo et al., the *pmt1* deletion mutant was constructed by replacement of the entire coding region of the *pmt1* in *A. fumigatus* strain CEA17 (*pyrG* $^-$) with a *pyrG* cassette [113]. Therefore, the genetic background of the *pmt1* null mutant is *pyrG*$^+$ *pmt*1$^-$, while in the report by Mouyna et al., the *pmt1* mutant was constructed by transformation of *A. fumigatus* strain ΔKU80 with a deletion cassette containing the *E. coli* phleomycin phosphotransferase gene (*PHLE*) [115]. Therefore, the major differences may be due to the different genetic background of the strains used in these two reports.

The single *pmt2* or double *pmt1pmt*4 deletion(s) are lethal [114, 115]. Fang et al. [114] reported that reduced expression of *pmt2* leads to retarded growth, cell wall defects, abnormal polarity, and reduced conidiation; however, no temperature-sensitive growth was found. Interestingly, this is the first time that Pmt2p is revealed to be involved in polarized growth. These observations suggest that *A. fumigatus* Pmt2p is required for cell wall synthesis and morphogenesis and its function is distinct from that of *A. fumigatus* Pmt1p.

Disruption of *A. fumigatus pmt4* leads to abnormal mycelial growth, poor conidiation, and abnormal polarity. Although an increased sensitivity to echinocandin, a β1,3-glucan synthase inhibitor, was observed in the *A. fumigatus pmt4* null mutant, glucan synthase activity and β1,3-glucan content were not affected [115]. In contrast to its counterpart in *C. albicans* [107], *A. fumigatus pmt4* is not required for full virulence.

The different functions associated with different *A. fumigatus* PMTs are likely due to their different substrate specificities. Further investigation of the *pmt* mutants will be helpful for understanding their molecular mechanism, which will not only increase our understanding of the function of O-mannosylation in *A. fumigatus*, but also may deepen our understanding of the molecular basis of the human Walker-Warburg Syndrome (WWS) which features mutations in POMT1, a homologue of *A. fumigatus* Pmt4p, and results in a failure of polarized growth during neuronal migration [13].

6. Biosynthesis and Function of GPI Anchoring in *A. fumigatus*

GPI anchoring is a conserved glycosylation process in eukaryotes, which enables many cell surface proteins such as cell surface enzymes, receptors, and adhesion molecules to be covalently anchored to the cell membrane [157]. The core structure of the GPI anchor consists of a lipid group, myoinositol, glucosamine, several mannose residues, and a phosphoethanolamine group, which ultimately connects the GPI anchor to the protein via an amide bond. Although the number of mannose groups and the position of side-chains on the GPI anchors vary widely between species, a common core structure of EtNMan$_3$GlcN-PI is conserved in all GPI-anchored proteins found in protozoa, yeast, plants, and mammals (Figure 6).

GPI anchoring is not essential in mammals at a cellular level as several GPI-deficient cell lines have been established [158]. However, an acquired GPI-anchoring deficiency in haematopoietic stem cells causes paroxysmal nocturnal haemoglobinuria [14], a rare but serious human disease. Also an overexpression of PIG-P, a protein of unknown function required for GPI anchor synthesis, has been noted in fetal Down syndrome brain [159]. In contrast to mammals, GPI anchor synthesis is essential in *S. cerevisiae* [116]. In *S. cerevisiae*, many GPI-anchored proteins are known to be involved in morphogenesis and cell wall organization. Two types of functions have been assigned to these proteins depending on their localization [160]. One type is the GPI-mannoproteins covalently linked to cell wall β-1,6-glucan which play important biological functions in filamentation, mating, flocculation, or adhesion to the external matrix [161–167]. The second type are the GPI proteins associated with the plasma membrane which possess enzymatic activities able to modify cell wall polymers and are involved in altering cell morphology, such as β-glucanase and β-glucosyltransferase [168–170]. Recent studies in *A. fumigatus* suggest that at least nine GPI-anchored proteins are common to filamentous fungi and yeast. Five of them are homologues

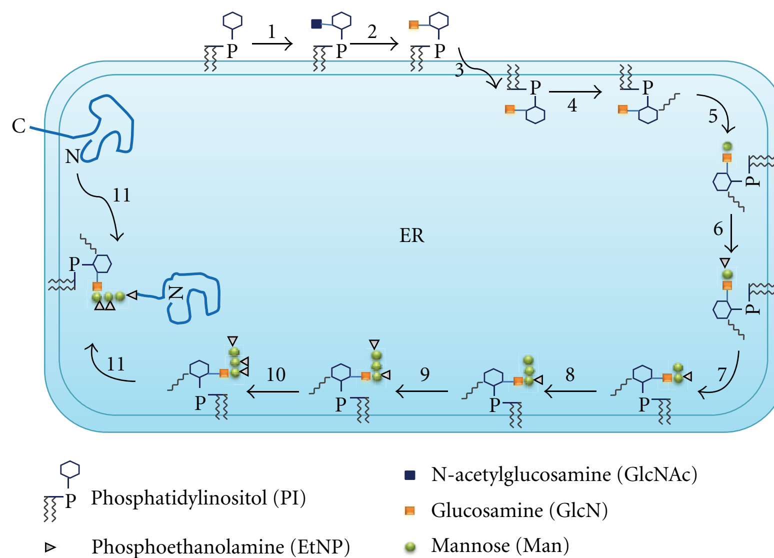

Figure 6: Schematic illustration of GPI biosynthesis in the ER of yeast and mammals. Biosynthesis of the GPI anchor begins at step 1; PI is glycosylated to generate GlcNAc-PI on the cytoplasmic face of the ER. GlcNAc-PI is then de-N-acetylated (step 2) to yield GlcN-PI. GlcN-PI is flipped (step 3) into the lumenal leaflet of the ER, where it is inositol acylated (step 4), inositol mannosylated, and modified by EtNP (steps 5–10). The EtNP-capped GPIs are attached (step 11) to ER-translocated proteins displaying a C-terminal GPI signal sequence. Step 11 is catalyzed by GPI transamidase.

of putative GPI-anchored yeast proteins that have been shown to play a role in cell wall morphogenesis [160].

The GPI anchor is assembled at the ER in multiple steps catalyzed by the concerted actions of approximately 20 proteins [171]. The first step of GPI anchor synthesis is initiated by the transfer of N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to phosphatidylinositol (PI), which is catalyzed by the glycosylphosphatidylinositol-N-acetylglucosaminyltransferase (GPI-GnT) complex. The mammalian GPI-GnT complex consists of seven proteins, including PIG-A, PIG-H, PIG-C, PIG-P, GPI1, PIG-Y, and DPM2 [172]. All except DPM2 have structural and functional counterparts in *S. cerevisiae*, where they are known as Gpi3p, Gpi15p, Gpi2p, Gpi19p, Gpi1p, and Eri1p, respectively [173]. PIG-A/Gpi3p is believed to possess the catalytic domain because Gpi3p binds a photoactivatable UDP-GlcNAc analog and is a member of glycosyltransferase Family 4 of retaining glycosyltransferases [171]. The roles of the other subunits in the GPI-GnT complex are as yet unclear, but they may mediate regulatory interactions.

In yeast, GPI anchoring is essential for viability and plays an important role in the biosynthesis and organization of the cell wall. A *gpi3* temperature-sensitive mutant is not viable at 37°C [116, 117]. Similar results have been observed in the filamentous fungus *N. crassa* [118]. In both cases, it is postulated that the introduction of mutations in *GPI3*/*gpig-1* genes allows for minimal level of product function and survival when growing the mutant cells below the restrictive temperatures. However, the mechanism, by which the defect in GPI anchoring leads to a lethal phenotype in these two species, is poorly understood.

It has been shown that *A. fumigatus* GPI anchors possess five mannose residues with a phosphoethanolamine linked on the first three residues [174, 175]. The *A. fumigatus pig-a* gene, the homologue of the *GPI3*/*pig-A* gene in yeast, has been investigated [119]. Deletion of Δ*afpig-a* results in a phenotype characterized by increased cell lysis. Also, an increased content of β-glucan and mannoprotein was observed in the mycelial cell wall of the Δ*afpig-a* mutant. Unlike the temperature-sensitive or conditional lethal phenotype seen in the yeast *GPI3* mutant, Δ*afpig-a* can survive at temperatures from 30°C to 50°C. Completely blocking GPI anchor synthesis in *A. fumigatus* Δ*afpig-a* leads to cell wall defects, abnormal hyphal growth, rapid conidial germination, and aberrant conidiation. *In vivo* assays reveal that the mutant exhibits reduced virulence in immunocompromised mice. Therefore, the GPI anchor seems not essential for viability, but required for cell wall integrity, morphogenesis and virulence in *A. fumigatus.* Indeed, this is the first report that a deficiency in GPI-anchor synthesis does not lead to a temperature-sensitive or conditional lethal phenotype in microbes, which provides an opportunity to identify the basic function of GPI anchoring in fungi.

