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The *Aspergillus fumigatus* Septins Play Pleiotropic Roles in Septation, Conidiation, and Cell Wall Stress, but are Dispensable for Virulence

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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 *aspB* strain was also sensitive to anti-cell wall agents. Infection with the *aspB* 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 *aspB* 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.

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Keywords

Aspergillus fumigatus; septins; septation; conidiation; cell wall; virulence

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; 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, CDC11 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; Blankenship et al., 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 defects (Kozubowski and Heitman, 2010). The *C. neoformans* *cdc3* and *cdc12* strains are capable of hyphal growth; however, they exhibit aberrant dikaryotic hyphal morphology and nuclear distribution and the *cdc3* strain fails to form long spore chains in the basidia. The *cdc3*, *cdc12*, and *cdc3 cdc12* 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., 2010l). 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 appressorium 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 *A. 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 pleiotrophic 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 *aspB* strain exhibited hypervirulence in a *Galleria 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* DH5 α 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 *aspE aspB* strain was obtained by replacing the 1.8 kb *aspB* gene with the *hph* cassette in the *aspE* background. The *aspE aspD* strain was obtained by replacing 1.3 kb *aspD* gene with the *hph* cassette, as described above, in the *aspE* 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 *aspE* strain, and transformants were selected in the presence of hygromycin B. The *aspE aspD aspB* triple deletion strain was obtained by replacing the 1.8 kb *aspB* gene with the 2.3 kb *ble* cassette, as described above, in the *aspE aspD* background. All the respective gene deletions were confirmed by both PCR (data not shown) and Southern analyses (Fig. S1, Fig. S2). The *aspB::aspB* complemented strain was obtained by replacing the deletion cassette present in *aspB* native locus with the *aspB* gene, using hygromycin B resistance as a marker. Approximately 1 kb of upstream region of *aspB*, 1.8 kb *aspB* gene and 1 kb of downstream region of *aspB* was PCR-amplified from AF293 genomic DNA and cloned into pUCGH. The resulting plasmid was digested with KpnI and XmaI, transformed into the *aspB* strain, and transformants were selected in presence of hygromycin B. Finally, the *aspB-egfp* strain was generated by cloning 1 kb of the *aspB* gene and terminator sequence into the pUCGH vector. The resulting plasmid was digested with KpnI and XbaI and transformed into the *akuB*^{KU80} strain.

2.3. Radial growth and conidial quantification

Conidia (10^4) from each strain were inoculated on GMM+UU agar, incubated at 37°C, and radial growth was measured every 24 h for 5 days. For conidial quantification, 10^4 conidia from each strain were inoculated on GMM+UU agar, incubated for 5 days at 37°C, and then conidia were harvested in 10 ml of 0.05% Tween-80 and quantified using a hemocytometer, as previously described (Lamoth et al., 2012). All assays were performed in triplicate. Statistical analyses were performed using Graph Pad Prism (San Diego, CA).

2.4 Scanning electron microscopy and transmission electron microscopy

For scanning electron microscopy, conidia were harvested from strains grown on GMM agar for 5 days and fixed with osmium tetroxide for 72 h. Fixed conidia were sputter coated with gold and visualized using an accelerating voltage of 7 kV on a FEI XL30 SEM-FEG. For transmission electron microscopy, conidia were harvested from strains grown on GMM agar for 5 days and then fixed with 4% formaldehyde and 2% glutaraldehyde in 1x PBS for 24 h at 4°C. Samples were washed twice with PBS for 10 min and then stained for an hour with osmium tetroxide. Conidia were washed twice with PBS and once with 0.1N acetate buffer for 10 min. Then, conidia were stained with 0.5% uranyl acetate for an hour. After staining, conidia were washed twice using 0.1N acetate buffer for 10 min each. Samples were dehydrated using serial washes of 30%, 50%, 70%, 90% and 100% ethanol twice for 10 min at each concentration. Samples were embedded using the SPURR Low Viscosity Embedding Kit. Samples were then thin sectioned and stained using 1% uranyl acetate and 0.4% lead citrate. Conidia were visualized using an accelerating voltage of 80 kV on a FEI Tecnai G² Twin.

2.5. Antifungal susceptibility testing

To determine the effect of antifungal agents on the septin deletion strains, 10^4 conidia from these strains and the *akuB*^{KU80} control strain were inoculated on GMM+UU agar supplemented with either caspofungin (1 or 4 µg/ml) or nikkomycin Z (2 µg/ml) and growth visualized after 3 days of incubation at 37°C. Additionally, 10^4 conidia of each strain were inoculated on GMM+UU agar containing 1 and 4 µg/ml of caspofungin to observe the caspofungin paradoxical effect after 5 days of incubation at 37°C. For liquid culture, growth was monitored after 48 h of incubation at 37°C. RPMI media was used to determine the minimal effective concentration (MEC) of the anti-cell wall agents on the strains (Fortwendel et al., 2009).

2.6 Fluorescence Microscopy

Conidia (10^4) of the AspB-EGFP strain were cultured on coverslips immersed in 10 ml of GMM+UU broth and incubated for 20 h at 37°C, as previously described (Juvvadi et al., 2011b). To examine localization after exposure to anti-cell wall agents, caspofungin (1 µg/ml) and nikkomycin Z (2 µg/ml) were added after 18 h of incubation and samples were imaged after 2 h following drug co-incubation at 37°C. Localization patterns were visualized using an Axioskop 2 plus microscope (Zeiss) equipped with AxioVision 4.6 imaging software.

