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The three *Aspergillus fumigatus* CFEM-domain GPI-anchored proteins (CfmA-C) affect cell-wall stability but do not play a role in fungal virulence

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ABSTRACT

Fungal cell-wall proteins containing the conserved fungal CFEM domain have been implicated in host-pathogen interactions and virulence. To determine the role of these proteins in the mold pathogen *Aspergillus fumigatus*, we deleted the entire family of three CFEM-containing genes (*CfmA-C*), singly and in all combinations. We found an additive increase in the susceptibility of the single, double and triple Δ*Cfm* mutants towards the chitin/β-glucan-microfibril destabilizing compounds Congo Red (CR) and Calcofluor White (CFW), indicating that the *A. fumigatus* CFEM proteins are involved in stabilizing the cell wall. No defects in growth or germination were observed, indicating that CFEM proteins do not have an essential role in the morphogenesis of *A. fumigatus*. Unlike in *Candida albicans*, the *A. fumigatus* CFEM proteins were not implicated in heme uptake or biofilm formation.

The ΔTriple-*Cfm* deletion strain did not exhibit altered virulence in either insect or murine models of infection, suggesting that cell-wall proteins containing the conserved fungal CFEM domain are not a significant virulence factor in *A. fumigatus*.

1. Introduction

Aspergillus fumigatus is the most common mold pathogen in immunocompromised patients. Infection is most commonly initiated by inhalation of airborne fungal spores or conidia, which germinate in the alveoli and whose hyphae subsequently grow invasively into the lungs. The mortality, despite advances in diagnosis and treatment remains unacceptably high at 50–80% of patients (Binder and Lass-Florl, 2013; Chai and Hsu, 2011). Numerous virulence determinants have been identified in *A. fumigatus*, including toxin production, the ability to withstand oxidative stress, hypoxia, nutrient deprivation, iron limitation and the ability to form a biofilm (Abad et al., 2010; Tekaia and Latge, 2005).

CFEM domains (Common in Fungal Extracellular Membrane) are fungal-specific, ~60 amino-acids long, and contain eight characteristically spaced cysteine residues (Kulkarni et al., 2003). They are found primarily in GPI-anchored cell-wall proteins (CWPs), usually near the N terminus. Most CFEM-containing CWPs studied to date are involved in host-pathogen interactions and virulence. In *Candida albicans* and *Candida parapsilosis* the CFEM-containing proteins Rbt5/Rbt51 and Cfem2/Cfem3/Cfem6 are involved in haemin and haemoglobin-iron utilization (Ding et al., 2011; Weissman and Kornitzer, 2004; Weissman et al., 2008). In *C. albicans*, deletion of three of five CFEM-encoding genes in the genome (Rbt5/Rbt51/Csa1) results in an increased sensitivity to cell-wall damaging agents and a reduced ability to form a biofilm (Perez et al., 2006, 2011). The *Coccidioides immitis* Ag2 CFEM-containing CWP is a major immunogenic antigen in this dimorphic fungal pathogen and Ag2-antigen immunization protected mice against a lethal fungal challenge (Zhu et al., 1996). In the plant pathogen *Magnaporthe grisea* the CFEM-containing protein Pth11 is required for appressorium development and subsequently plant infection (DeZwaan et al., 1999).

The *A. fumigatus* genome contains three CFEM-encoding genes (*CfmA*/*Af6g14090*, *CfmB*/*Af6g10580* and *CfmC*/*Af6g06690*) of unknown function. Here we describe the generation and phenotypic characterization of *A. fumigatus* deletion mutants lacking one, two or all three CFEM-containing genes.

2. Materials and methods

2.1. Strains and media

The strains used in this study are detailed in Table S1. *A. fumigatus* conidia were harvested in 0.2% (v/v) Tween 20, resuspended in double-distilled water (DDW) and counted with a hemocytometer.

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Growth was performed in either minimal medium (MM) containing 70 mM $NaNO_3$, 1% (w/v) glucose, 12 mM potassium phosphate pH 6.8, 4 mM $MgSO_4$ supplemented with trace elements and vitamins. 1.5% (w/v) agar (for MM agar plates) or rich YAG medium which consists of 0.5% yeast extract, 1% glucose, 10 mM MgCl2, supplemented with trace elements and vitamins. 1.5% agar was added for both MM and YAG agar plates. RPMI/MOPS used for the growth inhibition assays contains RPMI 1640 medium (Biological Industries, Beit Haemek, Israel) and 0.165 M MOPS (Sigma) buffer at pH 7.0. Brian medium used for the biofilm formation assay contains 1% (w/v) glucose, 1% asparagine, 1% KH_2PO_4, 0.2% $MgSO_4{\cdot}7H_2O$, 0.24% NH_4NO_3, supplemented with trace elements.

2.2. Generation and confirmation of gene deletion mutants

A ~5 kb DNA fragment flanking the *A. fumigatus CfmA-C* genes was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostic, Penzberg, Germany) and primers designed to contain an *Asc*I restriction site at their 5′ end. The fragments were cloned into the pGEM T/A cloning vector (Promega) to yield pGEM-*CfmA-C* (Table S2). The *Hph* resistance cassette was then cloned with primers to generate 5′*Hind*III-*Hph*-*Hind*III 3′, 5′*Hind*III-*Hph*-*Nhe*I 3′ and 5′*Avr*II-*Hph*-*Avr*II 3′ (Table S2). *CfmA* was removed by *Hind*III from pGEM-*CfmA* and replaced by 5′*Hind*III-*Hph*-*Hind*III 3′ to generate pGEM-Δ*CfmA-Hph*; *CfmB* was removed by *Hind*III + *Nhe*I from pGEM-*CfmB* and replaced by 5′*Hind*III *Hph Nhe*I 3′ to generate pGEM-Δ*CfmB-Hph*; *CfmC* was removed by *Avr*II from pGEM-*CfmC* and replaced by 5′*Avr*II-*Hph*-*Avr*II 3′ to generate pGEM-Δ*CfmC-Hph*. Deletion constructs pGEM-Δ*CfmA-C-Hph* were digested with *Asc*I and the fragments transformed into the $AkuB^{KU80}$ strain protoplasts as previously described (Levdansky et al., 2007). To generate the Δ*CfmA*/*CfmB* and Δ*CfmA*/*CfmC* double mutants, the Δ*CfmA* strain was transformed with either pGEM-Δ*CfmB-phleo*^{R} in which *CfmB* was removed and replaced by 5′*Hind*III *phleo*^{R}*NheI* 3′ or pGEM-Δ*CfmC-PtrA*^{R} in which *CfmC* was removed and replaced by 5′*Avr*II-*PtrA*^{R}-*Avr*II 3′. To generate Δ*CfmB*/*CfmC* the Δ*CfmC* strain was transformed with pGEM-Δ*CfmB-phleo*^{R} in which *CfmB* was removed and replaced by 5′*Hind*III-*phleo*^{R}*Nhe*I 3′. To generate the ΔTriple-*Cfm* mutant lacking all three CFEM genes the Δ*CfmA*/*CfmB* strain was transformed with pGEM-Δ*CfmC-PtrA*^{R} in which *CfmC* was removed and replaced by 5′*Avr*II-*PtrA*^{R} *Avr*II 3′. Three or more independent mutants were identified and verified for each strain by means of both PCR and Southern analysis as previously described (Levdansky et al., 2007).

