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EDITORIAL

Balancing iron and calcium: Flavin carrier family proteins in *Aspergillus fumigatus* virulence

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The air-borne opportunistic fungus, *Aspergillus fumigatus*, is a critically important human pathogen and the major cause of invasive pulmonary aspergillosis (IPA).[1,2] IPA is a severe disease in immunocompromised individuals,[3] transplant recipients,[4,5] and critically ill patients with chronic obstructive pulmonary disease.[6] The noninvasive forms of lung diseases this mold can cause are aspergilloma and allergic bronchopulmonary aspergillosis especially in asthma and cystic fibrosis patients.[7,8] With the few currently available treatment options and a high mortality rate, invasive aspergillosis, is a very significant clinical problem and is largely refractory to current therapies due to the emergence of resistance.[9-12] A bottle neck to developing effective antifungal therapeutics is the lack of in-depth knowledge on regulation of *A. fumigatus* growth and pathogenesis. Mechanistic studies, especially those focusing on fungal-specific processes or proteins that impact virulence are much needed and very significant for direct targeting of the pathogen with little or no effect on the host. In recent years several investigators have focused their research in pursuit of identifying novel antifungal targets with the hope to combat this devastating disease.

External milieu plays a pivotal role in shaping the enormous biodiversity of microorganisms and their acquisition of traits for survival, proliferation and even virulence. Although the evolutionary origin of virulence in microorganisms is not clearly deciphered there are hypotheses that relate microbial pathogenic traits to the environment and the selective pressure imposed by interactions within microbes.[13,14] Depending on their growth requirements different microorganisms have evolved differently to utilize important and essential nutrients for their survival from their surrounding or host environments. In addition to macronutrients, some metal ions functioning as micronutrients are known to promote growth of a pathogen despite the host nutritional immunity by binding to different proteins/enzymes within the cell and modulating their activity.[15] In this regard metal ions such as Ca^{2+}, Zn, and Fe (Fe^{2+} and Fe^{3+}) bind to proteins that act as their store houses, binders, modulators, chelators, and transporters. Prominent examples include the Ca^{2+}-binding proteins such as calmodulin and calcineurin which mobilize Ca^{2+} within the cell and activate the Ca^{2+}-dependent signal transduction cascade of events important for various cellular aspects of growth and metabolism.[16-18] Zinc being absolutely essential for proliferation of several microbial pathogens is sequestered through specific transporters whose expression is triggered by the zinc-responsive transcription factor under Zn limitation, and alternatively in the presence of excess Zn, the metal is efficiently detoxified.[15,19] Of note the deletion of *zafA*, the Zn transcriptional regulator in *A. fumigatus* resulted in avirulence.[20] Similarly microbial acquisition of Fe is facilitated through non-reductive and reductive mechanisms including direct uptake by Fe^{3+}-chelators that are prominently known as the "siderophores," then the low affinity and high-affinity Fe^{2+}-specific transporters, and the heme-bound iron uptake.[21,22]

"Nutrient sensing" and "nutrient uptake mechanism" pathways are therefore important avenues to be explored for the identification of organism-specific targets. In an effort to identify genes that are unique to fungi compared to other organisms, Hsiang and Ballie (2005),[23] compared the yeast proteome to other available fungal genomes and found 17 yeast genes with homologs in other fungal genomes but none in other organisms. Later on, Protchenko et al (2006)[24] in trying to identify genes involved in heme uptake from *Candida albicans*

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serendipitously discovered a fungal specific flavin carrier gene family (*FLC*) required for FAD transport into the endoplasmic reticulum (ER). Interestingly, these *FLC* genes happened to be represented among the 17 core fungal genes in the study of Hsiang and Ballie (2005).[23]

