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Regular Articles

The *Aspergillus fumigatus* septins play pleiotropic roles in septation, conidiation, and cell wall stress, but are dispensable for virulence

José M. Vargas-Muñiz [a], Hilary Renshaw [a], Amber D. Richards [b], Frédéric Lamoth [b,c,d], Erik J. Soderblom [e], M. Arthur Moseley [e], Praveen R. Juvvadi [b], William J. Steinbach [a,b,*]

[a] Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, United States
[b] Division of Pediatric Infectious Diseases, Department of Pediatrics, Duke University Medical Center, Durham, NC, United States
[c] Service of Infectious Diseases, Department of Medicine, Lausanne University Hospital, Lausanne, Switzerland
[d] Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland
[e] Duke Proteomics and Metabolomics Core Facility, Center for Genomic and Computational Biology, Duke University, Durham, NC, United States

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ABSTRACT

Septins are a conserved family of GTPases that regulate important cellular processes such as cell wall integrity, and septation in fungi. The requirement of septins for virulence has been demonstrated in the human pathogenic yeasts *Candida albicans* and *Cryptococcus neoformans*, as well as the plant pathogen *Magnaporthe oryzae*. *Aspergillus* spp. contains five genes encoding for septins (*aspA–E*). While the importance of septins AspA, AspB, AspC, and AspE for growth and conidiation has been elucidated in the filamentous fungal model *Aspergillus nidulans*, nothing is known on the role of septins in growth and virulence in the human pathogen *Aspergillus fumigatus*. Here we deleted all five *A. fumigatus* septins, and generated certain double and triple septin deletion strains. Phenotypic analyses revealed that while all the septins are dispensable in normal growth conditions, AspA, AspB, AspC and AspE are required for regular septation. Furthermore, deletion of only the core septin genes significantly reduced conidiation. Concomitant with the absence of an electron-dense outer conidial wall, the *ΔaspB* strain was also sensitive to anti-cell wall agents. Infection with the *ΔaspB* strain in a *Galleria mellonella* model of invasive aspergillosis showed hypervirulence, but no virulence difference was noted when compared to the wild-type strain in a murine model of invasive aspergillosis. Although the deletion of *aspB* resulted in increased release of TNF-α from the macrophages, no significant inflammation differences in lung histology was noted between the *ΔaspB* strain and the wild-type strain. Taken together, these results point to the importance of septins in *A. fumigatus* growth, but not virulence in a murine model.

1. Introduction

Septins are a conserved family of GTPases that form heteropolymeric complexes (Gladfelter, 2010) and function in a myriad of cellular processes, including cell division, cytoskeleton organization, vesicle trafficking, and cell wall maintenance (Alvarez-Tabares and Perez-Martin, 2010; Hernandez-Rodriguez et al., 2012; Kozubowski and Heitman, 2010; Li et al., 2012; Lindsey et al., 2010a,b; Momany et al., 2001). The number of septin-encoding genes varies greatly between organisms, from 2 in the nematode *Caenorhabditis elegans* to 13 in humans (Lindsey and Momany, 2006). *Candida albicans* contains 7 septin encoding genes, all of which have orthologs in the model yeast *Saccharomyces cerevisiae* (Warenda and Konopka, 2002). *C. albicans* septins CDC3 and CDC12 are essential for hyphal growth, while CDC10 and CDC11 are not; nonetheless, deletion of CDC10 and CDC11 yields aberrant hyphal morphology (Warenda and Konopka, 2002) and results in defective agar invasion and attenuation of virulence in a murine model (Warenda et al., 2003). Additionally, *C. albicans* CDC3, CDC10, CDCD11 and CDC12 have been implicated in cell wall regulation and response to the anti-cell wall antifungal caspofungin (Badrane et al., 2012; Blankenship et al., 2014, 2010). In *Cryptococcus neoformans*, septins CDC3, CDC11, and CDC12 are necessary for growth at 37 °C and proper localization of the other septins at the mother-bud neck, and deletion mutants exhibit cell wall

* Corresponding author at: Division of Pediatric Infectious Diseases, 427 Jones Building, Research Drive, Duke University Medical Center, Durham, NC 27710, United States. Tel.: +1 (919) 681 1504; fax: +1 (919) 668 4859.
E-mail address: bill.steinbach@duke.edu (W.J. Steinbach).