7. Outlook

During the past 50 years, proteins and nucleic acids have dominated the field of biology. Carbohydrates remained very much on the sidelines. Since the late 1980's, the enormous advances in the analysis of complex carbohydrates have enabled us to investigate the structure and function of carbohydrates, and the field has developed enormously. It is now known that carbohydrates play very important roles, especially in the regulation of development of higher organisms. However, the mechanisms by which carbohydrates play a role in development and diseases are still

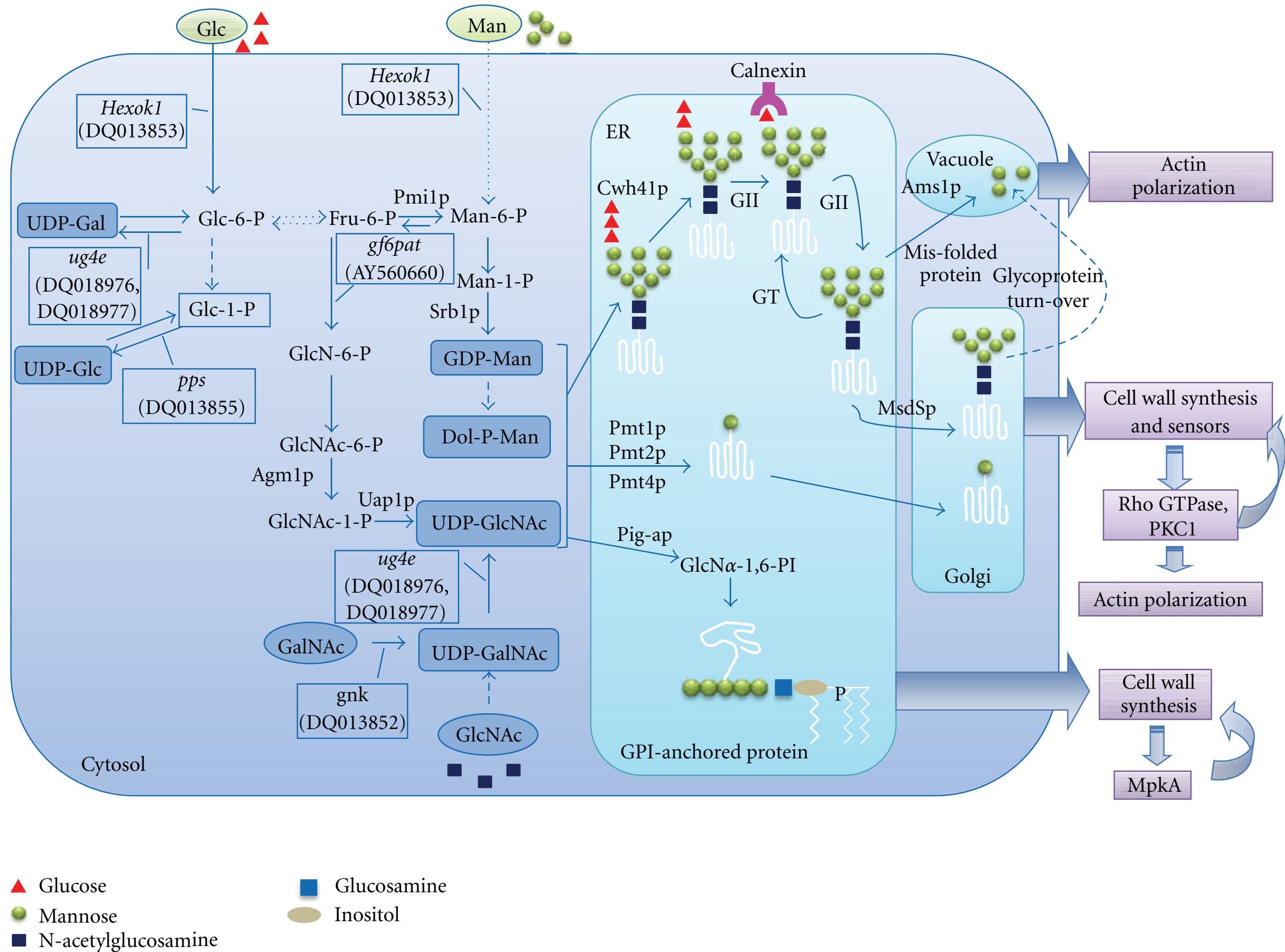

Figure 7: Pathways and functions of protein glycosylation in *A. fumigatus*. In *A. fumigatus*, the activation of mannose initiates from formation of mannose 6-phosphate (Man-6-P), which occurs by one of two routes: direct phosphorylation of mannose by hexokinase or interconversion from fructose 6-phosphate (Fru-6-P), the latter pathway requires three enzymes: phosphomannose isomerase (PMI), phosphomannomutase (PMM), and GDP-mannose pyrophosphorylase (GMPP). Pmi1p (AFUA_1G13280), Sec53p (AFUA_6G06580), and Srb1p (AFUA_6G07620) have been annotated as PMI, PMM, and GMPP, respectively. Functional analyses of Pmi1p and Srb1 imply that mannose activation is specifically crucial for the synthesis and organization of the cell wall and thus essential for survival of *A. fumigatus*. The N-glycosylation is initiated by transfer of Dol-PP-linked $Glc_3Man_9GlcNAc_2$ to an asparagine residue within an N-X-T/S consensus sequence of a nascent peptide, which is catalyzed by Stt3p (AFUA_8G04430), a putative catalytic subunit of the oligosaccharyltransferase (OST) complex. Subsequently, the N-glycan is processed sequentially in the ER and Golgi. N-Glycan processing is initiated by the removal of the glucose residues catalyzed by ER glucosidase I Cwh41p (AFUA_6G04210) and glucosidase II (AFUA_5G03500) to the monoglucosylated form, which is bound by calnexin (AFUA_4G12850). For many glycoproteins, the interaction with calnexin slows down the rate of folding but increases efficiency. GII-catalyzed removal of the third glucose residue follows the dissociation of folding substrates from calnexin and is required for the release of properly folded proteins from the ER and transport to the Golgi. When correct folding is not achieved, the folding sensor peptide:glucosyltransferase (GT) (AFUA_2G02360) adds back a terminal glucose to promote reassociation of nonnative polypeptides released from calnexin, thus prolonging their retention in the ER folding environment. Cycles of de-/reglucosylation might be protracted until the polypeptide released from calnexin fulfills quality control requirements. Misfolded proteins are removed by an ER-specific N-glycan-dependent pathway of degradation. Ams1p (AFUA_3G08200) has been identified as vacuole α-mannosidase that is involved in degradation of N-glycans. Once the native proteins are released from calnexin, the N-glycan is further processed by a yet-unknown ER mannosidase to form $Man_8GlcNAc_2$. Then N-glycosylated proteins are transported into the Golgi, where the N-glycan is trimmed by MsdSp (AFUA_1G14560) to yield $Man_6GlcNAc_2$. When N-glycosylation is inhibited in *A. fumigatus*, the most commonly observed effects are cell wall defects and abnormal polarity, which are likely due to the generation of misfolded, aggregated proteins that are required for cell wall synthesis. The O-mannosylation is catalyzed by Pmt1p (AFUA_3G06450), Pmt2p (AFUA_1G07690), and Pmt4p (AFUA_8G04500), which function independently and are required for cell wall synthesis, thermotolerance, and polarity. Pig-ap (AFUA_1G16950) has been identified as the catalytic subunit of the glycosylphosphatidylinositol-N-acetyl-glucosaminyltransferase (GPI-GnT) complex. GPI anchoring is required for cell wall synthesis, morphology, and virulence.

Table 2: Summary of the fungal genes studied in glycosylation pathways.

Pathway	Function	Gene	Species	Phenotypes	Reference
Mannose activation	Phosphomannose isomerase (PMI)	*PMI40*	*S. cerevisiae*	The *pmi*⁻ mutant only grows on media with exogenous mannose. Excess exogenous mannose leads to an accumulation of Man-6-P, which represses glycolysis, protein biosynthesis, and cell wall biogenesis	[52]
		MAN1	*C. neoformans*	Disrupted *MAN1* mutant displays poor capsule formation, reduced polysaccharide secretion, morphological abnormalities, and attenuated virulence	[50]
		manA	*A. nidulans*	The *manA1* mutant exhibits abnormal ballooning of hyphal tips and eventually ceases to grow.	[53]
		pmi1	*A. fumigatus*	Deletion of *pmi1* results in defects in cell wall integrity, conidiation, and morphology. Both lower and higher concentrations of mannose lead to a reduction in the levels of α-glucan in the cell wall and an accumulation of Man-6-P in the mutant	[54]
	GDP-mannose pyrophosphorylase (GMPP)	*SRB1*	*S. cerevisiae*	Cell lysis, failure of cell separation, impaired budding and hyphal switching, clumping and flocculation, and cell wall defects	[61]
		srb1	*A. fumigatus*	Defective cell wall and impaired polarised growth, as well as rapid germination and reduced conidiation	[63]
N-glycosylation	Oligosaccharyltransferase (OST)	*STT3*	*S. cerevisiae*	Essential gene	[72, 73]
		stt3	*A. fumigatus*	Repression of the *stt3* gene leads to a severe retardation of growth, a slight defect in cell wall integrity and UPR	[39]
	ER Glucosidase I	*CWH41*	*S. cerevisiae*	Mutational defects in the *CWH41* gene cause severe and selective instability of glycoprotein Kre6p, a putative Golgi glucan synthase required for β-l, 6-glucan synthesis	[23, 83, 84]
		cwh41	*A. fumigatus*	Deletion of *cwh41* leads to severe reduction in conidial formation, abnormalities of polar growth, septation and temperature-sensitive deficiency of cell wall integrity.	[37]
	Golgi mannosidase II	*MSDS*	*S. cerevisiae*	Disruption of t *MSDS* does not prevent outer chain synthesis	[96]
		msdS	*A. fumigatus*	Deletion of *msdS* gene leads to a defect in N-glycan processing, as well as a reduction of cell wall components (including α-glucan, β-glucan, mannoprotein, and chitin), reduced conidiation, abnormal polarity, and septation	[97]
	Cytosolic/vacuolar α-mannosidase	*AMS1*	*S. cerevisiae*	The Ams1p is involved in recycling macromolecular components of the cell under nutrient deprivation. Deletion of *AMS1* causes no visible effect on growth or morphology	[98–101]
		ams1	*A. nidulans*	oligosaccharide catabolism; no visible effect on growth or morphology	[102]
		ams1	*A. fumigatus*	Deletion of *ams1* leads to a severe defect in conidial formation (especially at a higher temperature), abnormalities of polarity, and septation	[103]

Table 2: Continued.

