Genes Involved in the Pathogenic Potential of *Mucorales*

Subjects: Genetics & Heredity

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The classification of *Mucorales* encompasses a collection of basal fungi that have traditionally demonstrated an aversion to modern genetic manipulation techniques. This aversion led to a scarcity of knowledge regarding their biology compared to other fungal groups. However, the emergence of mucormycosis, a fungal disease caused by *Mucorales*, has attracted the attention of the clinical field, mainly because available therapies are ineffective for decreasing the fatal outcome associated with the disease.

Mucorales mucormycosis antifungal resistance

1. Introduction

The progression of microorganisms embodies a persistent emergence of pathogens that affect humans, including new variants of bacteria and fungi that elude existing antibiotics and antifungal treatments. Among the fungi, the *Mucorales* order is a wellspring of highly resistant species responsible for mucormycosis, a deadly and emerging infection [1]. This disease is the third-most-common angio-invasive fungal infection, following candidiasis and aspergillosis, in patients with hematological and allogeneic stem cell transplantation [2]. With an aging population, the increased number of immunocompromised patients, and the recent COVID-19 pandemic, the number of individuals susceptible to Mucorales infections is on the rise [3]. The noticeable increase in mucormycosis cases, a mortality rate of 90% for disseminated infections, and the absence of effective antifungal treatments have triggered widespread concern regarding this emerging disease [4][5]. In addition, advancements in diagnostic techniques have uncovered an alarming number of cases of mucormycosis among immunocompetent/otherwise healthy individuals [6]. The *Mucorales* are often overlooked compared with other fungi, such as Ascomycetes and Basidiomycetes. The limited understanding of the genetics of *Mucorales* is due to their resistance to modern genetic manipulation techniques, as many cannot be transformed. However, the persistence of the scientific community has found a few species, such as *Mucor lusitanicus* (previously known as *Mucor circinelloides f. lusitanicus*) and *Rhizopus microsporus,* which are opening this field to genetic manipulation [7][8][9]. The increasing concern about emerging cases of mucormycosis, coupled with these genetic models, has sparked interest among the scientific community. As a result, the past decade has seen a surge in studies on genes, pathways, and mechanisms that directly correlate with virulence and antifungal resistance in *Mucorales*. One of the most thoroughly examined mechanisms has been the process of gene silencing or RNA interference (RNAi) in *M. lusitanicus*. Following the dissection of the gene-silencing machinery, knowledge of this mechanism led to the

discovery of a novel and specific type of antifungal resistance mediated by temporal epigenetic changes [10]. The application of gene silencing as a genetic tool has facilitated the development of functional genomic techniques, which have been used to identify several new virulence factors. Along with silencing, gene disruption through homologous recombination has also enabled the study of the specific role of virulence factors in *M. lusitanicus*, identified in other fungi, such as the role of a high-affinity iron-uptake mechanism, the CotH protein family, and the calcineurin pathway. Furthermore, the advent of new omics technologies has produced a long list of candidate genes not previously linked to virulence, providing promising targets for developing new treatments for mucormycosis. Finally, the diversity of molecular and cellular methodologies has allowed for the study of the genetic response during host–pathogen interactions, revealing the crucial role of several regulatory genes.

2. Genes Involved in the Pathogenic Potential of *Mucorales*

2.1. The High-Affinity Iron Uptake System in Mucorales

Iron is an essential micronutrient for the different organisms across the life kingdoms. Due to its fundamental functions in the physiology of living organisms, it has a substantial role in maintaining the virulence of pathogens. Conversely, hosts have evolved to hide their iron reservoirs from pathogens to diminish their virulence [11]. In the case of *Mucorales* and mucormycosis, several studies established an apparent increase in virulence when the host iron-hiding systems fail, and an abnormally high free iron level is observed in blood [12]. Moreover, *Mucorales* have developed their own systems to acquire iron from their hosts, highlighting the vital role of iron for these pathogens during infection [13][14][15].

