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# The *mtfA* Transcription Factor Gene Controls Morphogenesis, Gliotoxin Production, and Virulence in the Opportunistic Human Pathogen *Aspergillus fumigatus*

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***Aspergillus fumigatus* is the leading causative agent of invasive aspergillosis (IA). The number of cases is on the rise, with mortality rates as high as 90% among immunocompromised patients. Molecular genetic studies in *A. fumigatus* could provide novel targets to potentially set the basis for antifungal therapies. In the current study, we investigated the role of the transcription factor gene *mtfA* in *A. fumigatus*. Our results revealed that *mtfA* plays a role in the growth and development of the fungus. Deletion or overexpression of *mtfA* leads to a slight reduction in colony growth, as well as a reduction in conidiation levels, in the overexpression strain compared to the wild-type strain. Furthermore, production of the secondary metabolite gliotoxin increased when *mtfA* was overexpressed, coinciding with an increase in the transcription levels of the gliotoxin genes *gliZ* and *gliP* with respect to the wild type. In addition, our study showed that *mtfA* is also necessary for normal protease activity in *A. fumigatus*; deletion of *mtfA* resulted in a reduction of protease activity compared to wild-type levels. Importantly, the absence of *mtfA* caused a decrease in virulence in the *Galleria mellonella* infection model, indicating that *mtfA* is necessary for *A. fumigatus* wild-type pathogenesis.**

*Aspergillus fumigatus* is a ubiquitous saprotrophic fungus that is also an opportunistic human pathogen. Inhalation of *A. fumigatus* conidia can cause allergic bronchopulmonary aspergillosis, an allergic response to the spores that occurs in hypersensitive patients (1, 2). In immunodepressed patients *A. fumigatus* is the leading causative agent of invasive aspergillosis (IA). This group of patients includes those infected with HIV, cancer patients undergoing chemotherapy, organ transplant patients, individuals with genetic immunodeficiencies, and patients with hematological malignancies (3–8). A number of factors are contributing to the increasing proportion of the population affected by this disease (9), for example, the rise in HIV cases, more advanced technology to perform organ transplants, and more effective therapies for cancer patients and autoimmune diseases (3, 9–11). Once the fungal infection has been established, IA has a mortality rate ranging from 40 to 90% in these patients.

The primary route of infection by *A. fumigatus* is inhalation. The small size (2.5 to 3.0 μm) of the asexual spores, called conidia, allows them to reach the lung alveoli (12). The first point of contact is with the bronchial epithelial cells (1). These cells have dectin-1 receptors that can recognize the β-1,3-glucan on the cell surfaces of conidia (1, 13, 14), activating the production of reactive oxygen species (ROS) and of antimicrobial peptides and cytokines as part of the initial immune response. In healthy individuals, conidia that are able to evade mucociliary clearance are quickly removed by either alveolar macrophages or epithelial cells via phagocytosis. A proinflammatory response also includes the recruiting neutrophils that have the capability to eliminate hyphae, preventing further fungal colonization. The identification of genetic mechanisms that regulate *A. fumigatus* conidiation could contribute to strategies to decrease the primary source of the fungal inoculum.

Fungal morphological development, such as conidiation, and secondary metabolism have been shown to be genetically linked (15–18). *A. fumigatus* possesses the capacity to produce a large range of secondary metabolites, also called natural products, which are important for environmental fitness (15, 19). Some of these secondary metabolites are virulence factors during *A. fumigatus* infection. The best known is gliotoxin, which has been shown to inhibit phagocytosis by macrophages and to kill neutrophils (20–34).

Knowledge of genetic links connecting morphogenesis and biosynthesis of natural products in *A. fumigatus* is still limited. The most studied genes are those encoding components of the global regulatory *velvet* protein complex (17, 18, 35), in which VeA might act as a scaffold. Previously reported work from our laboratory has shown that *A. fumigatus veA* plays a role in growth and development, as well as in regulating protease activity (17). Furthermore, we found that *veA* regulates the expression of secondary-metabolite gene clusters and concomitant biosynthesis of associated compounds, such as gliotoxin, fumagillin, fumitremorgin G, and fumigaclavine C (17, 35).

Components of the conserved *velvet* complex, particularly VeA, have been characterized in greatest depth in the model fungus *Aspergillus nidulans* (15–18, 36–39). Recently, in a mutagenesis screening, we identified *mtfA*, a *veA*-dependent gene encoding a novel $C_2H_2$ finger domain transcription factor in this model organism (40). *A. nidulans mtfA* regulates both morphological development and secondary metabolism. Deletion of *A. nidulans mtfA* decreases conidiation and sexual development (40). Fur-

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**TABLE 1** Fungal strains used in the study

| Strain name | Pertinent genotype | Source |
|---|---|---|
| CEA10 | Wild type | Gift from Robert Cramer |
| CEA17 | *pyrG1* | Gift from Robert Cramer |
| tTDS1 | *pyrG1 gpdA*(p)::*mtfA*::*trpC*(t)::*pyrG*$^{A.fum}$ | This study |
| tTDS4.1 | *pyrG1* Δ*mtfA*::*pyrG*$^{A.para}$ | This study |
| tTDS10 | *pyrG1* Δ*mtfA*::*pyrG*$^{A.para}$ *mtfA*::*ptrA* | This study |
| tTDS5.1 | *pyrG1 mtfA*::*gfp*::*pyrG*$^{A.fum}$ | This study |

**TABLE 2** Primers used in the study

| Primer name | Sequence (5′→3′) |
|---|---|
| fumRM75p1 | GTGACAGGTAGAATATTCTGTCTCCAGAATCC |
| fumRM75p2 | GGTGTTTGCGCGAAAGTATGCA |
| fumRM73p3 | ACTGTGACTTTGTTTTGAACAACACAACCT |
| fumRM73p4 | TGCGGAGTATGACCATGCCAT |
| Afumparapyrf2 | CGGGCCGTTGCATACTTTCGCGCAAACACC GGATCCTATGGATCTCAGACAAT ATACC |
| Afumparapyrr2 | AGGTTGTGTTGTTCAAAACAAAGTCACAGTG TCGACATCACCCTTACCCAAAC |
| fumRM7OEp1 | AAAAAGGCGCGCCATGGATGTCGCAAGCCT CATCTC |
| fumRM7OEp2 | AAAAAAAAAAAAGCGGCCGCCTATTGGACC ATGGCGGTAGC |
| AfummtfAcompF | AAAAAAAAAAAGCGGCCGCCGGACAAGAC AGATCGAAGCAGCC |
| AfumRm7Cp2 | AAAAAAAAAAAGCGGCCGCCGTTTGCTGTT GCAATGGT |
| AfumRM7midF | ATGGATGTCGCAAGCCTCATCTCGCCT |
| AfumRM7rev | TTGGACCATGGCGGTAGCAACTGGGC |
| AfumRM7gfpLink | TTGGACCATGGCGGTAGCAACTGGGCGGAG CTGGTGCAGGCGCTGGAGCC |
| AfumRM7qrtPCRF | GCTTCGCCTGCTCCCAGCGTATCG |
| AfumRM7qrtPCRR | GTTGATGACCCGGGGAGGGAAGAG |
| 18s_qRTPCRF_849 | TAGTCGGGGGCGTCAGTATTCAGC |
| 18s_qRTPCRR_850 | GTAAGGTGCCGAGCGGGTCATCAT |
| brlA_qRTPCRF_1158 | TGCACCAATATCCGCCAATGC |
| brlA_qRTPCRR_1159 | CGTGTAGGAAGGAGGAGGGGTTACC |
| gliP_qRTPCR_F840 | AGTTACACCGACTCGCATCCAGCTGC |
| gliP_qRTPCR_R841 | CTGGGGCAGACCATGCGTAG |
| gliZ_qRTPCR_F838 | ACGACGATGAGGAATCGAACCCG |
| gliZ_qRTPCR_R839 | GGTGCTCCAGAAAAGGGAGTCGTTG |
| gdpApromoF | AAGTACTTTGCTACATCCATACTCC |
| Afu_wetA_f | TTGACTCGCTGTCAAGTGATTGTGG |
| Afu_wetA_r | TGGTGGATTTGTGGTGGGGAGTT |

thermore, absence of *mtfA* also completely prevents the expression of genes involved in the production of the mycotoxin sterigmatocystin, the antitumoral compound terrequinone, and the beta-lactam antibiotic penicillin (40). Our previous studies also revealed that *mtfA* orthologs are present in many fungal species, including *A. fumigatus* (40). It is possible that *mtfA* could play similar roles in *A. fumigatus* that might affect virulence. In our current study, we characterized the role of this conserved master transcription factor gene in this opportunistic pathogen, particularly its roles in growth, conidiation, production of gliotoxin, and protease activity, as well as direct assessment of its role in pathogenicity.

