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Aspergillus fumigatus: Growth and virulence

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Aspergillus fumigatus is a ubiquitous fungus that plays an important role in carbon and nitrogen recycling in nature. Because *A. fumigatus* is thermotolerant, it is a predominant organism during the high-temperature phase of the compost cycle. The ability to grow at elevated temperatures and to utilize numerous varied sources of both carbon and nitrogen to support its growth have made *A. fumigatus* an important opportunistic pathogen of humans as well as a vital part of the nutrient-recycling ecosystem. Data correlating the growth rate and germination potential of *A. fumigatus* at 37°C with its pathogenic potential suggest that these are related, both when viewed from a population standpoint and when analyzed on a single gene basis. Nutritional versatility has been cited as an important contributor to virulence as well. Indeed, perturbation of pathways involved with nitrogen or carbon sensing has been shown to reduce virulence in animal models, even when *in vitro* growth rates have not been altered. Therefore, the remarkable ability of *A. fumigatus* to grow efficiently under a variety of environmental conditions and to utilize a wide variety of substrates to meet its nutritional needs contributes to its role as the predominant mould pathogen of immunocompromised patients.

Keywords *Aspergillus fumigatus*, virulence, growth characteristics, thermotolerance, nutrient sensing

Introduction

Aspergillus fumigatus is the leading mould pathogen among immunocompromised patients, especially bone marrow and solid organ transplant recipients [1–5]. The increased number of patients at risk has contributed to the increasing incidence of this infection, but even patients outside of the traditional risk categories have shown a relatively high rate of infection [6]. Although *A. fumigatus* makes many 2–3 μm conidia that are easily airborne, its prevalence in the air does not explain its prevalence as an etiologic agent of invasive aspergillosis (IA) [3,7]. In a recent review of the virulence of the organism, it was suggested that *A. fumigatus* is a grass eater or saprophyte able to infect only immunocompromised hosts [8]. Although undoubtedly true, the authors concluded that future studies should concentrate on the host, rather than on the organism. However, those qualities that make *A. fumigatus* a highly competitive member of the compost microbiota, where temperature, pH, and nutrients may not be optimal for a broad range of organisms, are precisely the ones that may enable it to be a successful opportunistic pathogen [9–11]. For, were it not for those features, the spectrum of those fungi causing invasive fungal infections would mirror that of air surveys [12]. What are some of the growth characteristics of this grass eater that allow it to be a successful opportunistic pathogen? Thermotolerance, in the context of efficient growth and germination at temperatures of 37°C or higher, and nutritional versatility, the ability to sense and respond to various carbon and nitrogen sources, will be discussed as possible contributors to the opportunistic pathogenicity of *A. fumigatus*.

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Thermotolerance and growth rate

Germination rate

The reported growth rate of isolates of *Aspergillus* is often derived from measuring both the time required to

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germinate and the rate of apical extension of the hyphae. Germination initiates the asexual developmental program in the fungus. When the resting conidia of *A. fumigatus* are placed in an appropriate milieu and given nutrients and water, they begin the process of germination [13]. The conidia undergo a period of isotropic growth before switching to a polar growth program and producing a germ tube [14]. These are the first steps in growth of a new fungal colony, whether it is in the compost heap or the lung of a host. Therefore, it is important that these steps be carried out quickly, efficiently and that a compost organism is able to undergo this developmental program at elevated temperature. When conidia from clinical isolates of three *Aspergillus* species, *A. fumigatus*, *A. flavus*, and *A. niger*, were studied for their ability to germinate at 37°C, there was a strong correlation between germination rate and prevalence as opportunistic pathogens [15]. After 8 h in RPMI 1640, 58% of *A. fumigatus* conidia, 32% of *A. flavus* conidia, and 15% of *A. niger* conidia had germinated, with germtubes of *A. fumigatus* being visible an hour earlier than those from the other two species. To determine whether this simply represented a higher growth rate overall, the germination rate was measured at temperatures from 20–42°C. At temperatures up to and including 30°C, there was no difference among the isolates. However, at 37° and 42°C, the most common etiologic agent of invasive aspergillosis, *A. fumigatus*, showed a significantly higher percentage of germination, 50–60%, when compared with the other two species, $<40\%$ [15].