2.7. Protein extraction, AspB-EGFP fusion protein purification and LC-MS/MS Analysis

The *A. fumigatus* strain expressing the *aspB-egfp* fusion construct under the control of the *aspB* native promoter was grown in GMM liquid for 24 h at 37°C. Total cell lysate was obtained by homogenizing the fungal mycelia (600-650 mg wet weight) as previously described (Juvvadi et al., 2013). Total protein from the crude extract were quantified by the Bradford method and normalized to contain ~10 mg of protein in the sample before purification using the GFP-Trap[®] affinity purification (Chromotek). GFP-Trap[®] resin was equilibrated according to the manufacturer instruction and then resuspended in 100 µl ice-cold dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF, 1:100 protease inhibitor cocktail). GFP-Trap[®] resin suspension was then added to the crude lysate containing the ~10 mg of protein and incubated for 2 h at 4°C with gentle agitation. The suspension was centrifuged at 2000 rpm for 10 min at 4°C and the GFP-Trap[®] pellet was washed once with 500 µl of iced-cold dilution buffer and twice with 500 µl of wash buffer (10 mM Tris-HCl pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 1mM PMSF, 1:100 protease inhibitor cocktail). The resin was finally washed 3 times with 200 µl of 50 mM ammonium bicarbonate and resuspended in 50 µl of 50 mM ammonium bicarbonate.

Resin-bound proteins then were reduced with 5 mM dithiothreitol for 30 min at 37°C and then alkylated with 12 mM iodoacetamide for 45 min at room temperature. Trypsin digestion was allowed to proceed on-resin for 18 h at 37°C. Samples were then subjected to a 90 min chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C₁₈ 75 µm I.D. X 250 mm reversed-phase column as previously described (Juvvadi et al., 2013). The analytical column was coupled to a Thermo QExactive Plus high-resolution mass spectrometer through an electrospray ionization source. The instrument was operated in a data-dependent mode of acquisition with a precursor MS scan from m/z 375-1600 at r=70,000 followed by ten MS/MS spectra at r=15,000 using a 26% CID energy setting.

Mass spectra were processed with Mascot Distiller (Matrix Science) and were then submitted to Mascot searches (Matrix Science) against an NCBI_RefSeq_Aspgillus database at 5 ppm precursor and 0.02 Da product ion mass tolerances. Static mass modifications corresponding to carbamidomethylation on Cys residues and dynamic mass modifications corresponding to oxidation on Met residues and deamidation on Asn and Gln residues were included. Searched spectra were imported into Scaffold v4.3 (Proteome Software) and scoring thresholds were set to yield a 0.1% protein false discovery rate. A minimum of two unique peptides from each protein was required for identification.

2.8. Aniline blue, calcofluor white and propidium iodide staining

Aniline blue stain was used for detection of cell wall β-glucan. 10⁴ conidia of each strain were cultured on coverslips immersed in 10 ml of GMM+UU broth and incubated for 20 h at 37°C, as previously described (Juvvadi et al., 2011a). The coverslips were rinsed with GMM+UU broth, inverted over a 500 µl drop of aniline blue stain, and incubated for 5 min at 25°C. The coverslips were rinsed once more briefly with GMM+UU broth and observed by fluorescence microscopy.

Calcofluor white and propidium iodide was utilized to visualize chitin distribution and nuclei, respectively. Cultures on coverslips were washed with 50 mM PIPES (pH 6.7) for 5 min and fixed in 8% formaldehyde and 0.2% Triton X-100 for 40 min at 25°C. The coverslips were then washed twice for 10 min in 50 mM PIPES (pH 6.7) at 25°C, and then treated with 100 µg/ml RNase for 60 min at 37°C. Fixed samples were co-stained with 12.5 µg/ml of propidium iodide and 1 µg/ml calcofluor white in 500 µl of 50 mM PIPES (pH 6.7) for 5 min and observed under the fluorescent microscope.

2.9. Germling Septation assay

Conidia (10^5) of each strain were cultured on coverslips immersed in 600 µl of GMM+UU for 12 and 15 hours. The coverslips were rinsed once briefly with GMM+UU broth and the septation status of the germlings was quantified using an Axioskop 2 plus microscope (Zeiss). A chi-square test was used to determine if there was a difference in proportions between any of the groups. As the overall test was significant, to determine where the difference occurred between the groups, and avoid finding statistical significance by chance due to performing multiple comparisons, the marascuilo procedure was used. A p-value of 0.05 was used to determine significance.

2.10. Hypotonic shock

Conidia (0.5 µl of 10^4 spores/ml) were inoculated on a thin layer of yeast peptone dextrose agar (YPD) medium and incubated at 37°C for 18 h. Then 500 µl of distilled water was added and samples were incubated at room temperature for 1 h. Hyphal tip bursting was visualized using a Nikon Diaphot Phase Contrast microscope. Approximately 50 hyphal tips were counted in each strain.

2.11. *Galleria mellonella* and murine invasive aspergillosis virulence assays

Virulence of the septin deletion strains was assessed in an iterative fashion using an invertebrate and then a murine model of invasive aspergillosis. For the initial invertebrate model, we infected 20 larvae of the wax moth *Galleria mellonella* with each of the *A. fumigatus* septin deletion strains or the wild-type strain, delivering 5 µl of a 1×10^8 conidia/ml of suspension with a total inoculum size of 2×10^5 conidia. Infected larvae were incubated at 37°C and survival scored daily for 5 days (Steinbach et al., 2006). For the murine model, we utilized an intranasal delivery method (due to conidiation defects and difficulty in obtaining substantial amounts of conidia) with our standard persistent immunosuppressive protocol (Steinbach et al., 2004). Male mice (CD1, Charles River Laboratories, Raleigh, NC) were immunosuppressed with cyclophosphamide (175 mg/kg intraperitoneally on day -2 and +3) and triamcinolone acetate (40 mg/kg subcutaneously on days -1 and +6). Intranasal inoculations were performed with 40 µl of 10^8 conidia of the *aspB*, *aspB::aspB*, or the *akuB*^{KU80} wild-type strains into 10 mice per strain with a total inoculum of 4×10^6 conidia (Steinbach et al., 2006). Survival for the invertebrate and murine models was plotted on a Kaplan-Meier curve with log rank pair-wise comparison. Vertebrate animal experiments were conducted in compliance with the Animal Care and Use Program of the Duke University Medical Center.