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defects (Kozubowski and Heitman, 2010). The *C. neoformans cdc3Δ* and *cdc12Δ* strains are capable of hyphal growth; however, they exhibit aberrant dikaryotic hyphal morphology and nuclear distribution and the *cdc3Δ* strain fails to form long spore chains in the basidia. The *cdc3Δ*, *cdc12Δ*, and *cdc3Δcdc12Δ* strains were also less virulent in the heterologous host *Galleria mellonella* at 24 °C, indicating that *C. neoformans* septins contribute to virulence through a mechanism that is independent of high temperature growth (Kozubowski and Heitman, 2010).

The role of septins in morphogenesis and growth has also been explored in the model filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*. Deletion of *N. crassa* core septin genes *cdc-3*, *cdc-10*, *cdc-11* and *cdc-12* exhibited slower hyphal growth, defective conidiation, hyperbranching, and a reduction in the number of septa (Berepiki and Read, 2013). *A. nidulans* contains 5 septin genes: *aspA*, *aspB*, *aspC*, *aspD* and *aspE*. While *aspA*, *aspB*, *aspC* and *aspD* are orthologs of *S. cerevisiae* core septins *CDC11*, *CDC3*, *CDC12* and *CDC10*, respectively; *aspE* is absent in *S. cerevisiae* (Juvvadi et al., 2011b; Lindsey et al., 2010a; Pan et al., 2007). Deletion of *A. nidulans* core septins *aspA* or *aspC* resulted in early emergence of germ tubes, an increase in the number of germ tubes and branches, and a reduction in septation and conidiation (Lindsey et al., 2010a). Heterologous expression of *A. nidulans aspC* in *S. cerevisiae* induced anomalous pseudohyphal growth, suggesting that *Aspergillus* septins are a key component for hyphal growth and morphology (Lindsey et al., 2010a,b). Deletion of *aspB* in *A. nidulans* led to reduced conidiation, uninucleate or binucleate conidia, delayed septation, increased branching, and aberrant nuclear distribution (Hernandez-Rodriguez et al., 2012). Deletion of the non-core septin *aspE* resulted in reduced conidiation, delayed growth at 18 °C, and increased susceptibility to osmotic stress (Hernandez-Rodriguez et al., 2014). These septin deletion phenotypes in *A. nidulans* suggest their role in suppressing new growth foci in addition to their role in conidiophore development and organization.

While the deletion of the core septin genes of the filamentous fungal plant pathogen *Magnaporthe oryzae* led to aberrancies in germ tube and appresorium development, the deletion of *sep3*, an ortholog of AspB, completely abolished pathogenicity (Dagdas et al., 2012). However, in another phytopathogenic fungus, *Ustilago maydis*, despite the morphological and polarity defects noted, the septin deletion strains were still virulent, indicating probable functional diversity of septins among different fungi (Alvarez-Tabares and Perez-Martin, 2010). Apart from these reports of filamentous fungal core septins involved in morphogenesis and plant pathogenesis, no study has examined their role in a filamentous fungal human pathogen.

The *Aspergillus fumigatus* genome also contains 5 septin genes that are orthologous to the *A. nidulans* septins (Juvvadi et al., 2011b). In this study, we explored the role of AspA, AspB, AspC, AspD, and AspE in the hyphal morphology, septation, cell wall integrity, and virulence of *A. fumigatus* by generating single, double, and triple septin deletions of the *aspA*, *aspB*, *aspC*, *aspD*, and *aspE* genes. While the deletion of *aspA* affected septation and conidiation, and *aspB* or *aspC* deletion resulted in defects in septation, cell wall stress, and conidiation, the deletion of *aspD* exclusively resulted in defective conidiation, and the deletion of *aspE* only impacted septation but not conidiation. Concomitant to the pleiotropic effects observed with *aspB* deletion, aberrant conidial walls lacking the electron dense layer were also noted, implicating the importance of this core septin for the maintenance of the conidial cell wall. Interestingly, the *ΔaspB* strain exhibited hypervirulence in a *G. mellonella* model of invasive aspergillosis and increased TNF-α release from *ex vivo* macrophages, but was equally as virulent as the wild-type strain in a neutropenic murine model of invasive aspergillosis.

