Introduction

*Aspergillus fumigatus* is a saprophytic fungus that plays an important ecological role in recycling organic material [1,2]. It is also the primary causative agent of invasive aspergillosis, a fungal infection of immunocompromised patients with both high mortality and morbidity [3,4]. *A. fumigatus* depends largely upon its asexual life cycle to generate spores, termed conidia, for propagation and dissemination [5,6]. Like other *Aspergillus* species, the asexual life cycle of *A. fumigatus* has distinct developmental stages [7,8]. Initially, airborne conidia land upon a suitable substrate and germinate to form tubular hyphae which grow and branch to form a network of mycelium. The mycelia expand indefinitely forming a radially symmetric colony. Once the hyphae mature, multicellular conidiation structures are formed which can produce large numbers of conidia to allow the cycle to continue [9,10].

Conidiation in *Aspergillus* species has been studied extensively in the model organism *Aspergillus nidulans* and is regulated via the key core regulatory proteins BrkA, AhaA and WetA. Another regulatory protein, MedA, was identified as a temporal modifier of the expression of these core conidiation proteins [11–15]. Mutations in the *A. nidulans medA* gene resulted in abnormal and reduced conidiogenesis [11,16]. In other fungi, orthologues of MedA have been found not only to govern asexual reproduction, but also to play a role in virulence. For example, deletion of the *A. fumigatus medA* gene resulted in a strain with impaired biofilm production and a reduced capacity to adhere to pulmonary epithelial cells, endothelial cells and fibronectin *in vitro* [17]. The *ΔmedA* mutant also exhibited attenuated virulence in an invertebrate and murine model of invasive aspergillosis, suggesting that the downstream targets of *A. fumigatus* MedA mediate virulence [17]. Disruption of *ACR1*, the *Magnaporthe grisea* orthologue of *medA*, resulted in the production of conidia that fail to cause disease [18]. Similarly, Ren1, the MedA orthologue in *Fusarium oxysporum*, is essential for microconidia and macroconidia development and for the correct differentiation of conidiophores and phialides [19]. However, unlike in *A. fumigatus* and *M. grisea*, the *REN1* deletion mutant of *F. oxysporum* was fully virulent [19].

Previous studies have hypothesized that MedA, Acr1 and Ren1 are transcription regulators that govern fungal conidiogenesis and adherence to substrates. In support of this hypothesis, transcriptome studies in *A. fumigatus* have identified the dysregulation of over 142 genes in the *medA* deletion mutant (unpublished data). However, no DNA-binding domains or other conserved motifs have been identified within MedA or its homologues. Although two studies have reported nuclear localization of GFP-tagged *A. nidulans* MedA and *F. oxysporum* Ren1, a nuclear localization signal (NLS) has not been identified within these proteins, and the role of nuclear localization in governing asexual reproduction, adherence or virulence is unknown [19,20].
In this work, we hypothesized that nuclear localization is required for the function of MedA. To test this hypothesis, we performed a deletion and mutational analysis of *A. fumigatus* MedA. We identified a conserved domain located within the C-terminal portion of MedA which mediates nuclear localization. Further, we demonstrate that nuclear localization of this domain is both necessary and sufficient for MedA regulation of asexual reproduction, adherence and virulence.

**Materials and Methods**

**Oligonucleotides, Fungal Strains and Media**

The oligonucleotides used are fully described in Table 1. The *Aspergillus fumigatus* strain Af293 [21] was kindly provided by P. Magee (University of Minnesota, St. Paul, MN). All *A. fumigatus* strains were generated in this study by ectopic integration of plasmids. Transformation of *A. fumigatus* was achieved using protoplasting [17]. Conidia were harvested from mycelia grown for 6 days at 37°C on YPD agar (1% yeast extract, 2% peptone, 2% glucose, and 1.5% agar, pH 6.5) using PBS supplemented with 0.1% (v/v) Tween 80, pH 7.4 (PBST). For the *A. fumigatus* biofilm adherence assay, Sabouraud media was used, while for induction of *alcA* promoter, fungal strains were grown in *Aspergillus* minimal medium (AMM) [22] (0.152% KH2PO4, 0.052% KCl, 0.6% NaNO3, 0.05% MgSO4, 1 ml/l trace elements, 1% (v/v) EtOH, 2% Lactose, 0.05% Glucose, pH 6.5). For observation of conidia pigmentation, conidia were grown on Sabouraud agar for 6 days at 37°C. To study fungal hyphal growth, 10^6 conidia were used to inoculate the center of YPD plates and incubated at 37°C. Colony diameter was measured daily.