2.12. Bone marrow-derived macrophages isolation and TNF- α release assay

Bone marrow-derived macrophages (BMDM) were isolated from cleaned female C57BL/6 mice femurs and tibias. Bone marrow was flushed using 5 ml of PBS and centrifuged at 4000 RPM (5 min at 4°C). Contaminating red blood cells (RBC) were lysed using 1 ml of lysis buffer (0.15 M NH₄Cl, 1mM NaCO₃, pH 7.4). Remaining bone marrow cells were washed with 5 ml of DMEM containing 10% FBS. Cells were resuspended at a concentration of 100,000 cells/ml in macrophage medium (DMEM, 10% FBS, penicillin/streptomycin, 3 ng/ml GM-CSF) and plated into a tissue culture flask. On day 3, media was changed and macrophages were harvested on day 7 of culture.

For the ELISA, 62,500 BMDM were co-incubated with 250,000 conidia from each strain (multiplicity of infection [MOI] of 40) for a period of 6 h at 37°C. Cell culture supernatants were obtained and diluted 1:10. TNF- α released was measured using the Mouse TNF- α ELISA MAX Standard kit (BioLegend) following the manufacturer's instructions.

2.13. Histopathology analyses

To characterize *in vivo* histopathologic disease, three additional mice per each strain were infected as described in *section 2.11*. Mice were euthanized on day +3 after infection and lungs were harvested. Lungs were stained with Gomori's methenamine silver stain to characterize fungal invasion and hematoxylin and eosin stain to evaluate inflammation (Steinbach et al., 2006).

3. Results

3.1 Septins AspA, AspB, AspC and AspE coordinate interseptal distances in the apical and subapical hyphal compartments

Recent work on the non-core septin, AspE, expressed under the control of its native promoter in *A. nidulans*, revealed that the localization of AspE requires the three core septins, AspA, AspB, and AspC. However, the interaction of AspE with only one core septin (AspB) was noted during multicellular developmental stage (Hernandez-Rodriguez et al., 2014). Our previous proteomic interaction analysis of *A. fumigatus* AspE, expressed using a constitutively active promoter, revealed its interaction with all the core septins, including AspB and AspD as the major interactors (Juvvadi et al., 2013). Based on these interaction studies, we performed the deletion of all the septins and also generated the double, and triple deletions of *aspE*, *aspB* and *aspD* to understand the coordinated role between the core and non-core septins in *A. fumigatus* growth and development. All the deletion strains showed comparable radial growth to the *akuB*^{KU80} wild-type strain under basal conditions (Fig. 1A). Only deletion of *aspB* resulted in a modest 8-12% radial growth reduction (73-76 mm diameter compared to the 83 mm diameter of the *akuB*^{KU80} strain) that is statistically significant; nonetheless, this reduction in hyphal extension is likely not biologically relevant. These results suggest that septin genes are dispensable for *A. fumigatus* growth under normal conditions.

Although aniline blue staining of the septa in the respective deletion strains did not reveal significant morphological variations (data not shown) in comparison to the wild-type strain,

the *aspA*, *aspB*, *aspC*, and *aspE* strains exhibited significantly extended apical and subapical compartments (Fig. 1B, Fig. S3A). While the *aspA* strain showed a 3.5-fold ($p<0.001$) increase in the length of apical compartment, the apical compartments of the *aspB*, *aspC*, and *aspE* strains showed a 2.3-fold ($p<0.001$) increase in comparison to the wild-type strain. The *aspD* strain had a slight increase of 1.3 fold ($p=0.0025$) in the apical compartment length. The *aspE aspD* double deletion strain displayed a 2.1-fold increase ($p<0.001$) in apical compartment length compared to the wild-type strain, similar to that of the *aspE* strain. The *aspE aspB* and the *aspE aspD aspB* strains exhibited larger apical compartments than the single and other double deletion strains, with a more than 2.9-fold ($p<0.001$) increase in length compared to the wild-type strain. Furthermore, the apical compartments of the *aspE aspB* double deletion strain ($p=0.002$) and *aspE aspD aspB* triple deletion strain ($p=0.02$) were approximately 20% larger than the apical compartment of the *aspE* and *aspB* single deletion strains. The *aspA*, *aspB*, *aspC*, *aspE*, *aspE aspD*, *aspE aspB* and *aspE aspD aspB* strains, but not the *aspD* strain, exhibited a significant increase in inter-septal distances of the subapical compartments, with approximately a 3.3-, 2.6-, 4.2-, 1.9-, 4-, 2-, and 2.6-fold increase ($p<0.001$), respectively. In the *aspB::aspB* complemented strain apical and sub-apical compartment were similar to those of the wild-type strain. The hyphal width of the septin deletion strains ($3.56\text{ }\mu\text{m}$) were comparable to those of the wild-type strain ($3.12\text{ }\mu\text{m}$) ($p>0.05$). These results collectively suggest that septins AspA, AspB, AspC and AspE contribute to the formation of the apical and sub-apical septa. In order to determine if septation was in fact delayed, we quantified the number of septated germlings at 12 and 15 hours of incubation (Fig. 1C). After 12 hours of incubation, 65% of the *akuB*^{KU80} strain germlings had a visible septum while the only 10-30% of the septin deletion strains germlings examined had a visible septa ($p=0.0002$). After 15 hours of incubation, all of the *akuB*^{KU80} germlings had visible septum, in contrast only 50-65% septin deletion germlings with a visible septum ($p=0.0042$). Additionally, quantification of nuclei at the apical compartment revealed that *aspB*, *aspA* and *aspE aspD aspB* strains contain more nuclei per compartment compared to the *akuB*^{KU80} strain ($p<0.001$) (Fig. S3B). These results suggest that septation is delayed in the septin deletion strains, resulting in an increase in interseptal distances of both the apical and subapical compartments.

