

Aspergillus fumigatus: virulence genes in a street-smart mold

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Infections with the filamentous fungus *Aspergillus fumigatus* are among the most devastating of the systemic mycoses. Unlike most primary pathogens, which possess virulence traits that developed in association with a host organism, evidence suggests that the virulence of *A. fumigatus* entails a collection of 'street-smart' attributes that have evolved to resist the adverse selection pressures encountered in decaying vegetation. These features enhance the overall competitiveness of the organism in its environmental niche but are also thought to promote growth and survival in a human host. Although many of the genes that are responsible for these characteristics do not fit into the classical definition of a virulence factor, they are nonetheless important to the pathogenesis of aspergillosis and may therefore provide novel opportunities for antifungal development.

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Introduction

Aspergillus fumigatus is a saprophytic filamentous fungus that is the predominant mold pathogen of the immunosuppressed population [1]. The organism is acquired through the inhalation of asexual spores called conidia, which are widespread in the environment and small enough to reach the distal airways. The conidia are of minimal concern to healthy individuals because they are cleared by pulmonary defenses. However, when the immune system is compromised, the conidia may germinate into hyphae and establish a focus of infection within the lung. Although it is likely that conidial germination begins within the surfactant layer, both conidia and hyphae can be endocytosed by, and grow within, lung epithelial cells [2]. Since the ability of *A. fumigatus* to assimilate nutrients from a complex substrate requires the secretion of extracellular hydrolases [3], the progressive

growth of hyphae within the lung eventually damages the epithelial barrier, providing access to the interalveolar septum where the fungus can enter the vasculature by penetrating endothelial cells (Figure 1). Hyphal fragments are then free to migrate to distal sites, and the prognosis for disseminated infection is very poor [4,5]. Throughout the infection, *A. fumigatus* must continually adjust its physiology to survive in the host environment, and the genes that have been implicated in this adaptability are the subject of this review. The emphasis is on fungal gene products since the contribution of the host has been highlighted in several recent articles [6,7,8].

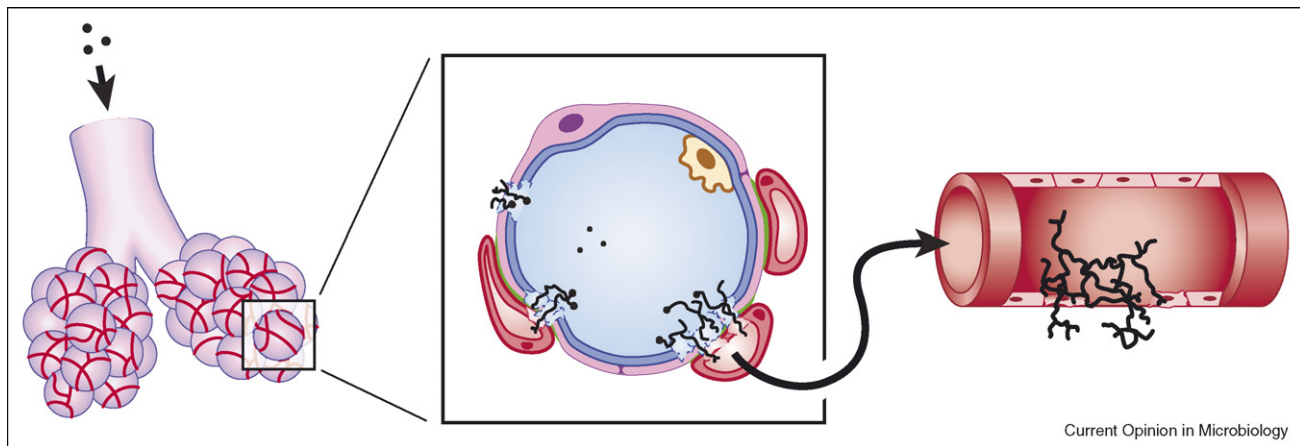
Sustaining growth at body temperature

A. fumigatus resides in compost, a dynamic environment that undergoes wide fluctuations in temperature as a consequence of intense microbial activity. The ability of *A. fumigatus* to thrive in this niche requires a substantial level of thermotolerance that has been speculated to contribute to virulence [9–11]. Although *A. fumigatus* displays a distinct pattern of gene expression at 37 °C [12], only three genes have been demonstrated to be necessary for thermotolerant growth: the ribosome biogenesis protein CgrA [13], the *O*-mannosyltransferase Pmt1 [14] and a protein of unknown function, ThtA [15]. Disruption of any of these genes influences thermotolerance to some degree. However, only the Δ cgrA mutant has impaired growth and virulence at 37 °C. The ribosome defect in the Δ cgrA mutant is present at 22 °C, even though the mutant grows normally at this temperature [16]. This suggests that the defect in ribosome biogenesis caused by loss of CgrA is compatible with the limited physiological demands at low temperature but not with the heightened metabolic requirements at 37 °C. The challenge for the future will be to determine whether the thermotolerance of *A. fumigatus* can be reduced further by disrupting other genes involved in ribosome biogenesis, with the goal of rendering the organism incapable of growth at 37 °C.