One of these systems is the high-affinity iron-uptake mechanism, a three-component-based mechanism depending on iron reduction, oxidation, and incorporation activities. These enzymatic activities are performed by the iron reductase Fre, the ferroxidase Fet3, and the permease Ftr1 [15][16][17]. A failure in the high-affinity iron-uptake system of *Mucorales* inevitably leads to a decrease in their virulence [16][18] These failures, induced by directed mutagenesis, were mainly studied in the genetic model *M. lusitanicus* [15]. This fungus has three paralogous ferroxidases genes, fet3a, fet3b, and fet3c, with fet3c being the key virulent factor, although a partial redundancy exists with the other two paralogs. However, the most intriguing result from this genetic analysis was the sub-functionalized role of the three paralogs during dimorphism. The genes fet3b and fet3c are highly expressed in the yeast form, whereas fet3a is only expressed in the mycelium [15]. Only the mycelial form is capable of developing mucormycosis, which settles the process of dimorphism as another determinant involved in virulence (elaborated below). The differential expression of the three fet3 genes in the two dimorphic states of *M. lusitanicus* was the first evidence connecting the high-affinity iron-uptake mechanism and dimorphism, linking two crucial processes involved in the virulence of *Mucorales*. Besides the role of iron ferroxidases in *M. lusitanicus*, the iron permease Ftr1 was studied in Rhizopus delemar, showing that a reduced expression correlated with decreased virulence [16] (**Table 1**).

Table 1. Genes recently involved in the virulence potential of *Mucorales*.

Study Model	Gene	Function	Reference
M. lusitanicus	*fet3a*	Iron uptake	[15]
M. lusitanicus	*fet3b*	Iron uptake	[15]
M. lusitanicus	*fet3c*	Iron uptake	[15]
R. delemar	*ftr1*	Iron uptake	[16]
Several	*cyp51 f1*	Ergosterol synthesis	[19]
Several	*cyp51 f5*	Ergosterol synthesis	[19]
M. lusitanicus and *R. delemar*	*cotH* family	Cell wall antigen	[20][21]
M. lusitanicus	ID112092	Secreted, unknown	[22]
M. lusitanicus	*wex1*	Exonuclease, unknown	[23]
M. lusitanicus	*atf1* and *atf2*	Transcription factors	[24]
M. lusitanicus	*mcplD*	Signaling	[25]
M. lusitanicus	*mcmyo5*	Intracellular transport	[25]
M. lusitanicus	*cnaA*, *cnaB*, *cnaC*, and *cnaR*	Calcineurin, pleiotropic	[26][27]
M. lusitanicus	*pkar1*, *pkar2*, and *pkar4*	Dimorphism	[28]
M. lusitanicus	*gpb1*	Dimorphism	[29]
M. lusitanicus	*r3b2*	RNAi	[30]
Rhizopus microsporus	*pyrF*	Uracile synthesis	[9]

Another system developed by pathogens to acquire iron from the host is based on small compounds with high-affinity iron-chelating activity known as siderophores. In the case of *Mucorales*, the most specific siderophore is rhizoferrin, but this is also the least studied in mucormycosis [13][14][31]. Fungi synthesize their own siderophores but can also use the siderophores produced by other microorganisms [32]. Moreover, Rhizopus spp. can use the synthetic siderophore deferoxamine, a siderophore used in dialysis patients with iron overload [33].

2.2. Azole Resistance in Mucorales: The Cytochrome P450

Azoles are a group of antifungal compounds commonly used in the clinic against fungal infections. Among them, those with three nitrogen atoms and a cyclic structure are known as triazoles. Their antifungal mechanism relies on suppressing the cytochrome P450 activity, which is mainly involved in synthesizing ergosterol [34][35][36]. Azole resistance spontaneously appears in fungi [37] by three different mechanisms: increasing the amount of P450, decreasing its inhibition, or increasing the azole transport out of the cell [38][39]. However, *Mucorales* present an

innate and general azole resistance higher than other fungi [1][40][41]. This resistance explains the lack of effective antifungal treatments against *Mucorales*, and it is the main reason forcing clinicians to continue using old compounds such as amphotericin B to treat mucormycosis infections [1][42][43]

Among the different cytochrome P450 enzymes, the lanosterol 14*α*-demethylase CYP51 (also known as Erg11) plays a critical role in azole antifungal resistance in Aspergillus [44][45][46][47][48]. Similarly, a recent study found a possible link between specific mutations in CYP51 and the innate triazole resistance observed in *Mucorales* [19][49]. *Mucorales* have two paralogues of CYP51, CYP51 F1 and CYP51 F5. The amino acid sequence analysis of different *Mucorales* showed only two conserved mutations in F5, which create a predicted structural change that might explain how short-tailed azoles cannot inhibit this version of CYP51 [49] (**Table 1**). However, this predicted model still requires experimental validation.