3.2. Deletion of core septins, but not *aspE*, reduced conidiation in *A. fumigatus*

In *A. nidulans*, it has been shown that AspA, AspB, and AspC are involved in conidiophore morphogenesis. Strains lacking these core septins have anomalous conidiophore organization, resulting in reduced conidial production (Hernandez-Rodriguez et al., 2012; Lindsey et al., 2010a). Recently, AspE in *A. nidulans* was also shown to be required for conidiation, as the *aspE* strain revealed a 8-50 fold reduction in conidiation under different growth conditions in comparison to the wild-type strain (Hernandez-Rodriguez et al., 2014). Conidial quantification in the *aspA*, *aspB*, *aspC*, *aspD*, *aspE aspD*, *aspE aspB* and *aspE aspD aspB* septin deletion strains showed an average of a 8.4-fold reduction in conidiation compared to the wild-type strain ($p<0.001$) (Fig. 1D). Interestingly, only in the *aspE* strain was conidial production similar to the wild-type strain ($p=0.552$), implicating the requirement of the core septins, but not AspE, for conidiation. Our previous results on localization patterns of *A. fumigatus* septins revealed the localization of septins AspB, AspC

and AspD at the junction of the vesicle and phialides during conidiophore development, and AspA localized at the cytosol of the conidiophore (Juvvadi et al., 2011b). However, we did not observe such a localization pattern for AspE, further supporting our present findings.

Next, in order to distinguish any variation in conidial morphology, scanning electron microscopy of the conidia was performed for the *aspB*, *aspB::aspB*, and wild-type strains (Fig. S4). As shown at 10,000x magnification, the *aspB* conidia remained clumped together, in contrast to the more dispersed conidia seen in both the wild-type and the *aspB* complemented strains. Additionally, in comparison to the wild-type conidia, *aspB* conidia were more heterogeneous in morphology and in appearance of the rodlet layer on the conidial surface. To more clearly visualize the conidial wall surface, transmission electron microscopy was performed. As shown in Fig. 2, in contrast to the wild-type strain, the *aspB* conidial cell wall lacked the electron dense outer layer (indicated by a black arrow) and had a more translucent inner layer (indicated by a white arrow). The outer electron dense layer was restored in the *aspB* complemented strain, indicating that the septin AspB may have a role in maintenance of the conidial cell wall architecture. Moreover, the clumping of conidia observed in scanning electron microscopy may be due to the extracellular release of the cell wall material between the *aspB* conidia.

3.3. Deletion of *aspB* and *aspC* causes hypersensitivity to anti-cell wall agents

The noted increase in the apical and subapical hyphal compartments of the septin deletion strains and the aberrant conidial cell wall architecture observed in the *aspB* strain may be indirectly attributed to defects in cell wall biosynthesis. In order to test this hypothesis, the respective septin deletion strains were treated with cell wall perturbing agents. All of the septin deletion strains were more susceptible to the cell wall perturbing agent Congo Red (100 µg/ml) (Fig. S5). In order to further characterize this susceptibility to cell wall perturbing agents, we treated the strains with the β-glucan synthase inhibitor caspofungin (1 µg/ml) and the chitin synthase inhibitor nikkomycin Z (2 µg/ml). Deletion of *aspB* or *aspC* genes resulted in hypersensitivity to caspofungin (Fig. 3). Another β-glucan synthase inhibitor, micafungin, yielded similar results (data not shown). As this hypersensitivity phenotype could be due to an increase in susceptibility to osmotic stress, we verified if supplementation with the osmotic stabilizer sorbitol (1M) remediated caspofungin hypersensitivity when the strains were grown in presence of sorbitol and caspofungin (1 µg/ml). However, sorbitol supplementation did not remediate the hypersensitivity of the *aspB*, *aspC*, *aspE aspB* and *aspE aspD aspB* septin deletion strains to caspofungin (data not shown). Minimum effective concentration (MEC) of caspofungin for the septin deletion strains (*aspB*, *aspC*, *aspE aspB*, and *aspE aspD aspB*) was determined to be 0.5 µg/ml, slightly lower in comparison to the wild-type strain (MEC 1.0 µg/ml). In addition, all the septin deletion strains except for *aspE* exhibited a moderate increase in susceptibility to the chitin synthase inhibitor, nikkomycin Z (Fig. 3), with the greatest effect seen with strains harboring the *aspB* and *aspC* deletion. To confirm this cell wall defect phenotype, the *aspB*, *aspC*, *aspE aspB*, and *aspE aspD aspB* strains were also cultured in the presence of calcofluor white (5 and 10 µg) and these strains exhibited increased susceptibility to this cell wall stress (Fig. S5).

Due to the observed aberrant cell wall architecture of the conidia in the *aspB* strain and hypersensitivity of the *aspB*, *aspE aspB* and *aspE aspD aspB* strains to both glucan- and chitin-specific anti-cell wall agents, we hypothesized that the deletion of septin genes may cause a reduction in the major components of the cell wall. To verify this, the β -1,3-glucan content of the septin deletion strains was measured (data not shown). Although the septin deletion strains showed greater susceptibility to caspofungin, we did not observe significant alterations in the total β -1,3-glucan content under basal conditions. However, in the presence of caspofungin, we observed a slight increase in β -glucan content in the septin deletion strains compared to the expected and well-defined reduction in the wild-type strain following caspofungin treatment (data not shown) (Kahn et al., 2006). We also tested the strength of the cell wall by exposing the hyphae to hypotonic shock and quantifying hyphal tip bursting in the *aspA*, *aspE aspD aspB*, and wild-type strains. No significant increase in hyphal tip bursting was observed, but an increase in the amount of cytoplasm leakage was noted in the *aspA* and *aspE aspD aspB* strain compared to wild-type strain (Fig. S6). This may be attributed to the extended apical compartment of the *aspA* and *aspE aspD aspB* strains.