Pathway	Function	Gene	Species	Phenotypes	Reference
O-glycosylation	mannosyltransferase	*PMT1* *PMT2* *PMT3* *PMT4* *PMT5* *PMT6* *PMT7*	*S. cerevisiae*	Single *pmt1* mutants fail to grow in anaerobic conditions on some media. The *pmt1,2,3*-triple mutants grow only in osmotically stabilized medium, whereas the *pmt1,2,4*-and *pmt2,3,4*-triple mutants are not viable in any conditions	[104, 105]
		PMT1 *PMT2* *PMT4* *PMT5* *PMT6*	*C. albicans*	The *pmt1* mutants are viable, but defective in undergoing cellular differentiation from yeast to a true hyphal growth form under some conditions. The virulence of the *pmt1* null mutant is significantly attenuated. *pmt1,4*-double mutants are not viable. The *pmt* phenotypes are closely linked to alterations in cell wall components, including cell wall mannoproteins and polysaccharides	[106, 107]
		*oma*1$^+$, *oma*2$^+$ *oma*4$^+$	*S. pombe*	Deletion of *oma*2$^+$, as well as simultaneous deletion of *oma*1$^+$ and *oma*4$^+$, is lethal. The viable *oma1*D and *oma4*D single mutants show abnormal cell wall and septum formation	[108]
		PMT1 *PMT2* *PMT4*	*C. neoformans*	Pmt4p is essential for morphogenesis and virulence. *PMT2* is an essential gene, and the double *pmt1pmt4* deletion is synthetically lethal	[109, 110]
		pmtA *pmtB* *pmtC*	*A. nidulans*	All single *pmt* mutants are viable but show reduced growth at elevated temperatures and defects in morphogenesis. Double deletion of *pmtA/pmtC* and *pmtB/pmtC* is lethal	[111, 112]
		pmt1 *pmt2* *pmt4*	*A. fumigatus*	Deletion of *pmt1* results in temperature- sensitive phenotypes including retarded growth, cell wall defects, deficient ability of conidiation, and reduced germination	[113–115]
				Single *pmt2* or double *pmt1pmt*4 deletion(s) are lethal. Repression of *pmt2* leads to retarded growth, cell wall defects, abnormal polarity, and reduced conidiation	
				Disruption of *pmt4* leads to abnormal mycelial growth, poor conidiation, and abnormal polarity.	
GPI-anchoring	Glycosylphosphatidyl-inositol-N-acetyl-glucosaminyltrans-ferase (GPI-GnT)	*GPI3*	*S. cerevisiae*	A *gpi3* temperature-sensitive mutant is lethal at 37°C	[116, 117]
		gpig-1	*N. crassa*	Temperature-sensitive phenotypes	[118]
		pig-a	*A. fumigatus*	Deletion of *pig-a* results in a number of phenotypes including increased cell lysis, cell wall defects, abnormal hyphal growth, rapid conidial germination, aberrant conidiation, and reduced virulence	[119]

poorly understood. Our knowledge of protein glycosylation comes mainly from the investigation of *S. cerevisiae* and mammalian cells. Although investigations of the model yeast and mammalian cells have been very useful in elucidating the biochemical features of protein glycosylation, these investigations at the cellular level cannot reflect the complicated functions of glycosylation in the development of multicellular eukaryotes. Therefore, more model systems have been introduced, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice. However, these model systems are still too complex since deletion of individual glycosyltransferase genes in these systems sometimes leads to fetal death or nonvisible phenotypes. As compared with *S. cerevisiae, C. elegans*, *D. melanogaster*, or mice, *A. fumigatus* seems to be an ideal model for investigation of the function of glycosylation since *A. fumigatus* is a multicellular

eukaryote with a relative simple life cycle, in which it undergoes a series of developmental events that require polarized growth. Recent progress shows that *A. fumigatus* has evolved an intact N-glycan-dependent QC system, which is present in mammalian cells but not in yeast. Disruption of either processing or degradation of N-glycan in *A. fumigatus* leads to phenotypes such as cell wall defects and abnormal polarity.

Based on investigations of *S. cerevisiae* and filamentous fungi, it is likely that glycosylation first evolved to ensure synthesis of the fungal cell wall and only later did the N-glycan-dependent QC system evolve to ensure precisely controlled cell wall synthesis and polarized growth which are important for multicellular development. However, this hypothesis is controversial. Recently, based on investigations of numerous protists and fungi, Banerjee et al. [85] showed that the N-glycan-dependent QC system is functional in *Entamoeba*, *Trichomonas*, *Cryptococcus*, and *S. pombe*, but is not functional in some fungi such as *Giardia* and *Plasmodium*, *Theileria*, *Encephalitozoon*, *Toxoplasma*, *Cryptosporidium*, and *Tetrahymena*. They proposed that the N-glycan-dependent QC system was likely present in the common ancestor of extant eukaryotes and was secondarily lost from some eukaryotes. For example, the *S. cerevisiae* Kre5 is believed to be the GT ortholog that no longer glucosylates misfolded glycoproteins but is instead thought to be involved in β-1,6-glucan synthesis [85]. Of course, the possibility that the *S. cerevisiae* Kre5 is the ancestor of GT cannot be excluded. It remains unclear where is the evolutionary origin of glycosylation, what is the basic function of glycosylation at the early stages of evolution, and how glycosylation is regulated. Definitely, the answers to these questions will enable us to understand the basic function and regulation of glycosylation in the development of multicellular eukaryotes and help to understand more complex functions in higher eukaryotes. On the other hand, the investigation of *A. fumigatus* is also a key to understanding complex compensatory mechanisms of cell wall biosynthesis and may provide a new strategy for drug development.

During the past few years, the framework of the biosynthetic pathways of glycosylation in *A. fumigatus* has been delineated. Functional analyses of some of the genes in this pathway have shown that glycosylation is required for cell wall synthesis, polarity, morphogenesis, and cellular function in *A. fumigatus* (Figure 7 and Table 2). However, a detailed understanding of this pathway remains unknown, such as details regarding the synthesis of the N-glycan precursor, the precise molecular mechanism of N-glycan processing, QC of protein folding, and modification of the GPI anchor. Moreover, the molecular mechanisms by which glycosylation plays a role in morphogenesis and development of *A. fumigatus* are vaguely understood. Therefore, the future direction would be looking for those key proteins that are affected by glycosylation and identifying the signal transduction pathways that link glycosylation and development, through genetic, biochemical, cell biological, and proteomic studies.

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Review Article

Communication in Fungi

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We will discuss fungal communication in the context of fundamental biological functions including mating, growth, morphogenesis, and the regulation of fungal virulence determinants. We will address intraspecies but also interkingdom signaling by systematically discussing the sender of the message, the molecular message, and receiver. Analyzing communication shows the close coevolution of fungi with organisms present in their environment giving insights into multispecies communication. A better understanding of the molecular mechanisms underlying microbial communication will promote our understanding of the "fungal communicome."

1. Introduction

Any form of communication requires the existence of three obligatory components: a sender, a message, and a receiver. The process starts with the release of a message by a sender and ends with the understanding of the message by a receiver. This type of cycle has been developed with different degrees of complexity from prokaryote to higher eukaryotes optimizing fitness and adaptation for individual members and populations. The nature and mode of action of communication is as diverse as the response to the information it carries. Inter- and intraspecies communication has been widely studied analyzing the exchange of information between fungi and bacteria or fungi and plant cells [1, 2]. This review will focus predominantly on intraspecies fungal communication addressing key biological functions including mating, growth, morphological switching, or the regulation of virulence factor expression (Figure 1). We will show that in the fungal kingdom most of these mechanisms are controlled by a variety of messengers including small peptides, alcohols, lipids, and volatile compounds.

2. Peptides: Pheromones

Pheromones have been known to act as an informative molecule since 1959 [3] and were reported to be involved in the sexual cycle of fungi in 1974 [4]. In the fungal kingdom, they are involved in the reconnaissance of compatible sexual partner to promote plasmogamy and karyogamy between two opposite mating types followed by meiosis. Taking the example of the extensively described sexual cycle of *Saccharomyces cerevisiae*, pheromones are diffusible peptides called a-factor (12 aa) when produced by a cells, and α-factor (13 aa) when produced by α cells. Each mating type responds to the opposite factor, and is able to produce only one of the two peptide pheromones depending on the alleles present at the *MAT* locus. Indeed, *MATa* or *MAT*α controls the expression of a and α specific genes, respectively, such as genes encoding the prepro-factor and the pheromone receptor (for a comprehensive description of the *MAT* locus, see review [5]). In the example of α cells, *MF*α*1* encodes the pheromone precursor, prepro-α-factor, which undergoes several proteolytic reactions in the classical secretory pathway before releasing the mature pheromone.

