

Disruption of the *Aspergillus fumigatus* ECM33 homologue results in rapid conidial germination, antifungal resistance and hypervirulence

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The ECM33/SPS2 family of glycosylphosphatidylinositol-anchored proteins plays an important role in maintaining fungal cell wall integrity and virulence. However, the precise molecular role of these proteins is unknown. In this work, *AfuEcm33*, the gene encoding the ECM33 homologue in the important pathogenic fungus *Aspergillus fumigatus*, has been cloned and its function analysed. It is shown that disruption of *AfuEcm33* results in rapid conidial germination, increased cell–cell adhesion, resistance to the antifungal agent caspofungin and increased virulence in an immunocompromised mouse model for disseminated aspergillosis. These results suggest that the protein encoded by *AfuEcm33* is involved in key aspects of cell wall morphogenesis and plays an important role in *A. fumigatus* virulence.

INTRODUCTION

Aspergillus fumigatus is the most common opportunistic mould pathogen of humans, causing invasive diseases in immunocompromised patients (Latgé, 1999). Invasive pulmonary aspergillosis is caused by inhalation of *A. fumigatus* spores and growth of the fungus inside the lungs, which often spreads from the initial site of infection to attack various organs in the body. It is the leading cause of death in leukaemia and bone-marrow-transplant patients, with between 10 and 20% of patients contracting this disease. Left untreated, mortality rates from this disease are extremely high (>90%), and even following aggressive antifungal treatment, fatality rates of 50 to 70% are common (Steinbach & Stevens, 2003). Therefore, there is an urgent need for a deeper understanding at the molecular level of the interaction of *A. fumigatus* with the infected host.

The fungal cell wall plays a crucial role in infection. In *A. fumigatus*, as in other pathogenic fungi, the cell wall is in continuous contact with the host, acting as a reservoir for displayed and secreted antigens and enzymes. The cell wall protects the fungus and interacts directly with the host immune system. It is an elastic, dynamic and highly regulated structure, and is essential for growth, viability and infection. The cell wall of *A. fumigatus* is composed of a

polysaccharide skeleton interlaced and coated with cell wall proteins (CWPs). The main building blocks of the polysaccharide skeleton are an interconnected network of glucan, chitin and galactomannan polymers (Latgé *et al.*, 2005). The cell wall of *A. fumigatus* differs considerably from that of yeast such as *Saccharomyces cerevisiae* and *Candida albicans*. It contains a much higher level of chitin and unique polysaccharides such as 1,3- α -glucan, (1,3)(1,4)- β -glucan and galactomannan (Latgé *et al.*, 2005).

The major class of fungal CWPs is the glycosylphosphatidylinositol (GPI)-modified proteins (de Groot *et al.*, 2003; Eisenhaber *et al.*, 2004). They contain an N-terminal hydrophobic signal peptide sequence that targets them to the endoplasmic reticulum (ER), and a C-terminal hydrophobic domain that is cleaved off in the ER and replaced with a GPI anchor. The GPI anchor directs the attachment of these proteins to the plasma membrane. Subsequently, the GPI anchor may be processed and attached to 1,6- β -glucan in the cell wall. ECM33/SPS2-family proteins have the typical features of GPI-anchored proteins, with a signal peptide, a serine and threonine-rich region and a potential C-terminal domain for GPI-anchor attachment (Percival-Smith & Segall, 1987; Caro *et al.*, 1997; Tougan *et al.*, 2002; Terashima *et al.*, 2003). They play an important role in fungal cell wall organization. Deletion of *S. cerevisiae* ECM33 results in a weakened and disorganized cell wall, defects in glycosylation, and activation of the cell wall integrity pathway (Pardo *et al.*, 2004). The *C. albicans* CaEcm33 protein is required for normal cell wall architecture and expression of cell-surface proteins. *CaEcm33*-deleted mutants display a

Abbreviations: CWP, cell wall protein; DDW, double-distilled water; GPI, glycosylphosphatidylinositol.

Figures showing sequence alignments and a phylogenetic tree are available as supplementary data with the online version of this paper.

reduced ability to invade and damage epithelial cells, and show decreased virulence in a murine model of systemic candidosis (Martinez-Lopez *et al.*, 2004, 2006).

Proteomic analysis of *A. fumigatus* membrane preparations identified nine GPI-anchored proteins, including the protein encoded by *AfuEcm33* (*Afu4g06820*), the homologue of *S. cerevisiae* *ECM33* (Bruneau *et al.*, 2001). In light of the importance of the ECM33/SPS2-family proteins in cell wall organization and virulence, we have undertaken the characterization of the *A. fumigatus* *ECM33* homologue, *AfuEcm33* (*Afu4g06820*). This is believed to be the first time that such a characterization has been undertaken in a pathogenic filamentous fungus. Interestingly, we show that disruption of *AfuEcm33* in *A. fumigatus* results in rapid conidial germination, increased cell–cell adhesion, resistance to the antifungal caspofungin and increased virulence in an immunocompromised mouse model for disseminated aspergillosis.

METHODS

Strains and culture conditions. *A. fumigatus* strain AF293, originally isolated at autopsy from a patient with invasive pulmonary aspergillosis, and *A. fumigatus* strain AF293.1, a derivative of the AF293 strain (Pain *et al.*, 2004), were used throughout this study. The AF293.1 strain is deficient in *pyrG* (encoding OMP-decarboxylase) and, consequently, is auxotrophic for uridine/uracil (Oshero *et al.*, 2001). AF293.1 was grown on YAG UU medium, which consists of 0.5% (w/v) yeast extract, 1% (w/v) glucose, 10 mM MgCl₂, 10 mM uracil and 5 mM uridine, supplemented with trace elements, vitamins and 1.5% (w/v) agar when needed (Bainbridge, 1971). For phenotypic analysis, mutants were grown on minimal medium (MM) containing 70 mM NaNO₃, 1% glucose, 12 mM potassium phosphate pH 6.8, 4 mM MgSO₄, 7 mM KCl, trace elements and 1.5% agar (for MM agar plates). Conidia were harvested in 0.2% (v/v) Tween 80, resuspended in double-distilled water (DDW) and counted with a haemocytometer. *Escherichia coli* strain DH10B (Invitrogen) was used to clone and replicate the genes in the pGEM-T/A and pGPS1/*pyr4* transposon-containing vectors (Jadoun *et al.*, 2004).