3.4. Septin AspB exhibits altered localization patterns after exposure to caspofungin and nikkomycin Z

In *C. albicans* it has been shown that septins redistributed after exposure to caspofungin, affecting chitin distribution (Badrane et al., 2012). To determine if exposure to anti-cell wall drugs alters *A. fumigatus* septin localization, we generated a strain that expressed the AspB-EGFP fusion protein under the control of the *aspB* native promoter. Under normal growth conditions, we observed similar localization patterns as previously described (Juvvadi et al., 2011c). AspB localized transiently at the septum, and a similar localization pattern (septal-like) was observed in regions that did not contain a visible septum, which may be preparing the future septation site. Ring-like structures, double bars of different lengths, and filaments were also observed (Fig. 4). After 2 hours of exposure to caspofungin, AspB localization was altered. Although septal, septal-like, and ring-like structures persisted in this stress condition, ring-like structures were frequently found as clumps, and two novel localization patterns (X-like and ring & bar) were observed. After 2 hours of exposure to nikkomycin Z, AspB rarely localized at the septum; however, septal-like localization was predominant. We observed similar localization patterns to those observed under normal growth condition, with the addition of X-like and ring & bar structures. We noted that there was an increase in the frequency of structures after exposure to caspofungin, and quantified the different structures in 20 hyphae apical regions. We observed an average of 1-2 structures per hyphae under normal growth condition and after exposure to nikkomycin Z, while exposure to caspofungin lead to an average of 7 structures per hyphae (Table 2). One caveat of this approach is that we only quantified structures observed in the apical region instead of more basal areas, and because of this we likely missed some structures such as the ring & bar or X-like structures in the nikkomycin Z exposure condition.

3.5. Proteomic analysis revealed that AspB interacts with all of the other septins

In order to understand the overlapping phenotypes obtained with regard to conidiation in the background of *aspB* and *aspD* deletion and the septation defect in the *aspB* and *aspE*

deletion background, the AspB protein was purified by expressing the *aspB-egfp* construct under the control of its native promoter. The GFP-Trap[®] affinity purified AspB-bound protein complex was subjected to LC-MS/MS analysis to identify interactions between the septins. As summarized in Table 3, all of the core septins, including the non-core septin AspE, co-purified with AspB. Earlier proteomic analysis, performed by utilizing a constitutively expressed AspE, also demonstrated the interaction of this non-core septin with all of the core septins, including AspB (Juvvadi et al., 2013). Based on the number of peptides bound to AspB, septins AspC, AspD and AspA may be strong interactors of AspB. Nevertheless, it is interesting to note that septins AspB and AspD play a concerted role in conidiation, while AspB and AspE play overlapping roles in septation. While in *A. nidulans* the interaction of AspB with AspE was previously confirmed at the multicellular stage (Hernandez-Rodriguez et al., 2014), the phenotypes of the *A. nidulans aspE* deletion differ from the *A. fumigatus aspE* deletion, indicating an important point of divergence between the closely related species and how septins may function differently.

3.6. Deletion of *aspA*, *aspB* or *aspC* results in hypervirulence in *Galleria mellonella*, but not in the intranasal murine model of invasive aspergillosis

In *C. albicans*, deletion of septins CDC10 and CDC11 impaired hyphal invasion and reduced virulence (Warenda et al., 2003). Deletion of the *S. cerevisiae* CDC3 ortholog, *sep3*, in the plant fungal pathogen *Magnaporthe oryzae* also resulted in reduced tissue invasion and avirulence (Dagdas et al., 2012). Considering the phenotypic alterations noted with the deletion of *A. fumigatus aspB*, which is orthologous to the CDC3 and *sep3* genes, each of our three *aspB* deleted strains were examined for virulence in the heterologous invertebrate host *Galleria mellonella*. Surprisingly, the *aspA*, *aspB*, *aspC*, *aspE aspB* and *aspE aspD aspB* strains all showed significantly greater mortality (80-90%) by day +2 post infection, compared to only 40% mortality with the wild-type and the *aspB::aspB* control strains (Fig. 5A, 5B). Notably, larvae infected with the *aspA*, *aspB*, *aspC*, *aspE aspB* and *aspE aspD aspB* strains all showed signs of melanization as early as 1 h post infection (hpi), a phenomenon not observed in the wild-type or the *aspB::aspB* complemented strain (Fig. 5C). Melanization persisted following infection with heat-killed conidia (Fig. 5D); however, it did not result in increased mortality when the larvae were infected with heat-killed conidia (data not shown). Similar *Galleria* infection experiments performed with the *aspE*, *aspD*, and *aspE aspD* strains showed no hypervirulence and yielded survival results comparable to the wild-type strain (data not shown), confirming that the absence of *aspB* was responsible for this effect in the *aspE aspB* and *aspE aspD aspB* strains.

Following the virulence screen with the *Galleria* invertebrate model, the *aspB*, *aspB::aspB*, and wild-type strains were tested in an intranasal persistently immunosuppressed murine model of invasive aspergillosis. In contrast to the hypervirulence of the *aspB* strain consistently noted in the *Galleria* model, there were no significant virulence differences observed with the three strains in the murine model ($p=0.5680$) (Fig. 6A). When macrophages were co-incubated with *aspB* conidia for 6 h, we observed a significant increase in TNF- α released compared to both the *aspB::aspB* ($p=0.002$) and the wild-type strains ($p<0.001$) (Fig. 6B). Based on this increase in TNF- α release, we

hypothesize that the mice might be dying from an over activation of the immune system. However, Gomori's methenamine silver stain and hematoxylin and eosin staining revealed that there was not a discernable difference in fungal burden or lung inflammatory response between the *aspB* and the *aspB::aspB* or wild-type strains (Fig. 6C).