## MATERIALS AND METHODS

**Strains and culture conditions.** The *A. fumigatus* strains used in this study are listed in Table 1. The strains were grown on Czapek-Dox medium (Difco) plus the necessary supplements (41) unless otherwise indicated. For solid medium, 10 g/liter of agar was added. Stocks were stored in 30% glycerol at −80°C.

**Generation of *mtfA* deletion, complementation, and overexpression mutant strains. (i) *mtfA* deletion strain.** The *mtfA* deletion (Δ*mtfA*) strain, tTDS4.1, was obtained by gene replacement using the *A. fumigatus* CEA17 strain, a *pyrG* auxotroph derived from the CEA10 isolate (Table 1). The deletion cassette was generated by fusion PCR as described by Szewczyk et al. (42). Briefly, 1.5-kb fragments corresponding to the *mtfA* 5′ and 3′ untranslated regions (UTRs) were PCR amplified from *A. fumigatus* genomic DNA using fumRM75p1/fumRM75p2, and fumRM73p3/fumRM73p4, respectively (Table 2). The intermediate fragment corresponding to *Aspergillus parasiticus* was PCR amplified from the genomic DNA of the fungus using primers Afumparapyrf2 and Afumparapyrr2 (Table 2). The three fragments were joined by fusion PCR using primers fumRM75p1 and fumRM73p4 (Table 2). Confirmation of double crossover and gene replacement was carried out by Southern blotting. quantitative reverse transcription (qRT)-PCR analysis was used to confirm the absence of *mtfA* expression in the deletion mutants (Δ*mtfA*) using primers AfumRM7qrtPCRF and AfumRM7qrtPCRR (Table 2). The resulting deletion strain was denoted tTDS4.1 (Table 1).

**(ii) Complementation strain.** A complementation strain was generated as follows. A 5.8-kb fragment containing the *mtfA* wild-type locus (including the *mtfA* coding region plus 3.3 kb of the 5′ UTR and 1.5 kb of the 3′ UTR) was PCR amplified with primers AfummtfAcompF and AfumRM7cp2 (Table 2). The primers contained engineered NotI sites to facilitate ligation to pTDS3, a PJET-based plasmid containing the fungal transformation marker *ptrA*. The fragment was digested with NotI and ligated into pTDS3 previously digested with the same enzyme to generate the complementation vector pTDS10. This plasmid was then transformed into the Δ*mtfA* strain, tTDS4.1 (Table 1). Integration of the plasmid was confirmed via PCR, and expression levels were evaluated using qRT-PCR. The resulting complementation strain was denoted tTDS10 (Table 1).

**(iii) *mtfA* overexpression strain.** The *mtfA* overexpression (OE*mtfA*) strain, tTDS1, was generated by transforming *A. fumigatus* CEA17 with the plasmid pTDS1. This overexpression plasmid was generated first by PCR amplification of the *mtfA* coding region from *A. fumigatus* genomic DNA with primers fumRM7OEp1 and fumRM7OEp2 (Table 2), containing AscI and NotI sites. Then, the fragment was digested with AscI and NotI and ligated into pSD21 (17) previously digested with the same enzymes. pSD21 contains the *A. nidulans gpdA* promoter, *gpdA*(p), the *A. nidulans trpC* terminator, *trpC*(t), and the *A. fumigatus pyrG* transformation selection marker. Integration of the overexpression vector into the genome was confirmed by PCR using primers gdpApromoF and fumRM7OEp2 (Table 2). Overexpression of *mtfA* was confirmed by qRT-PCR using primers AfumRM7qrtPCRF and AfumRM7qrtPCRR (Table 2).

**Morphological studies.** Fungal growth was evaluated as colony diameter. *A. fumigatus* wild-type (CEA10), Δ*mtfA*, complementation, and OE*mtfA* strains were point inoculated on Czapek-Dox medium and incubated at 37°C. The experiments were carried out with three replicates. The strains were grown for 5 days in the dark, and the colony diameter was measured.

To determine the role of *mtfA* in *A. fumigatus* conidiation, spores ($1 \times 10^7$ spores/ml) of *A. fumigatus* CEA10, Δ*mtfA*, complementation, and OE*mtfA* strains were inoculated in 25 ml of liquid Czapek-Dox medium. The cultures were grown at 37°C. At 72 h, the mycelial mats were collected and homogenized in 10 ml of distilled water ($dH_2O$). The conidia were counted using a hemacytometer under a light microscope.

**Fluorescence microscopy.** The subcellular localization of the *mtfA* gene product was determined in *A. fumigatus* strain tTDS5.1, where the

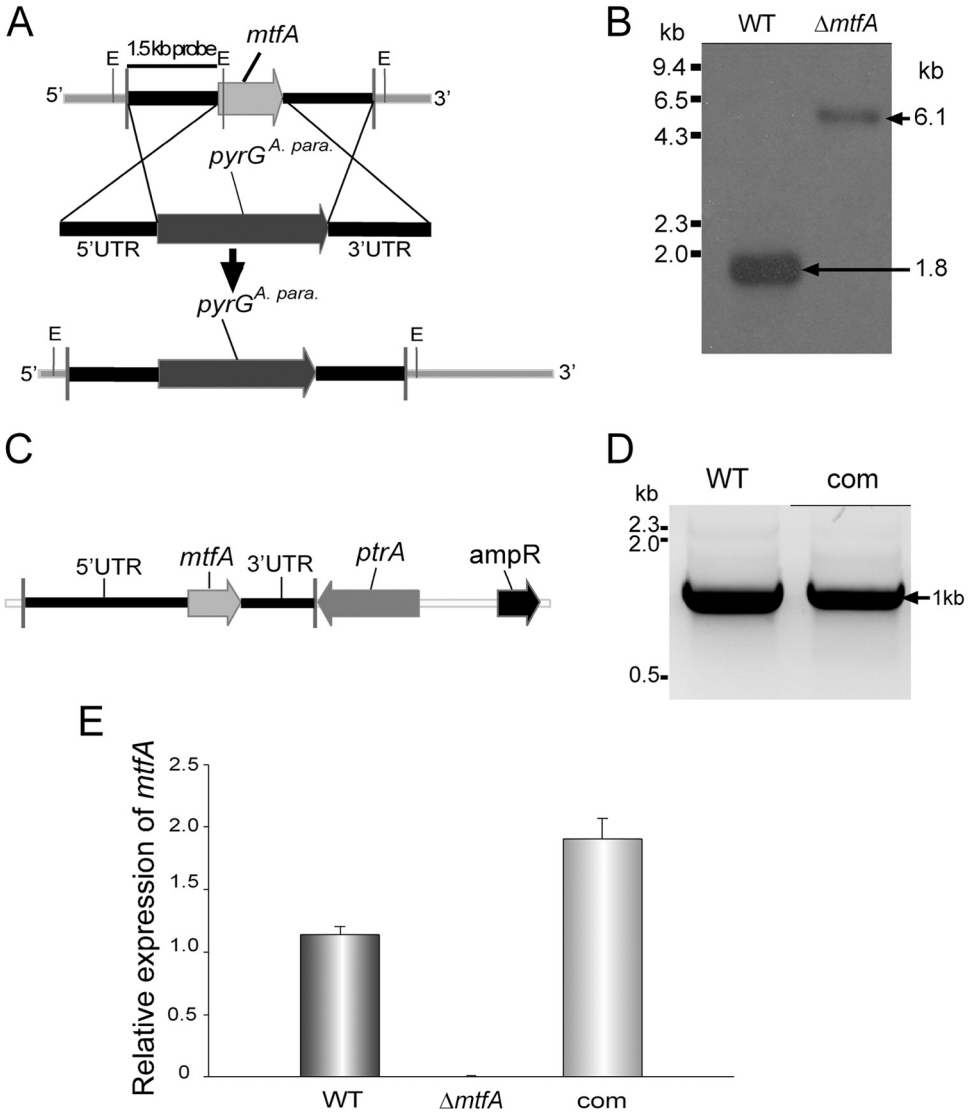


**FIG 1** Generation of *A. fumigatus mtfA* deletion and complementation strains. Confirmation of *mtfA* deletion (Δ*mtfA*) and complementation (com) strains by Southern blotting, PCR, and qRT-PCR. (A) Diagram showing the replacement of *mtfA* with the marker gene *pyrG* by a double-crossover event. EcoRI restrictions sites (E) and probe template are shown. (B) X-ray image showing the Southern blot results confirming the deletion of *mtfA*. Genomic DNA samples were digested with EcoRI. The expected band sizes were 1.8 kb (wild type [WT]) and 6.1 kb (Δ*mtfA*). (C) Linear representation of plasmid pTDS10 used for complementation. (D) PCR confirmation of the *mtfA* wild-type allele integration in the Δ*mtfA* genome. The wild-type strain was used as a control. The PCR yielded the predicted 1-kb PCR product. (E) qRT-PCR expression analysis of *mtfA* with primers AfumRM7qrtPCRF and AfumRM7qrtPCRR (Table 2). *mtfA* expression is recovered in the complementation strain. The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (70). Expression of 18S rRNA was used as an internal reference gene. Values were normalized to the expression levels in the wild type, considered 1. The error bars represent standard errors.