RNA analysis. AF293 and disrupted strains *AfuEcm33-D1–4* were grown for the indicated time in liquid MM at 37 °C. Total RNA was extracted by the ‘hot SDS’ protocol (May & Morris, 1988). Northern blot analysis was performed as described previously (Oshero *et al.*, 2002). Briefly, 5 µg fungal total RNA was run on a 1% (w/v) agarose gel under denaturing conditions, transferred to a Nytran N

nylon membrane (Schleicher & Schuell) and hybridized with an [α -³²P]dCTP radiolabelled *AfuEcm33* full-length probe at 47 °C. For RT-PCR, total RNA was treated with DNase (Ambion) according to the manufacturer’s instructions. The RNA concentration was assessed and 3 µg were taken for the RT reaction using PowerScript reverse transcriptase (Clontech). PCR was performed using ReddyMix PCR master mix (ABgene) with the following designed primer pairs (Table 1): *AfuEcm33* forward and reverse primers were used to identify the *AfuEcm33* transcript, and *AfugpdA* forward and reverse primers were used as a loading control. The PCR was carried out according to the manufacturer’s instructions. PCR products were analysed by gel electrophoresis.

Construction and verification of the *A. fumigatus* *AfuEcm33* disruption mutant. Disruption of *AfuEcm33* was performed using an *in vitro* transposon-based mutagenesis approach as described previously (Jadoun *et al.*, 2004). Briefly, this approach utilizes a modified transposon containing the *Neurospora crassa* *pyr4* gene, which is randomly inserted *in vitro* into a target sequence of interest. Clones in which the gene of interest has been disrupted are identified by PCR and used to transform a *pyrG*-deficient strain of *A. fumigatus*. Primers were designed to contain an *AscI* restriction site at their 5’ end (Table 1) and generated a ~4 kb fragment using the Expand high-fidelity PCR system (Roche Diagnostics). The primer pairs used to amplify the fragments for the *AfuEcm33* gene were *AfuEcm33*+*AscI* site forward and *AfuEcm33*+*AscI* site reverse (Table 1). *A. fumigatus* AF293 CsCl₂-purified genomic DNA (1 µg per reaction) was used as a template in the reactions. PCR conditions were as recommended by the manufacturer. The PCR fragment produced was gel purified by the Wizard SV gel and clean-up system (Promega), and cloned into the pGEM T/A cloning vector (Promega), according to the manufacturer’s instructions, to obtain pGEM-*AfuEcm33*. Inactivation of the *AfuEcm33* gene was performed using the GPS-1 genome priming system (New England Biolabs) with the modified GPS-1/*pyr4* transposon (Jadoun *et al.*, 2004) according to the manufacturer’s instructions. Clones carrying transposon-disrupted genes were identified by PCR using *AfuEcm33* forward and reverse primers (Table 1). Sequencing indicated that the transposon inserted 624 bp downstream of the *AfuEcm33* gene start codon. The gene fragment with the transprimer 1-*pyr4* disruption, and its flanking sequence, was released by cleavage with *AscI*, purified with the Wizard SV gel and clean-up system and used to transform *A. fumigatus* strain AF293.1 (Jadoun *et al.*, 2004). Rapid initial screening for positive disrupted mutants was performed by PCR amplification of crude conidial genomic DNA. Conidia from transformed colonies were collected and transferred to 500 µl DDW to give a conidial concentration of ~10⁷ conidia ml⁻¹. The tubes were snap frozen in liquid nitrogen for 10 min, heated at 95 °C for 5 min and used for PCR with the *AfuEcm33* forward and reverse primers (Table 1). Amplification of the housekeeping *gpdA* gene was used as a positive control. Colonies negative for gene amplification

Table 1. Primers

Name of primer	Sequence	Product length (bp)
<i>AfuEcm33</i> forward	5'-CCATTGAGATCGACGAGCACCT-3'	
<i>AfuEcm33</i> reverse	5'-CATGCTGATGTTAGTGAGCTTGCT-3'	635
<i>AfugpdA</i> forward	5'-TCTCCAACGTTCTTGCACC-3'	
<i>AfugpdA</i> reverse	5'-CCACTCGTTGTGCTACCAGG-3'	600
<i>AfuEcm33</i> + <i>AscI</i> site forward*	5'-ATGGCGCGCCCCGTAGTCCAGGTGATGAC-3'	
<i>AfuEcm33</i> + <i>AscI</i> site reverse*	5'-ATGGCGCGCCGACCGTTCGGTCTTGTA-3'	4037

*Italics indicate the *AscI* restriction site included in the primer sequence.

but positive for *gpdA* amplification were chosen for further analysis. For further Southern blot analysis, *A. fumigatus* genomic DNA was extracted with a MasterPure yeast DNA purification kit (Epicenter), with modifications for *A. fumigatus* as described by Jin *et al.* (2004). Southern hybridization analysis was performed as described previously (Jadoun *et al.*, 2004). Briefly, 10 µg fungal genomic DNA were digested with *Hind*III and run on a 1% (w/v) agarose gel. The cleaved DNA was transferred to a Nytran N nylon membrane (Schleicher & Schuell) and hybridized with an α^{32} [dCTP] radio-labelled *N. crassa pyr4* probe at 65 °C. The AF293.1 *pyr4* complemented strain was obtained by transforming the auxotrophic AF293.1 strain with the *N. crassa pyr4* gene. The *AfuEcm33 KI* strain was prepared by complementing the *AfuEcm33-D1* strain with plasmid pGEM-AfuEcm33 containing the pTrpC-*hyg* cassette (Punt *et al.*, 1987). Three *AfuEcm33 KI* strains were obtained and verified for *AfuEcm33* mRNA expression by RT-PCR. All three strains were phenotypically identical to the control wild-type strain AF293 as assessed by clumping, aerosolization and germination experiments (data not shown).