Maintaining a rigid yet permeable barrier to the environment

In addition to providing structural integrity, the cell wall represents the major interface between the internal physiology of the fungus and the hostile environment of either compost or human tissue. The wall of *A. fumigatus* comprises branched and linear β -(1,3) and β -(1,4) glucan, α -(1,3) glucan, chitin, chitosan, and galactomannan [17]. There are three predicted α -(1,3) glucan synthase genes in the *A. fumigatus* genome, but only a disruption of *ags1* resulted in a decrease in cell wall α -(1,3) glucan [18]. The Δ ags1 mutant retained virulence, despite

Figure 1



Schematic illustration of the pathogenesis of invasive aspergillosis (not drawn to scale). *A. fumigatus* conidia are small enough (2–3 μm in diameter) to reach the distal airways when inhaled (left). A cross-section of the alveolar space is enlarged in the center panel, showing the close proximity of adjacent blood vessels in the interalveolar septum. In a susceptible host, the conidia are able to germinate and damage the blood–air interface. This barrier comprises a surfactant layer (blue), a type I pulmonary epithelial cell (pink), and an underlying microvascular endothelial cell (red). Loose interstitial tissue can sometimes be found between the epithelial and endothelial cells, but when the two cells are closely apposed the basal laminae fuse (green), making the barrier only 0.1–1.5 μm in thickness. The growing hyphae eventually penetrate this barrier and hyphal fragments are released into the blood, providing access to other organs by extravascular invasion (right panel).

suffering a 50% reduction in α -(1,3) glucan content, indicating that the fungus can tolerate a considerable loss of this polysaccharide and still maintain cell wall homeostasis. Surprisingly, deletion of *ags3* increased virulence without affecting α -(1,3) glucan content [18]. The normal level of α -(1,3) glucan in this mutant is likely to be a consequence of redundancy between *ags1* and *ags3* since *ags1* levels were dramatically upregulated in the Δ *ags3* strain. The hypervirulence of Δ *ags3* was speculated to involve the observed increase in melanin, resistance to oxidative stress, and more rapid germination of the mutant conidia, although the mechanism by which loss of *Ags3* induces this phenotype remains to be elucidated.

Chitin is a polymer of *N*-acetyl-glucosamine that confers high tensile strength upon the wall [19]. Of the three chitin synthase genes that have been examined in *A. fumigatus*, only the double Δ *chsC*/ Δ *chsG* mutant showed a reduction in virulence, a phenotype that was attributed to *chsG* because the Δ *chsG* mutant had the same morphological abnormalities as the Δ *chsC*/ Δ *chsG* mutant [20]. At least seven chitin synthase genes can be found in the *A. fumigatus*, suggesting that considerable redundancy among these proteins has evolved to ensure that cell wall homeostasis is maintained when the organism is confronted with adverse conditions that interfere with wall integrity.

Glycophosphatidyl-inositol (GPI)-linked proteins anchored to the plasma membrane also play important roles in fungal cell wall organization. The family of GPI-anchored β -1,3-glucanosyltransferases are thought to

participate in the elongation of β -(1–3) side chains in the *A. fumigatus* cell wall, and at least one of these genes, *gel2*, is required to support the virulence of *A. fumigatus* [21]. By contrast, deletion of the GPI-linked Ecm33 protein increased virulence, possibly resulting from the increased germination rate of this mutant [22]. A more global block of GPI-anchored protein function was accomplished by disrupting *Aspig-a*, encoding the catalytic subunit of an enzyme involved in GPI anchor biosynthesis [23]. The absence of this protein was associated with reduced cell wall integrity and attenuated virulence, demonstrating the importance of this general protein class in both cell wall function and pathogenesis.

The wall of *A. fumigatus* conidia is distinguished by the presence of melanin, a pigment that is thought to defend the genome from the adverse effects of ionizing radiation in the environment. Pigment biosynthesis is catalyzed by a polyketide synthase, and mutants lacking this enzyme have white conidia and attenuated virulence when inoculated intravenously into mice [24,25]. Pigmentless conidia are more susceptible to phagocytic killing than wild-type conidia, which may explain their lack of virulence in this model.