MtfA protein was tagged with the green fluorescent protein (GFP). This strain was generated by transforming CEA17 with an *mtfA*::*gfp*::*pyrG*$^{A.fum}$ cassette. The cassette was generated by fusion PCR as previously described by Szewczyk et al. (42). First, the 3′ end of the *mtfA* coding region was PCR amplified from genomic DNA with primers AfumRM7midF and AfumRM7rev (Table 2). The 3′ UTR of the *mtfA* gene was PCR amplified with primers fumTM73p3 and fumRM73p4, also from genomic DNA (Table 2). The plasmid p1439 (36) was used as the template to PCR amplify the intermediate DNA fragment containing *gfp*::*pyrG*$^{A.fum}$. The three fragments were fused using primers AfumRM7midF and fumRM73p4 (Table 2). Correct integration of the cassette into the genome was verified by PCR with primers fumRM75p1 and fumRM73p4 (Table 2).

Conidia from tTDS5.1 were inoculated on coverslips submerged in Watch minimal medium (43) and incubated at 37°C in both light and dark for 16 h. Then, the slides were washed with 1× phosphate-buffered saline (PBS) and stained for 5 min with DAPI (4′, 6-diamidino-2-phenylindole) (1:1,000) under gentle agitation. Samples were then viewed using a Nikon Eclipse E-600 microscope equipped with Nomarski optics and fluorochromes for GFP, with excitation at 470 nm and emission at 525 nm and ×600 magnification. Micrographs were obtained using a Hamamatsu Orca-ER high-sensitivity monochrome digital charge-coupled-device (CCD) camera with Microsuite 5 image capture and optimization software. The exposure time was 500 ms for DAPI images and 2 s for GFP images.

**Protease activity.** *A. fumigatus* wild-type, Δ*mtfA*, complementation, and OE*mtfA* strains were point inoculated on plates containing Czapek-

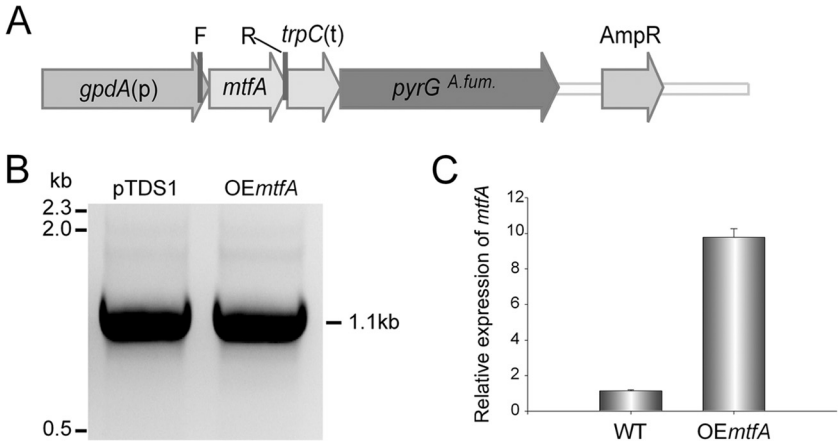


**FIG 2** Generation of the *mtfA* overexpression strain. The *mtfA* overexpression (OE*mtfA*) strain was confirmed by PCR and qRT-PCR analyses. (A) Linear representation of the overexpression plasmid pTDS1. The primers utilized for the diagnostic PCR are shown: F (gdpApromoF) and R (fumRM7OEp2) (Table 2). (B) PCR results, indicating integration of the overexpression plasmid into the genome, obtaining the expected 1.1-kb PCR product. pTDS1 was used as a positive control. (C) Expression of *mtfA* analyzed by qRT-PCR, showing greater accumulation of *mtfA* transcripts in the OE::*mtfA* strain than in the wild type. The qRT-PCR primers used were AfumRM7qrtPCRF and AfumRM7qrtPCRR (Table 2). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (70). The expression of 18S rRNA was used as an internal reference gene. Values were normalized to the expression levels in the wild type, considered 1. The error bars represent standard errors.

Dox medium (1% agar) and 5% skim milk (Difco). The plates were incubated at 37°C in the dark. After 3 days, the cultures were blended in 25 ml distilled water and collected in a 50-ml Falcon tube. The tubes were centrifuged at 3,500 rpm at 4°C, and 1 ml of supernatant was transferred to an Eppendorf tube, where it was centrifuged again at 10,000 rpm for 10 min at 4°C. An azocasein assay was performed as previously described by Reichard et al. (44) with slight modifications. One-hundred microliters of supernatant was mixed with 400 μl of azocasein (Sigma) at a concentration of 5 mg/ml; dissolved in 50 mM Tris buffer (pH 7.5), 0.2 M NaCl, 5 mM $CaCl_2$, 0.05% Brij 35, and 0.01% sodium azide; and incubated at 37°C for 90 min. One hundred and fifty microliters of 20% trichloroacetic acid was then added to stop the reaction, and the samples were left at room temperature for 30 min. The tubes were spun at 8,000 rpm for 3 min, and 500 μl of the supernatant was mixed with 500 μl 1 M NaOH. Two-hundred microliters from each sample was placed into a 96-well plate (BD Falcon) in duplicate, and the absorbance of the released azo group was read at 436 nm using a plate reader (Epoch by Biotek). A negative control was used with sterile distilled water mixed with azocasein.

In a separate experiment and to test for protease activity in cell-free culture supernatants, *A. fumigatus* CEA10, Δ*mtfA*, complementation, and OE*mtfA* strains were inoculated in 50 ml of liquid Czapek-Dox medium containing 2.5% skim milk (Difco). Cultures were grown at 37°C at 200 rpm. Culture supernatant was collected at 48 h and 72 h. The azocasein assay was performed on the culture supernatants as described above.

**Gliotoxin analysis.** Plates containing 25 ml of liquid Czapek-Dox medium were inoculated with $1 \times 10^7$ conidia/ml of *A. fumigatus* wild-type, Δ*mtfA*, complementation, and OE*mtfA* strains. Liquid stationary-phase cultures were incubated at 37°C in the dark. The supernatants were collected by filtration at 120 h after inoculation, using Miracloth (Calbiochem) to remove the mycelium. Fifteen milliliters of supernatant was collected from each plate. An equal amount of chloroform was added to extract the gliotoxin. The gliotoxin extracts were then resuspended in 200 μl of methanol and filtered through a 0.22-μm-pore-size membrane. Quantification of gliotoxin was performed by high-performance liquid chromatography (HPLC), as previously described by Cramer et al. (45) with some modifications. A Waters 1525 HPLC apparatus was used for the analysis. Injections of 20 μl of the methanol extract were applied. The flow rate was 1 ml/min, using a mobile phase of water-acetonitrile-trifluoroacetic acid (65:34.9:0.1). UV detection was performed at 264 nm (Waters 2487 dual λ absorbance detector). Gliotoxin peak areas were compared to those of standard gliotoxin samples of known concentrations (Sigma-Aldrich, St. Louis, MO).

**Gene expression analysis.** Conidia ($1 \times 10^7$ spores/ml) of *A. fumigatus* wild-type, Δ*mtfA*, complementation, and OE*mtfA* strains were inoculated in 25 ml of liquid Czapek-Dox medium and incubated in the dark at 37°C. Total RNA was collected at 72 h using Pig-B, as described by Weber and collaborators (46).

cDNA was generated from the extracted total RNA samples, and qRT-PCR was performed using an Mx3000p thermocycler (Agilent Technologies) with SYBR green Jumpstart *Taq* Ready mix (Sigma). Expression of *brlA*, *wetA*, *gliP*, and *gliZ* was examined. The PCR primers used to analyze the expression of these genes are listed in Table 2.

**Pathogenesis analysis using *G. mellonella* as an infection model.** Spore suspensions of *A. fumigatus* wild-type, Δ*mtfA*, complementation, and OE*mtfA* stains were generated in 1× PBS (with 0.1% Tween). The spore suspensions were quantified using a hemacytometer and diluted in 1× PBS to a concentration of $1 \times 10^6$ spores/10 μl. The infection procedure was done as previously described by Fuchs et al. (47). Briefly, *G. mellonella* larvae with a weight range between 275 and 300 mg and lacking gray markings were selected for the experiment. Groups of 30 larvae were selected for each *A. fumigatus* strain, and PBS injections and noninjected larvae were used as a control. The larvae were injected behind the last left proleg with $1 \times 10^6$ spores, using a Hamilton syringe. The larvae were transferred into a petri dish (90 mm by 15 mm) wrapped in aluminum foil. The plates were placed at 37°C in the dark. After 16 h, the larvae were checked every 2 h for mortality. Mortality curves were generated using PRISM software. Statistical analysis was performed using a log rank test to generate pairwise comparisons of the survival of the larvae infected with different strains.