4. Discussion

As cytoskeletal components, septins contribute to a myriad of morphological events that are relevant for fungal development, such as cytokinesis, hyphal morphology, and cell wall integrity (Alvarez-Tabares and Perez-Martin, 2010; Boyce et al., 2005; Hernandez-Rodriguez et al., 2012; Kozubowski and Heitman, 2010; Li et al., 2012; Lindsey et al., 2010a; Warena et al., 2003; Warena and Konopka, 2002). Previously, it has been demonstrated that filamentous fungal septins interact with each other and cytoskeletal components, such as actin and microtubules (Berepiki and Read, 2013; Hernandez-Rodriguez et al., 2014; Juvvadi et al., 2013). Here, we explored the role of all five septins in *A. fumigatus* growth, development, and pathogenesis. Deletion of septin genes did not affect hyphal extension, suggesting that these genes are dispensable for hyphal growth under basal conditions. In *N. crassa* and *A. nidulans*, septin deletions result in an increase in branching, suggesting their role in suppressing the emergence of new growth foci (Berepiki and Read, 2013; Hernandez-Rodriguez et al., 2012; Lindsey et al., 2010a). Although *A. fumigatus* core septins are >90% homologous to the respective *A. nidulans* and *N. crassa* core septin genes, none of the *A. fumigatus* septin deletion strains reproduced the hyperbranching phenotype.

Deletion of *A. fumigatus aspA*, *aspB*, *aspC* or *aspE* resulted in a significant increase in apical and sub-apical compartment lengths. As suggested by the septal localization pattern we previously described for AspE (Juvvadi et al., 2011b), it is possible that AspE is also required for proper septation. However, all septa from the *aspB*, *aspE*, *aspE aspB* and *aspE aspD aspB* strains appeared morphologically similar to the wild-type strain, suggesting that septins AspA, AspB and AspE do not participate in septum formation per se, but are required for coordinating the interseptal distances in the actively growing hyphal regions such as the apical and subapical compartments. It is possible that deletion of these septins resulted in delayed septation, similarly to what is described for *N. crassa* and *A. nidulans* core septins (Berepiki and Read, 2013; Hernandez-Rodriguez et al., 2012; Lindsey et al., 2010a). To our surprise, deletion of the *aspA* gene resulted in a higher apical compartment length, greater than that observed in the *aspE* or *aspB* single deletion strains, but comparable to the *aspE aspB* strain. Previously, we found that AspA did not localize at the septum when it is overexpressed ectopically (Juvvadi et al., 2011b). Due to the increment in the apical compartment, we suggest three possible hypotheses: 1) AspA may be involved in transporting other septins to the future septation site, 2) the GFP tag and previous overexpression may have altered the localization pattern of AspA, and AspA actually localizes at the septa, in a similar manner as *A. nidulans* (Lindsey et al., 2010a) where it coordinates septation, or 3) AspA might be involved in the trafficking of the molecular machinery needed for the progression of septation.

Of all the core septins, we observed less of a septation defect in the *aspD* strain, where also the apical and sub-apical compartments were larger than the wild-type strain but smaller

than the *aspE* or *aspB* strains. In *S. cerevisiae*, it has been suggested that the core septins form a heterooctameric complex in the following order: Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11. Cdc11 homodimerizes to allow the end-to-end interaction of the septin complex (Bertin et al., 2008). Furthermore, in *A. nidulans* a similar organization has been suggested, where the complex is organized as: AspA-AspC-AspB-AspD-AspD-AspB-AspC-AspA (Hernandez-Rodriguez et al., 2012). Septins also mislocalize in the absence of other septins, suggesting that interdependency between septins exists (Hernandez-Rodriguez et al., 2012; Hernandez-Rodriguez et al., 2014; Lindsey et al., 2010a). In the *A. nidulans aspD* strain, it was suggested that AspB homodimerizes, allowing the formation of the septin complex in the absence of AspD (Hernandez-Rodriguez et al., 2012). This homodimerization of AspB could account for the less severe septation defect observed in the *A. fumigatus aspD* strain. However, although we noted binding between AspB and AspE, AspE does not fit into this model and this non-core septin may be a peripheral septin. In *N. crassa*, it has been suggested that all core septins interact with each other to form a oligomeric complex and that ASP-1, an AspE ortholog, may interact with this complex even if it does not constitute a core septin of the polymer (Berepiki and Read, 2013). Similar results have been recently suggested in *A. nidulans*, where AspE is not part of the core septin complex, but interacts with AspB to form higher order structures during multicellular growth (Hernandez-Rodriguez et al., 2014). The *aspE aspB* double deletion strain contains apical compartments that are larger than the wild-type strain, *aspE*, or *aspB* septin single deletion strains. This exacerbation in interseptal distances suggests that AspE and AspB might have some overlapping roles in septation. Furthermore, the observed binding of AspE to AspB supports this notion. In the *A. fumigatus aspE aspD* strain, apical and sub-apical lengths were similar to those of the *aspE* strain, suggesting that AspD does not participate in the regulation of compartment length. Thus, in the *aspE aspD* strain, we do not observe the increase of apical compartment length observed in the *aspE aspB* strain. Previous work done in *A. nidulans* shows that deletion of the core septins *aspA*, *aspC* or *aspB* resulted in delayed septation (Hernandez-Rodriguez et al., 2012; Lindsey et al., 2010a). We observed a similar phenotype in our *aspB*, *aspA*, and *aspE aspD aspB* deletion strains where only 50-65% of the germlings had visible septa, compared to a 100% of the wild-type strain after 15 hours of incubation. We also noted an increase in the number of nuclei contained in the apical compartment of the septin deletion strains compared to those of the wild-type strain, further supporting septins' role in septation.