Despite the considerable redundancy among cell wall synthesis genes in *A. fumigatus*, the wall remains an attractive target for therapy because of its fungal specificity and the fact that an intact cell wall is essential to the organism. The challenge will be to identify new strategies that can disrupt cell wall synthesis in a more global fashion so that overlapping pathways are unable to protect the organism.

Secretion of damaging products

A. fumigatus secretes numerous secondary metabolites into its environment [26[•]], which is thought to provide a chemical shield against competing or predatory species [27,28]. The secondary metabolite gliotoxin has attracted the most interest in *A. fumigatus* because of its potent immunosuppressive and cytotoxic properties and the fact that it can be readily detected during experimental infection and in sera from patients with aspergillosis [29–32]. Two studies have shown that blocking gliotoxin production by disrupting the *gliP* gene had no effect on virulence in neutropenic mice, arguing against a major role for this toxin in the pathogenesis of aspergillosis [33,34]. However, recent studies have shown that the contribution of gliotoxin to virulence is host-strain-dependent and requires an immunosuppression protocol that does not cause neutropenia [35[•],36]. These results imply that gliotoxin augments virulence only when some neutrophil function is present, raising the possibility that neutrophils are the major target of this toxin. A more global repression of secondary metabolite production was accomplished by disrupting *laeA*, encoding a predicted protein methyltransferase that regulates the expression of secondary metabolite clusters [37]. Although the Δ *laeA* mutant lacked gliotoxin, and was hypovirulent in mice, the mechanism for the attenuated virulence cannot be identified with certainty because *LaeA* influences the expression of ~10% of the *A. fumigatus* genome [38].

Signaling and responding to stress

The ability of *A. fumigatus* to reprogram its physiology in response to the environment requires an effective communication strategy that is mediated by signal transduction pathways [39]. The cAMP-dependent protein kinase (PKA) is key to this signaling, particularly with respect to the sensing of carbon source and environmental stress. The central messenger of the pathway is cAMP, produced by the action of adenylate cyclase, an enzyme that is under the regulation of the G-protein- α -subunit GpaB. Accumulating levels of cAMP bind to the regulatory subunit of PKA, PkaR, thereby liberating the catalytic subunits PkaC1 and PkaC2, which then phosphorylate downstream targets and trigger the appropriate adaptive responses. Dysregulation of the PKA pathway, either by disrupting its activity (Δ *gpaB*, or Δ *pkaC1*) [40], or allowing unrestrained PKA activity (Δ *pkaR*) [41], attenuates virulence in mice. This suggests that an imbalance in *A. fumigatus* PKA signaling, in either direction, is deleterious to the pathogenesis of aspergillosis, presumably by disrupting the ability of the fungus to sense and adequately respond to host-specific stressors.

Calcineurin is a Ca^{2+} -calmodulin-activated protein phosphatase that is an important mediator of calcium signaling and stress responses in eukaryotic organisms [42]. Deletion of *cnaA*, encoding the catalytic A subunit of calcineurin, profoundly impaired the growth of *A. fumigatus* hyphae and

rendered the organism almost avirulent in multiple infection models [43[•],44]. By contrast, a mutant lacking the calcineurin-dependent transcription factor CrzA grew normally *in vitro* but was still attenuated for virulence [45^{••}]. These findings argue that the impaired virulence of the Δ *cnaA* mutant is not simply due to reduced hyphal growth. Since calcineurin inhibitors are already in use for the treatment of other diseases, it may be possible to manipulate this pathway to improve outcome in patients with invasive aspergillosis.

Virulence studies have been reported on one of the four mitogen-activated (MAP) kinases in the *A. fumigatus* genome, *mpkA* [46]. Despite a heightened sensitivity to cell wall stress, and a considerable growth defect on standard laboratory medium, the Δ *mpkA* mutant was as virulent as wild-type *A. fumigatus*. Interestingly, deletion of an upstream regulator of the high osmolarity glycerol (HOG)-MAPK pathway, *sho1*, also impaired *in vitro* growth rate without affecting virulence [47]. The lack of correlation between *in vitro* growth rate and virulence in the Δ *mpkA* and Δ *sho1* mutants suggest that using growth rate to predict virulence potential is perhaps an oversimplification of a complex phenotype.

Oxidative damage generated by host immune responses is one of the major sources of stress encountered by pathogens. Although infections with *A. fumigatus* occur primarily in immunocompromised patients, the severity of the immunosuppression varies widely, so it is likely that residual host defenses can influence the progression of the disease. Several *A. fumigatus* genes have been reported to modify the sensitivity of the organism to oxidative stress, including catalases (*catA*, *cat1*, *cat2*) [48,49], the PKA regulatory subunit PkaR [41], the MAPK pathway (*mpkA*, *sho1*) [46,47], and two transcription factors that mediate oxidative stress responses (*yap1* and *skn7*) [50[•]]. However, only the Δ *pkaR* and Δ *cat1*/ Δ *cat2* mutants showed a reduction in virulence. These results argue against a major role for these anti-oxidant responses in an immunocompromised host, although it is conceivable that they could influence virulence under conditions of less severe host immunosuppression, analogous to the findings reported for the gliotoxin mutant [35[•],36].