**Amino Acid and Nucleic Acid Sequences**

The 683 amino acid sequence of MedA (GenBank: EAL93620.1) and the amino acid sequences of MedA orthologues, i.e. MedA from *A. nidulans* (GenBank: AAC31205.1), ACON-3 from *N. crassa* (GenBank: BDG10820.1), Acr1 from *M. grisea* (GenBank: BAC41196.1), and Ren1 from *F. oxysporum* (GenBank: BAC500115.1), were obtained from the National Center for Biology Information (www.ncbi.nlm.nih.gov). Identification of the conserved region in MedA was performed using BLASTp search of the NCBI Reference Proteins (refseq_protein) database, and the *Aspergillus fumigatus* MedA 683 amino acid sequence as...
a query sequence after restricting the BLAST search to fungi only. To ensure best accuracy of the BLAST output, amino acid sequences of hypothetical proteins annotated as MedA-homologous proteins were selected, neglecting hypothetical and incomplete proteins, as well as those not annotated as MedA-homologous proteins. MedA and MedA-homologous proteins were then aligned using ClustalX multiple alignment application [23]. Prediction of the NLS1 motif for MedA was performed using DNA-binding protein prediction PSORT II (psort.hgc.jp/form2.html) [24]. DNA-binding motif searches were performed using DNA-binding protein prediction server DNABIND (www.enzim.hu/~szia/dnabind.html) [25] and BLASTp search of the NCBI MedA secondary structure analysis was performed using HHpred [26] and Jpred3 [27], and fold prediction using PHyre [28]. Query results in DSSP format were manually aligned against the MedA amino acid sequence and compared.

**RNA Extraction and RT-PCR**

Conidia were grown at 37°C for 24 hr in Sabouraud medium and RNA was extracted using NucleoSpin® RNA Plant kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer instructions. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen). MedA gene expression was measured by quantitative real-time RT-PCR using the fluorescent reporter SYBR Green (Fermentas) and ABI 7300 thermocycler (Applied Biosystems). RT-PCR was performed for medA using primer pair RT-Meda-F and RT-Meda-R. The endogenous reference gene, tef1, was quantified using the primer pair TEF1-sense and TEF1-antisense [17]. Quantification of mRNA level of medA was performed using 2^ΔΔCt method [29,30].

**Construction of GFP-tagged MedA Truncations**

The medA open reading frame was PCR amplified from genomic DNA using the primers MedA-F and MedA-R and then cloned into plasmid pGFP-Phleo [31] using EcoRV and NotI, resulting in plasmid pMedA-GFP. Next, the alcA promoter was amplified from plasmid pAL4 by PCR using the primers AlcAp-For and AlcAp-Rev and used to replace the gpdA promoter in the plasmid pMedA-GFP after AgeI and EcoRV digestion. The resulting plasmid was designated pAlcA-Meda-GFP. Individual medA truncations were amplified by PCR and cloned into the plasmid pAlcA-Meda-GFP using EcoRV and NotI, replacing the full-length medA gene. The constructed plasmids, the combinations of oligonucleotides used to clone these plasmids, and the corresponding generated A. fumigatus strains are listed in Table 2.

**Site-directed Deletion Mutagenesis of MedA Putative Nuclear Localization Signals**

Deletion of NLS1, NLS2, and NLS3 of medA was done by fusion PCR [32]. Briefly, medA was amplified from the plasmid pAlcA-
MedA-GFP using the primers MedA-F and MedA-R while deletions spanning NLS1, NLS2, and NLS3 in medA, i.e. medA\(_{\Delta NLS1}\), medA\(_{\Delta NLS2}\), and medA\(_{\Delta NLS3}\), were obtained using the primer pairs that carry the desired deletion mutation (Table 2). The PCR products were then ligated into the EcoRV-NotI site of plasmid pAlcA-MedA-GFP to replace the intact medA gene as above. Deletion of NLS4 in medA, designated medA\(_{\Delta NLS4}\), was achieved by PCR amplification of 1518 bp medA fragment using the primers MedA-F and NLS4-R. The PCR product was then cloned into pAlcA-MedA-GFP using NheI and SmaI, generating plasmid pMedA-GFP-\(\Delta\)NLS4. The constructed plasmids and the corresponding A. fumigatus expression strains are listed in Table 2.