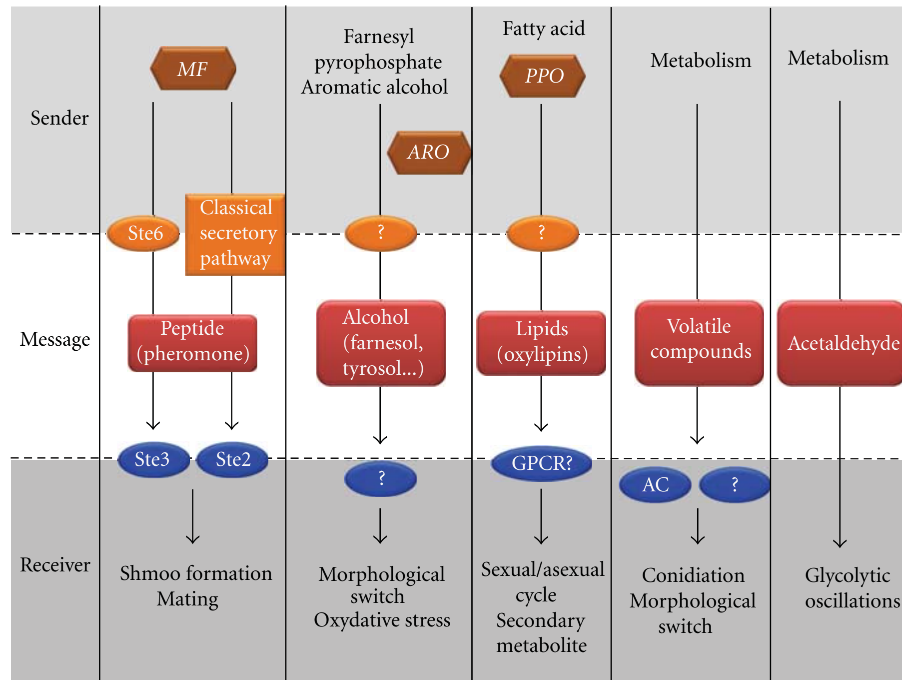

Figure 1: Schematic representation of fungal intra and interspecies communication. The "sender" is an organism from the fungal kingdom and the "receiver" can be from any kingdom. Genes involved in messenger synthesis are represented as brown hexagons. Proteins involved in secretion or receiving the message are in orange and blue.

Contrary to the α-factor, the ABC transporter Ste6p is required to secrete a-factor [6]. This difference could be due to the fact that a-factors are farnesylated [7]. Once released, pheromones freely diffuse in the environment and create a concentration gradient. These peptides are subsequently recognized by a 7 transmembrane receptor present on the surface of cells: Ste2p on a cells binds the α-factor and Ste3p on α cells binds the a-factor. Ste2p and Ste3p are G-protein coupled receptors (GPCR) and the binding of pheromone induces the separation of the associated heterotrimeric G-protein into a monomeric α subunit GTPase (Gpa1p) and a $\beta\gamma$ dimer (Ste4p–Ste18p). This mechanism results in the recruitment of Ste5p by Ste4p to the membrane, which activates a protein kinase cascade ultimately resulting in the phosphorylation of the MAP kinases Fus3p and Kss1p [8]. Once phosphorylated Fus3p migrates to the nucleus were it activates the transcriptional factor Ste12p leading to the expression of pheromone responsive genes. Phenotypically, the morphological response of cells to opposite mating pheromone is the development of a shmoo, that is, directional cell growth in response to the pheromone gradient. As each opposite cell develops a shmoo, the plasmogamy between the two cells occurs when the shmoos establish contact, starting the first step of the sexual cycle.

Contrary to *S. cerevisiae*, where karyogamy is followed by meiosis to end the sexual cycle, *Candida albicans* has not yet been described to undergo meiosis. This yeast displays only an imperfect sexual cycle, where karyogamy results in the formation of tetraploid cells that restore natural diploidy via random loss of chromosomes [9]. This process occurs only after *C. albicans* has undergone a so-called white-to-opaque switch (see review [10]), where opaque cells are the sole mating competent form of this yeast. The opaque cells morphologically respond to pheromone by producing a shmoo, like in *S. cerevisiae*, but the mating incompetent form, white cells, is also sensitive to pheromone [11]. Indeed, *C. albicans* α-factor but also a-factor promotes the formation of biofilm by white cells via enhancing their adhesiveness. A process which uses the same receptor (Ste2p or Ste3p) and transduction pathway as the response of opaque cells to pheromone [12]. The formation of fungal biomass by white cells facilitates the establishment of a pheromone gradient in a population of individual cells and assists the mating process of opaque cells. This process involves another molecule, farnesol, as the production of this molecule under aerobic conditions induces the death of the mating competent opaque cells. Anaerobic conditions that prevent production of farnesol facilitate mating between *C. albicans* opaque cells. These observations suggest that the gastrointestinal tract of humans could promote *C. albicans* mating [13]. The mechanism of pheromone communication has a broad significance in diverse fungi including ascomycetes like *Histoplasma capsulatum* [14] or *Aspergillus fumigatus* [15, 16], to basidiomycetes such as *Cryptococcus neoformans* [17] and *Ustilago maydis*, which possess a tetrapolar mating system [18]. Pheromone communication appears to be a critical mechanism for fungi as it supports the exchange of genetic material between cells and by extension the ability of the organism to evolve in response to their environment.

3. Alcohols: Quorum Sensing

Quorum sensing is a mechanism of communication based on the accumulation of a messenger molecule in the medium of culture [19]. As the production of messenger molecules increases with cell number, this system reflects population size. Initially discovered in bacteria, quorum sensing in fungi became relevant for the control of virulence factor expression in *C. albicans*. In 1979, Hazen and Cutler showed that the supernatant from a 48 h culture of *C. albicans* prevents the yeast to hyphae switch of a fresh culture [20]. The quorum sensing molecule (QSM) responsible for this effect has since been identified as an acyclic sesquiterpene alcohol called farnesol [21].

C. albicans produces farnesol at a rate of 0.12–0.133 mg/g of cells dry weight [22] from an intermediate of the mevalonate pathway (sterol biosynthesis), farnesyl pyrophosphate [23]. At concentrations of 10–250 μM farnesol inhibits the formation of hyphae when induced with proline, N-acetylglucosamine, and serum, but does not suppress further elongation of preexisting hyphae [24]. Farnesol-dependent quorum sensing involves the histidine kinase Chk1p [25] and the Ras1-Cyr1 pathway [26] but the receptor for farnesol remains to be identified. Farnesol regulates the expression of several genes and induces *TUP1,* a transcriptional cofactor repressing filamentation [27], while repressing *CPH1* and *HST7* expression, which are both activators of the morphological switch [28]. The oxidized form of farnesol, farnesoic acid, has also been reported to inhibit hyphal growth by acting via *PHO81* [29]. However, morphological inhibition is stronger with farnesol, although farnesoic acid is less toxic at high concentration [30], it displays only 3% of farnesols QSM activity [31]. While the function of farnesol as a cell density regulator remains to be established, farnesol has been described to inhibit *C. albicans* biofilm formation due to its repressing function on the morphological switch [32]. Additionally it has been shown to increase resistance to oxidative stress by suppressing the Ras1-cAMP pathway [33].

Notably, farnesol also acts as an interspecies QSM that impacts on growth of other *Candida* species including *Candida tropicalis* or *Candida parapsilosis* [34] as well as *S. cerevisiae* or the mould *Aspergillus nidulans* and *A. fumigatus* [35–38]. In the case of *A. fumigatus*, farnesol has been described to alter the localization of AfRho1p and AfRho3p, proteins involved in the cell wall integrity (CWI) pathway and cytoskeleton regulation [35]. This phenotype is explained by the fact that farnesyl derivatives interfere with prenylated proteins such as the two Rho GTPases [39, 40]. The CWI pathway implies the activation of AfPkcA by AfRho1p, which leads on to the MAP kinase cascade and subsequent AfMpkA phosphorylation. Dichtl et al. showed that in the presence of only 40 μM farnesol, phosphorylation of AfMpkA in response to Congo red was completely inhibited [35]. In *S. cerevisiae*, farnesol prevents growth via a different mechanism, which involves an increase of mitochondrial reactive oxygen species (ROS) [37]. The latter observation was also reported for *A. nidulans* where ROS augmentation induced cellular apoptosis but had no role on hyphal morphogenesis [38]. Two proteins have been identified in this response; the Gα subunit FadA of a heterotrimeric G protein, where hyperactivation leads to a strong increase in farnesol sensitivity [38], and the kinase PkcA. Mutation of PkcA increases resistance to farnesol while overexpression results in a higher rate of cell death in response to the QSM [41]. Finally, farnesol has also been described to induce apoptosis of cancerous cells *in vivo* (see review [42]), as well as increasing antibiotic sensitivity of *Staphylococcus aureus* [43]. Thus, farnesol appears to function as both an intraspecies and inter-species communication molecule.