Deletion of any *A. fumigatus* core septin caused a similar reduction in conidial production, suggesting that for this process the possible homodimerization of AspB is not enough to partially rescue the absence of AspD. This reduction in conidia production could be due to disorganization of the conidiophores, as previously described in *A. nidulans* (Hernandez-Rodriguez et al., 2012; Lindsey et al., 2010a). In *A. fumigatus*, the absence of septin AspE did not alter conidial production under basal conditions, a phenotype that was also observed in *N. crassa* when this septin ortholog is absent, but not in *A. nidulans* (Berepiki and Read, 2013; Hernandez-Rodriguez et al., 2014). Furthermore, deletion of *aspB* resulted in aberrant morphology of the conidia and a disorganized conidial cell wall. This provides further evidence that AspB has an important role in *A. fumigatus* conidiation.

The role of septins in cell wall stress response has not been addressed before in filamentous fungi. We observed that all of the *aspB* septin deletion strains were hypersensitive to cell wall inhibitors, indicating that there might be a cell wall defect in these strains. However, the β -1,3-glucan and chitin distribution and cell wall β -1,3-glucan content of the septin deletion strains remained unaltered under basal conditions when compared to the wild-type strain. When the septin deletion strains were exposed to caspofungin, we observed an increase in β -1,3-glucan released from the cell wall. The β -1,3-glucan quantification assay used in this study measures the amount of β -1,3-glucan released from the cell wall as equivalents of curdlan, and this increase in β -1,3-glucan from the septin deletion strains could be due to aberrant cell wall assembly, therefore resulting in greater release of the β -1,3-glucan following stress. In *C. neoformans*, the septin deletion strains were more susceptible to cell wall damaging agents SDS and caffeine (Kozubowski and Heitman, 2010). Also, a *C. albicans* core septin mutant showed a significant increase in susceptibility to caspofungin (Blankenship et al., 2014; Blankenship et al., 2010). Based on screening with calcofluor white staining that did not show any altered distribution of chitin in any of the *A. fumigatus* septin deletion strain under normal growth condition, we did not biochemically quantify total chitin content. Here, we observed that not only are septins involved in response to the echinocandin, caspofungin, but also to nikkomycin Z, suggesting a unifying link with septins and cell wall stress response in fungi.

In addition to controlling morphogenesis, septins have also been shown to have a role in pathogenesis. In *C. albicans*, deletion of septins CDC11 and CDC12 results in defective invasive growth and avirulence in a murine model (Warenda et al., 2003). In the basidiomycete *C. neoformans*, absence of CDC3 or CDC12 results in decreased virulence in *G. mellonella* (Kozubowski and Heitman, 2010). In the plant pathogen *M. oryzae*, the absence of septin Sep3 (ortholog of CDC3) abolishes virulence by impairing the ability of the fungus to penetrate the leaf cuticle. Deletion of the other core septins also reduced virulence in this plant pathogen (Dagdas et al., 2012). Nonetheless, septin deletions did not reduce or abolish virulence of *A. fumigatus*, and deletion of *aspA*, *aspB*, or *aspC* resulted in an increased mortality in the *G. mellonella* model. Due to the rapid melanization of the larvae, this increase in mortality could be due to a failure in masking cell wall components that then trigger the immune reaction of the larvae. We subsequently used our persistently immunosuppressed murine model to assess if this hypervirulence was seen in a host with a more complex immune system. However, *aspB* virulence was not significantly different from the *aspB:aspB* or the wild-type strains. Based on the disorganization of the *aspB* conidial cell wall and the hypervirulence in the *Galleria* model, we characterized the *ex vivo* interaction of the conidia with macrophage TNF- α release. We observed that the *aspB* conidia had a higher amount of TNF- α secretion compared to the *aspB:aspB* complemented or the wild-type strains. It is possible that AspB contributes to the organization of the rodlet layer and other immune evasion molecules of the conidia, contributing to host-pathogen interaction (Carrion Sde et al., 2013). However, histopathological analyses of the murine lungs showed that the fungal tissue invasion and inflammation were comparable to the wild-type and complemented strains. It is possible that the aggressive immune suppression used in the intranasal murine model suppresses the inflammatory response that we expected to observe. While individual septins may be

dispensable for virulence due to possible overlapping roles, it is still possible that the whole septin complex *per se* is required for full virulence of *A. fumigatus*. Future studies will address this hypothesis.

In conclusion, as an exploration of *A. fumigatus* septins' role, we generated 8 deletion strains, including double and triple deletions, and determined that *A. fumigatus* septins are not only involved in septation and conidia production, but also play an important role in cell wall stress and possibly immune evasion. We observed that the *aspB* or *aspC* deleted strains exhibited an increased susceptibility to echinocandin, and deletion of any septin gene increased susceptibility to nikkomycin Z, suggesting that these septin mutants have a defect in cell wall assembly or cell wall maintenance. Because the cell wall of the septin deletion strains is similar in β -1,3-glucan content to the wild-type strain, and cell wall component distribution and morphology are also similar to wild-type strain, it is possible that *A. fumigatus* septins only participate in the response to cell wall stress. *C. albicans* regulates septin organization by direct interaction of the septin complex with phosphatidylinositol-(4,5)-biphosphates [PI(4,5)P₂], contributing to the maintenance of cell wall integrity (Badrane et al., 2012). In response to caspofungin, *C. albicans* redistributes PI(4,5)P₂, altering localization of the septin complex and creating aberrant chitin patches in the cell wall (Badrane et al., 2012). In this study differential localization of AspB after exposure to anti-cell wall drugs suggests that a similar response to caspofungin and nikkomycin Z could be occurring in *A. fumigatus*. Further studies directed towards understanding the role of *A. fumigatus* septins in response to cell wall stress will help to reveal the mechanism by which septins mediate this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- All the 5 septin encoding genes in *Aspergillus fumigatus* were deleted
- Septins AspA, AspB, AspC and AspE are required for regular septation
- Core Septins AspA, AspB, AspC and AspD are required for conidiation
- Deletion of *aspB* or *aspC* caused increased susceptibility to anti-cell wall drugs
- *aspB* strain was hypervirulent in *Galleria mellonella* but not in a murine model