### Figure 1. Subcellular localization of the MedA-EGFP fusion truncation constructs in A. fumigatus.

(A) Schematic overview of the various truncated medA-egfp gene fusions. Full-length medA and different medA truncations were fused in-frame to egfp under the control of alcA promoter. (B) The cellular localization of various MedA-EGFP fusion proteins expressed in A. fumigatus AF293. Nuclei were stained by Draq5 and mycelia were analyzed by light microscopy. Left, center, and right columns show the GFP, Draq5, and DIC (Differential Interference Contrast), respectively. The MedA-EGFP constructs are indicated on the left side. Vertical lines, from left to right, represent amino acids 1, 346, 557, and 683 respectively.

doi:10.1371/journal.pone.0049959.g001

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region and the first 282 bp of \textit{medA} coding sequences was amplified from \textit{A. fumigatus} genomic DNA using the primers PmedA-F and MedA-NheI-R. This PCR product was digested with NheI and NruI and subcloned upstream of the \textit{medA} open reading frame of the pMedA-GFP plasmid, after NheI and EcoRV digestion. The resulting plasmid was designated pPmedA-MedA-GFP. The \textit{A. fumigatus} \textit{D} \textit{medA} mutant was then transformed with these plasmids. The plasmids and the corresponding \textit{A. fumigatus} expression strains are listed in Table 2.

Generation of \textit{A. fumigatus} Strain that Encodes medA\textsuperscript{346-557} under the Expression of the \textit{medA} Endogenous Promoter

The plasmids, pPmedA-MedA(1036–1671), which encodes medA\textsuperscript{346–557}, was constructed using fusion PCR \cite{32}. The 1.5 kb promoter region of \textit{medA} and the 636 bp \textit{medA}\textsuperscript{346–557} region were PCR amplified using the primers PmedA-AgeI-F and MedA-1671-R while the primer pairs PM(1036)-R and PM(1036)-F provided the 5' complementary sequences for hybridization. The two PCR products were hybridized and digested with NheI and NruI to

\begin{figure}[h]
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\caption{Amino acid sequence alignment of \textit{A. fumigatus} MedA putative NLS 1–4 with other MedA homologues. (A) Sequence alignment and motif prediction using PSORT II identified NLS1 sequence among orthologues of MedA. (B) Sequence alignment of the MedA minimal nuclear localization domain, MedA\textsuperscript{346–557} with other MedA orthologues. The sequences representing the putative NLSs 2, 3, and 4 are boxed. The basic amino acids within the putative NLSs of \textit{A. fumigatus} MedA and the corresponding amino acids in MedA orthologues are highlighted in gray. The presence of an asterisk or a colon below the basic amino acids indicates a fully or strongly conserved residue, respectively. Numbers indicate the amino acid position within the primary amino acid sequence of the protein. \textit{Af}_\textit{MedA}: \textit{A. fumigatus} MedA (GenBank: EAL93620.1), \textit{An}_\textit{MedA}: \textit{A. nidulans} MedA (GenBank: AAC31205.1), \textit{Nc}_\textit{ACON-3}: \textit{N. crassa} ACON-3 (GenBank: ADL28820.1), \textit{Mg}_\textit{Acr1}: \textit{M. grisea} Acr1 (GenBank: BAC41196.1), and \textit{Fo}_\textit{Ren1}: \textit{F. oxysporum} Ren1 (GenBank: BAC55015.1).}
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\end{figure}
replace the gpdA promoter of the pGFP-phleo plasmid, which was digested with NheI and the compatible blunt end EcoRV. The plasmid was used to transform the DmedA strain and the resulting strain was designated Dm-M(346–557).

Fungal Biofilm Adherence Assay

Fungal biofilm adherence assay was performed using 6-well non-tissue culture-treated plates as described previously [33]. Wells were inoculated with 1 ml of Sabouraud media containing 10^5 conidia and incubated for 24 hr at 37°C. Biofilms were washed 3 times with 3 ml of PBS containing Ca & Mg (Thermo Scientific) and stained with 3 ml 0.05% (w/v) crystal violet solution for 24 hr.

Hydrophobicity Assay of Conidia

Aerial conidia were collected with a cotton swab from cultures grown for 6 days on YPD agar. Conidia were then resuspended in 3 ml mineral oil-water mix. The conidia located in oil phase were considered hydrophobic while conidia resuspended in water were considered hydrophilic.

Fluorescence Microscopy

A. fumigatus conidia were grown in liquid AMM for 48 hr at 37°C. Slides were prepared by mixing 20 μl of sample with 0.5 μl of 5 mM Draq5. Subcellular localization studies were performed under a confocal laser scanning microscope (x40) Fluoview FV1000 (Olympus, Tokyo, Japan) equipped with 488 nm and 633 nm lasers for GFP and Draq5 excitation, respectively. Images were processed for optimal presentation using GIMP (GNU Image Manipulation Program) software.