Farnesol is not the only continuously released messenger molecule by *C. albicans*. Tyrosol, an aromatic alcohol, is produced from aromatic amino acids undergoing the processes of transamination (*ARO8, ARO9*), decarboxylation (*ARO10*), and reduction by alcohol dehydrogenase (*ADH*) [44]. This synthesis pathway is strongly dependant on growth conditions including environmental pH, availability of aromatic amino acids, oxygen levels, or presence of ammonium salts [44]. Similar to farnesol, tyrosol's sensor has not yet been identified. Fungal responses to tyrosol include the induction of germ tubes in planktonically growing cells and during the early stages of biofilm formation, as well as a reduction in the lag phase of *C. albicans* growth following dilution of a highly concentrated culture to fresh minimal medium [45, 46]. The latter phenotype occurs predominantly at low concentrations of cells (5×10^3 cell/mL) by promoting the expression of genes involved in DNA replication, chromosome segregation, and cell cycle processes [45].

Aromatic alcohol synthesis is not exclusive to *C. albicans* but can also be found in *S. cerevisiae*, which produces phenylethanol and tryptophol via a similar pathway involving AROs genes [47]. Both molecules stimulate diploid pseudohyphal growth at concentrations above 20 μM on low-ammonium agar (SLAD) by inducing the PKA pathway resulting in *FLO11* induction [48]. Recently, response to phenylethanol and tryptophol has been proposed to involve two main transcriptional regulators: Cat8p and Mig1p [49]. Interestingly, *C. albicans* is insensitive to phenylethanol and tryptophol [48]. *H. capsulatum* and *Ceratocystis ulmi* are two fungi also displaying quorum sensing phenotypes. However, the messenger molecule is not yet characterized [50, 51]. At low density, *H. capsulatum* cells have low amounts of α-(1,3)-glucan in their cell walls and addition of supernatant from a stationary phase culture induces α-(1,3)-glucan incorporation into the cell wall [50]. Similarly addition of *C. ulmi* spent medium to a fresh culture promotes a switch from hyphae to yeast growth [51].

4. Lipids: Oxylipins

Oxylipins are oxygenated fatty acids used as cell messengers and have been intensely studied in plants and mammalian cells (see review [52]). They also appear to be widely synthesized and secreted by fungi. *A. nidulans* was reported to produce one of the first oxylipins called psi factor (precocious sexual inducer), which is composed of a series of different oxylipin derivates from oleic acid (C18:1), linoleic

acid (C18:2), and linolenic acid (C18:3) called psiA, psiB, and psiC, respectively [53]. The genes involved in the production of psi factor are called *Ppo*s (for psi-producing oxygenases) [54]. In the case of *A. nidulans*, *PpoA* is involved in psiBα synthesis and *PpoB* and *PpoC* contribute to psiBβ biogenesis [54, 55]. Inactivation of these genes results in perturbations not only of psi factor production but also mycotoxins production, as well as in the ratio between the development of sexual and asexual ascospores [54, 55]. The latter phenotype is due to the fact that oxylipins control the expression of *NsdD* and *BrlA*, transcription factors required for meiotic and mitotic sporulation, respectively [54, 56]. Overexpression or addition of psiBα or psiCα to the culture medium stimulates sexual sporulation and represses asexual spore development while an opposite effect is observed for psiAα and psiBβ [55]. Secondary metabolite mycotoxin sterigmatocystin (ST) and antibiotic penicillin (PN) production are also dependent on oxylipin [57]. Indeed, inactivation of the *Ppo*s genes results in the inability to secrete ST as a result of downregulation of ST biosynthesis genes including *aflR* and *stcU*, and the overproduction of PN through induction of the gene involved in its biosynthesis: *ipnA* [57]. Interestingly, the exact opposite observations are found when FadA, an α subunit of a GPCR, is constitutively activated due to a G42R mutation, a reaction which is mediated by the PkaA enzyme [58]. This result, in addition to the fact that oxylipins in mammalian cells are sensed via GPCR complexes [59] led to the hypothesis that fungi use the same system to detect oxylipin, ultimately activating the cAMP/PKA pathway [58]. The *ppo* and GPCR encoding genes have been identified in the genomes of several filamentous fungi predicting a broader role of oxylipin in fungal biology [60]. In fact, inactivation of the *ppo* genes in *Aspergillus flavus* and *Fusarium sporotrichioides* has already been shown to perturb mycotoxin and spore production [61, 62]. Finally, using confocal laser scanning microscopy, oxylipins have been described to accumulate in the capsule of *C. neoformans* before being released into the external medium under the form of hydrophobic droplets that are transported via tubular protuberances [63].

Recently, Nigam et al. have described a 3(*R*)-Hydroxy-tetradecanoic acid, a derivate of linoleic acid as a novel QSM of *C. albicans* [64]. Previously known to be produced during the sexual phase of *Dipodascopsis uninucleata* [65], this oxylipin increases filamentation in *C. albicans* in response to N-acetylglucosamine at a concentration of 1 μM. Although the receptor of 3(*R*)-Hydroxy-tetradecanoic is not known, this QSM induces *HWP1* and *CAP1* mRNA transcripts [64]. Interestingly, 3(*R*)-Hydroxy-tetradecanoic is metabolized inside cells to generate two new compounds that could also act as messenger molecules [64].

Another family of oxylipin are the eicosanoids, which are molecules containing a 20 carbon backbone [66]. PGE_2 is produced by *C. albicans* from exogenous arachidonic acid via enzymes not yet characterized [67]. PGE_2 is also produced by humans, similar to other prostaglandins (PG), and it appears that fungal PGE_2 can enter in competition with human PG impacting on the host's immune response [67]. Indeed, PGE_2 is known to balance Th1/Th2 differentiation as this molecule decreases the expression of IL-12R and inactivates Th1 differentiation while activating the Th2-related immune responses [68]. PGE_2 also enhances the production of IgE in stimulated B cells [69].

5. Volatile Compounds and Gas

In addition to releasing mediators into solution or onto solid growth media, organisms also exchange information via the liberation of messenger molecules into air. For example, insects have been thoroughly studied for their secretion of pheromones into air to attract mating partners [70]. In the fungal kingdom, as early as in the 1970s, volatile compounds from fungi and others organism have been described to impact on fungal growth (review [71, 72]). More recently, Palkova et al. observed that *S. cerevisiae* colonies grown on complex agar form a turbid path in the vicinity of another colony. Subsequently, they discovered that this reaction is induced by the small volatile messenger molecule, later described as ammonia [73], which also required amino-acid uptake for its production. Indeed, inactivation of *SHR3*, which is responsible for the correct localization of several amino-acid permeases, disrupts the turbid path between colonies [73].

Trichoderma species have been described to produce the volatile molecule 6-Pentyl-α-pyrone, a secondary metabolite with antifungal activity [74]. However, more recently the induction of conidiation in *Trichoderma* species, which is known to be regulated by a circadian cycle, has also been shown to be controlled via a volatile agent. Solid-phase microextraction linked with gas chromatography and mass spectrometry has allowed the identification of the chemical profiles of volatile molecules produced from nonconidiated colonies grown in darkness and conidiating colonies grown in light [75]. Comparison of the two profiles identified production of the 8-carbon compounds molecules 1-octen-3-ol, 3-octanol and 3-octanone specifically during conidiation [75]. Each of these three compounds induces conidiation in colonies placed in the dark. This regulation could involve a calcium-dependant signaling pathway as it has been shown that high concentration of calcium can induce conidiation of *Penicillium isariaeform* in darkness [76]. 1-octen-3-ol is the most efficient molecule being active at concentrations of only 0.1 μM. Interestingly, concentrations above 500 μM of any of the three compounds suppress conidiation and growth of *Trichoderma* species. These observations are consistent with a previously described putative fungistatic and fungicidal role of the molecules [77, 78]. Notably, the same compounds have previously been shown to function as insect attractants improving fungal spore dispersal [77, 78], and inter-species communication has already been described between *Epichloë* species and the female *Botanophila* flies [79].

Fungi are not only responsive to volatile compounds that they produce but also, in at least one example, to a gas liberated during respiration: carbon dioxide (CO_2). As early as 1961, Vakil et al. demonstrated that the optimum CO_2 concentration for the germination of *Aspergillus niger* conidiospores is reached not under normal atmospheric

concentrations of CO_2 (0.033%) but at 0.5% [80]. Since then several additional phenotypes in fungi have been attributed to changes in the concentration of environmental CO_2 including the sporulation of *Alternaria crassa* and *Alternaria cassiae* [81], conidiation of *Neurospora crassa* [82], or capsule formation and mating in *C. neoformans* [83, 84].