2.3. RNA extraction and analysis

Total RNA was isolated from each strain using the Qiagen RNeasy plant kit (Qiagen, Valencia, CA) following the filamentous fungus protocol. For RT-PCR analysis, 3 μg of total RNA from each sample was digested by Dnase (DNA free; Ambion, Austin, TX) according to the manufacturer's instructions. 1 μg RNA was taken for the RT reaction with AffinityScript multiple-temperature reverse transcriptase (Stratagene, TX). The first-strand cDNA was PCR amplified with gene-specific primers for 300- to 500-bp fragments of *CfmA-C* (Table S2). *GpdA* was used as a normalizing control.

2.4. Electron microscopy

For transmission electron microscopy (TEM), dormant conidia (0 h) and germinated conidia (6 h) were fixed in 2.5% glutaraldehyde in PBS. They were then washed, postfixed in 1% OsO4 in PBS and washed again. After dehydration in graded ethanol solutions, the cells were embedded in glycid ether 100 (Serva Electrophoresis Gmbh, Heidelberg, Germany). Ultra-thin sections were stained by uranyl acetate and lead citrate and observed under a Jeol 1200 EX TEM.

2.5. MIC determination

MIC (Minimal Inhibitory Concentration – the lowest drug concentration to completely inhibit fungal growth) was determined with the twofold microdilution method in a 96-well plate. 2.5×10^3 *A. fumigatus* conidia per well were used in a total volume of 200 μl RPMI/MOPS medium per well. The plates were incubated at 37 °C for 48 h unless otherwise specified. MIC values were determined by light microscopy.

2.6. Droplet assay

Freshly harvested conidia of each strain were diluted to 10^7, 10^6, 10^5, 10^4 and 10^3 spores/ml. A 10 μl drop of spore suspension was placed on MMV plates containing 0, 25 or 50 μg/ml Congo Red (Sigma) or 0, 25, 50, 100 or 200 μg/ml Calcoflour White (Fluorescent Brightener 28, Sigma). The plates were incubated for 48 h at 37 °C.

Enzymatic protoplasting and regeneration assays. The protoplasting assay was performed as previously described (Levdansky et al., 2010). Briefly 6 h germinated conidia underwent protoplasting for between 0 and 120 min at 30 °C. At each time point protoplasts were resuspended in liquid YAG containing 1 M sorbitol, and spread on either YAG or YAG-sorbitol agar plates. Colonies counted on YAG-sorbitol plates represented all cells that had undergone treatment (intact osmotically stable cells and osmotically sensitive protoplasts). Colonies counted on YAG plates represented only osmotically insensitive cells (i.e., cells with relatively undamaged cell walls).

Protoplaszt regeneration was performed on conidia that had germinated in liquid YAG for 6 h and had then undergone 120 min of protoplasting. These protoplasts were then allowed to undergo cell wall regeneration for up to 8 h by incubation in YAG-sorbitol liquid medium. At each time point the regenerating protoplasts were spread on either YAG or YAG-sorbitol agar plates and the results analyzed as described above.

2.7. Glass bead conidial-disruption assay

A suspension of 10^8 *A. fumigatus* conidia/ml was prepared. A volume of 500 μl conidial suspension was placed in an eppendorf tube with an equal volume of glass beads (Sigma, glass beads 150–212 μm). The tubes were then vortexed at medium speed for between 0 and 360 s. At each time point, a 10 μl sample was collected, diluted to a final concentration of ~10^4 spores/ml, and 10–30 μl spread on YAG agar plates. The plates were incubated at 37 °C overnight and the colonies were counted. Survival rates were calculated by the following formula: [(number of colonies at time *X*)/(number of colonies at time zero)] × 100.

2.8. Surface carbohydrate composition by lectin staining

Fluorescence microscopy was used to determine differences in expression of cell-wall surface protein glycosidic residues. Two different FITC-labeled lectins, at a 10 μg/ml concentration, were used; concanavalin A (ConA) and wheat germ agglutinin (WGA), that specifically label alpha-mannosyl groups or N-acetylglucosamine (a component of chitin) residues, respectively. Samples were incubated for 1 h at room temperature in the dark in the presence of lectins, washed twice in DDW, resuspended in 300 μl PBS and then observed under fluorescence microscopy.

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2.9. Galactomannan release from the cell-wall

2.5×10^7 conidia were incubated in 50 ml of RPMI/MOPS medium for 72 h at 37 °C, while rotating at 200 rpm. The medium was collected and filtered through a 0.22 μm filter. The filtrate was diluted 1:10 and 1:100 and used to assess galactomannan release according to the BioRad platelia™ Aspergillus EIA (BioRad) kit protocol.

2.10. Biofilm formation

Biofilm formation was assessed by two methods: First, as described in Alcazar-Fuoli et al. (2011) 0.5×10^4 spores/ml were suspended in Brian medium and incubated at 37 °C overnight in a 6-well plate. The wells were washed 3 times with PBS and stained by 0.5% (w/v) crystal violet dye (750 μl/well) for 5 min at room temperature. After 3 washes with 1 ml PBS, 750 μl of 100% ethanol was added to each well for 5 min of incubation at room temperature. OD values were read at 570 nm. In the second method, previously described by Gravelat et al. (2010)), 10^5 spores/ml suspended in 50 ml of RPMI/MOPS medium, in a 250 ml glass Erlenmeyer were incubated at 37 °C with 250 rpm shaking for 24 h. Biofilm formation on the glass rim was estimated visually.

2.11. Zinc(II)Mesoporphyrin IX (ZnMP) assay

10^5 spores/ml suspended in 10 ml of MM were incubated at 37 °C overnight in a petri dish containing submerged glass coverslips. 50 μM of ZnMP alone or with 100 μM of hemin was added for between 0 and 180 min. The slides were removed, washed 5 min in saline and fluorescence microscopy was performed on an Olympus fluorescent microscope under 400× magnification.

2.12. Galleria mellonella larval model of infection

Sixth instar stage *G. mellonella* larvae were injected through the last pro-leg with 5×10^6 *A. fumigatus* conidia, suspended in 10 μl of PBS as previously described (Fallon et al., 2012). The larvae were kept for 120 h at 37 °C and death was monitored by visual inspection and prodding with a toothpick.

2.13. Murine model of infection

Six week old ICR female mice were used in this work in two models of infection. In the neutropenic model, neutropenia was induced by intraperitoneal injection with cyclophosphamide (150 mg/kg) 3 days prior to infection and 1 and 7 days after it. In addition these mice were treated with cortisone acetate (150 mg/kg) three days prior to infection by subcutaneous injection. To induce an immunocompromised state but without neutropenia (non-neutropenic model), mice were injected subcutaneously with cortisone acetate (300 mg/kg) three days prior to infection, on the day of infection, and 3, 7 and 11 days post-infection. The mice were infected intranasally with 5×10^5 dormant spores, suspended in 20 μl of PBS + 0.2% Tween 20 (10 μl in each nostril). Mortality was monitored for 18 days.