## RESULTS

***mtfA* affects growth rate and conidiation in *A. fumigatus*.** In order to elucidate the role of *mtfA* (accession number XP_747808.1) in *A. fumigatus*, deletion (Δ*mtfA*), complementation, and overexpression (OE*mtfA*) strains were generated as specified in Materials and Methods. The strains were confirmed by Southern blotting or diagnostic PCR (Fig. 1 and 2). *mtfA* expression levels in wild-type, deletion, complementation, and overexpres-

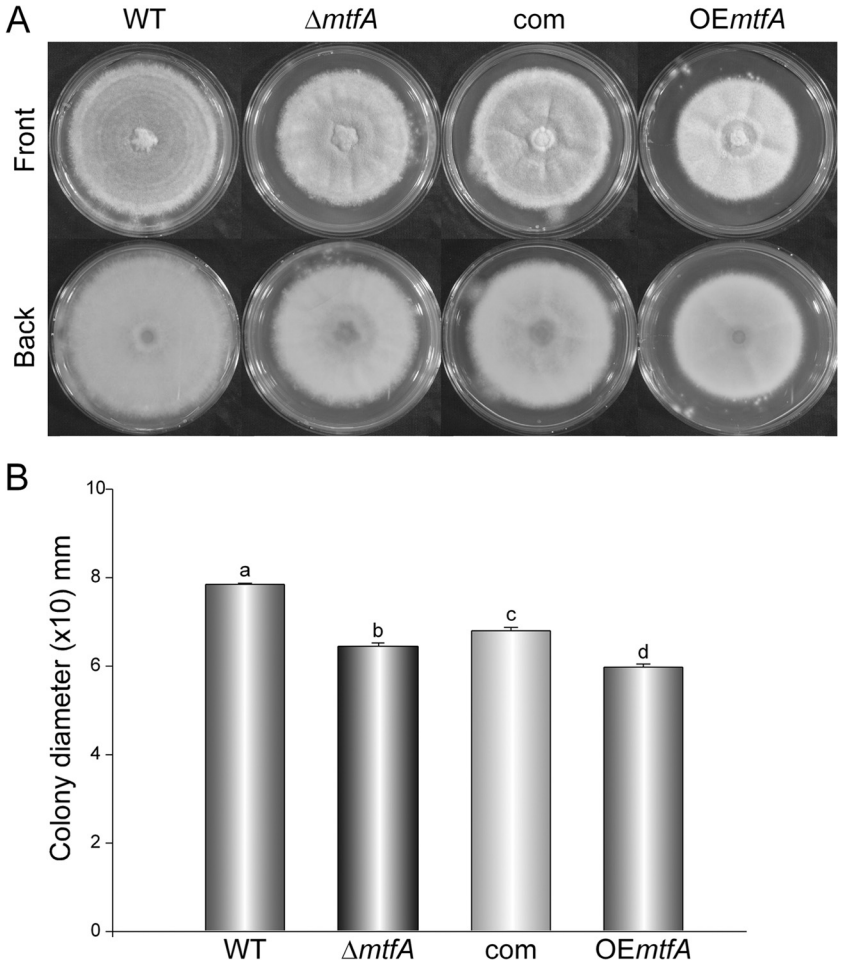


**FIG 3** Effect of *mtfA* on *A. fumigatus* colony growth. (A) *A. fumigatus* wild-type, Δ*mtfA*, complementation (com), and *mtfA* overexpression (OE*mtfA*) strains were point inoculated on solid Czapek-Dox medium, and the cultures were incubated at 37°C in the dark for 5 days. The experiment was carried out with three replicates. (B) Quantification of colony diameters in 5-day-old cultures. The error bars represent standard errors. Different letters above the bars represent significantly different values ($P \leq 0.05$).

sion analysis were also analyzed (Fig. 1E and 2C). As expected, the Δ*mtfA* strain did not show expression. Expression of *mtfA* in the OE*mtfA* strain was approximately 10 times greater than in the wild-type strain, indicating that the overexpression cassette was functional in the strain (Fig. 2).

Our results showed a slight reduction in colony growth in the Δ*mtfA* strain (18%) compared to the wild-type control (Fig. 3A and B). Complementation with the *mtfA* wild-type allele was able to partially recover radial growth. Overexpression of *mtfA* resulted in a more pronounced reduction in colony diameter (Fig. 3). The indicated differences in colony growth were statistically significant (Fig. 3).

In addition to its role in vegetative growth, our study revealed that *mtfA* also influences morphological differentiation. Overexpression of *mtfA* resulted in a significant reduction in conidiation in comparison to the wild type (68% reduction) (Fig. 4). Two genes in the asexual development pathway, *bristle A* (*brlA*), which encodes a known transcription factor essential for the initiation of conidiophore formation (48), and *wetA*, the last gene in this signaling pathway leading to conidiation, were both drastically downregulated in the overexpression strain (Fig. 4).

**MtfA localizes in the nucleus.** Our previous work showed that in *A. nidulans* the MtfA ortholog localizes in the nucleus. In the current study, we investigated whether the subcellular localization of MtfA is conserved in *A. fumigatus*. With this goal, a strain with *mtfA* fused to *gfp* was generated (Fig. 5A and B). A single copy of the fusion cassette was integrated at the *mtfA* locus. The resulting *mtfA*::*gfp* strain presented a wild-type phenotype (data not shown). Our results verified that MtfA also localizes in the nuclei when grown in the dark, as determined by comparison of the green fluorescent images with those from DAPI staining (Fig. 5C). A similar experiment carried out under white light yielded similar results, indicating that the localization of MtfA is not light dependent (see Fig. S1 in the supplemental material).

**MtfA acts as a positive regulator of protease activity in *A. fumigatus*.** The ability to secrete hydrolytic enzymes into its environment contributes to the success of *A. fumigatus* in colonizing its natural niches (49). Our results showed that *mtfA* acts as a positive regulator of protease production in *A. fumigatus*. The quantitative azocasein assay showed a significant decrease in protease activity in the deletion mutant colonies, with reduction of 50% compared to the wild type (Fig. 6). Furthermore, analysis of

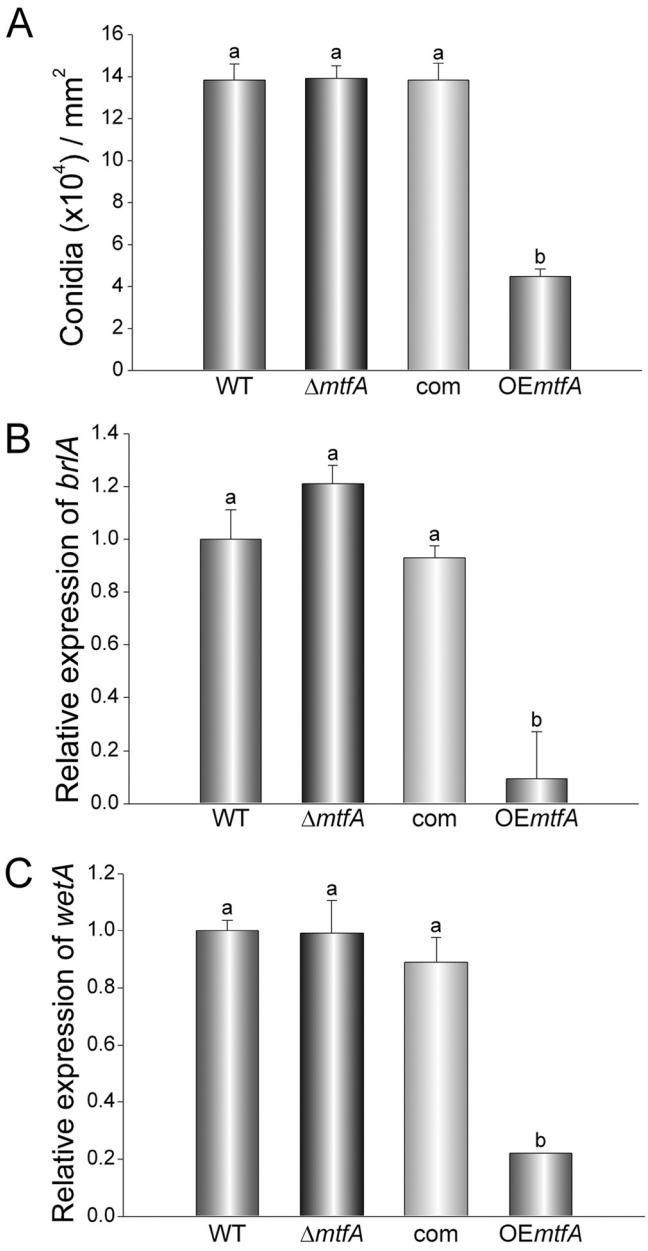


**FIG 4** Effect of *mtfA* on *A. fumigatus* asexual development. *A. fumigatus* wild-type, Δ*mtfA*, complementation (com), and *mtfA* overexpression (OE*mtfA*) strains were inoculated in Czapek-Dox liquid medium ($1 \times 10^7$ spores/ml), and the cultures were grown for 72 h at 37°C. (A) Mycelial mats were homogenized in water, and conidia were quantified using a hemacytometer. (B and C) Expression analysis of *brlA* (B) and *wetA* (C). The error bars represent standard errors. Different letters above the bars represent significantly different values ($P \leq 0.05$).

protease activity in culture supernatants showed a pattern similar to that observed in solid cultures, indicating a reduction in secreted protease activity in the absence of *mtfA* (see Fig. S2 in the supplemental material).