Phenotypic analysis of the *AfuEcm33* disrupted mutant

Growth assay. *A. fumigatus* AF293 and disrupted *AfuEcm33* mutant isolates were grown at a concentration of 10^4 conidia ml⁻¹ in MM without glucose supplemented with 1% (w/v) raffinose, sucrose, glycerol, ethanol or BSA as sole carbon sources. For growth experiments at 37 °C and 42 °C, MM containing 1% glucose (w/v) was used. For growth analysis at different pH values, conidia were grown in MM buffered to pH 6 and pH 9 with 50 mM BIS or citrate buffers, respectively. For analysis of growth at reduced osmolarity, conidia were grown in MM containing 7 mM NaNO₃, 0.4 mM MgSO₄ and 0.7 mM KCl. For growth experiments in a high osmolarity environment, MM containing 1.0 M NaCl was used.

Qualitative assay for conidial clumping and aerosolization. For assessment of conidial clumping, freshly harvested *A. fumigatus* AF293 and disrupted *AfuEcm33* mutant conidia grown for 3 days on YAG agar plates were harvested in 0.2% (v/v) Tween 80, resuspended in DDW and counted with a haemocytometer. A total of 2×10^8 conidia ml⁻¹ were transferred to a sterile glass tube, vortexed for 30 s, allowed to stand for 15 min and photographed. For qualitative assessment of conidial aerosolization, strains were grown on YAG agar plates for 3 days. During harvesting of conidia, four sterile YAG agar plates were placed uncovered around and immediately adjacent to the harvested plate. The four plates were subsequently incubated for 2 days at 37 °C to allow growth of aerosolized conidia. The extent of colonization, correlating to the degree of conidial aerosolization during harvesting, was assessed visually.

Microscopic analysis. Conidia at a concentration of 10^3 conidia ml⁻¹ were grown in 1 ml liquid MM on glass disks in a stationary 24-well plate at 37 °C. Before microscopic examination, the conidia were stained with 8 µl Fluorescent Brightener 28 (0.5 µg ml⁻¹) (Sigma-Aldrich) and observed under an Olympus IX50 fluorescent microscope at a magnification of $\times 400$. Hyphal growth rate and germination studies were performed by plating 10^3 – 10^5 freshly harvested spores ml⁻¹ onto 96-well plates in 200 µl liquid YAG at 37 °C. At various time points, growth was observed under a grid-mounted Olympus CK inverted microscope at a magnification of $\times 200$. The percentage of germinated conidia ($n=200$) was assessed, and the lengths of the germlings ($n=50$) were measured in microns.

Sensitivity to reagents and antifungals. *A. fumigatus* AF293 and disrupted *AfuEcm33* mutant isolates were grown in 96-well plates at a concentration of 10^4 conidia ml⁻¹ in MM supplemented with reagents and antifungals in 96-well plates. MICs (the lowest drug

concentrations to completely arrest germination and growth) were evaluated after 24 h incubation at 37 °C. Unless otherwise specified, all reagents were from Sigma-Aldrich. The concentration ranges of the reagents and antifungals were: Congo red 1–160 µg ml⁻¹; caspofungin (Merck) 1–160 µg ml⁻¹; Calcofluor white 10–320 µg ml⁻¹; hygromycin B 5–320 µg ml⁻¹; amphotericin B 1–80 µg ml⁻¹; itraconazole 0.25–8 µg ml⁻¹; tunicamycin 1–80 µg ml⁻¹; trifluoroperazine 10–160 µg ml⁻¹.

For sensitivity testing on agar plates, conidia from the mutant and AF293 wild-type strain (10^8 conidia ml⁻¹) were point inoculated on MM plates containing either 80 µg Congo red ml⁻¹ or 40 µg caspofungin ml⁻¹ or no drug control. The strains were grown for 48 h at 37 °C.

Murine model for systemic aspergillosis. Six-week-old female ICR mice were injected intraperitoneally with 200 mg cyclophosphamide kg⁻¹ at 3 days prior to conidial infection. Mice were inoculated intravenously via the tail vein with a 2.5×10^5 conidia per mouse inoculum of freshly harvested AF293 wild-type, *AfuEcm33-D1* disrupted, *AfuEcm33 KI* (complemented) or AF293.1 *pyr4* (*pyr4* complemented) conidia. To prolong neutropenia, additional cyclophosphamide (70 mg kg⁻¹) was administered 2 and 5 days after infection. Systemic aspergillosis was followed up for 30 days. Statistical analysis of mouse survival was performed with GraphPad Prism 4 software (GraphPad Software). *P* values of <0.05 were considered significant in this analysis. Animal studies were performed in accordance with Tel-Aviv University institutional policies.