Survival Assay

Virulence was tested using Galleria mellonella as described previously [17,34]. Sixth instar of G. mellonella larvae, were injected with 10 μl of 10^7 swollen conidia ml^-3, by insertion of a Hamilton needle through the last pseudopod. Uninfected worms were sham infected with 10 μl of YPD media. After injection, worms were incubated at 37°C in the dark and surviving worms were counted daily. The Wilcoxon sum test was used to test for significant differences in survival between groups.

Results

MedA Nuclear Localization is Mediated via Sequences within the Conserved C-terminal Domain

Previous analyses have demonstrated that MedA in hyphae and conidiophores of A. nidulans is located predominately within the nucleus, however the sequences governing nuclear localization remain unknown [20]. To identify candidate regions within MedA...
YPD agar plates were spotted inoculated with the indicated strains and the colony diameter measured daily. doi:10.1371/journal.pone.0049959.g004

amino acids 349–549 of MedA) resulted in a construct that decreasing the size of this construct minimally (encompassing 557) and examined its subcellular distribution. As predicted, this minimal nuclear localization domain identified above (aa 346–349) resulted in a predominant accumulation of GFP within the nucleus (Figure 1B). Deletion of up to 345 N-terminal amino acids of MedA did not affect the nuclear localization of MedA, suggesting that these sequences are dispensable for nuclear localization and subsequent function of MedA.

To investigate the role of this domain in mediating MedA nuclear localization, we generated a series of GFP-tagged MedA truncations constructs, and expressed these constructs in the A. fumigatus wild type strain AF293 under the control of the inducible A. nidulans alcA promoter (Figure 1A). Consistent with reports of nuclear localization in other MedA orthologues, induction of the intact A. fumigatus medA-GFP expression construct resulted in a predominant accumulation of GFP within the nucleus (Figure 1B). Deletion of up to 345 N-terminal amino acids of MedA did not affect the nuclear localization of MedA, suggesting that these sequences are dispensable for nuclear localization (Figure 1B). Extending this deletion to include the first 348 amino acids resulted in predominately cytoplasmic accumulation of the MedA-GFP construct (Figure 1B). Similarly, while deletion of the C-terminal amino acids 558–683 had no effect on nuclear localization, extending this deletion to begin at amino acid 550 resulted in a strain with predominately cytoplasmic accumulation of MedA-GFP (Figure 1B). Collectively, these results suggest that the nuclear localization domain(s) of MedA lie between amino acid 346 and 557. To confirm these results, we constructed an expression construct in which GFP was fused to this putative nuclear localization domain identified above (aa 346–557) and examined its subcellular distribution. As predicted, this domain alone was sufficient to mediate nuclear localization while decreasing the size of this construct minimally (encompassing amino acids 349–549 of MedA) resulted in a construct that remained predominately cytoplasmic (Figure 1B). Of note, the MedA346–557 fragment which localizes to the nucleus spans the conserved sequences identified by sequence alignment of the MedA orthologues. Interestingly, although the most highly conserved region identified in this alignment was also contained within the smaller MedA349–549 fragment, this construct failed to localize to the nucleus (Figure 1B). Thus, other sequences immediately bordering the core conserved region are likely required for normal nuclear localization or maintaining normal protein structure.

Additionally, differences in the intensity of nuclear staining were evident between the various constructs. Full-length MedA, and the C-terminal deletion constructs MedA1–557 and MedA1–549 required two days of alcA induction for high level fluorescence to be evident. In contrast, all MedA N-terminal truncations studied showed a strong fluorescence after only one day of induction, suggesting that N-terminal sequences of MedA might contain sequences important for the regulation of MedA nuclear expression levels.

MedA Requires the Entire Nuclear Localization Domain for Nuclear Localization

Importin-mediated nuclear localization is classically facilitated by cluster(s) of positively charged amino acids, i.e. lysine (K) and arginine (R). These clusters may be present singly (monopartite) or duplicate (bipartite) in which case they are classically separated by 10–12 amino acids [35,36].