3. Results

3.1. Identification of a family of three CFEM-containing genes in A. fumigatus

We analyzed the *A. fumigatus* genome for the presence of CFEM-domain containing genes. BLASTP searches identified three genes containing a CFEM domain with an *E* value of <10E−4, *Afu6g14090*, *Afu6g10580* and *Afu6g06690* that we named *CfmA-C* respectively. All three encode for putative GPI-anchored CWPs containing an N-terminal CFEM domain and a ~100 amino-acid long Ser/Thr/Pro (S/T/P) – rich C-terminal region (Fig. 1A). As we showed previously, *CfmA* contains a 6 bp internal tandem repeat which varies in copy number among clinical *A. fumigatus* isolates (Levdansky et al., 2007). Sequence alignment of the *A. fumigatus*, *C. albicans*, *M. grisea*, *C. immitis* and *Saccharomyces cerevisiae* CFEM domains revealed the presence of the conserved eight-cysteine amino-acid sequences (Fig. 1B, highlighted in black). Dendrogram cluster analysis by the ClustalX neighbor-joining algorithm shows that the *A. fumigatus* CfmA-C CFEM domains occupy distinct and divergent branches, with CfmB-CFEM most closely related to the Ag2 *C. immitis* CFEM domain. Notably, all *C. albicans* CFEM domains are closely related and occupy a distinct branch (Fig. 1C). Cluster analysis of all available CFEM-containing proteins from the genus *Aspergillus* shows that they assign to three distinct groups, each containing a single representative (CfmA-C) from *A. fumigatus* (Fig. 1D). This implies a conserved, evolutionarily distinct role for each of these three proteins.

To investigate the temporal expression pattern of *CfmA-C*, total RNA was extracted from the parental $AkuB^{KU80}$ strain after different time points of growth in liquid YAG medium and analyzed by RT-PCR. *CfmA* was expressed during germination and hyphal growth, *CfmB* was primarily expressed during late mycelial growth (24 h) and *CfmC* was constitutively expressed (Fig. 1E).

3.2. Generation and verification of the ΔCfmA-C single, double and triple mutant strains

The *CfmA-C* genes were singly disrupted in the $AkuB^{KU80}$ strain using the pGEM-Δ*CfmA/B/C-hyg*^{R} constructs as described in the Materials and methods (Supplemental Fig. 1). Initial phenotypic characterization, including growth in liquid and agar MM and YAG medium indicated that there was no observable difference between the mutants and the parental strain (data not shown). Therefore, we constructed double deletion mutants (Δ*CfmA*/*CfmB*, Δ*CfmA*/*CfmC* and Δ*CfmB*/*CfmC*) and a triple deletion mutant Δ*CfmA*/*CfmB*/*CfmC* heretofore designated ΔTriple-*Cfm*, using pGEM-Δ*CfmB-phleo*^{R} and pGEM-Δ*CfmC-ptrA*^{R} (see Materials and Methods and Supplemental Fig. 1). Mutants were verified by both PCR and Southern analysis. PCR was performed with primers for the targeted *Cfm* gene and for the correctly inserted selectable marker (Supplemental Fig. 2A and B). Southerns were performed with the corresponding radiolabeled selectable marker (*hph*/*phleo*/*ptrA*) (Supplemental Fig. 2C). The double *Cfm* deletion mutants showed no observable growth difference compared to the parental $AkuB^{KU80}$ strain (data not shown). Therefore all further phenotypic analyses were undertaken on the ΔTriple-*Cfm* mutant strain.

3.3. Phenotypic characterization of the ΔTriple-Cfm mutant

Initially, we compared between the ΔTriple-*Cfm* and parental $AkuB^{KU80}$ strain for morphological changes in growth under various temperatures (37 °C and 48 °C), on rich medium (YAG) and on defined medium (MM) in agar (Fig. 2A). No differences were observed. Omission of the vitamins PABA, pyroxidine, riboflavin, thiamine, and biotin or the trace elements iron, zinc, copper and manganese from the defined medium did not affect mutant growth compared to wild-type (data not shown). These results support the conclusion that the three *A. fumigatus* CFEM genes do not affect growth properties under extreme heat stress and are probably not involved in the uptake of vitamins or trace elements.

We then compared the germination and hyphal growth rates of the ΔTriple-*Cfm* strain to the parental $AkuB^{KU80}$ strain (Fig. 2B and

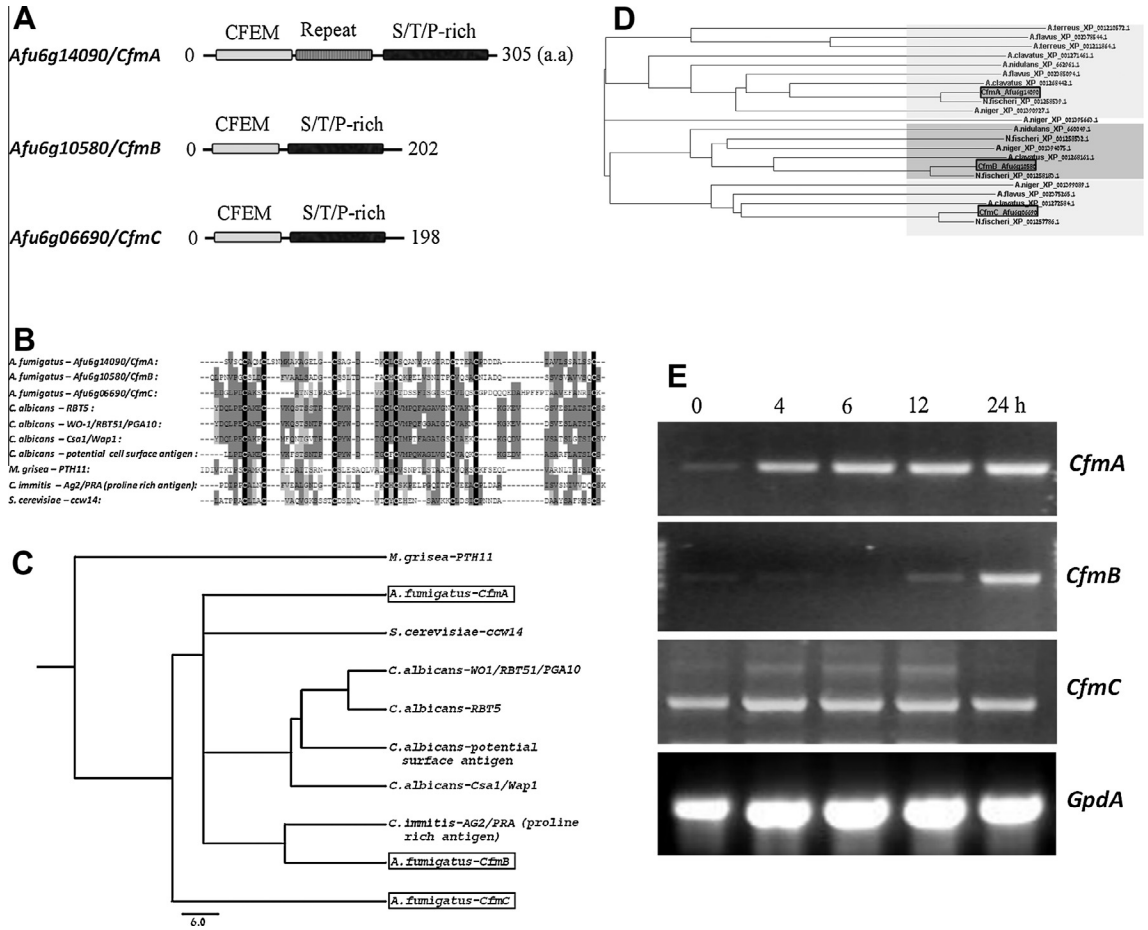

Fig. 1. Sequence and expression analysis of the *A. fumigatus CfmA-C* genes. (A) Schematic representation of CFEM (gray shading), repeat rich (diagonal lines) and serine/threonine/proline-S/T/P (black) domains in the CfmA-C proteins. (B) Amino-acid alignment comparison between the CFEM domains of CfmA-C and those of *C. albicans*, *M. grisea*, *C. immitis* and *S. cerevisiae* CFEM proteins. (C) Dendogram showing relatedness between the gene products described above. The tree was generated by the ClustalX program with the neighbor-joining algorithm for cluster analysis. (D) Dendogram showing relatedness among CFEM proteins within the genus Aspergillus. (E) Developmental expression of *A. fumigatus CfmA-C*. Conidia were grown in rich YAG liquid medium followed by RNA extraction. Gene expression was examined at different time points by RT-PCR and normalized to *gpdA* expression.