**Gliotoxin production is dependent on *mtfA*.** Gliotoxin is a secondary metabolite produced by *A. fumigatus* that has been shown to have immunosuppressive properties. The mycotoxin has been associated with pathogenicity and has been isolated from the lungs of infected mice (20–34). In the model fungus *A. nidulans*, it was shown that *mtfA* controlled the production of several secondary metabolites, such as penicillin and the mycotoxin sterigmatocystin (40). For this reason, we investigated whether *mtfA* controls gliotoxin production in *A. fumigatus*. Cultures of *A. fumigatus* wild-type, Δ*mtfA*, complementation, and OE*mtfA* strains were analyzed for gliotoxin content by HPLC after 120 h of incubation time. Our results indicated an increase in the production of the toxin in the OE*mtfA* strain (Fig. 7A). At 120 h, the overexpression strain showed a 4-fold increase in the production of gliotoxin.

The gene cluster responsible for the production of gliotoxin has been identified (50). This gene cluster includes a putative $Zn_2Cys_6$ binuclear transcription factor gene, *gliZ* (21), and a nonribosomal peptide synthase gene, *gliP* (45, 51–53), both necessary for gliotoxin production. We investigated whether *mtfA* affects gliotoxin production in *A. fumigatus* by regulating the transcription of *gliZ* and *gliP*. The overexpression mutant showed an increase in *gliZ* and *gliP* expression at 72 h (Fig. 7B and C). The increase in the transcription of both of these genes is concomitant with the increase in the production of gliotoxin observed in the OE*mtfA* strain.

***mtfA* is necessary for normal virulence in a *G. mellonella* infection model.** *G. mellonella* larvae are commonly used as a nonvertebrate host for virulence studies of fungal species (54–58). The insect presents both cellular and humoral responses to the invading fungal pathogen (59–62). Our pathogenicity assay indicated that absence of *mtfA* results in a decrease in virulence (Fig. 8). Thirty-three percent of the larvae infected with the Δ*mtfA* strain survived the infection at the end of the 30-h monitoring time. Overexpression of *mtfA* showed no differences in virulence with respect to the wild-type control.

## DISCUSSION

The filamentous fungus *A. fumigatus*, the leading causative agent of IA, efficiently disseminates by producing abundant conidia. The small size of the asexual spores allows them to reach the lung alveoli, resulting in devastating infections in immunosuppressed hosts (12, 63). Our current study revealed that conidiation in *A. fumigatus* is regulated by the *mtfA* gene. *mtfA* encodes a putative $C_2H_2$ zinc finger domain-type transcription factor first described in the model organism *A. nidulans* (40), where this master transcription factor gene was shown to regulate both development and secondary metabolism (40). Similarly to the case in *A. nidulans*, absence of *mtfA* in the opportunistic pathogen *A. fumigatus* resulted in a slight decrease in colony growth, and forced overexpression of *mtfA* resulted in a greater decrease in colony growth, along with severe reduction in conidiation. The decrease in conidial formation in the *mtfA* overexpression strain coincided with a significant decrease in the expression of *brlA* with respect to the wild-type strain. BrlA is another $C_2H_2$ zinc finger transcription factor that acts as a primary regulator of asexual development, controlling the developmental switch from vegetative growth to conidiophore formation (48, 64). Expression of the *wetA* gene is also downregulated in the *A. fumigatus mtfA* overexpression strain. WetA, also present during conidiogenesis, is required for the synthesis of a layer in the cell wall that results in impermeable conidia (65). Additionally, it is known that WetA also regulates the expression of additional genes expressed at a late stage in the formation of the asexual spores (65).

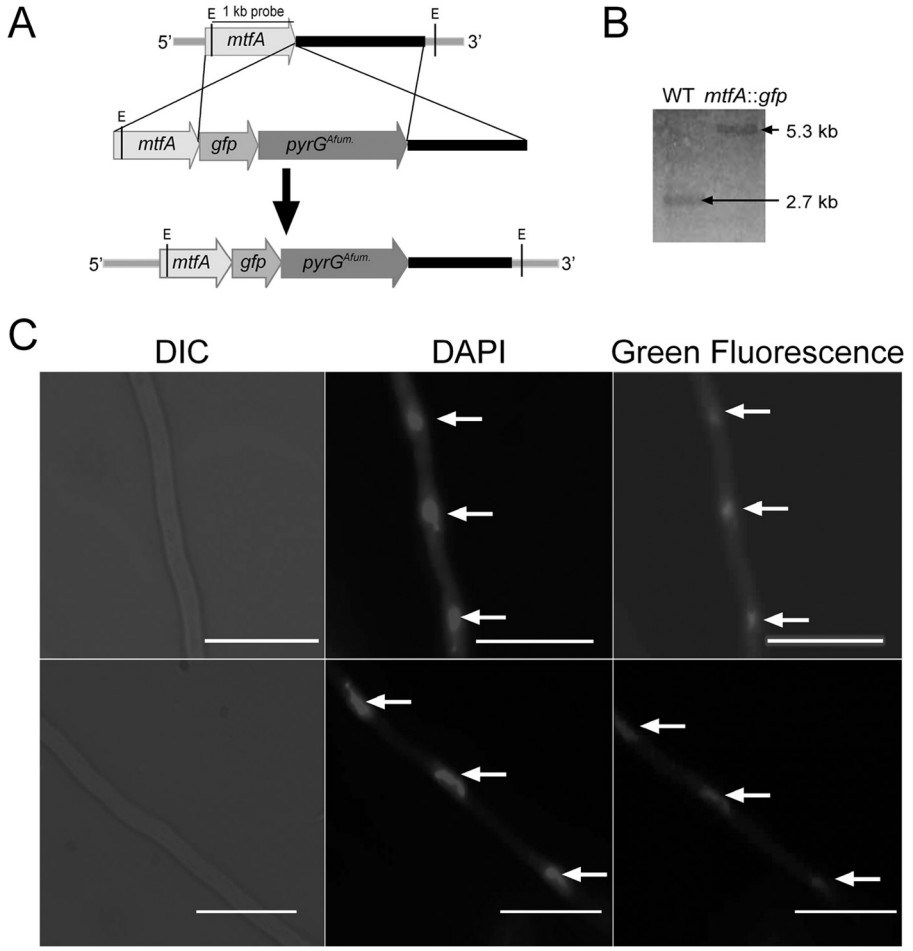


**FIG 5** Nuclear localization of *A. fumigatus* MtfA. (A) Diagram showing the strategy used to fuse *mtfA* with *gfp*. The tag was integrated into the *mtfA* locus via double-crossover events, using *pyrG* as the selection marker. (B) Southern analysis results confirming the integration of the *mtfA*::*gfp* cassette at the *mtfA* locus. Genomic DNA samples were digested with EcoRI. The expected bands corresponded to 2.7-kb (WT) and 5.3-kb (*mtfA*::*gfp*) DNA fragments. (C) Micrographs showing the nuclear localization of MtfA::GFP grown in the dark. From left to right are Nomarski images (differential interference contrast [DIC]), DAPI images, and green fluorescent images. The scale bars represent 20 μm. The arrows indicate nuclei.

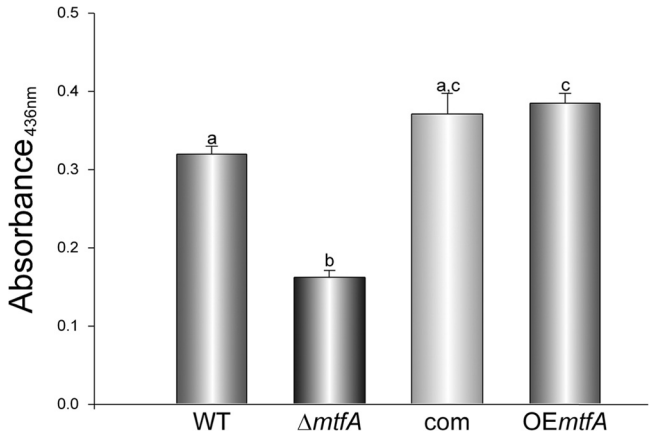


**FIG 6** Protease activity is controlled by *mtfA*. *A. fumigatus* wild-type, Δ*mtfA*, complementation (com), and *mtfA* overexpression (OE*mtfA*) strains were point inoculated on solid Czapek-Dox medium supplemented with 5% skim milk (Difco), and the cultures were incubated for 4 days at 37°C in the dark. Proteolytic activity was quantified by using an azocasein assay as described in Materials and Methods. The experiment was carried out with three replicates. Standard errors are shown. Different letters above the bars represent significantly different values ($P \leq 0.05$).