2.3. The cotH Gene Family, an Important Source of Virulence Factors in Mucorales

The cotH gene family encodes unconventional protein kinases that are found in spores of different organisms, both prokaryotes and eukaryotes [50][51]. They are related to the regulation of the spore integrity, and mutants affected in their sequence are frequently associated with defective germination [52]. The genomes of different *Mucorales* contain several copies of cotH genes, and the proteins are found in the spore surface [52][53]. Usually, mucoralean species lacking these proteins are avirulent, and a higher number of cotH gene copies is associated with more aggressive species [53][54]. The mechanism relating CotH proteins to virulence is associated with adherence and tissue invasion. Thus, CotH proteins have a conserved motif that interacts with the host endothelial cells, initiating the tissue invasion [53][55][56].

A recent study found 17 cotH-like genes in the genome of Mucor lusitanicus. The disruption of five of them led to defects in temperature adaptation and cell wall development. More importantly, their role in virulence was confirmed in a mouse model [20] (**Table 1**). Besides some bacteria and *Mucorales*, CotH proteins are not found in other fungi such as Candida and Aspergillus, making them a specific target for therapeutic and diagnostic approaches [21][57].

2.4. Genomic Approaches to Identify New Virulence-Related Genes

Although most of the genetic analyses described in the previous sections dissected the role of genes identified in other pathogenic fungi, the current omic technologies allow more ambitious projects to search for new virulence factors in *Mucorales*. Two approaches explored mucoralean genomes, trying to find new virulence determinants that were not previously described in other pathogenic organisms.

The first one performed a comparative genomic approach confronting the genomes of two highly related strains of *M. lusitanicus*: CBS277.49 and NRRL363 [22]. These two strains share identical features in most of their fungal physiology except for a critical aspect: their pathogenic potential. The strain CBS277.49 is virulent and kills most of the hosts in survival assays, whereas NRRL363 is avirulent and is usually chosen as a negative control. Thus, the comparison between their genomes is expected to find key differences that might be involved in the pathogenic

phenotype. This approach identified 543 absent genes and 230 discontiguous protein-coding sequences in the avirulent strain [22]. The functional screening of those genetic differences identified a secreted protein with unknown functions that was highly involved in the virulence of CBS277.49 [22] (**Table 1**). As expected, other differences between CBS277.49 and NRRL363 strains, such as the gene ID108920 (hypothetical g-glutamyltranspeptidase), had no impact on the virulent phenotype. Another study with a different perspective also compared mucoralean genomes, but in this case, all of them were from mucormycosis-causing isolates searching for similarities. This approach revealed that a higher copy number of cotH genes correlates with strong virulence and clinical prevalence [53].

In a similar comparative approach but at the RNA level, a transcriptomic analysis comparing the avirulent NRRL3631 and the virulent strain CBS 277.49 led to the identification of Wex1, a new exonuclease involved in virulence [23]. Moreover, transcriptomic analysis during macrophage–spore interaction allowed for the identification and further genetic dissection of two Atf transcription factors and their regulated targets [24] (**Table 1**).

The second approach developed a methodology for identifying new virulence factors at the genomic scale. This methodology developed a functional genomic strategy using an RNAi high-throughput library that allowed for the fast screening of new virulence factors. Briefly, a collection of plasmids capable of silencing all the genes of *M. lusitanicus* enabled the isolation of transformants with interesting phenotypes. Later, a fast screening in Galleria mellonella selected only the transformants with phenotypes related to virulence. Then, the plasmids in the selected transformants were rescued and sequenced, unveiling the genes responsible for the virulence-related phenotype. Finally, deletion mutants were generated and validated in a murine survival assay. The first “proof of concept” application of this methodology identified two previously unknown virulence determinants: the genes mcplD and mcmyo5 [25] (**Table 1**).

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