C). Dormant conidia were incubated in liquid YAG medium at 37 °C. At each time point a sample was observed under a light microscope and the percent of conidia germinating as well as the length of the growing hypha were determined (100 germlings analyzed per time point). No difference between the control strain and three independent isolates of the ΔTriple-*Cfm* mutant was seen, indicating that germination and hyphal growth rates were not affected by deletion of all three CFEM genes (Fig. 2B). Hypothesizing that CfmA-C are involved in the uptake of nutrients through the cell-wall, we tested the ability of ΔTriple-*Cfm* to use carbon sources other than glucose. We replaced the carbon source (1% glucose) in the defined MM with alternative sources at 1%, including raffinose, sucrose, glycerol, trehalose, sorbitol, maltose, lactose, starch, pectin, ethanol, propionate, glutamate and albumin. Plates were incubated for 48 h at 37 °C. We found no notable changes in growth patterns, appearance or growth rates of the mutant compared to the parental *AkuB*^{KU80} strain (data not shown). The growth rate of the ΔTriple-*Cfm* mutant in acidic (pH-3) or alkaline (pH-9) MM or RPMI/MOPS liquid media was also tested and was identical to that of the parental *AkuB*^{KU80} strain (Data not shown).

3.4. Deletion of the CFEM genes results in increased sensitivity to wall destabilizing agents

We hypothesized that CfmA-C, because they contain motifs targeting them to the plasma membrane and cell-wall, are involved in aspects of membrane or cell-wall integrity. We therefore tested the ΔTriple-*Cfm* mutant (three independent isolates) relative to the parental *AkuB*^{KU80} strain, for differences in sensitivity to different cell-wall and membrane stressors (sodium dodecyl sulfate/SDS, Calcofluor White/CFW, Congo Red/CR, caspofungin/CAS, nikkomycin Z/NKZ, amphotericin B/AMB, voriconazole/VORI), oxidative and reductive stressors (menadione/MND, H_2O_2) ER stressors (tunicamycin/TUNI, Dithiothreitol/DTT) or the calcium chelator EGTA by broth microdilution. Freshly harvested conidia were incubated in liquid MM in the presence of different concentrations of each compound for 48 h at 37 °C, followed by determination of minimal inhibitory concentration (MIC) values. A small twofold increase in sensitivity of the ΔTriple-*Cfm* mutant was observed for CFW and CR. (Table 1). We therefore analyzed the susceptibility of the single, double and triple CFEM mutants against CR and CFW by microbroth dilution, droplet dilution on agar, colony diameter measurements and microscopy. The results revealed that the CFEM single and double mutants also showed a similar increase in sensitivity to CFW and CR by broth microdilution (Table 1), and all the deletion strains showed a larger increase in sensitivity in the droplet dilution assay (Fig. 3A and B), indicating that this phenotype was more severe on agar as compared to submerged culture. Measurement of colony diameter on CR agar plates revealed that all CFEM mutants were markedly more sensitive to CR than the parental *AkuB*^{KU80} strain (P value $<10^{-11}$), the double mutants more sensitive than the single mutants (P value $<10^{-11}$)

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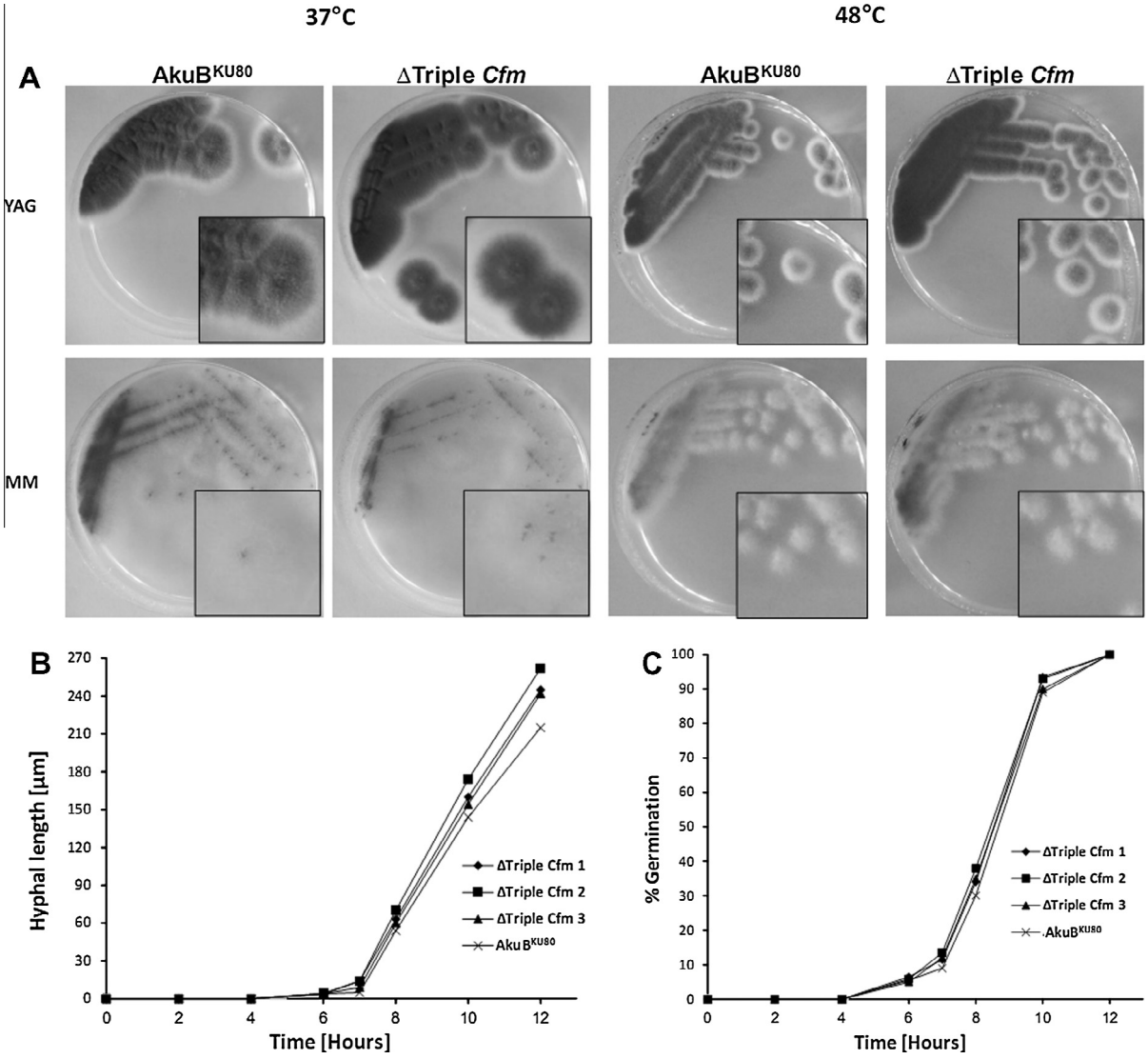