*A. fumigatus* MtfA was found to localize mainly in nuclei, similar to the case in *A. nidulans* (40), where *mtfA* was also shown to control the expression of biosynthetic genes involved in the production of secondary metabolites, some with beneficial properties, such as penicillin, and others detrimental, such as the mycotoxin sterigmatocystin (40). For this reason, we evaluated whether *A. fumigatus mtfA* influences the production of gliotoxin, a compound known for its negative impact on the immune system, including inhibition of phagocytosis and killing of neutrophils (20–34), as well as acting as an antioxidant (66), facilitating *A. fumigatus* infection in the lung (67). Overexpression of *A. fumigatus mtfA* resulted in an increase in gliotoxin production with respect to the control strains. Furthermore, our study indicated that expression of *gliZ*, encoding a putative $Zn_2Cys_6$ binuclear transcription factor that controls the expression of the gliotoxin gene cluster (21), and *gliP*, encoding a non-ribosomal peptide synthase essential for gliotoxin biosynthesis (45, 51–53), were upregulated when *mtfA* was overexpressed, suggesting that the observed increase in gliotoxin production could, at least in part, result from the *mtfA*-dependent increase in *gliZ* expression, leading to greater expression of gliotoxin biosynthetic genes. Based on our previous

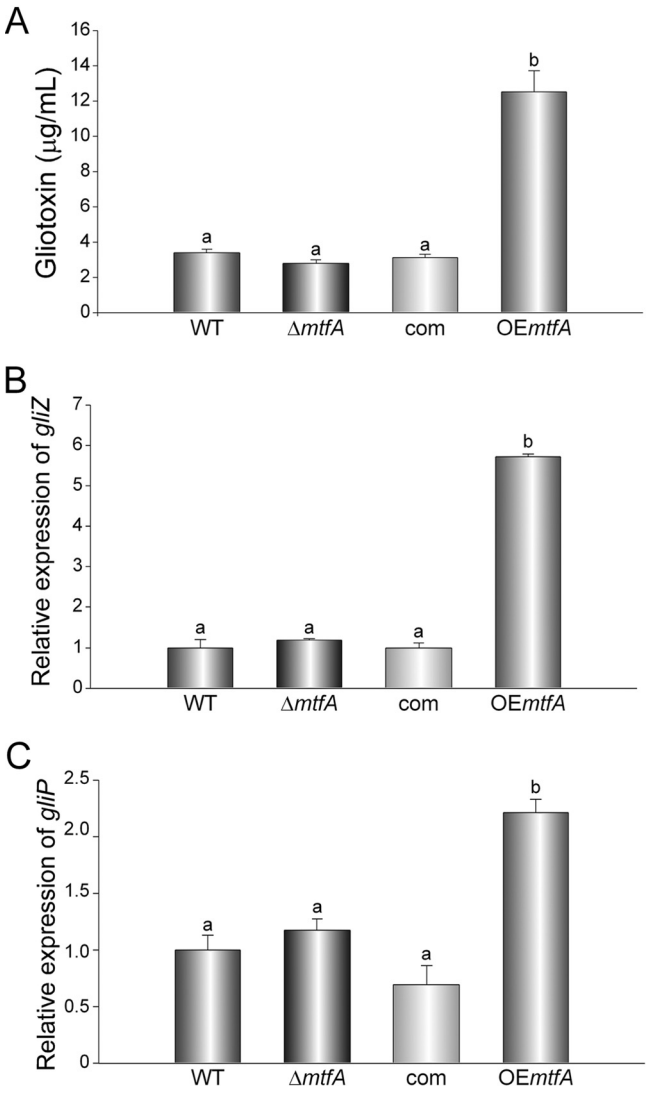


**FIG 7** Gliotoxin production is regulated by *mtfA*. *A. fumigatus* wild-type, Δ*mtfA*, complementation (com), and *mtfA* overexpression (OE*mtfA*) strains were grown in liquid Czapek-Dox stationary-phase cultures, and mycelial samples were collected at 72 h for RNA analysis. qRT-PCR was used to determine the expression levels of *gliZ* (A) and *gliP* (B) using primer pairs gliZ_qRTPCR_F838 and gliZ_qRTPCR_R839, and gliP_qRTPCR_F840 and gliP_qRTPCR_R841, respectively (Table 2). (C) Quantification of gliotoxin in filtrates from 120-h liquid Czapek-Dox stationary-phase cultures by HPLC. The bars represent the averages of three replicates. The error bars represent standard errors. Different letters above the bars represent significantly different values ($P \leq 0.05$).

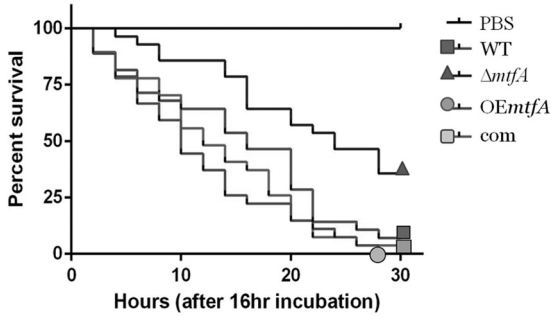


**FIG 8** Deletion of *mtfA* decreases virulence in a *Galleria* model of infection. Larvae of *G. mellonella* were infected with *A. fumigatus* wild-type, Δ*mtfA*, complementation (com), and *mtfA* overexpression (OE*mtfA*) strains. After a 16-h incubation period, survival was monitored every 2 h up to 30 h. Survival rates are shown. The experiments included 30 larvae per group. Statistical analysis was carried out by pairwise comparison using a log rank test.

studies in *A. nidulans*, it is possible that the regulatory scope of *mtfA* on *A. fumigatus* could be broader, including the biosynthesis of other natural products. Future studies in our laboratory will focus on elucidating whether *mtfA* regulates the biosynthesis of other secondary metabolites produced by the fungus.

In addition to fungal secondary metabolites, the activities of hydrolytic enzymes could also influence fungal infection. For example, an association between the production of fungal proteases and pathogenicity has been previously shown (49, 68). Secreted proteases could cause cytokine release, mucin secretion, and cell peeling (release of epithelial cells), which may contribute to increasing the ability of *A. fumigatus* to colonize the lung environment (1). Recently, our group also demonstrated that protease activity is regulated by *veA* in *A. fumigatus* (17) and other fungi, such as *Aspergillus flavus* (69). Due to the fact that in *A. nidulans* *mtfA* and *veA* are functionally connected (40), during the course of the present work we also examined the possible role of *mtfA* in regulating protease activity. Cultures of the Δ*mtfA* strain showed a decrease of protease activity compared to the wild type. These results provide evidence that *mtfA* is a positive regulator of protease activity in *A. fumigatus*. Additional experiments examining cell-free culture supernatants yielded similar results, with significant differences in the protease activity in the secreted proteins, depending on the presence or absence of *mtfA*.

Because *mtfA* plays a role in growth, conidiation, gliotoxin biosynthesis, and protease activity, it is possible that *mtfA* could also affect *A. fumigatus* pathogenesis. Using a *G. mellonella* infection model, we found that deletion of *mtfA* resulted in a decrease in the mortality rate. This indicates that *mtfA* is a virulence factor necessary to achieve normal pathogenesis levels in this commonly used infection system, showing potential as a promising target against *A. fumigatus* infection.

In conclusion, our studies revealed that *mtfA* affects several important cellular processes in the opportunistic pathogen *A. fumigatus*, such as growth and conidiation, the most important form of dissemination in this fungal species, affecting the expression of developmental genes. We also demonstrated that *mtfA* regulates the expression of gliotoxin genes, *gliZ* and *gliP*, and concomitant gliotoxin biosynthesis, as well as protease activity in the secretome. Importantly, deletion of *mtfA* resulted in a reduction in virulence in a *G. mellonella* infection model, suggesting that *mtfA* and *mtfA*-dependent factors may be used as possible genetic targets against invasive aspergillosis.