In this issue de Castro and colleagues[25] for the first time characterize 3 potential members of the FLC family of proteins (FlcA, FlcB and FlcC) in *A. fumigatus* and reveal their requirement for growth, calcium and iron homeostasis, and more importantly for virulence of this pathogen. Interestingly, results from de Castro and colleagues emphasize the role of FLC family proteins in the maintenance of both calcium and iron homeostasis. The genes encoding FLC family proteins were picked from a list of candidate genes they obtained through ChIP sequence data as part of identifying the targets of the CrzA[26] (a fungal ortholog of mammalian NFAT). Crz1/CrzA is a well characterized calcineurin-dependent transcription factor activated in response to increases in $[Ca^{2+}]_c$ in *Aspergillus* species.[27,28-30] The importance of calcineurin for various cellular processes, including growth, sexual development, pathogenesis and stress-dependent regulation, has been well documented from yeasts to other fungi.[31] Regulation of gene expression in response to Ca^{2+} signaling is one of the most explored functions of calcineurin wherein it primarily dephosphorylates Crz1/CrzA and enables its translocation from the cytoplasm to the nucleus in response to stress induction and activates the transcription of several genes.[26-29,32] In this study after confirming the calcium and CrzA-dependence of *flcA* expression by quantitative PCR, the 2 other paralogs *flcB* and *flcC* were identified through BLAST searches from the *Aspergillus* genome database. Pfam analyses identified potential domains for lipid binding and FAD transport into the ER in the FlcA-C proteins with predicted ER-targeting signal sequences. The membranous nature of the FLC proteins was also confirmed bioinformatically.

By generating the 3 respective deletion strains (Δ*flcA*, Δ*flcB* and Δ*flcC*) and their respective complementation strains the roles of these proteins in growth, response to calcium inhibitors, cell wall stressors, oxidative stress and other metal ions were systematically characterized. Finally their role in virulence in a murine model was examined pointing to their importance for virulence. Extensive phenotypic analyses revealed that only the *flcA* mutant was highly restricted in growth in comparison to the *flcB* and *flcC* mutants under standard growth conditions on solid nutrient media. As previously observed with the calcineurin deletion strains,[33,34] tip-splitting or hyperbranching was clearly noted in the *flcA* mutant. While it is definitive that the *flcA* is a major player, the difference of morphology between the *flc* deletion strains to some extent may be attributable to the compensatory transcriptional upregulation of individual genes in the absence of the other as revealed by the mRNA data from the 3 mutants. Supporting this observation a double mutant of *flcB* and *flcC* could not be obtained suggesting their interdependency in function. Significant increase in the expression of *flcA* in the Δ*flcB* or Δ*flcC* was evident which probably contributes to the lesser growth defect observed in these mutants. Testing the sensitivity of these mutants with different compounds and stressors revealed the specific function of the genes under different stress conditions and metal homeostasis. In particular, the *flcA* mutant was sensitive to the Ca^{2+} chelation and Ca^{2+} supplementation improved growth indicative of its role in Ca^{2+} homeostasis. Considering the previous observation of *S. cerevisiae FLC2* (a TRP-like transporter on the ER membrane required for FAD transport) involvement in negative regulation of calcium release in response to hypotonic stress,[35] it is possible that the FLC proteins may have a role in calcium transport as well. The Δ*flcA* strain was also sensitive to other metals (Fe, Li and Mn) but could grow in the Fe-deplete media. Furthermore, measuring FAD import and accumulation into the microsomes supported their conserved role as FLC proteins. Although the FLC proteins are known to be involved in FAD import activity, their importance has been well recognized for cell wall integrity pathways in the yeast.[24] In this regard the Δ*flcA* strain was also similarly sensitive to cell wall inhibitors (Congo Red and CFW) and also oxidative stress. Although the ER targeting signal was predicted the localization of FlcA was only observed in vesicle-like structures and in the vacuoles. Interestingly, increased apical localization of FlcA was noted in germlings which indicated its function at these active points of hyphal tip growth. The observation of bipolar branching at the tips in the *flcA* mutant further strengthens this probability.

Although the growth of the *flcA* mutant was restricted in comparison to the *flcB* and *flcC* mutants, all the mutants were equally avirulent in a persistently neutropenic murine model of invasive aspergillosis. This study establishing the relevance of FLC proteins for virulence in *A. fumigatus* opens the avenue for future studies on using them as potential targets. In this regard screening for the effects of FAD transport inhibitors on growth and virulence might be interesting. A recent computational modeling of IA in the lung revealed that the iron levels in blood and lung tissue of the host play a key role in the ability of the pathogen to proliferate and cause infection.[36] Considering the fact that majority of the available iron in the mammalian host is in the heme-bound form it is possible that the *FLC* mutants are

lacking the ability to use heme as their nutritional source of iron and therefore remain avirulent. It is quite possible that the deletion of the *flc* genes causes the accumulation of misfolded proteins in the ER and activated the unfolded protein response pathway. Certainly further research to identify the exact mechanism of how these proteins regulate metal ion homeostasis and virulence is required.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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