Fig. 2. Deletion of all three *A. fumigatus CfmA-C* genes does not alter radial growth, conidial germination or hyphal growth rates. (A) Conidia of the ΔTriple-*Cfm* mutant and parental *AkuB*^{KU80} strains were streaked out on rich YAG or defined MM agar plates and grown at 37 °C or 48 °C for 48 h. (B) Hyphal length measurements. (C) Germination rates were compared microscopically between three independent ΔTriple-*Cfm* mutant strains and the parental *AkuB*^{KU80} strain. Three biological repeats of these experiments gave similar results. A representative experiment is shown.

Table 1
MICs of antifungal drugs and inhibitors.

Stressor compound	MIC (µg/ml)							
	AkuB^{KU80}	ΔTriple *Cfm*	ΔCfmA/B	ΔCfmA/C	ΔCfmB/C	ΔCfmA	ΔCfmB	ΔCfmC
SDS	500	500						
CFW	200	100	100	50	100	100	100	100
CR	25	12.5	12.5	6.25	12.5	12.5	12.5	12.5
CAS[a]	0.06	0.06						
AMB	2	4						
VORI	0.5	0.5						
TUNI[a]	80	80						
MND	10	5						
H_2O_2	12.5 mM	12.5 mM						
DTT	6.25 mM	6.25 mM						
EGTA[a]	0.255 µg/ml	0.255 µg/ml						

[a] MEC – Minimal Effective Concentration – the lowest drug concentration to visibly inhibit fungal growth was determined for caspofungin, tunicamycin, nikkomycin Z and EGTA.

and the ΔTriple-*Cfm* mutant more sensitive than either the double or single CFEM deletion mutants (*P* value <0.05), (Fig. 3C). These results imply that *CfmA-C* contribute additively to wall stability.

Colonies of the parental *AkuB*^{KU80} strain grown on CR-agar formed a dense circular conidiating mycelium with normal hyphal tips. In contrast, the ΔTriple-*Cfm* strain formed small irregular and

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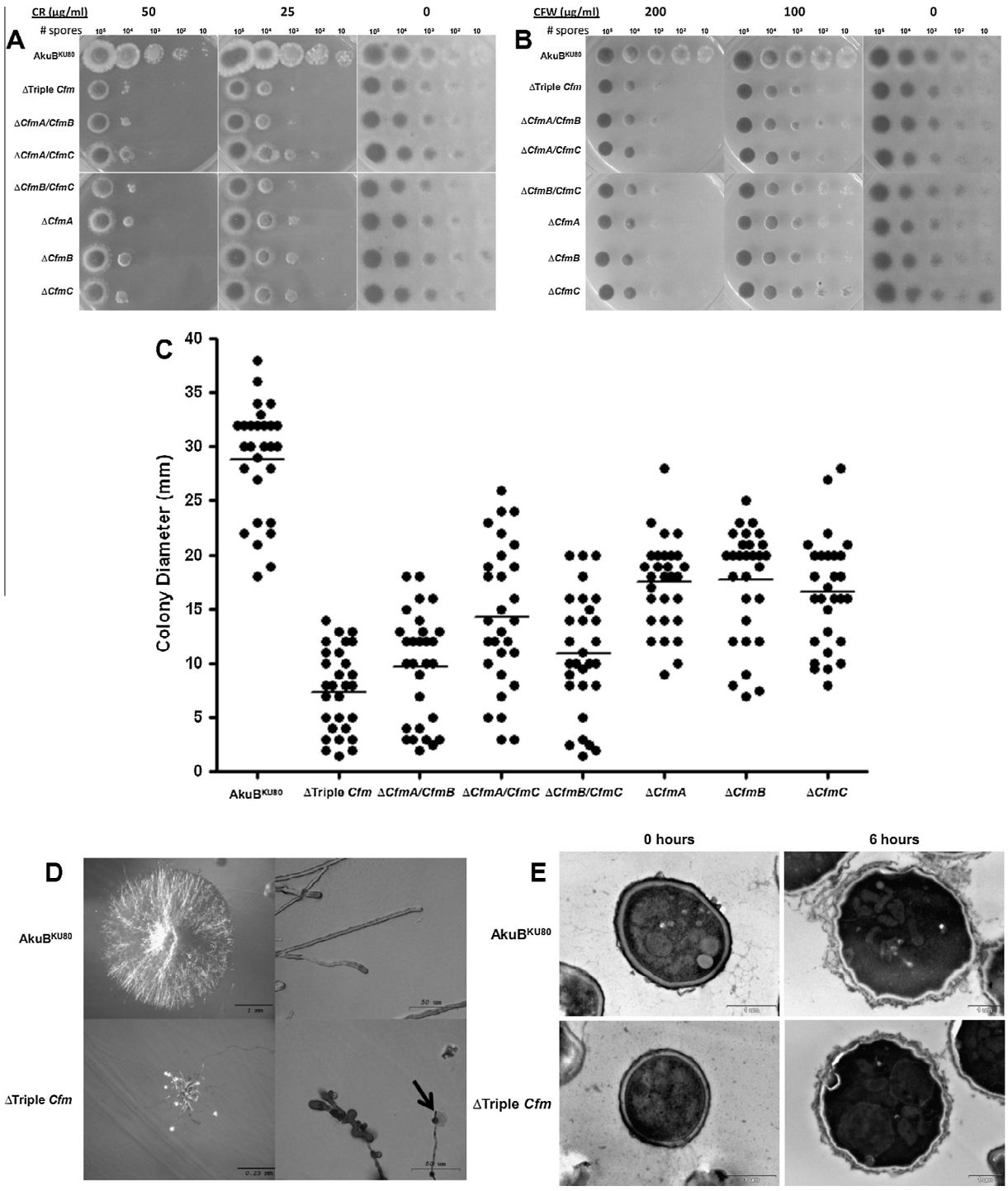

Fig. 3. Deletion of *CfmA-C* results in increased sensitivity to the cell wall disrupting agents CR (A) and CFW (B) by droplet dilution on MM agar plates. (C) Colony diameter measurements of parental *AkuB*^{KU80} and mutant strains grown at 37 °C for 120 h on MM agar plates containing 50 µg/ml CR. (D) Microscopy of the parental *AkuB*^{KU80} and ΔTriple-*Cfm* mutant strains after 48 h of growth at 37 °C on MM agar plates containing 50 µg/ml CR. The mutant strain shows decreased growth, hyphal swelling and lysis (arrow). (E) Transmission electron microscopy (TEM) of dormant and germinating (6 h) parental *AkuB*^{KU80} and ΔTriple-*Cfm* mutant strains conidia. No changes in ultrastructure are apparent.

poorly conidiating colonies characterized by ballooning compartments and hyphal tips, some of which had undergone spontaneous lysis (Fig. 3D). Similar results were seen for the double and single CFEM deletion mutants (not shown). These findings prompted us to compare additional parameters of cell wall stability. We looked for changes in the microscopic ultrastructure of the cell-wall by TEM analysis of the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain. Both dormant (0 h) and germinating conidia (6 h) were compared. We saw no substantial ultrastructural differences between the cell-wall of the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain (Fig. 3E).