RESULTS

Primary sequence analysis of *AfuEcm33p*

The *AfuEcm33* gene is 1300 nucleotides in length and contains two introns (at positions 61–131 bp and 972–1029 bp). *AfuEcm33p* (gene product of *Afu4g06820*, TIGR assembly) is 398 amino acids in length, rich in serine/threonine amino acid residues (21%), and contains a predicted signal peptide sequence at its N-terminus and a GPI-anchor sequence at its C-terminus. The ω -site of *AfuEcm33p* is predicted to be at sequence position ASN372, with the typical consensus of two alanines ($\omega + 1$, $\omega + 2$) and serine ($\omega - 1$) (Eisenhaber *et al.*, 2004). The most likely signal peptide cleavage site is predicted to be between ALA19 and ALA20. Based on multiple sequence alignment, *AfuEcm33p* has significant sequence identity to *Aspergillus nidulans* hypothetical protein AN4390.2 (60% identity in 398 amino acids), *Magnaporthe grisea* Ecm33-like AAX07654 (36% identity in 326 amino acids), *N. crassa* Sps2-like CAD70996 (32% identity in 333 amino acids), *C. albicans* CaEcm33p (31% identity in 318 amino acids), *S. cerevisiae* Ecm33p (31% identity in 319 amino acids) and *S. cerevisiae* Sps2p (28% identity in 304 amino acids). A search of the *A. fumigatus* database yielded a single additional predicted protein sequence with a low but significant degree of similarity to *AfuEcm33p*, the gene product of *Afu6g10290* (29% identity in 141 amino acids). *Afu6g10290* exhibits significant similarity to *S. cerevisiae* PstIp (31% identity in 172 amino acids, probability value 2×10^{-7}), a protein secreted by regenerating protoplasts and a member of the ECM33/SPS2 family of proteins (Pardo *et al.*, 1999).

A complete evolutionary analysis of *AfuEcm33p* using 25 sequence homologues with the ConSeq web server (<http://conseq.bioinfo.tau.ac.il/>) (Berezin *et al.*, 2004) revealed a significant conservation of large amino acid blocks between the *Aspergillus* species, and a low conservation towards other fungal species (see the supplementary figures available with the online journal). This is characteristic of serine/threonine-rich CWP containing large numbers of nucleotide repeat units.

We showed by RT-PCR, using primers *AfuEcm33* forward and reverse (Table 1), that *AfuEcm33* is not significantly expressed in dormant conidia, but is expressed throughout germination and hyphal growth (Fig. 1).

Disruption of *AfuEcm33* results in abnormal clumping, reduced aerosolization and rapid germination of conidia

To investigate the effect of loss of function of the *AfuEcm33* gene in *A. fumigatus*, a disruption plasmid (p*AfuEcm33-D*) was constructed by transposon mutagenesis as described in Methods (Fig. 2a). After transformation of p*AfuEcm33-D* into AF293.1, 20 *pyrG*⁺ transformants were purified and screened by PCR for putative insertion mutants. Four putative mutants were identified and further characterized by Southern blotting (Fig. 2b). Based on this analysis, all four transformants were disrupted in the *AfuEcm33* gene alone (*AfuEcm33-D1-4*). The indication based on Northern blot analysis is that a truncated *AfuEcm33* mRNA is expressed in all four disrupted strains (~1.4 kb in size as compared to 2.5 kb in the wild-type, Fig. 2c). RT-PCR indicated that this mRNA contains the first 624 bp of *AfuEcm33*, a short portion of the transposon, but not the 676 bp of coding sequence downstream of the transposon (our unpublished results). Although it cannot be formally ruled out that a truncated form of *AfuEcm33p* protein is expressed, this form would lack the C-terminal GPI-anchor motif targeting it to the cell membrane, and therefore it is highly unlikely that it would remain functional (Terashima

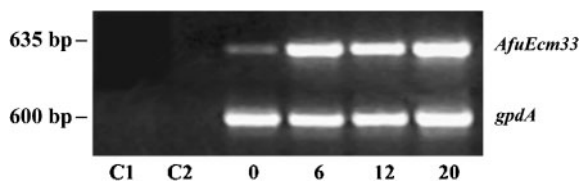


Fig. 1. Expression of the *A. fumigatus* *AfuEcm33* (*Afu4g06820*) gene. Expression was determined by RT-PCR using primers *AfuEcm33* forward and reverse (Table 1). Dormant conidia (0 h) were incubated for 6, 12 and 20 h in MM at 37 °C, after which total RNA was isolated and used in RT-PCR. Control reactions: C1, PCR was performed on RNA following DNase treatment; C2, no DNA control. RT-PCR with primers for *gpdA*, a housekeeping gene, was performed as a control for the loading (lower bands).

et al., 2003). However, to formally rule out the possibility that a phenotype is resulting from expression of a truncated form of *AfuEcm33p* protein, complete gene deletion mutants are being generated and their phenotype will be compared to that of the disrupted mutants described in this report.