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## REFERENCES

1. **Osherov N.** 2012. Interaction of the pathogenic mold fungus *Aspergillus fumigatus* with lung epithelial cells. Front. Microbiol. **3**:346. http://dx.doi.org/10.3389/fmicb.2012.00346.
2. **Knutsen AP, Slavin RG.** 2011. Allergic bronchopulmonary aspergillosis

in asthma and cystic fibrosis. Clin. Dev. Immunol. **2011:**843763. http://dx.doi.org/10.1155/2011/843763.
3. **Denning DW.** 1998. Invasive aspergillosis. Clin. Infect. Dis. **26:**781–803. http://dx.doi.org/10.1086/513943.
4. **Kliasova GA, Petrova NA, Parovichnikova EN, Gotman LN, Isaev VG, Mikhailova EA, Ustinova EN, Khoroshko ND, Vishnevskaia ES, Kremenetskaia AM, Kravchenko SK, Kaplanskaia IB, Kokhno AA, Ptitsin SA, Liubimova LS, Mendeleeva LP, Mitish NE, Galstian GM, Ryzhko W, Tochenov AV, Savchenko VG.** 2005. Invasive pulmonary aspergillosis. Ter. Ark. **77:**65–71.
5. **Marr KA, Carter RA, Boeckh M, Martin P, Corey L.** 2002. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood **100:**4358–4366. http://dx.doi.org/10.1182/blood-2002-05-1496.
6. **Pagano L, Girmenia C, Mele L, Ricci P, Tosti ME, Nosari A, Buelli M, Picardi M, Allione B, Corvatta L, D'Antonio D, Montillo M, Melillo L, Chierichini A, Cenacchi A, Tonso A, Cudillo L, Candoni A, Savignano C, Bonini A, Martino P, Del Favero A.** 2001. Infections caused by filamentous fungi in patients with hematologic malignancies. A report of 391 cases by GIMEMA Infection Program. Haematologica **86:**862–870.
7. **Post MJ, Lass-Floerl C, Gastl G, Nachbaur D.** 2007. Invasive fungal infections in allogeneic and autologous stem cell transplant recipients: a single-center study of 166 transplanted patients. Transpl. Infect. Dis. **9:**189–195. http://dx.doi.org/10.1111/j.1399-3062.2007.00219.x.
8. **Wiederhold NP, Lewis RE.** 2003. Invasive aspergillosis in patients with hematologic malignancies. Pharmacotherapy **23:**1592–1610. http://dx.doi.org/10.1592/phco.23.15.1592.31965.
9. **Latge JP.** 1999. *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. **12:**310–350.
10. **Hohl TM, Feldmesser M.** 2007. *Aspergillus fumigatus*: principles of pathogenesis and host defense. Eukaryot. Cell **6:**1953–1963. http://dx.doi.org/10.1128/EC.00274-07.
11. **Sherif R, Segal BH.** 2010. Pulmonary aspergillosis: clinical presentation, diagnostic tests, management and complications. Curr. Opin. Pulm. Med. **16:**242–250. http://dx.doi.org/10.1097/MCP.0b013e328337d6de.
12. **Ruchel R, Reichard U.** 1999. Pathogenesis and clinical presentation of aspergillosis. Contrib. Microbiol. **2:**21–43. http://dx.doi.org/10.1159/000060302.
13. **Cunha C, Di Ianni M, Bozza S, Giovannini G, Zagarella S, Zelante T, D'Angelo C, Pierini A, Pitzurra L, Falzetti F, Carotti A, Perruccio K, Latge JP, Rodrigues F, Velardi A, Aversa F, Romani L, Carvalho A.** 2010. Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. Blood **116:**5394–5402. http://dx.doi.org/10.1182/blood-2010-04-279307.
14. **Sun WK, Lu X, Li X, Sun QY, Su X, Song Y, Sun HM, Shi Y.** 6 May 2012. Dectin-1 is inducible and plays a crucial role in Aspergillus-induced innate immune responses in human bronchial epithelial cells. Eur. J. Clin. Microbiol. Infect. Dis. http://dx.doi.org/10.1007/s10096-012-1624-8.
15. **Calvo AM, Wilson RA, Bok JW, Keller NP.** 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66:**447–459. http://dx.doi.org/10.1128/MMBR.66.3.447-459.2002.
16. **Calvo AM.** 2008. The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet. Biol. **45:**1053–1061. http://dx.doi.org/10.1016/j.fgb.2008.03.014.
17. **Dhingra S, Andes D, Calvo AM.** 2012. VeA regulates conidiation, gliotoxin production, and protease activity in the opportunistic human pathogen *Aspergillus fumigatus*. Eukaryot. Cell **11:**1531–1543. http://dx.doi.org/10.1128/EC.00222-12.
18. **Park H, Bayram O, Braus G, Kim SC, Yu J.** 2012. Characterization of the *velvet* regulators in *Aspergillus fumigatus*. Mol. Microbiol. **86:**937–953. http://dx.doi.org/10.1111/mmi.12032.
19. **Tekaia F, Latge JP.** 2005. *Aspergillus fumigatus*: saprophyte or pathogen? Curr. Opin. Microbiol. **8:**385–392. http://dx.doi.org/10.1016/j.mib.2005.06.017.
20. **Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R.** 1995. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect. Immun. **63:**3266–3271.
21. **Bok JW, Chung D, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Kirby KA, Keller NP.** 2006. GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. Infect. Immun. **74:**6761–6768. http://dx.doi.org/10.1128/IAI.00780-06.
22. **Comera C, André K, Laffitte J, Collet X, Galtier P, Maridonneau-Parini I.** 2007. Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signaling pathways in human neutrophils. Microbes Infect. **9:**47–54. http://dx.doi.org/10.1016/j.micinf.2006.10.009.
23. **Frame R, Carlton WW.** 1988. Acute toxicity of gliotoxin in hamsters. Toxicol. Lett. **40:**269–273. http://dx.doi.org/10.1016/0378-4274(88)90050-1.
24. **Mullbacher A, Eichner RD.** 1984. Immunosuppression in vitro by a metabolite of a human pathogenic fungus. Proc. Natl. Acad. Sci. U. S. A. **81:**3835–3837. http://dx.doi.org/10.1073/pnas.81.12.3835.
25. **Pan XQ, Harday J.** 2007. Electromicroscopic observations on gliotoxin-induced apoptosis of cancer cells in culture and human cancer xenografts in transplanted SCID mice. In Vivo **21:**259–265.
26. **Piva TJ.** 1994. Gliotoxin induces apoptosis in mouse l929 fibroblast cells. Biochem. Mol. Biol. Int. **33:**411–419.
27. **Stanzani M, Orciuolo E, Lewis R, Kontoyiannis DP, Martins SL, St John LS, Komanduri KV.** 2005. *Aspergillus fumigatus* suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes. Blood **105:**2258–2265. http://dx.doi.org/10.1182/blood-2004-09-3421.
28. **Suen YK, Fung KP, Lee CY, Kong SK.** 2001. Gliotoxin induces apoptosis in cultured macrophages via production of reactive oxygen species and cytochrome c release without mitochondrial depolarization. Free Radic. Res. **35:**1–10. http://dx.doi.org/10.1080/10715760100300541.
29. **Tsunawaki S, Yoshida LS, Nishida S, Kobayashi T, Shimoyama T.** 2004. Fungal metabolite gliotoxin inhibits assembly of the human respiratory burst NADPH oxidase. Infect. Immun. **72:**3373–3382. http://dx.doi.org/10.1128/IAI.72.6.3373-3382.2004.
30. **Waring P.** 1990. DNA fragmentation induced in macrophages by gliotoxin does not require protein-synthesis and is preceded by raised inositol triphosphate levels. J. Biol. Chem. **265:**14476–14480.
31. **Yamada A, Kataoka T, Nagai K.** 2000. The fungal metabolite gliotoxin: immunosuppressive activity on CTL-mediated cytotoxicity. Immunol. Lett. **71:**27–32. http://dx.doi.org/10.1016/S0165-2478(99)00155-8.
32. **Yoshida LS, Abe S, Tsunawaki S.** 2000. Fungal gliotoxin targets the onset of superoxide-generating NADPH oxidase of human neutrophils. Biochem. Biophys. Res. Commun. **268:**716–723. http://dx.doi.org/10.1006/bbrc.2000.2192.
33. **Eichner RD, Waring P, Geue AM, Braithwaite AW, Mullbacher A.** 1988. Gliotoxin causes oxidative damage to plasmid and cellular DNA. J. Biol. Chem. **263:**3772–3777.
34. **Richard JL, Dvorak TJ, Ross PF.** 1996. Natural occurrence of gliotoxin in turkeys infected with *Aspergillus fumigatus*, Fresenius. Mycopathologia **134:**167–170. http://dx.doi.org/10.1007/BF00436725.
35. **Dhingra S, Lind AL, Lin HC, Tang Y, Rokas A, Calvo AM.** 2013. The fumagillin gene cluster, an example of hundreds of genes under *veA* control in *Aspergillus fumigatus*. PLoS One **8:**e77147. http://dx.doi.org/10.1371/journal.pone.0077147.
36. **Stinnett SM, Espeso EA, Cobeno L, Araujo-Bazan L, Calvo AM.** 2007. *Aspergillus nidulans* VeA subcellular localization is dependent on the importin alpha carrier and on light. Mol. Microbiol. **63:**242–255. http://dx.doi.org/10.1111/j.1365-2958.2006.05506.x.
37. **Kato N, Brooks W, Calvo AM.** 2003. The expression of sterigmatocystin and penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. Eukaryot. Cell **2:**1178–1186. http://dx.doi.org/10.1128/EC.2.6.1178-1186.2003.
38. **Bayram O, Krappmann S, Ni M, Bok JW, Helmstaedt K, Valerius O, Braus-Stromeyer S, Kwon NJ, Keller NP, Yu JH, Braus GH.** 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. Science **320:**1504–1506. http://dx.doi.org/10.1126/science.1155888.
39. **Bayram O, Braus GH.** 2012. Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol. Rev. **36:**1–24. http://dx.doi.org/10.1111/j.1574-6976.2011.00285.x.
40. **Ramamoorthy V, Dhingra S, Kincaid A, Shantappa S, Feng X, Calvo AM.** 2013. The putative C2H2 transcription factor MtfA is a novel regulator of secondary metabolism and morphogenesis in *Aspergillus nidulans*. PLoS One **8:**e74122. http://dx.doi.org/10.1371/journal.pone.0074122.
41. **Käfer E.** 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. Adv. Genet. **19:**33–131. http://dx.doi.org/10.1016/S0065-2660(08)60245-X.
42. **Szewczyk E, Nayak T, Oakley CE, Edgerton H, Xiong Y, Taheri-Talesh**