Fig. 1. Septins are required for conidiation and proper septation, but dispensable for radial growth

(A) Septin deletions do not result in a significant radial growth defect after 5 days of incubation. Conidia (10^4) from each septin deletion strain and the *akuB^{KU80}* wild-type strain were spotted on GMM+UU agar and incubated for 5 days at 37°C. Radial growth was measured every 24 h. (B) Deletion of *aspA*, *aspB*, *aspC*, or *aspE* results in an increase in length of the apical. The apical compartments of the septin deletion strains and the *akuB^{KU80}* wild-type strain were measured utilizing aniline blue staining, which stains the cell wall component β -glucan, and visualized using fluorescent microscopy. (C) *aspA*, *aspB*, and *aspE* *aspD* *aspB* strains have delayed septation. Presence of visible septa was quantified after 12 hours and 15 hours of incubation at 37°C. Conidia (10^4) were inoculated onto coverslips immersed in GMM+UU. (D) Deletion of *aspA*, *aspB*, *aspC*, or *aspD* results in a reduction in conidial production. Conidia were harvested after 5 days of growth using 10 ml of 0.05% Tween-80 and counted using a hemocytometer.

Fig. 2. Disorganization of the conidial cell wall in the *aspB* strain

Conidia from the wild-type and *aspB::aspB* strains have the typical two layers of the cell wall: an electron dense outer layer and a translucent inner layer. The *aspB* strain's conidia have the inner translucent layer but a disorganized outer layer. Black arrows indicates the electron dense outer layer, white arrows indicate the inner translucent layer. Scale bar, 100 nm. Magnification 50,000x.

Fig. 3. Deletion of *aspB* or *aspC* increased susceptibility to anti-cell wall drugs caspofungin or nikkomycin Z

Conidia (10^4) were inoculated into GMM+UU agar and GMM+UU broth, containing 1 μg/ml of caspofungin or 2 μg/ml of nikkomycin Z. Strains inoculated into agar were incubated 37°C for 3 days.

Fig. 4. AspB localization patterns after exposure to caspofungin and nikkomycin Z AspB-EGFP strain was grown in GMM broth for 20 h. Anti-cell wall drugs were added 3 hours prior visualization. AspB-EGFP localization was visualized by fluorescent microscopy. White arrows indicate the septum.

Fig. 5. *aspB*, *aspE aspB*, *aspE aspD aspB* strains are hypervirulent in the heterologous host *G. mellonella*

(A-B) Conidia (2×10^5) from each strain were inoculated into 20 larvae and survival was scored every 24 h post infection for 5 days. Deletion of the *aspB* gene, in any of the three strains missing *aspB*, resulted in hypervirulence in the *Galleria* model. (C) Infection with *aspB* conidia resulted in rapid melanization of the larvae, which does not occur in the *akuB^{KU80}* wild-type. (D) Rapid melanization also occurs after infection with heat-killed *aspB* conidia.

Fig. 6. The *aspB* strain remains virulent in a persistently immunosuppressed intranasal murine model of invasive aspergillosis

(A) 10 mice were infected intranasally with a total of 4×10^6 conidia from each strain and survival monitored daily for 14 days. There was no significant virulence difference noted between mice infected with the *aspB*, *aspB::aspB*, or wild-type strains. (B) Bone marrow derived macrophages (BMDM) release higher amount of TNF- α after exposure to *aspB* conidia. BMDM were co-incubated with conidia using a MOI of 40 for 6 h, and TNF- α release measured. (C) Histopathological examination of lungs after 3 days post infection shows that there is no significant difference in fungal invasion or inflammatory response.

Table 1

Strains Used in the Present Study

Strain	Parent Strain	Genotype	Reference on Source
<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	N/A		(da Silva Ferreira et al., 2006)
<i>akuB</i> ^{KU80}	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻		(da Silva Ferreira et al., 2006)
<i>aspA</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspA::pyrG</i>	This study
<i>aspB</i>	<i>akuB</i> ^{KU80}	<i>aspB::ble</i>	This study
<i>aspC</i>	<i>akuB</i> ^{KU80}	<i>aspC::hph</i>	This study
<i>aspD</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspD::hph</i>	This study
<i>aspE</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspE::pyrG</i>	This study
<i>aspE aspB</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspE::pyrG aspB::hph</i>	This study
<i>aspE aspD</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspE::pyrG aspD::hph</i>	This study
<i>aspE aspD aspB</i>	<i>akuB</i> ^{KU80} <i>pyrG</i> ⁻	<i>aspE::pyrG aspD::hph aspB::ble</i>	This study
<i>aspB::aspB</i>	<i>aspB</i>	<i>aspB::aspB-promo-aspB-hph</i>	This study
<i>aspB-egfp</i>	<i>akuB</i> ^{KU80}	<i>aspB::aspBpromo-aspB-egfp-hph</i>	This study

Table 2
Quantification of AspB Localization Patterns under Basal Conditions and after Caspofungin or Nikkomycin Z Exposure.

Conditions	Septal	Septal-Like	Filaments	Double Bar (Short)	Double Bar (Long)	Ring	Clumped-Rings	Ring & Bar	X-Like	Total
Basal	3	1	4	5	1	7	0	0	0	21
Caspofungin (1 µg/ml)	7	9	0	4	0	20	50	19	32	141
Nikkomycin Z (2 µg/ml)	1	14	1	1	1	4	0	0	0	22

Table 3List of AspB-Binding Septins in *Aspergillus fumigatus*

Septin	NCBI Accession Number	Molecular Weight (kDa)	Identified Peptides
AspB	gi 70986973	59	41
AspD	gi 70994952	39	30
AspA	gi 70998104	40	26
AspC	gi 70985058	44	30
AspE	gi 71000419	62	15