Preliminary experiments conducted with the four independent disrupted strains confirmed that all of them exhibited the mutant phenotype, characterized by clumping, aerosolization and rapid germination. This suggests that the mutant phenotype is associated with disruption of the gene and is not due to an unlinked mutation resulting from transformation. The four strains displayed the mutant phenotype in both the absence and presence of uracil/uridine, indicating that the mutant phenotype is not a result of positional silencing of the *pyr4* marker (Greenstein *et al.*, 2006). A representative strain, *AfuEcm33-D1* was chosen for further characterization. The *AfuEcm33-D1* mutant strain grew normally on rich YAG medium or defined MM agar plates, indicating that the *AfuEcm33* gene is not essential for growth (Fig. 3a). Freshly harvested *AfuEcm33*-disrupted conidia formed large aggregates in DDW, suggesting an increase in cell–cell adhesive properties (Fig. 3b). *AfuEcm33*-disrupted conidia showed a greatly decreased ability to aerosolize during harvesting. Sterile YAG agar plates left uncovered in the direct vicinity of harvested plates showed very high levels of colonization by conidia from wild-type plates as compared to plates containing the mutant *AfuEcm33-D1* strain (Fig. 3c). Microscopic analysis of freshly harvested conidia indicated that there is no statistically significant difference in size or shape between the mutant and wild-type conidia (AF293 conidial diameter 3.2 ± 0.5 microns, *AfuEcm33-D1* conidial diameter 4 ± 0.6 microns, $n=100$). Microscopic analysis in liquid MM demonstrated that the *AfuEcm33-D1* strain germinates earlier than the wild-type strain and forms large ‘star-shaped’ clusters of germinating conidia, in which the conidial cell bodies adhere to each other and the germ tubes grow outwards, suggestive of an alteration in cell–cell adherence (Fig. 3d). The time needed for 50% of conidia to germinate was 3 h 45 min for the mutant *AfuEcm33-D1* strain and 6 h 15 min for the AF293 control wild-type strain (Fig. 3e). However, the growth rates of the AF293 wild-type and mutant *AfuEcm33-D1* strain were similar (compare the slopes in Fig. 3e). After 12 to 24 h there was no significant difference in hyphal length, colony size or morphology of the *AfuEcm33-D1* strain compared to the wild-type AF293 parental strain.

The *AfuEcm33-D1* mutant is resistant to Congo red and caspofungin

We tested the effects of different sole carbon sources (raffinose, sorbitol, glucose, glycerol, ethanol, casein and albumin), osmolarity, temperature (24 and 42 °C), pH (6.0 and 9.0), and various compounds and drugs, including caffeine, trifluoperazine, hygromycin B, Calcofluor white, SDS, amphotericin B and itraconazole on the growth of the

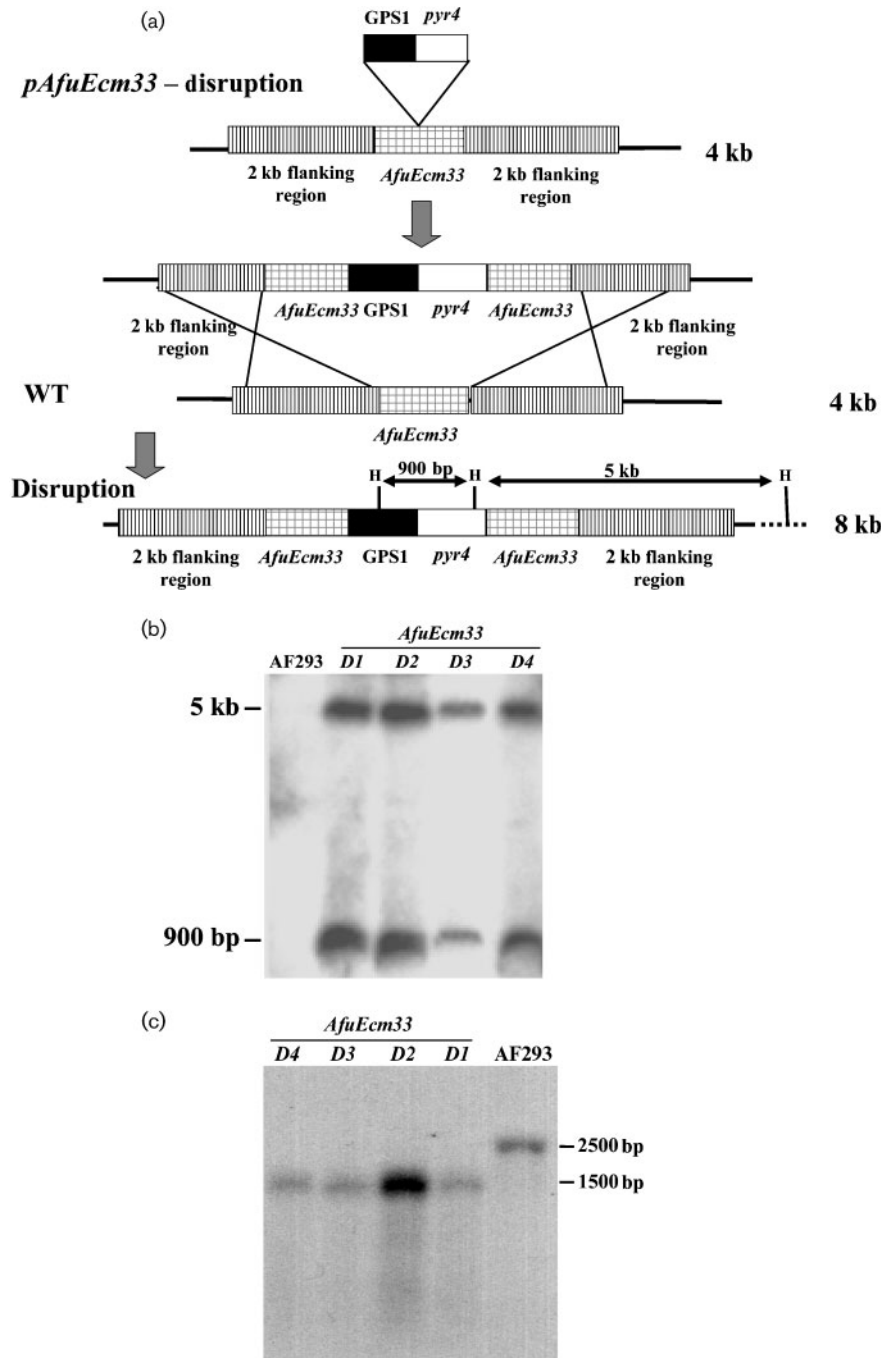
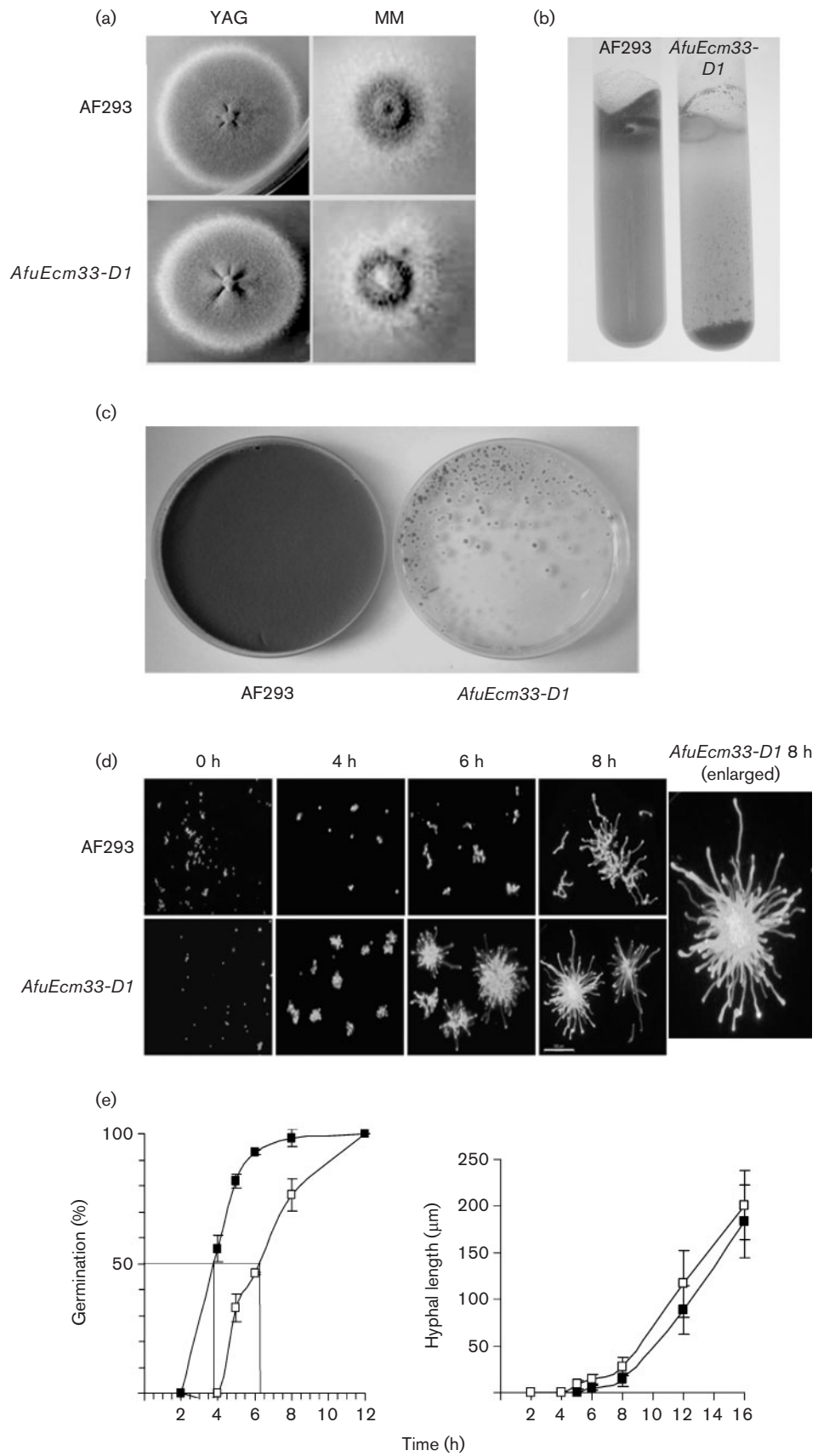