Recently, significant advances have been made in the understanding of CO_2 sensing in fungi. It was already known that the yeast to hyphae morphological switch in *C. albicans* is triggered by elevated environmental CO_2 [85]. Furthermore, the frequency of white-to-opaque switching can be increased 16-fold in hypercapnic conditions as opposed to atmospheric CO_2 [86]. Two different studies show that both phenotypes involve the *C. albicans* adenylyl cyclase Cyr1, first fungal CO_2 sensor. This enzyme generates the secondary messenger cAMP, which in the context of the cAMP/PKA signaling pathway has a fundamental impact on *C. albicans* morphogenesis [86, 87]. CO_2 activation of Cyr1p depends on the concentration of bicarbonate, the hydrated form of CO_2 [87]. CO_2 hydration occurs naturally at a very low rate, but is enhanced by the enzyme carbonic anhydrase [88]. Inactivation of *CYR1* results in a loss of filamentation and white to opaque switching frequency in response to hypercapnia [86, 87]. Hall et al. have now demonstrated that Lysine 1373 of the Cyr1 catalytic domain is essential for CO_2 sensing in *C. albicans* as mutation of this amino acid leads to a loss of filamentation in response to CO_2 but not to serum, another morphological cue [89]. These data show that in fungi environmental CO_2 is sensed via the adenylyl cyclase, which transduces the message via the regulation of the cAMP/PKA pathway. Hall et al. also showed that hypercapnia is not a condition solely encountered inside the host but can also establish itself as a population event, such as the center of a colony grown under normal atmospheric conditions [89]. Another study demonstrated that *C. albicans* produces CO_2 via the conversion of arginine to urea. Urea is ultimately degraded to generate CO_2 by the enzyme urea amidolyase (Dur1,2). Inactivation of the latter interferes with *C. albicans* filamentation in response to arginine and urea compared to the control strain but not to elevated CO_2 [90].

Control of the white to opaque switch-frequency in *C. albicans* by environmental CO_2 also involves the GTPAse Ras1 and the transcriptional factor Wor1. Indeed, Ras1, Cyr1, and Wor1 are critical for increasing the white to opaque switch in response to concentrations of CO_2 at 1%, but Ras1 and Cyr1 become optional for the induction at higher concentrations (20%). However, Wor1 remains essential for the switch even at high CO_2 [86]. These results imply that an alternative CO_2 sensing pathway is involved in the regulation of Wor1 at high CO_2 in *C. albicans*. However, it is important to note that under this condition a significant increase of the internal pH may occur which could also be a component of this alternative CO_2 sensing pathway.

6. Small Molecule: Acetaldehyde

Acetaldehyde, an organic compound involved in several cellular pathways, has been shown to impact on cell-density-dependent glycolytic oscillations of *S. cerevisiae* [91]. In 1964, Chance et al. described that the level of NADH in yeast oscillated when starved cells endure a pulse of glucose after a switch to anaerobic conditions [92]. Since then other metabolites have been described to oscillate in yeast including glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-biphosphate, AMP, ADP, and ATP (for a comprehensive review see [93]). Interestingly, at a population level these oscillations are not chaotic but appear to be subject to synchronization. The most striking observation was achieved when mixing two populations with a 180° out-of-phase oscillation showing that within minutes the oscillation of the new population were synchronized [91]. Acetaldehyde was identified as the active molecule in the synchronization of these oscillations, as the use of acetaldehyde traps induced the oscillation to be damped and addition of acetaldehyde to the medium produced a phase shift in the oscillation [91]. Acetaldehyde is a small molecule that can passively diffuse through the cell membrane. No specific target for acetaldehyde is known; however, this compound has an important impact on the NAD^+/NADH balance [94].

Acetaldehyde is also a volatile molecule, a property used to study inter and intraspecies communication in a synthetic ecosystem [95]. By engineering sender cells that liberate volatile acetaldehyde and receiver cells that contain a construct under an acetaldehyde-inducible promoter, it was possible to study volatile cell communication in a controlled environment. Using mammalian (CHO-K1), bacterial (*Escherichia coli*), yeast (*S. cerevisiae*), or plant (*Lepidium sativum*) cells, all combination of sender/receiver for inter and intraspecies resulted in a positive communication between cells [95]. These results show that virtually all cells can communicate with themselves or different species. Clearly such models could bring new insight in the understanding of communication in complex living systems.

7. Concluding Remarks and Outlooks

We are currently at an interesting stage in the understanding of fungal communication. Many essential compounds of the communication process have been identified, the sender (in our case fungi), the message (protein, alcohol, lipid, gas), and the receiver (bacteria, fungi, plant, mammalian). However, in most cases the actual molecular mechanism of such communication remains for most parts unknown. The determination of these pathways is of substantial significance as molecular messengers control the expression of fungal virulence determinants including the yeast-to-hyphae switch and biofilm formation in *C. albicans*, capsule formation in *C. neoformans*, or mycotoxin synthesis in *A. nidulans*, but also the propagation of these organisms via the regulation of their sexual and asexual cycle. A better knowledge of fungal communication is now required to permit the development of innovative strategies aiming to control disease or toxin production of these organisms.

Fungi have already taken advantage of the different communication processes and particularly inter-species communication to gain competitive advantages over other species. Good examples are the production of pollinators attracting insects to give phytopathogenic fungi a better chance for

dispersal of their spores [79]. Additionally, synthesis of PGE_2 by the human pathogens *C. albicans* and *C. neoformans* modify the host immune response and may enhance fungal survival [67]. Such mechanisms reveal the close coevolution of fungi with their environmental partner and give insights into multispecies communication. The remarkable versatility of communication in the fungal kingdom also raises the question how these organisms integrate intra- and interspecies messages that can have opposing effects. As the molecular mechanisms of fungal communication unravel further, they will promote our understanding of the highly attractive but challenging topic of the fungal "communicome."

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Review Article

Candida albicans versus *Candida dubliniensis*: Why Is *C. albicans* More Pathogenic?

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Candida albicans and *Candida dubliniensis* are highly related pathogenic yeast species. However, *C. albicans* is far more prevalent in human infection and has been shown to be more pathogenic in a wide range of infection models. Comparison of the genomes of the two species has revealed that they are very similar although there are some significant differences, largely due to the expansion of virulence-related gene families (e.g., *ALS* and *SAP*) in *C. albicans*, and increased levels of pseudogenisation in *C. dubliniensis*. Comparative global gene expression analyses have also been used to investigate differences in the ability of the two species to tolerate environmental stress and to produce hyphae, two traits that are likely to play a role in the lower virulence of *C. dubliniensis*. Taken together, these data suggest that *C. dubliniensis* is in the process of undergoing reductive evolution and may have become adapted for growth in a specialized anatomic niche.

1. Introduction

Fungi are an important cause of human infection, and yeast species of the genus *Candida* are the most pathogenic fungi. While most *Candida* species are found in the environment, approximately a dozen or so are associated with colonization and infection of humans [1]. *Candida* species are common commensals of the oral cavity, intestinal tract and vagina, with newborns being colonized soon after birth. While these species are innocuous in most individuals, under certain circumstances they can opportunistically overgrow and cause a variety of diseases [2]. These diseases range from superficial infections of the vaginal and oral mucosae, to life-threatening systemic infections that can spread via the bloodstream to organs throughout the body. The risk factors for candidal vaginitis are poorly understood; however, other candidal infections are largely the result of host-related defects. These include depletion of CD4 T cells in HIV-infected individuals, which predisposes to oropharyngeal candidosis, or neutropenia and intestinal surgery, both of which are significant risk factors for systemic infection [1–3].

Candida albicans is widely recognized as being the most pathogenic yeast species and in the majority of epidemiological studies has been found to be the most common cause of superficial and systemic infections. Other species, such as *Candida glabrata, Candida parapsilosis*, and *Candida tropicalis* have also been associated with most forms of candidiasis and the relative distribution of each species can vary depending on geographic location, patient cohort, and previous exposure to antifungal drugs [2, 4]. In 1995, a new *Candida* species was identified in HIV-infected individuals with oropharyngeal candidosis in Dublin, Ireland [5]. This species, which was subsequently named *Candida dubliniensis*, is very closely related to *C. albicans* with which it shares many phenotypic properties, including the ability to produce hyphae and chlamydospores, traits previously specifically associated only with *C. albicans* [6–8]. Phylogenetic studies indicate that *C. dubliniensis* is the species that is most closely related to *C. albicans*, and it is often quite difficult to discriminate between the two species in clinical samples [9, 10]. Indeed it was only when DNA fingerprinting techniques were applied to the large-scale analysis of *C. albicans* populations in epidemiological studies that the first isolates of *C. dubliniensis* were originally identified [5]. Surprisingly, despite the close phylogenetic relationship of the two species epidemiological data show that *C. albicans* is far more prevalent than *C. dubliniensis*. In particular, in most analyses of systemic infection, *C. albicans*

is found in >50% of cases, while if it is identified at all, *C. dubliniensis* has only been found in at most 2-3% of cases [11–13]. This apparent discrepancy between the ability of the two species to cause infection is also reflected in data obtained from comparative studies in a wide range of infection models (e.g., systemic and mucosal) which clearly show that *C. albicans* is significantly more pathogenic than *C. dubliniensis* [10, 14–18].