Growth rate

Growth rate in filamentous fungi can be measured in a number of different ways, and the results from different methods don't always agree [16]. One way to measure the growth of a filamentous fungus is to measure the apical growth rate of hyphae as a function of colony diameter. A specified number of conidia may be spotted into the center of a plate or a plug from a mature colony may be placed in the center of a plate [16–19]. Colony diameters are measured with time and the colony diameter itself or the rate in mm h^{-1} can be plotted. The biomass of the fungus can also be determined, either using absorbance or dry weight [16,20]. Using absorbance to measure growth in microtiter plates, different isolates of *A. fumigatus* were tested for growth rate and LD90 in a mouse model of IA [20]. When rank order of growth rate was compared with rank order of LD90, they were highly correlated, with an $r^2 = 0.6315$. This suggests that factors that promote faster growth rates *in vitro* may also contribute to the organism's ability to infect a host. Using dry weight to measure growth of an isogenic set of organism for the gene *rasB*, the mutant was shown to produce equivalent biomass to the wild type and reconstituted mutant strains, approximately 30 mg [16]. However, when the apical growth rate was measured on this same isogenic set, the growth rate of the mutant (<0.5 mm h^{-1}) was significantly reduced when compared with the wild type (>0.8 mm h^{-1}). Further, when the isolates were tested *in vivo*, the $\triangle$*rasB* mutant had attenuated virulence. In this case the apical growth rate seemed a better predictor of *in vivo* results than did biomass. But in the plant pathogen *Trichoderma virens*, a mutant in a MAP kinase, TmkA, had both reduced biomass and reduced ability to grow in plants [21]. Although the data suggest that it may be difficult to predict which method is better, the overall picture from these studies on growth rate supports the conclusion that *in vitro* growth rate at 37°C and ability to cause disease are correlated in *A. fumigatus*.

To look more specifically at thermotolerant growth and virulence, the nucleolar protein CgrA, which plays a role in ribosome biogenesis, may be instructive [22]. Mutants in which *cgrA* has been deleted have wild type germination and growth rates at 22–25°C. However, when the same experiments are carried out at 37°C, the mutants have a considerable growth detriment. The isogenic set of the wild type, $\triangle$*cgrA* and the complemented mutant were tested in two different models of IA, immunosuppressed mice and fruit flies. As one might predict from the *in vitro* growth rates, the mutant lacking CgrA showed a loss of virulence in mice, which have a body temperature of 37°C, whereas there was no significant reduction in virulence in the fruit flies, which were held at 25°C. But is there an upper temperature limit beyond which the ability to thrive no longer offers an increased *in vivo* advantage? In the only study examining mutants of *A. fumigatus* selected for their impaired growth above 42°C, a gene of unknown function, *thtA*, was identified [18]. *In vitro*, a mutant lacking *thtA* did not show any significant growth detriment at 37°C or 42°C, but was unable to grow at 48°C. However, when tested in two different murine models of IA, no difference in virulence between the mutant and the wild type was seen. Since this is an unknown gene, all that can be concluded is that ThtA, although required for high temperature growth, is not required for *in vivo* growth and virulence.

Based on the studies discussed here, it does seem clear that the ability to germinate and grow efficiently at 37°C is required to be an opportunistic pathogen. Since *A. fumigatus* thrives in the compost pile, where temperatures exceed 50°C, its metabolic program must

be adaptable to high temperature growth, which is not necessarily true of other species of *Aspergillus*.

Nutritional versatility

In the compost pile, *A. fumigatus* plays an important role in carbon and nitrogen recycling, primarily from non-woody plant material [8]. In this capacity, the organism must be able to respond to nutrient limitations, both qualitative and quantitative. This ability to sense carbon and nitrogen and to have the physiologic versatility to utilize different compounds contributes to the ability of *A. fumigatus* to compete with the other fungi and bacteria in compost and to be able to colonize and infect immunocompromised mammalian hosts [3,23]. A number of genes have been shown to be important in nitrogen utilization or to be regulated by nitrogen: *rhbA*, *cpcA*, *areA*, and *sakA* [24–27]. In carbon utilization, the cAMP-dependent protein kinase (PKA) pathway is known to be regulated, in part, by glucose availability in *Saccharomyces cerevisiae*, as well as in *A. fumigatus* [28,29]. The third aspect upon which this discussion will touch is the effects of auxotrophies on virulence. The overall theme is that the fewer limitations there are on an organism's ability to obtain or manufacture nutrients, the more competitive it will be, regardless of the growth environment. A second observation is that the availability of nutrients in an environment may not be obvious.

Nitrogen sensing and response

Since the role played by nitrogen metabolism in pathogenicity of *A. fumigatus* was recently reviewed in detail by Krappmann and Braus [30], RhbA will be used as an example to illustrate some key features of nitrogen sensing and utilization. RhbA is a transcriptionally controlled ras homologue that is upstream of TOR in the TOR nutrient sensing and growth pathway [19,26,31]. As such, levels of RhbA message are increased *in vitro* following nitrogen starvation, but not carbon starvation, differentiating it from the mitogen-activated protein kinase SakA [26,27]. Mutants that lack *rhbA* show wild type growth on complex media or on minimal medium containing the good nitrogen source, ammonium tartrate, but they have reduced growth when nitrate, histidine or proline serve as the sole nitrogen source. RhbA was first linked to virulence in *A. fumigatus* in an *in vitro* screen, when it was found to be up-regulated during growth on endothelial cells [32]. When the mutant was tested in an intranasal murine model of IA, there was a significant decrease in virulence when compared with the wild type and the complemented mutant. Subsequently, competitive RT-PCR was used to show that message levels of *rhbA* were markedly up-regulated following 24 and 72 h of growth in mouse lung [33]. Although counter-intuitive, these data together suggest that the mammalian lung, like the compost pile, may be limited in nitrogen either qualitatively, quantitatively, or both [34]. Therefore, *A. fumigatus* must be able to quickly sense and respond to these conditions in order to be an opportunistic pathogen.