Fig. 2. Disruption of the *AfuEcm33* gene in *A. fumigatus*: (a) schematic representation of the *AfuEcm33* wild-type locus and the plasmid *pAfuEcm33-K/O* *Ascl*-cut insert used for disruption, (b) Southern blot and (c) Northern blot verification of the *AfuEcm33*-D1-4 isolates and control AF293 wild-type strain. For the Southern blot analysis, genomic DNA (10 µg per well) was digested with *Hind*III, blotted and hybridized with a ³²P-labelled *pyr4* DNA probe, resulting in ~0.9 kb and 5 kb fragments for the *AfuEcm33* disrupted strains, and no hybridization with the AF293 control strain. For the Northern blot analysis, total RNA (5 µg per well) was hybridized with a ³²P-labelled *AfuEcm33* DNA probe. H, *Hind*III site.

AfuEcm33-D1 strain in comparison to the wild-type AF293 strain (see Methods). The analysis was performed on MM agar plates and in liquid culture. Freshly harvested conidia

were inoculated under the various conditions and grown for 24 h at 37 °C. We found no appreciable growth differences between the mutant and wild-type strains. In contrast,



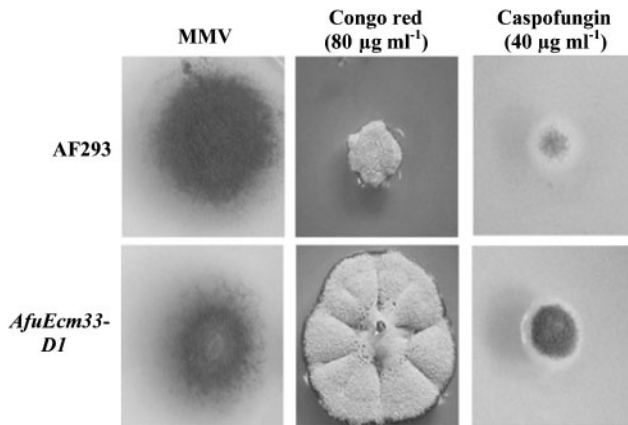


Fig. 4. The *AfuEcm33-D1* mutant is resistant to the cell wall-stabilizing agents Congo red and caspofungin. Conidia from the mutant and the AF293 wild-type strain were point inoculated on MM plates containing either 80 µg Congo red ml⁻¹ or 40 µg caspofungin ml⁻¹ or no drug (control). The strains were grown for 48 h at 37 °C.