The identification of virulence-associated factors in *Candida* species is complicated by the fact that they are opportunistic pathogens that usually exist in harmony with the human host as part of the commensal flora and only cause infection when host deficiencies permit. Since it is by far the most pathogenic *Candida* species, *C. albicans* is the best-studied member of the genus in terms of pathogenesis. The most commonly cited *C. albicans* virulence factors include adhesins (e.g., Hwp1 [19] and the Als family [20]), extracellular enzymes (e.g., the secreted aspartyl proteinase (Sap) family [21] and phospholipases [22]), and most importantly of all, the ability to alternate between unicellular yeast and filamentous hyphal forms of growth [23]. Both morphological forms have been shown to be essential for virulence. Hyphae have been proposed to play a major role in adhesion, invasion, and biofilm formation while yeast cells are likely to be important for dissemination and initial colonization of host surfaces [24]. Comparative phenotypic analysis of *C. albicans* and *C. dubliniensis* has suggested that *in vitro* isolates of *C. dubliniensis* exhibit higher levels of proteinase activity, are more adherent to buccal epithelial cells, and undergo phenotypic switching at a higher rate than *C. albicans* [10, 25–27]. In addition, as described earlier, *C. dubliniensis* is the only *Candida* species, other than *C. albicans* that is able to produce hyphae [5, 6]. Given the close relationship between the two species and the fact that they are so alike phenotypically, at first glance, it is difficult to understand why there is such disparity in the capacity of *C. albicans* and *C. dubliniensis* to colonise and cause disease in humans. This short review appraises recent findings that help to clarify this conundrum and explain how *C. albicans* appears to have evolved to be a better commensal and opportunistic pathogen than *C. dubliniensis*.

2. Comparative Genomic Analysis of *C. albicans* and *C. dubliniensis*

The *C. albicans* genome sequence was first published in 2004 [28], with improved annotation and analysis subsequently reported in 2005 [29] and 2007 [30]. In an early attempt to identify genomic differences that might serve to explain the disparity in the virulence of *C. albicans* and *C. dubliniensis*, Moran et al. cohybridized genomic DNA from each species to *C. albicans* whole genome microarrays to identify genes that are only present in *C. albicans* [31]. This relatively crude experiment suggested that there are 247 (approx. 4%) *C. albicans* genes that are either absent or highly (i.e., >60%) divergent in the *C. dubliniensis* genome. Interestingly, several genes strongly associated with *C. albicans* virulence are included in the list of absent/divergent genes. In 2009, in order to further investigate the genetic differences between the two species the Wellcome Trust Sanger Institute sequenced the entire *C. dubliniensis* genome [32]. Comparison of the two genome sequences revealed that, despite major karyotypic differences, the genomes of the two species are remarkably similar with 96.3% of genes exhibiting >80% identity, while 98% of genes are syntenic, thus, confirming the very close phylogenetic relationship and the relatively recent divergence of the two species (estimated to have occurred approx. 20 million years ago [33]). When transposable elements were discounted, comparison of the two genome sequences revealed that there are 29 *C. dubliniensis*-specific genes and 168 *C. albicans*-specific genes. The majority of the differences observed between the two species can be accounted for by the expansion of gene families in *C. albicans*, many of which have been previously associated with virulence. In particular, genes missing from the *C. dubliniensis* genome include those encoding hypha-specific virulence factors, such as the cell surface proteins Hyr1 and Als3 and two members of the secreted aspartyl proteinase family (i.e., Sap5 and Sap6), while the gene encoding the well-characterized epithelial adhesin Hwp1 is highly divergent [31, 32]. Hyr1 has been shown to confer resistance to neutrophil killing activity [34] and, along with Hwp1, has been shown recently to play an important role in oral mucosal biofilm formation [35]. Als3 has been shown to play an important role in adhesion to host cells and has been shown to have invasin-like [36] and iron-sequestering [37] activity, while the Saps are well-known virulence factors [21]. The biggest difference in gene family size between the two species is the TeLOmere-associated (TLO) family which is comprised of 14 genes in *C. albicans,* but only two genes in *C. dubliniensis*. Sequence comparisons suggest that the TLO genes encode transcriptional regulators, and preliminary analysis of the phenotype of *C. dubliniensis* Δ*tlo* mutants suggests that these genes may play a role in the control of hypha formation [32]. In addition to these differences, a range of genes appear to be in the process of being lost by *C. dubliniensis*. There are 78 *C. dubliniensis* pseudogenes with intact positional orthologs in *C. albicans*, including genes identified as filamentous growth regulators (FGR) in haploinsufficiency studies [38]. These findings suggest that *C. dubliniensis* is undergoing a process of reductive evolution leading to the loss of genes that have been associated with *C. albicans* virulence. Interestingly, many of these genes are only expressed by the hyphal form of growth and are likely to play a prominent role in host-pathogen interaction.

One of the most prominent phenotypic differences between *C. albicans* and *C. dubliniensis* is their different capacity to tolerate environmental stress, with the former being far more tolerant of thermal, osmotic, and oxidative stress [5, 14, 39, 40]. Indeed, comparative growth at 45°C is commonly used as a simple diagnostic test to discriminate between the two species [41]. Comparative transcriptional profiling analysis revealed that although the two species express similar core stress responses, *C. dubliniensis* mounts a more robust response to thermal stress and a very poor transcriptional response to oxidative and osmotic stress [39]. Forward genetic screens using a *C. albicans* library to try and identify genes that might increase the tolerance of *C.*

dubliniensis to environmental stress failed to identify any single gene that could complement oxidative and thermal sensitivity, suggesting that these are likely to be polygenic traits. However, the *C. albicans ENA21* gene, which encodes a sodium efflux pump, was found to increase the salt tolerance of *C. dubliniensis* [39]. Since the *C. dubliniensis* ortholog of this gene appears to be functional but not upregulated in response to the presence of salt, it is likely that the differential salt stress susceptibility of the two species is due to differences in stress-related transcriptional regulatory pathways.

3. Comparative Analysis of Hypha Formation by *C. albicans* and *C. dubliniensis*

One of the most important and best-studied virulence factors of *C. albicans* is its ability to switch between yeast and filamentous growth forms (i.e., dimorphism), a trait also shared by *C. dubliniensis* [5]. However, although *C. dubliniensis* is capable of producing germ tubes and true hyphae, it does so far less efficiently than *C. albicans,* both *in vivo* and under a wide range of *in vitro* conditions [16, 42, 43]. Given the perceived importance of dimorphism in *C. albicans* virulence, we have previously suggested that the lower virulence of *C. dubliniensis* may, at least in part, be related to its relatively poor ability to switch between yeast and hyphal forms [16]. Evidence in support of this was obtained from murine systemic infection model studies [14, 15] and the neonatal orogastric infection model [16]. In the latter, stomach and kidney samples in infected animals contained only *C. dubliniensis* yeast cells, while *C. albicans* cells were found in both the yeast and hyphal forms [16].

We have used the RHE model of superficial infection [44] to compare the invasive potential of both species (Figure 1). In particular, in this model, *C. albicans* grows as both yeast and hyphae and invades the tissue causing major damage. In contrast, *C. dubliniensis* grows exclusively in the yeast form in this model, therefore, causing relatively limited tissue invasion and damage [16, 17]. In order to investigate why the two species differ so markedly in virulence in this model and in order to identify novel virulence-associated genes; Spiering et al. compared their global gene expression profiles during the early stages of RHE infection [17]. Both species showed similar expression profiles for ribosomal and general metabolic genes, however, unsurprisingly, *C. albicans* showed increased expression of hypha-specific virulence genes (e.g., *ECE1, HWP1, HYR1, and ALS3*) within 30 minutes of infection. In contrast, *C. dubliniensis* showed a far less robust transcriptional response and no expression of hypha-specific genes. In addition, several genes with unknown function were found to be specifically upregulated in *C. albicans* that are absent from or very divergent in the *C. dubliniensis* genome. One of these genes, named *SFL2* due to its sequence similarity to the transcription factor-encoding gene *SFL1*, encodes a putative DNA-binding heat shock factor protein. When the gene was deleted in *C. albicans*, it resulted in the failure to produce hyphae under a wide range of growth conditions, including the RHE infection model. Interestingly, the Δ*sfl2* mutation had no effect on survival in the murine systemic infection model, although histological analysis revealed that the kidneys of infected mice were infected only with yeast cells, while the kidneys of mice infected with the wild-type parental strains contained both yeast and predominantly hyphal cells [17]. In a subsequent study, it has been shown that the Δ*sfl2* mutant exhibits reduced virulence in a mouse model of gastrointestinal infection, suggesting that Sfl2 is required for the penetration of the gut wall and subsequent dissemination throughout the body [45]. The *C. dublinensis* ortholog of *SFL2* is only 50% identical and is not expressed under the same conditions as the *C. albicans* gene, therefore it is possible that the divergence of this gene and its apparent lack of expression may be partly responsible for its lower virulence.