**N, Osmani SA, Oakley BR.** 2006. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. **1:**3111–3120. http://dx.doi.org/10.1038/nprot.2006.405.
43. **Peñalva MA.** 2005. Tracing the endocytic pathway of *Aspergillus nidulans* with FM4-64. Fungal Genet. Biol. **42:**963–975. http://dx.doi.org/10.1016/j.fgb.2005.09.004.
44. **Reichard U, Monod M, Odds F, Ruchel R.** 1997. Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. J. Med. Vet. Mycol. **35:**189–196. http://dx.doi.org/10.1080/02681219780001131.
45. **Cramer RA, Gamcsik MP, Brooking RM, Najvar LK, Kirkpatrick WR, Patterson TF, Balibar CJ, Graybill JR, Perfect JR, Abraham SN, Steinbach WJ.** 2006. Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. Eukaryot. Cell **5:**972–980. http://dx.doi.org/10.1128/EC.00049-06.
46. **Weber K, Bolander M, Sarkar G.** 1998. PIG-B: a homemade monophasic cocktail for the extraction of RNA. Mol. Biotechnol. **9:**73–77. http://dx.doi.org/10.1007/BF02752699.
47. **Fuchs B, O'Brien E, Khoury J, Mylonakis E.** 2010. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. Virulence **1:**475–482. http://dx.doi.org/10.4161/viru.1.6.12985.
48. **Adams TH, Boylan MT, Timberlake WE.** 1988. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. Cell **54:**353–362. http://dx.doi.org/10.1016/0092-8674(88)90198-5.
49. **Richie DL, Hartl L, Aimanianda V, Winters MS, Fuller KK, Miley MD, White S, McCarthy JW, Latge PJ, Feldmesser M, Rhodes JC, Askew DS.** 2009. A role for the unfolded protein response (upr) in virulence and antifungal susceptibility in *Aspergillus fumigatus*. PLoS Pathog. **5:**e1000258. http://dx.doi.org/10.1371/journal.ppat.1000258.
50. **Gardiner D, Howlett BJ.** 2005. Bioinformatic and expression analysis of the putative gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*. FEMS Microbiol. Lett. **248:**241–248. http://dx.doi.org/10.1016/j.femsle.2005.05.046.
51. **Kupfahl C, Heinekamp T, Geginat G, Ruppert T, Hartl A, Hof H, Brakhage AA.** 2006. Deletion of the *gliP* gene of *Aspergillus fumigatus* results in loss of gliotoxin production but has no effect on virulence of the fungus in a low-dose mouse infection model. Mol. Microbiol. **62:**292–302. http://dx.doi.org/10.1111/j.1365-2958.2006.05373.x.
52. **Spikes S, Xu R, Nguyen CK, Chamilos G, Kontoyiannis DP, Jacobson RH, Ejzykowicz DE, Chiang LY, Filler SG, May GS.** 2008. Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence. J. Infect. Dis. **197:**479–486. http://dx.doi.org/10.1086/525044.
53. **Sugui JA, Pardo J, Chang YC, Zarember KA, Nardone G, Galvez EM, Mullbacher A, Gallin JI, Simon MM, Kwon-Chung KJ.** 2007. Gliotoxin is a virulence factor of *Aspergillus fumigatus*: *gliP* deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot. Cell **6:**1562–1569. http://dx.doi.org/10.1128/EC.00141-07.
54. **Reeves EP, Messina CG, Doyle S, Kavanagh K.** 2004. Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella*. Mycopathologia **158:**73–79. http://dx.doi.org/10.1023/B:MYCO.0000038434.55764.16.
55. **Mylonakis E, Moreno R, El Khoury JB, Idnurm A, Heitman J, Calderwood SB, Ausubel FM, Diener A.** 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. Infect. Immun. **73:**3842–3850. http://dx.doi.org/10.1128/IAI.73.7.3842-3850.2005.
56. **Mylonakis E.** 2008. *Galleria mellonella* and the study of fungal pathogenesis: making the case for another genetically tractable model host. Mycopathologia **165:**1–3. http://dx.doi.org/10.1007/s11046-007-9082-z.
57. **Dunphy GB, Oberholzer U, Whiteway M, Zakarian RJ, Boomer I.** 2003. Virulence of *Candida albicans* mutants toward larval *Galleria mellonella* (Insecta, Lepidoptera, Galleridae). Can. J. Microbiol. **49:**514–524. http://dx.doi.org/10.1139/w03-064.
58. **Brennan M, Thomas DY, Whiteway M, Kavanagh K.** 2002. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. FEMS Immunol. Med. Microbiol. **34:**153–157. http://dx.doi.org/10.1111/j.1574-695X.2002.tb00617.x.
59. **Jackson JC, Higgins LA, Lin X.** 2009. Conidiation color mutants of *Aspergillus fumigatus* are highly pathogenic to the heterologous insect host *Galleria mellonella*. PLoS One **4:**e4224. http://dx.doi.org/10.1371/journal.pone.0004224.
60. **Kavanagh K, Reeves EP.** 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. **28:**101–112. http://dx.doi.org/10.1016/j.femsre.2003.09.002.
61. **Ratcliffe NA.** 1985. Invertebrate immunity—a primer for the non-specialist. Immunol. Lett. **10:**253–270. http://dx.doi.org/10.1016/0165-2478(85)90100-2.
62. **Tojo S, Naganuma F, Arakawa K, Yokoo S.** 2000. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. J. Insect. Physiol. **46:**1129–1135. http://dx.doi.org/10.1016/S0022-1910(99)00223-1.
63. **Samson RA.** 1999. The genus *Aspergillus* with special regard to the *Aspergillus fumigatus* group. Contrib. Microbiol. **2:**5–20. http://dx.doi.org/10.1159/000060298.
64. **Han S, Adams TH.** 2001. Complex control of the developmental regulatory locus *brlA* in *Aspergillus nidulans*. Mol. Genet. Genomics **266:**260–270. http://dx.doi.org/10.1007/s004380100552.
65. **Marshall MA, Timberlake WE.** 1991. *Aspergillus nidulans wetA* activates spore-specific gene expression. Mol. Cell. Biol. **11:**56–62.
66. **Choi HS, Shim JS, Kim JA, Kang SW, Kwon HJ.** 2007. Discovery of gliotoxin as a new small molecule targeting thioredoxin redox system. Biochem. Biophys. Res. Commun. **359:**523–528. http://dx.doi.org/10.1016/j.bbrc.2007.05.139.
67. **Scharf DH, Heinekamp T, Remme N, Hortschansky P, Brakhage AA, Hertweck C.** 2012. Biosynthesis and function of gliotoxin in *Aspergillus fumigatus*. Appl. Microbiol. Biotechnol. **93:**467–472. http://dx.doi.org/10.1007/s00253-011-3689-1.
68. **Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P, Ceselski S, Fox B, Stein B, Copelan EA.** 1993. Evidence for possible involvement of an elastolytic serine-protease in aspergillosis. Infect. Immun. **61:**2357–2368.
69. **Duran RM, Gregersen S, Smith TD, Bhetariya PJ, Cary JW, Harris-Coward PY, Mattison CP, Grimm C, Calvo AM.** 4 March 2014. The role of *Aspergillus flavus veA* in the production of extracellular proteins during growth on starch substrates. Appl. Microbiol. Biotechnol. http://dx.doi.org/10.1007/s00253-014-5598-6.
70. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **24:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.