growth tests conducted with the cell wall-disrupting agents Congo red or caspofungin indicated that the *AfuEcm33-D1* strain is more resistant to these compounds (Fig. 4). The MIC of both agents was eightfold higher in the *AfuEcm33-D1* strain as compared to the AF293 control strain and was specific for these agents alone (Table 2). This result suggests that changes in the composition of the cell wall in the *AfuEcm33-D1* strain may render it more resistant to damage by these agents.

Disruption of *AfuEcm33* increases virulence

To determine whether the protein encoded by *AfuEcm33* is involved in pathogenicity, we tested the *AfuEcm33-D1* and *AfuEcm33-D2* disrupted strains for virulence in an immunocompromised murine model for disseminated aspergillosis. As a control we included AF293, the parental *A. fumigatus* strain, *AfuEcm33 KI*, an *AfuEcm33* complemented strain and AF293.1 *pyr4*, a derivative of AF293.1 that is complemented with the *N. crassa pyr4* gene (see Methods). Freshly harvested conidia were carefully counted and adjusted to the same density (2.5×10^5 conidia per mouse) before injection into the lateral tail vein. The number of mice alive in each group was recorded every day over a 30 day study period. Fig. 5 shows survival curves

Table 2. MIC values (µg ml⁻¹) for *AfuEcm33-D1* and the AF293 wild-type strain incubated for 24 h with Congo red (CR), caspofungin (CAS), Calcofluor white (CF), itraconazole (ITZ), amphotericin B (AMB), tunicamycin (TNM) and hygromycin B (HygB)

	CR	CAS	CF	ITZ	AMB	TNM	HygB
AF293	10	10	160	0.5	5	40	20
<i>AfuEcm33-D1</i>	80	80	80	0.5	5	40	40

obtained during the course of the experiment. By day 7, all 14 mice injected with the *AfuEcm33-D1* ($n=10$) and *AfuEcm33-D2* ($n=4$) disrupted strains had died [mean survival time (MST) = 4.1 days]. In contrast, 60 % of the mice injected with the wild-type AF293 strain or AF293.1 *pyr4* strains were still alive after 30 days (MST = 21 days, $n=10$) while 30 % of mice infected with the *AfuEcm33 KI* strain remained alive after 30 days (MST = 13.2 days, $n=10$). Statistical analysis of these data by the Wilcoxon rank sum test showed a highly significant survival difference

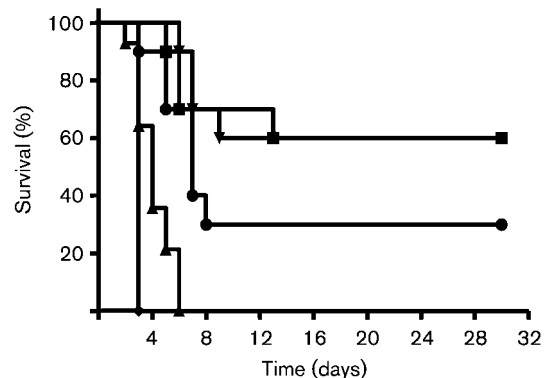


Fig. 5. The *AfuEcm33* disrupted mutant shows increased virulence in a murine model of invasive aspergillosis. Survival curves are shown of mice intravenously injected with an inoculum of 2.5×10^5 conidia per mouse of the wild-type AF293 strain (■) ($n=10$ mice), *AfuEcm33* complemented strain *AfuEcm33 KI* (●) ($n=10$), *pyr4* complemented AF293.1 *pyr4* (▼) ($n=10$), *AfuEcm33-D1* (▲) ($n=10$ mice) or *AfuEcm33-D2* (◆) ($n=4$ mice) disrupted strains. Percentage survival was monitored daily over the 30 day study period.

Fig. 3. Phenotypic characterization of the *AfuEcm33-D1* mutant. (a) Conidia were point inoculated on solid rich YAG and MM agar plates and incubated for 24 h at 37 °C. (b) Abnormal clumping of *AfuEcm33* mutant conidia (right tube) compared to the wild-type strain (left tube) was found after harvesting and resuspending conidia in DDW. (c) Aerosolization was reduced for the *AfuEcm33* mutant conidia (right plate) compared to the wild-type strain (left plate) after harvesting (see Methods). A representative result of three independent experiments is shown. (d) *AfuEcm33-D1* mutant germination and early growth in liquid MM was analysed by microscope. Note the early germination and cell-cell clumping in the mutant strain (lower panel) as compared to the control AF293 wild-type strain (bar, 100 µm). (e) A quantitative analysis of percentage mutant germination and hyphal growth was carried out; each time point was calculated as the mean \pm SD of 200 conidia or 50 hyphae, respectively. ■, AF293; □, *AfuEcm33-D1*.

between the mouse groups infected with the wild-type AF293, control AF293.1 *pyr4*, *AfuEcm33* *KI* strains and the *AfuEcm33* disrupted *A. fumigatus* strains ($P < 0.0005$), supporting a conclusion that the *AfuEcm33* disrupted strains exhibit hypervirulence in the tested mouse model. The survival difference between the wild-type AF293 strain and control *AfuEcm33* *KI* strain was not statistically significant ($P = 0.19$).