Recent studies by our group have been directed towards investigating the molecular basis for differences in the signaling pathways responsible for filamentation in the two species. Comparative genome analysis suggests that orthologs of the known components of the major *C. albicans* morphogenetic pathways (e.g., Cph1-mediated MAPK and the Efg1-mediated Ras1-cAMP pathways) are highly conserved in *C. dubliniensis*, so the reduced capacity of *C. dubliniensis* to produce hyphae and express hypha-specific genes such as *SFL2* is unlikely to be due to the absence of regulators involved in these pathways. Forced stimulation of the Ras1-cAMP pathway with a hyperactive $RAS1^{G113V}$ allele did not result in increased true hypha formation in *C. dubliniensis*, suggesting strong repression of the RAS1-cAMP pathway itself or downstream regulators [43]. One of the most important transcriptional regulators involved in the control of morphogenesis in *C. albicans* is Nrg1, which Staib and Morschhäuser. showed that it is differentially expressed by *C. dubliniensis* when grown on media such as Staib agar [46]. In *C. albicans* this protein targets the negative regulator Tup1 to specific sequences in the promoters of genes involved in hypha formation. *NRG1* expression is rapidly downregulated in *C. albicans* cells incubated under hypha-inducing conditions, including when cells are phagocytosed by murine macrophages, which results in germination and escape from the phagocytes. However, in *C. dubliniensis, NRG1* expression remains high under these conditions, preventing hypha formation and causing cells to remain in the yeast phase, which in the murine macrophage model results in failure to escape from the phagocytes and the death of the fungus [43]. Deletion of the *NRG1* gene in *C. dubliniensis* resulted in an increase in the rate of hypha and particularly pseudohypha formation, which in turn led to increased survival when exposed to murine macrophages as well as increased virulence in the reconstituted human epithelial (RHE) cell model of oral candidosis. Surprisingly, the *C. dubliniensis* Δ*nrg1* mutant was no more virulent than its parent strain in the murine systemic infection model and formed mainly pseudohyphae in infected kidneys, suggesting an additional level of repression preventing true hypha formation *in vivo* [43].

Recent investigations by O'Connor et al. have suggested that repression of filamentation in *C. dubliniensis* is mediated by nutrients. In order to improve our understanding of how environmental signals trigger these pathways O'Connor

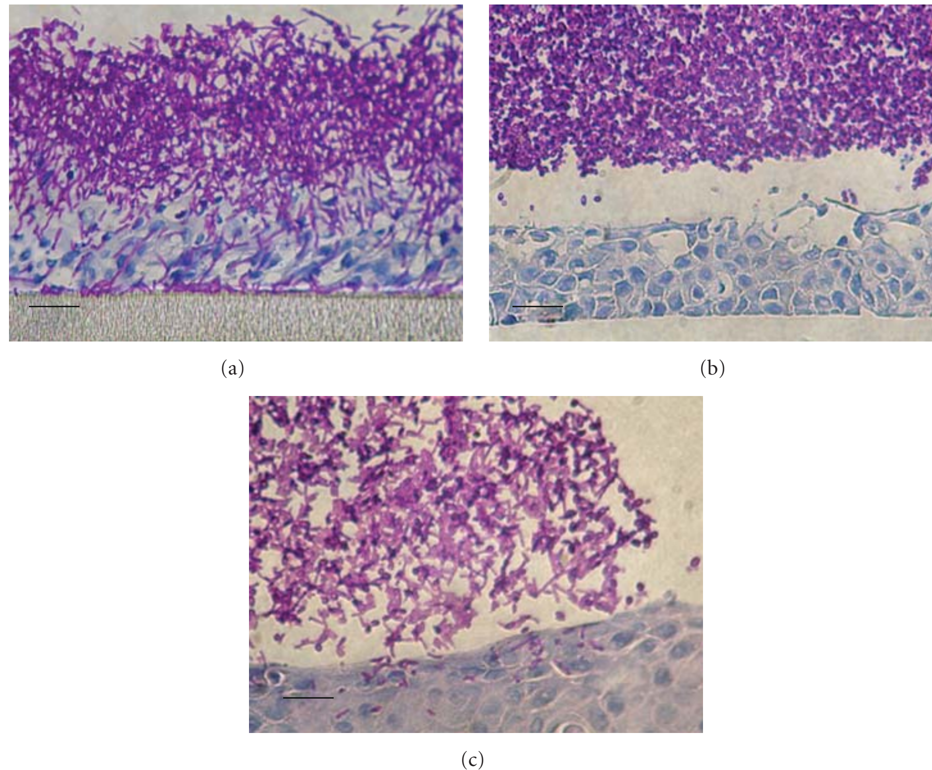

Figure 1: Photomicrograph of *C. albicans* SC5314 and *C. dubliniensis* CD36 infecting oral reconstituted human epithelial (RHE) tissue. (a) *C. albicans* originally grown in nutrient-rich YPD, note the presence of hyphae and extensive tissue invasion and damage; (b) *C. dubliniensis* originally grown in YPD, note the absence of hyphae and the limited level of invasion and tissue damage; (c) *C. dubliniensis* originally grown in Lee's medium, note the increased level of filamentation and invasion. Scale bars, approximately 25 μm.

et al. investigated the effects of nutrient availability on the rate of hypha formation [42]. One of the most common incubation conditions for inducing hypha formation in *C. albicans* is incubation in the nutrient-rich medium YPD supplemented with 10% (vol/vol) fetal calf serum at 37°C. Under these conditions >80% of *C. albicans* cells produced germ tubes/filaments within two hours, in contrast only ~20% of *C. dubliniensis* cells were observed to produce hyphae under the same induction conditions. However, when *C. dubliniensis* cells were incubated in water supplemented with 10% (vol/vol) fetal calf serum (WS) at 37°C, the level of hypha producing cells increased to 90% (i.e., similar to the level of hypha formation by *C. albicans*), suggesting that a nutrient-rich environment, in particular the presence of complex mixtures of peptides, suppressed hypha formation in this species. This was confirmed when the addition of peptone and peptone and glucose was found to significantly reduce the levels of hyphae, suggesting that nutrient starvation is a prerequisite for hypha formation by *C. dubliniensis* [42]. These morphological changes were coupled with changes in the expression of genes encoding key transcriptional regulators, such as *NRG1* and *UME6*, which were significantly altered in WS, with the former downregulated by 70% and the latter upregulated 30-fold. Overexpression of the *UME6* gene (which encodes a protein required for hyphal extension), using a doxycycline-inducible promoter led to *C. dubliniensis* cells being able to produce true hyphae, even in nutrient-rich media such as YPD. Similarly, preculture of *C. dubliniensis* cells in nutrient poor media, such as Lee's medium, pH 4.5, prior to the induction of hyphae in YPDS also resulted in a transient ability of *C. dubliniensis* cells to produce hyphae which increased the ability of these cells to adhere to and invade epithelial tissue in the RHE model (see Figure 1) and increased survival in the murine macrophage infection model [42].

These data suggest that factors controlling *UME6* expression in *C. dubliniensis* are repressed by the presence of nutrients, and unlike *C. albicans*, this repression cannot be lifted by a shift to alkaline pH, which occurs when serum is added to the medium. *UME6* is likely to be regulated by Efg1 and Eed1 and is therefore under the control of the Ras1-cAMP pathway. Few studies have investigated how nutrients regulate this pathway in *C. albicans* or *C. dubliniensis*. Preliminary investigations in our laboratory have shown that rapamycin, an inhibitor of the nutrient sensing kinase Tor1 (a kinase that plays a central role in the control of responses to nutrient availability [47]), can stimulate transient hypha formation in *C. dubliniensis* in nutrient-rich YPD serum [48]. This derepression of hypha formation in the presence of nutrients is concomitant with a reduction of *NRG1* and an increase in *UME6* expression. These data suggest that differences in Tor1 activity may play a role in the differential ability of *C. albicans* and *C. dubliniensis* to form hyphae. The

Table 1: Comparison of *C. albicans* and *C. dubliniensis*.

	C. albicans	*C. dubliniensis*	References
Growth and morphology			
Growth at ≥42°C	Yes	No	[39, 41]
Growth in high salt media	Yes	No	[39, 40]
Hypha formation in YPD + serum	Yes	Poor	[16, 42]
Hypha formation in water + serum	Yes	Yes	[42]
RHE infection model	yeasts and hyphae	yeasts only	[17]
Genome			
Chromosome number	8	9–11 chromosome-sized fragments	[7]
No. of species-specific genes	168	29	[32]
ALS3	Present	Absent	[32]
HYR1	Present	Absent	[32]
SAP4, 5 and 6	All three genes	One gene	[32]
HWP1	Present	Divergent	[32]
TLO family	14 genes	2 genes	[32]

molecular basis for the difference in the activity of Tor1 in the two species is currently under investigation.

4. Conclusions

Candidal pathogenicity involves the complex interplay of a wide range of virulence-associated factors. Comparative analysis of *C. albicans* and *C. dubliniensis* genomic and transcriptomic data has revealed that the reasons for the differences in the capacity of these two species to cause disease are also complex and are not due to a simple defect in *C. dubliniensis*. Instead these studies have revealed genetic differences in the two species, which, at least in part, may explain the differences in their capacity to tolerate stress and to filament. It is clear that the *C. dubliniensis* genome is missing important virulence genes (e.g., *ALS3* and *HYR1*), is in the process of losing others (e.g., the *FGR* genes), has failed to expand certain gene families (e.g., the *SAP* and *TLO* families), and has undergone some degree of transcriptional rewiring (e.g., the Tor pathway and Sfl2). All of these differences suggest that the main discrepancy between these two closely related species relates to differences in hypha formation and the expression of hypha-specific products (summarized in Table 1). We propose that *C. dubliniensis* is in the process of undergoing reductive evolution, whereby its genetic repertoire is diminishing in comparison with *C. albicans* and their common ancestor. One of the main phenotypic manifestations of this is the narrowing of environmental conditions permissive for hypha formation, perhaps as a result of specialization for survival in a specific (as yet unidentified) anatomic niche where hyphae are not required for colonization or growth. By further investigating the molecular basis for the differences between *C. albicans* and *C. dubliniensis*, we hope to improve our understanding of candidal virulence, in particular the relative contribution of hyphae and hypha-specific proteins to the pathogenesis of candidal infections.

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