Carbon sensing and response

In *S. cerevisiae*, the transcription of gene encoding enzymes involved in the utilization of alternate carbon sources is repressed in the presence of glucose, a process referred to as carbon catabolite repression (CCR) [35]. The cAMP-dependent protein kinase is one of a number of nutrient regulated protein kinases that function in the control of CCR in yeast [36]. In *A. fumigatus*, it has been reported that PKA activity varies based on the carbon compound in which the organism is grown, with activity being much higher in glucose-grown than glycerol-grown cultures [28]. Since *S. cerevisiae* mutants that lack the regulatory subunit of PKA are unable to grow on alternate carbon sources, it suggested that CCR was deregulated in those mutants [37]. Therefore, in *A. fumigatus*, we looked at the transcriptional response of an enzyme known to be a target of CCR, alcohol dehydrogenase (*alcA*), in a PKA regulatory subunit deletion ($\triangle pkaR$) mutant. Within 30 min of a shift from glucose to ethanol, the steady state *alcA* message increased over 10-fold in the wild type. However, there was no change in message abundance in the $\triangle pkaR$ mutant under the same conditions (Panepinto, JC and Rhodes, JC, unpublished data). This is compatible with loss of regulation of CCR, which could markedly reduce the ability of the mutant to utilize alternate carbon sources in the absence of glucose. Indeed, radial growth on glycerol is reduced in the $\triangle pkaR$ mutant, when compared with the wild type or with the mutant grown on glucose. When mutants in the PKA pathway have been tested *in vivo*, they have shown markedly attenuated virulence, whether or not they have reduced growth *in vitro* [38]. These results are compatible with an interpretation that a favored carbon source may also be limiting *in vivo*, and that mutations that interfere with an organism's ability to readily engage pathways for alternate carbon source utilization may suffer from reduced competitiveness, leading to reduced virulence.

Auxotrophies

The wild type *Aspergillus fumigatus* is a prototroph, able to grow on minimal medium, making all required nucleotides, amino acids and vitamins from simple precursors. Studies have looked at the ability of mutant strains that are unable to make some of these compounds to be effective opportunistic pathogens. Auxotrophic mutations yielding decreased virulence have been reported for uracil (*pyrG*), folate (*pabaA*), and lysine (*lysF*) [39–42]. A mutant of *A. fumigatus* in which the *lysF* gene encoding homoaconitase was deleted failed to grow *in vitro* on minimal medium unless it was supplemented with lysine or amino adipate [41]. The mutant was also hypovirulent in an immunosuppressed mouse model of IA. Although these results would suggest that the murine lung is limiting in lysine for growth, *in vivo* data fails to show up-regulation of *lysF* following infection with wild type conidia [33]. The requirement for uracil prototrophy for virulence has been reported in numerous fungal pathogens, *Candida albicans*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*, for example [43–45]. In *A. fumigatus*, mutation in the *pyrG* gene leads to uracil-uridine auxotrophy, failure to germinate without supplemental uracil-uridine, and loss of virulence in a mouse model of IA [40]. Specificity of the defect *in vivo* was shown by adding uridine to drinking water of the mice, which restored the ability to germinate and virulence. A similar pattern was shown using *A. fumigatus* mutants unable to make folate due to a defect in *p*-aminobenzoic acid synthase, encoded by *pabaA* [39,42]. In a mouse model of IA, the PABA auxotrophs were avirulent, but supplementation of the drinking water of the mice with PABA restored the virulence of the isolate. As reported for *lysF*, however, *in vivo* data using wild type conidia failed to show up-regulation of *pabaA* upon infection of mice [33]. These results may be reconciled by assuming that, although *in vivo* levels for some of these metabolites are limiting for growth and, therefore, will not support the growth of auxotrophic strains, the baseline levels of the synthetic enzymes are sufficient to do so.

Conclusions

Aspergillus fumigatus is a ubiquitous saprophytic fungus that recycles carbon and nitrogen in the competitive environmental niche of the compost pile. Although it is a grass eater, numerous growth characteristics that allow it to thrive in its ecological milieu, rapid and efficient germination and growth at elevated temperatures and responsive metabolic programming to various levels of quality and quantity in carbon and nitrogen sources, also provide the characteristics required to become an opportunistic pathogen of man. Thermotolerance and nutritional versatility, when combined with the production of many easily airborne, small conidia, set the stage for the grass eater to expand its menu.

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