Deletion of the single CFEM-domain containing gene *CCW14/ICWP* in *S. cerevisiae* leads to increased susceptibility to protoplasting enzymes (Moukadiri et al., 1997). We therefore tested the ability of freshly harvested conidia from the parental *AkuB*^{KU80} and ΔTriple-*Cfm* mutant to (ii) survive osmotic shock after prolonged protoplasting (ii) regenerate after protoplasting and subsequently withstand osmotic shock (Supplemental Fig. 3A and B). No

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significant differences were found. We also tested the ability of freshly harvested conidia from the parental *AkuB*^{KU80} ΔTriple-*Cfm* and control cell-wall defective Δ*Ecm33* strain (Romano et al., 2006) to (iii) survive repeated cycles of freezing in liquid nitrogen, (iv) remain viable during storage in 1% SDS, or (v) resist physical shearing in the presence of rapidly agitated glass bead (Supplemental Fig. 3C–E). In all cases, no appreciable differences were seen between the parental *AkuB*^{KU80} strain and the ΔTriple-*Cfm* mutants, whereas the control wall defective Δ*Ecm33* strain exhibited increased sensitivity. Conidial adhesion to polystyrene or laminin coated surfaces was also similar between the mutant and control strains (data not shown). To identify changes in wall composition during hyphal growth, two tests were undertaken. (i) surface staining of elongating hyphae with the fluorescently labeled lectins concavalin A/ConA or wheat germ agglutinin/WGA which bind cell-wall α-mannosyl residues (surface mannose and mannoproteins) and N-acetylglucosamine (chitin) respectively or with Calcofluor White which binds both external and internal chitin fibrils. No differences in fluorescence between the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain were seen (Supplemental Fig. 3F and G). (ii) galactomannan (GM) release into the growth medium. GM release is increased by cell-wall damage. The results (GM-indexes of 2.46 and 2.57 for the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strains, respectively) indicate that there was no significant difference in the release of soluble galactomannan between the mutant and parental *AkuB*^{KU80} strains. In summary, *CfmA-C* appear to play a narrowly defined role in stabilizing the cell wall towards CR and CFW, without affecting additional morphological and physiochemical parameters.

3.5. The ΔTriple-Cfm mutant is not impaired in biofilm formation

In *C. albicans*, deletion of the CFEM genes results in an inability to form a biofilm. We therefore tested biofilm formation in the ΔTriple-*Cfm* mutant relative to the parental *AkuB*^{KU80} strain and to a Δ*StuA* strain that has a known defect in biofilm formation (Gravelat et al., 2010). Two tests were performed. (i) Biofilm formation after growth in Brian medium and crystal violet colorimetric assessment. (ii) Biofilm-ring formation by hyphae incubated in swirling glass Erlenmeyers in RPMI/MOPS medium. Hyphae adhering to the air-surface interface form a biofilm ring. In both assays the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain formed similar biofilms, whereas the control Δ*StuA* strain did not, indicating that *CfmA-C* do not affect biofilm formation in *A. fumigatus* (Fig. 4A and B).

3.6. The ΔTriple-Cfm mutant is not impaired in iron and heme utilization

In *C. albicans*, the CFEM-domain CWPs Rbt5 and Rbt51 bind extracellular heme–iron and undergo endocytosis. Both heme and iron are subsequently utilized by the fungus (Weissman et al., 2008). We therefore tested the ΔTriple-*Cfm* mutant for defects in iron and heme–iron utilization. We first determined the MICs for ferrozine, a free iron chelator, in minimal medium (lacking Fe). There were no differences in MIC values (MIC-ferrozine = 625 μM) between the ΔTriple-*Cfm* and control *AkuB*^{KU80} strain. However, this is probably because unlike *C. albicans*, *A. fumigatus* produces secreted siderophores (triacetyl fusarinine – TAFC) that can acquire the iron chelated to the ferrozine and overcome any loss in the activity of other uptake systems. Several approaches were used to test the hypothesis that the ΔTriple-*Cfm* mutant is defective in heme uptake. In the first, we used sampangine, a novel inhibitor of hemin biosynthesis (Agarwal et al., 2008) to block endogenous biosynthesis pathways in both the ΔTriple-*Cfm* mutant and control strain. We reasoned that in the presence of exogenous hemin and sampangine (i.e. the fungus is unable to synthesize its own hemin and therefore dependent on exogenously

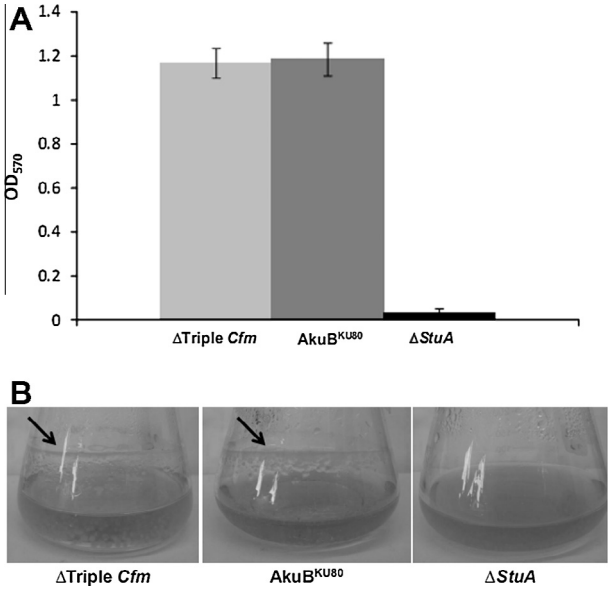

Fig. 4. Biofilm formation is not impaired in the ΔTriple-*Cfm* mutant strain. (A) Biofilm formation after growth in Brian medium and crystal violet colorimetric assessment. (B) Biofilm formation by hyphae incubated in swirling glass Erlenmeyers in RPMI/MOPS medium. Note the formation of a biofilm ring (arrow) at the air-medium interface. In both assays, the Δ*StuA* strain defective in biofilm formation was used as a negative control. These experiments were repeated three times with similar results.

supplied hemin), if the ΔTriple-*Cfm* mutant lacks the ability to take up exogenous hemin, it will be more sensitive to sampangine compared to the control strain in the presence of exogenous hemin (because it is being starved for both internal heme by the drug, and for external heme because it presumably lacks the ability to uptake it). Inhibition was tested under both normoxic and hypoxic conditions, as we reasoned that cells starved for oxygen will be more sensitive to inhibition of heme biosynthesis. However, no differences in sampangine MICs were found between the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain under increasing hemin concentrations in either normoxia or hypoxia (Table 2). In another approach, we used a fluorescent analogue of heme (Zn(II)Mesoporphyrin IX – ZnMP (Rajagopal et al., 2008) to directly visualize whether an exogenous fluorescent heme analog can travel through the cell-wall into the germinating spores and hyphae. We reasoned that if CfmA-C are involved in heme uptake, we would expect the ΔTriple-*Cfm* mutant to show slower or decreased uptake of fluorescent ZnMP. ZnMP at a concentration of 50 μM was added to germinating conidia. ZnMP with 100 μM of hemin was added as a negative control (exogenous hemin in excess abolishes ZnMP uptake). Slides were viewed under fluorescence microscopy for up to 3 h. The ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain showed comparable rapid uptake of ZnMP after 30 min of incubation, whereas addition of hemin abolished ZnMP uptake, as expected (Fig. 5). In addition, we expressed the recombinant CfmA-CFEM domain in *Pichia pastoris* and analyzed the binding of the purified protein to hemin-agarose or agarose (control) beads. The recombinant CfmA-CFEM domain bound weakly to both the hemin and agarose beads and was primarily released unbound into the supernatant (data not shown). Taken together, the results imply that the three *A. fumigatus* CFEM genes are not involved in the uptake of exogenous hemin.