2. Materials and methods

2.1. Strains, media, and culture conditions

The *A. fumigatus akuB*^{KU80} *pyrG*$^{-}$ uracil/uridine auxotroph was used for deletion analyses as well as served as the control strain. Cultures were grown on glucose minimal media (GMM) supplemented with 5 mM uracil and 5 mM uridine (GMM + UU) at 37 °C, except where otherwise specified. For some experiments, GMM + UU supplemented with 1.2 M sorbitol was used. *Escherichia coli* DH5α competent cells were used for cloning.

2.2. Construction of septin deletion strains

Deletion of *aspA* was obtained by replacing the 1.6 kb *aspA* gene (Afu5g08540; www.aspergillusgenome.org) with the 2.4 kb *pyrG* gene from *Aspergillus parasiticus*. Approximately 1 kb of upstream and downstream flanking regions of *aspA* were PCR-amplified from AF293 genomic DNA. *pyrG* was amplified from the pJW24 plasmid. The *aspA* deletion construct was generated by fusion PCR and transformed into *akuB*^{KU80} *pyrG*$^{-}$ strain, all as previously described (Steinbach et al., 2006). Transformants were selected for growth in the absence of uracil/uridine. Deletion of *aspB* was achieved by replacing the 1.8 kb *aspB* gene (Afu7g05370; www.aspergillusgenome.org) with the 2.3 kb phleomycin resistance gene (*ble*). Approximately 1 kb of upstream and downstream flanking regions of *aspB* were PCR-amplified from AF293 genomic DNA and cloned into pUCnGPhleo (Juvvadi et al., 2011a). The resulting plasmid was digested with KpnI and NdeI, transformed into *akuB*^{KU80}, and transformants selected in the presence of phleomycin. Deletion of *aspC* was performed by replacing the 1.5 kb *aspC* gene (Afu5g03080; www.aspergillusgenome.org) with the 4.4 kb hygromycin B resistance (*hph*) cassette. Approximately 1 kb of upstream and downstream regions of *aspC* were PCR-amplified from AF293 genomic DNA and cloned into pUCGH (Langfelder et al., 2001). The resulting plasmid was digested with KpnI and HindIII and transformed into *akuB*^{KU80}. Transformants were selected for growth in presence of hygromycin B. Deletion of *aspD* was attained by replacing the 1.3 kb *aspD* gene (Afu1g08850; www.aspergillusgenome.org) with the 4.4 kb hygromycin B resistance (*hph*) cassette. Approximately 1 kb of upstream and 500 bp of the downstream regions of *aspD* were PCR-amplified from AF293 genomic DNA and cloned into pUCGH (Langfelder et al., 2001). The resulting plasmid was digested with KpnI and HindIII and transformed into *akuB*^{KU80} *pyrG*$^{-}$. Transformants were selected for growth in presence of hygromycin B. Deletion of *aspE* was performed by replacing the 1.7 kb *aspE* gene (Afu3g07015; www.aspergillusgenome.org) with the 2.4 kb *pyrG* gene from *A. parasiticus*. Approximately 1 kb of upstream and downstream flanking regions of *aspE* were PCR-amplified from AF293 genomic DNA. pJW24 was used as a template for amplification of the *A. parasiticus pyrG* gene. The *aspE* deletion construct was generated by fusion PCR and transformation was performed into *akuB*^{KU80} *pyrG*$^{-}$ strain. Transformants were selected for growth in the absence of uracil/uridine. The *ΔaspEΔaspB* strain was obtained by replacing the 1.8 kb *aspB* gene with the *hph* cassette in the *ΔaspE* background. The *ΔaspEΔaspD* strain was obtained by replacing 1.3 kb *aspD* gene with the *hph* cassette, as described above, in the *ΔaspE* background. Approximately 1 kb of upstream and downstream region of *aspB* was PCR-amplified from AF293 genomic DNA and cloned into pUCGH. The resulting plasmid was digested with KpnI and HindIII, transformed into the *ΔaspE* strain, and transformants were selected in the presence of hygromycin B. The *ΔaspEΔaspDΔaspB* triple deletion strain was obtained by replacing the 1.8 kb *aspB* gene with the 2.3 kb *ble* cassette, as