Meeting nutritional requirements

In order to compete effectively in the environment, *A. fumigatus* must be able to adjust its metabolism to ensure that its needs are met during periods of fluctuating nutrient availability. Accumulating evidence indicates that this metabolic versatility is of particular importance in the host environment. One of the most striking examples of this is illustrated by iron. *A. fumigatus* uses siderophores for both iron acquisition and intracellular iron storage [51^{••}], and disrupting the siderophore biosynthetic pathway at multiple steps has revealed that *A.*

fumigatus relies heavily on these low-molecular weight chelators for growth in the iron-limited environment of the host [51^{••},52,53]. Zinc also has limited bioavailability *in vivo*, and the zinc-responsive transcriptional activator ZafA is required to support virulence, presumably by enhancing zinc uptake mechanisms [54].

Several studies have shown that mutants of *A. fumigatus* that are auxotrophic for *p*-aminobenzoic acid [55–57], uridine/uracil [58] or lysine [59] are avirulent, suggesting that these nutrients cannot be acquired in sufficient quantities from host tissues, at least during the initial part of the infection. A nitrogen source is also required for fungal growth, and the transcription factor AreA and the Ras-related protein RhbA have been implicated as signaling molecules that respond to nitrogen availability [60–62]. Both Δ *areA* and Δ *rhbA* mutants are hypovirulent in mice, suggesting that the nitrogen that is available in the host environment may require one or both of these proteins for optimal utilization by the fungus. The CpcA transcriptional activator of the cross-pathway control (CPC) system of amino acid biosynthesis is also required for the virulence of *A. fumigatus* [63]. This suggests that available amino acid pools in the host may be imbalanced, or alternatively that CpcA influences the expression of one or more virulence factors. Surprisingly, deleting the upstream signaling sensor kinase of the CPC system, CpcC, was dispensable for pathogenicity [64]. Since CpcC is responsible for upregulating the CPC pathway in response to stress, it appears that basal expression of the CPC system, rather than induced expression, supports virulence. It will be of interest to identify the target genes that are downstream of both CpcA and CpcC signaling.

A. fumigatus must continually extract nutrients from host tissues throughout the infection, which requires the secretion of degradative enzymes such as proteases. Deletion of several genes encoding secreted proteases has yet to demonstrate a role for such enzymes in virulence [65]. However, it is premature to discount the importance of protease secretion since only a limited number of them have been explored to date, and at least 99 secreted proteases are predicted in the *A. fumigatus* genome [12^{••},66]. Nevertheless, the progressive destruction of host tissues by *A. fumigatus* is likely to release amino acids that can be used by the fungus to support growth. An adverse consequence of amino acid metabolism is the toxic accumulation of propionyl-CoA, a problem that is countered by methylcitrate synthase, McsA, involved in the methylcitrate cycle [67^{••},68]. The striking reduction in virulence of a Δ *mcsA* mutant suggests that *A. fumigatus* relies upon protein degradation as a food source *in vivo*, making the fungus vulnerable to propionyl-coA accumulation [67^{••},68]. Since fungi and mammals handle propionyl-coA metabolism differently, an important implication of this finding is that it may be possible to design strategies to selectively interrupt the

fungal pathway. Taken together, each of these metabolic studies has revealed that although the nutritional environment of the host is not ideal for *A. fumigatus*, the fungus is well equipped to optimize its metabolism for the utilization of host tissues as a food supply. Further understanding of metabolic traits that are required for virulence may offer exciting new prospects for antifungal development.

Conclusions

It is becoming increasingly clear that the virulence of *A. fumigatus* is multifactorial, involving networks of genes that have probably evolved to support the organism in its primary ecological niche. The functions of these genes are diverse, influencing the integrity of the cell wall, the signaling pathways that detect and respond to environmental changes, and the adaptive responses that enhance overall fitness, most notably in the area of nutritional versatility. Although we have gained important insights into aspects of fungal physiology that support the growth of *A. fumigatus* in the host, the virulence determinants identified so far are not unique to this species and we have yet to determine what makes this fungus a more potent opportunistic pathogen than other commonly encountered environmental molds. The challenge for future research will be to obtain a comprehensive understanding of the requirements of this fungus in the host environment so that more effective strategies can be developed to interrupt these pathways.

Acknowledgements

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