3.7. The ΔTriple-Cfm mutant is not altered in virulence in infected G. mellonella larvae or in mice

The virulence of the ΔTriple-*Cfm* mutant was compared to that of the parental *AkuB*^{KU80} strain in both insect and murine infection

Table 2
Effect of sampangine on fungal growth in the presence of hemin.

Hemin concentration (μg/ml)	0 MIC (μg/ml)	1 MIC (μg/ml)	10 MIC (μg/ml)	30 MIC (μg/ml)	100 MIC (μg/ml)
ΔTriple *Cfm* – Normoxia	2	2	4	4	8
AkuB^{KU80} – Normoxia	2	2	4	4	8
ΔTriple *Cfm* – Hypoxia[a]	4	4	4	8	16
AkuB^{KU80} – Hypoxia	4	4	4	8	16

[a] Strains were grown under hypoxia in 1% O_2, 5% CO_2 and 94% N_2.

models. In the insect larva model of infection, conidia were injected into the last proleg of *G. mellonella* larvae (10 per group). The larvae were kept for 120 h at 37 °C and monitored daily for mortality. Kaplan–Meier survival curves were produced. No significant differences in virulence were found (Fig. 6A). Two murine infection models were tested: neutropenic (cyclophosphamide-induced) and non-neutropenic (cortisone-acetate induced). In each model ten mice in two groups were infected intranasally either with 5×10^5 dormant mutant or control *AkuB*^{KU80} conidia. Mouse mortality was monitored for 18 days. Kaplan–Meier survival curves were produced. No significant differences in virulence were found between the ΔTriple-*Cfm* mutant and control strain, supporting the conclusion that in *A. fumigatus* the CFEM family of genes is not involved in determining virulence (Fig. 6B).

4. Discussion

CFEM-domain containing fungal genes have been intensively studied in the model yeast *S. cerevisiae* and the pathogenic yeasts *C. albicans* and *C. parapsilosis* (Ding et al., 2011; Moukadiri et al., 1997; Perez et al., 2006, 2011; Weissman and Kornitzer, 2004; Weissman et al., 2008). However, a comprehensive analysis of their role in filamentous fungi is lacking. We therefore analyzed, singly and in all combinations, the roles all three CFEM domain-containing genes (*CfmA-C*) in the pathogenic mold *A. fumigatus*.

CfmA-C, like most fungal CFEM proteins, are relatively short (200–300 amino-acids) and contain an N-terminal CFEM domain, a serine-threonine-proline rich region that undergoes extensive O-linked glycosylation and a C-terminal GPI anchor motif that targets them to the plasma membrane and cell-wall. Sequence analysis of the CfmA-C CFEM domains shows that they diverge significantly, unlike those of *C. albicans*. Interestingly, comparison of CfmA-C to CFEM proteins of other *Aspergilli* revealed that each assigns to one of three conserved clusters implying that they retain an evolutionary distinct role.

To identify the function of *CfmA-C* in *A. fumigatus*, we generated strains harboring single, double and triple gene deletions. Because no obvious phenotypic changes resulted from the single and double gene deletions, further analysis was performed with the ΔTriple-*Cfm* mutant. Preliminary phenotypic analysis (hyphal growth and germination rates, effect of elevated temperature, extreme pH, different carbon sources, absence of trace elements and vitamins) did not reveal any differences between the ΔTriple-*Cfm* mutant and control *AkuB*^{KU80} strain.

Next, based on the findings that deletion of the single CFEM-domain containing gene *CCW14/ICWP* in *S. cerevisiae* leads to increased susceptibility to wall-disrupting agents and protoplasting enzymes (Moukadiri et al., 1997), and deletion of *C. albicans PGA10/RBT5/CSA1* results in alterations to the cell-surface, decreased adhesion and biofilm formation (Perez et al., 2006, 2011), we performed comparable experiments in the *A. fumigatus*

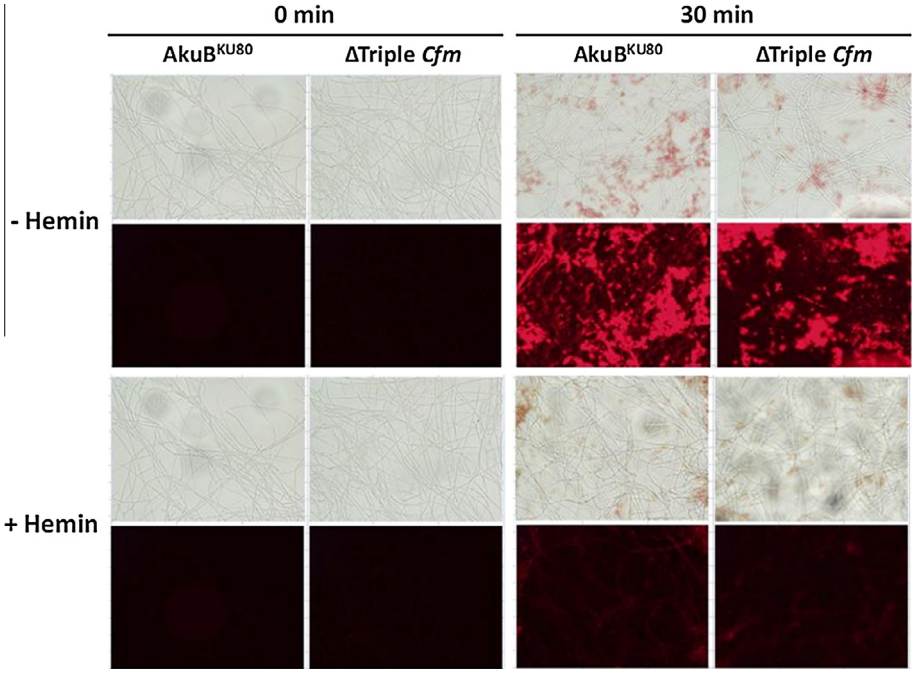

Fig. 5. Uptake of ZnMP (Zn(II) Mesoporphyrin IX), a fluorescent analogue of hemin is not impaired in the ΔTriple-*Cfm* mutant strain. Mycelium was incubated in the presence of 50 μM of ZnMP alone or with 100 μM of hemin, washed and viewed by fluorescence microscopy. This experiment was repeated twice with similar results.