DISCUSSION

In this report, we have described the disruption of the *A. fumigatus* *ECM33* homologue, *AfuEcm33*, and the phenotypic analysis of the mutant. We chose to study this gene because (i) deletion of *S. cerevisiae* *ECM33* and *C. albicans* *CaECM33* suggested that the proteins they encode play a complex but as yet poorly understood role in the maintenance of cell wall integrity, fungal morphogenesis and virulence (Martinez-Lopez *et al.*, 2004; Pardo *et al.*, 2004), (ii) biochemical studies had already identified *AfuEcm33p* as a genuine GPI-anchored protein in *A. fumigatus* (Bruneau *et al.*, 2001) and (iii) *ECM33*-family proteins have not been previously characterized in the filamentous fungi.

AfuEcm33 is involved in conidial adherence and morphogenesis

Disruption of the *AfuEcm33* gene in *A. fumigatus* resulted in subtle and unusual morphological changes, including rapid germination and conidial clustering during harvesting and germination. *C. albicans* *CaECM33*-deleted cells also exhibit a marked tendency to flocculate (cluster) extensively (Martinez-Lopez *et al.*, 2004). We hypothesize that cell wall alteration in the *AfuEcm33* disrupted mutant may lead to greater exposure of the cell-surface proteins involved in adherence, leading to increased cell clustering. However, the *AfuEcm33* disrupted strains exhibited no substantial differences in adherence to polystyrene (a measure of changes in cell wall hydrophobicity), laminin (a component of the extracellular matrix) or A549 lung-cell extracellular matrix as compared to the wild-type AF293 strain using either dormant or germinating conidia (our unpublished observations). This indicates that the increased cell-cell clustering we observed in the mutant is distinct from its ability to interact with the matrix.

The precocious germination of the *AfuEcm33* disrupted mutant is intriguing. *S. cerevisiae* *ECM33* deleted cells exhibit marked disorganization of the cell wall, and in particular the mannoprotein outer layer (Pardo *et al.*, 2004). Disruption of *MEU10*, an *ECM33*/*SPS2*-like gene in *Schizosaccharomyces pombe* results in the formation of fragile spores containing a weakened cell wall (Tougan *et al.*, 2002). Perhaps disruption of *AfuEcm33* leads to the formation of a softer, more pliable cell wall, enabling germination to proceed more rapidly.

Disruption of *AfuEcm33* leads to increased resistance to cell wall-disrupting agents

Surprisingly, we found that the *AfuEcm33* disrupted strain is more resistant to the cell wall-disrupting agents Congo red and caspofungin (Fig. 4). This result is in contrast to that found in *S. cerevisiae* and *C. albicans* *ECM33* deletion mutants, which display increased sensitivity to Congo red and Calcofluor white (Martinez-Lopez *et al.*, 2004; Pardo *et al.*, 2004). This could be a consequence of the considerable differences in polymer organization and cell wall content between *S. cerevisiae* or *C. albicans* and *A. fumigatus* (Bernard & Latgé, 2001; Latgé *et al.*, 2005). Resistance to the antifungal drug caspofungin typically results from mutations in its target, (1,3)- β -glucan synthase (Kartsonis *et al.*, 2003). Our work is believed to be the first to demonstrate a connection between caspofungin resistance in *A. fumigatus* and the loss of function of a defined gene. A plausible explanation for our finding is that disruption of *AfuEcm33* may lead to increased levels of chitin and (1,3)- α -glucan in the cell wall, thereby compensating for the reduced synthesis of (1,3)- β -glucan by glucan synthase (Reinoso-Martin *et al.*, 2003).

The *AfuEcm33* disrupted *A. fumigatus* mutant is hypervirulent in a murine model of disseminated aspergillosis

We demonstrate that disruption of *AfuEcm33* in *A. fumigatus* leads to hypervirulence in a mouse model of disseminated aspergillosis. This is in contrast to the results obtained using the *C. albicans* *CaECM33* disrupted mutant, which exhibited decreased virulence in a similar model system. A number of mechanisms may explain the increased virulence of the *AfuEcm33* mutant, including physical occlusion of blood vessels by clumps of conidia, enhanced resistance to phagocytes due to rapid germination and hyphal growth, or hyperstimulation of the immune system, leading to septic shock. Very few examples of fungal hypervirulence resulting from gene knockout have been described to date. Partial silencing of the *A. fumigatus* *AfppoA*, *AfppoB* and *AfppoC* genes encoding fatty acid dioxygenases resulted in increased resistance to oxidative stress and hypervirulence in mice (Tsitsigiannis *et al.*, 2005). It was proposed that Ppo reaction products (prostaglandins and other oxylipins) may serve as activators of host immune defences. A decrease in prostaglandin production by the *AfppoA-C* silenced mutant might lead to a weaker host response resulting in hypervirulence. Disruption of the *Candida glabrata* *ACE2* gene encoding a transcription factor and the *S. cerevisiae* *SSD1* gene encoding a CWP of unknown function leads to hypervirulence in mice by inducing severe septic shock in infected animals (Wheeler *et al.*, 2003; Kamran *et al.*, 2004). This has been tentatively attributed to an increase in the exposure of fungal cell-surface antigens, which hyperstimulate the immune system of the infected animals (Wheeler *et al.*, 2003). We are now performing experiments to determine whether similar mechanisms can explain the hypervirulence of the *AfuEcm33* disrupted mutant.

In summary, our findings suggest that the *A. fumigatus* *AfuEcm33* gene is involved in key aspects of cell wall architecture. The increased conidial aggregation, precocious germination, resistance to cell wall-destabilizing drugs and increased virulence resulting from the disruption of *AfuEcm33* suggest that significant changes in the cell wall have occurred. Further elucidation of the mechanisms responsible for these changes may shed new light on the pathogenesis of *A. fumigatus* at the molecular level.

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