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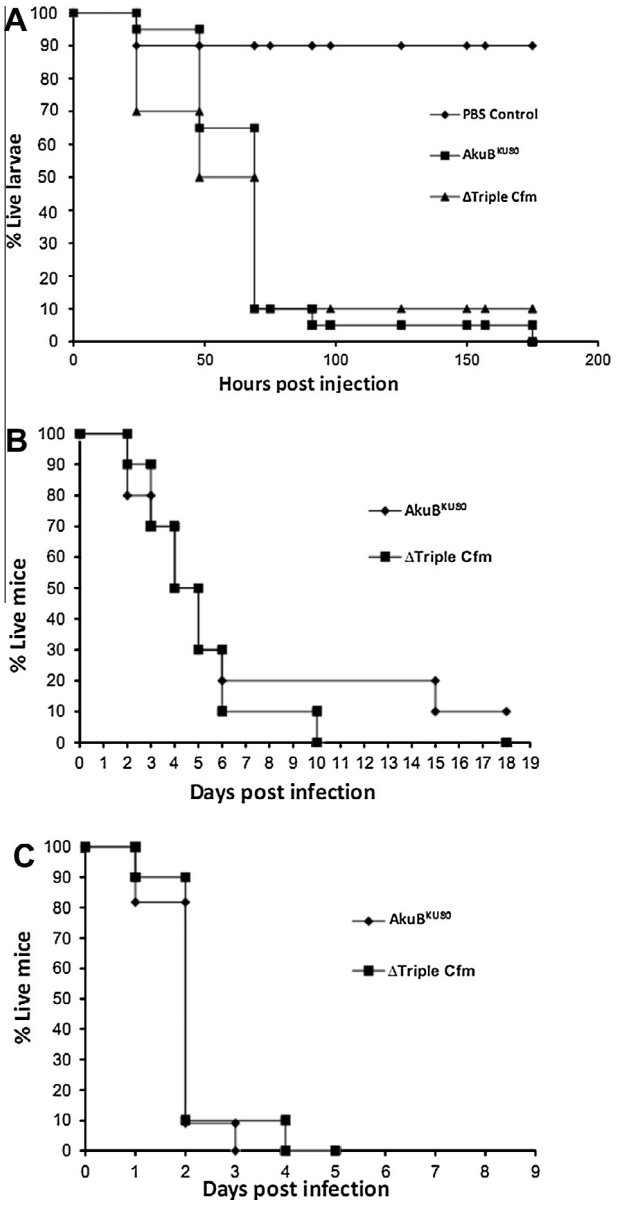

Fig. 6. The ΔTriple-*Cfm* mutant strain shows normal virulence in insect and murine models of infection. (A) *Galleria mellonella* insect larvae injected with 5×10^6 conidia/larva and monitored for viability over 3 days. (B) Murine pulmonary aspergillosis. Two regimens were used to immunocompromise mice: cortisone acetate alone (steroid model) and cortisone acetate plus cyclophosphamide (neutropenia model). Survival curves are shown for mice infected intranasally with an inoculum of 5×10^5 conidia per mouse. Percent survival was monitored throughout the 18-day study period.

ΔTriple-*Cfm* mutant. We found increased susceptibility to the chitin/β-glucan-microfibril destabilizing compounds CR and CFW following single, double or triple deletion of *CfmA-C*. Susceptibility was much more pronounced on agar as compared to submerged growth. The ΔTriple-*Cfm* mutant exhibited the greatest sensitivity, suggesting that *CfmA-C* contribute additively towards cell wall stability. Surprisingly, deletion of *CfmA-C* did not alter cell-wall morphology and surface characteristics, conidial stability under physical and chemical stress, conidial adherence, or the ability to form a biofilm. This implies that *CfmA-C* have a very tightly defined role in cell wall morphogenesis.

It is also noteworthy that deletion of *CfmA-C*, while increasing sensitivity towards CR and CFW, did not affect susceptibility towards the cell wall destabilizing antifungals CAS and Nikkomycin Z. A possible explanation is that CR and CFW primarily inhibit chitin/glucan microfibril cross-linking by binding to nascent extruded polymer chains. This interferes with the formation of large bundles of fibers from single fibrils, i.e. with the process of cell wall modeling. In contrast, both CAS and Nikkomycin Z, by targeting glucan and chitin synthase activity respectively, inhibit polysaccharide biosynthesis (Roncero and Duran, 1985). Perhaps CfmA-C are involved in stabilizing microfibril chains, allowing them to form bundles more efficiently. In the absence of CfmA-C, bundle formation may be more susceptible to interference by CR and CFW.

Considerable evidence indicates that the CFEM-containing genes *RBT5* and *RBT51* in *C. albicans* and *CFEM2/CFEM3/CFEM6* in *C. parapsilosis* are involved in the acquisition of iron from hemoglobin and hemin (Ding et al., 2011; Weissman and Kornitzer, 2004). Rbt5 directly binds hemin and undergoes endocytosis into the acidic endosome. There, the protoporphyrin rings are degraded by heme oxygenase, and the released iron is transported into the cytoplasm by a vacuolar iron-permease and utilized (Weissman et al., 2008). However, for the following reasons, our results do not support a role for *A. fumigatus CfmA-C* in hemin uptake: (i) There are no differences between the ability of the ΔTriple-*Cfm* mutant and wild-type strains to internalize the fluorescently labeled heme analog ZnMp, (ii) in the presence of exogenously added hemin, both strains are equally susceptible towards the heme biosynthesis inhibitor sampangine, (iii) recombinant *C. albicans* Rbt5 and Rbt51 CFEM domains bind tightly to hemin agarose beads (Weissman and Kornitzer, 2004), whereas CfmA-CFEM does not. This suggests that, unlike *C. albicans* and *C. parapsilosis* that employ a dedicated CFEM-dependent heme-uptake system, *A. fumigatus* depends on secreted siderophores and siderophore iron transporters (SITs) to directly strip ferric iron from hemin and transport the ferri-siderophore into the cell. Our results showing a lack of involvement of the *A. fumigatus* CFEM genes in biofilm formation and heme uptake, unlike in *C. albicans* can be explained by the totally different niches each of these organisms has evolved to exploit. *C. albicans* is a commensal human pathogen that has evolved complex mechanisms to attach to its host and to effectively utilize the unique nutritional sources, such as hemin, found in its environment. In contrast, *A. fumigatus* is an environmental saprophyte with generalist capabilities that enable it to adhere to diverse substrates and extract iron from numerous sources.

Despite their clear connection to heme–iron uptake, CFEM-encoding genes from *C. albicans* and *C. parapsilosis* have not been implicated in affecting virulence. Deletion of *RBT5* does not result in reduced virulence, although this could be due to redundancy with *RBT51* and *PGA7*. The results of our *in vivo* studies imply that the *A. fumigatus CfmA-C* genes are not involved in virulence. The ΔTriple-*Cfm* mutant was as virulent as the parental *AkuB^{KU80}* strain in both an insect and two murine models of invasive aspergillosis.

In summary, a detailed and comprehensive phenotypic analysis of an *A. fumigatus* mutant lacking all three CFEM-containing genes indicates that *CfmA-C* do not appear to be involved in biofilm formation or hemin uptake but do play a role in cell-wall integrity possibly by stabilizing the formation of polysaccharide fibers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.12.005.

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