To identify possible nuclear localization signal (NLS) sites in MedA, we performed a sequence analysis of the entire MedA amino acid sequence using PSORT II. The analysis predicted the presence of a putative nuclear localization signal, designated NLS1. However, NLS1 was located outside the 212 aa minimal nuclear localization domain, MedA346–557, and was not conserved when was aligned with other MedA orthologues (Figure 2A). To identify other possible non-canonical NLS regions, we performed a sequence alignment of the minimal nuclear localization domain of MedA346–557, with ACON-3 from N. crassa, Acr1 from M. grisea, Ren1 from F. oxysporum, and MedA from A. nidulans. This region was observed to be rich in positively charged residues, containing 29 K and L residues among its 212 amino acids (13.5%). Among these, 19 were fully conserved in all five studied MedA orthologues, and 4 were partially conserved in that all exchanges were either K to R or R to K (Figure 2B). Although this region lacked classical NLS sequence, 3 sets of sequences rich in positively charged residues were identified and designated NLS2, NLS3, and NLS4. NLS2 contains sequences consistent with the monopartite class, while NLS3 and NLS4 are complex regions containing elements consistent with both monopartite and bipartite classes. NLS3 contains a monopartite cluster of KNRRIR and a bipartite two clusters of RR and KAK separated by 11 amino acids. NLS4 is composed of a bipartite cluster surrounded by a number of R and K residues within 6 amino acids of each cluster (Figure 2B).

To investigate the role of NLS1, NLS2, NLS3, and NLS4 in nuclear localization, a functional analysis of the four NLSs was performed. Site-directed deletion mutagenesis of NLS1, NLS2, NLS3, and NLS4 of MedA was performed, generating constructs medAΔNLS1, medAΔNLS2, medAΔNLS3, and medAΔNLS4 which were expressed in A. fumigatus AF293 under the inducible alcA promoter. The effect of mutagenesis on MedA-GFP subcellular localization was then determined using confocal microscopy. As predicted from the truncation studies, the deletion of NLS1 did not affect the nuclear localization of MedA (Figure 3B). Surprisingly, however,
deletion any one of the three other NLS abrogated the nuclear localization of MedA (Figure 3B). Importantly, expression of the cytoplasmic MedA\textsuperscript{D NLS2}, MedA\textsuperscript{D NLS3}, or MedA\textsuperscript{D NLS4} with wild type MedA in the A. fumigatus Af293 strain did not affect adherence, conidiation or conidial pigment (data not shown) suggesting that these constructs do not result in dominant negative effects. Collectively, these results suggest that MedA nuclear localization is facilitated by the entire nuclear localization domain, rather than any single NLS.

**Figure 5. Effect of MedA nuclear localization on restoring wild type phenotype.** (A) Schematic overview of the medA-egfp fusion constructs under the control of the 1.5 kb medA promoter, medA(p) used for complementation of the ΔmedA strain. (B) Vegetative growth of strains on Sabouraud agar for 6 days at 37°C; hydrophobicity of conidia; and biofilm formation before and after washing. (C) Survival assay of G. mellonella larvae. 40 worms/strain were infected with 10⁵ swollen conidia. Af293 indicates the A. fumigatus wild type strain; MedA, MedA\textsuperscript{D NLS1}, MedA\textsuperscript{D NLS2}, MedA\textsuperscript{D NLS3}, and MedA\textsuperscript{D NLS4} indicate expression of the corresponding construct under the control of the medA(p) in the ΔmedA strain. Virulence of strains expressing cytoplasmic and nuclear MedA was compared to Af293 and ΔmedA strain, respectively, using the log rank test. For all comparisons, P was ≤0.05.

doi:10.1371/journal.pone.0049959.g005
MedA Nuclear Localization is Required for Adherence, Conidiation and Virulence in A. fumigatus

Previous studies revealed that MedA plays an essential role in conidial hydrophobicity, mycelial adherence to substrates, and fungal pathogenicity [17–19]. We therefore tested the role of nuclear localization of MedA in mediating these phenotypes using the NLS deletion mutant constructs of MedA. For these studies, a 1.5 kb region upstream of the medA ORF encompassing the predicted promoter sequences was cloned upstream of each of the medA<sup>NLS</sup> constructs. These constructs were then expressed in the ΔmedA mutant strain, and the resulting phenotypes were assayed.

As predicted, no difference in hyphal growth between wild type A. fumigatus, the ΔmedA mutant, or any of the MedA construct complemented strains was observed, regardless of their subcellular localization (Figure 4). In contrast, nuclear localization of MedA was required for normal conidia development. Conidia obtained from the ΔmedA strain and ΔmedA strains expressing MedA constructs with cytoplasmic localization (cytoplasmic MedA) were bright green, and markedly hydrophilic, while all strains expressing MedA which localized to the nucleus (nuclear MedA) phenocopied the A. fumigatus wild type strain and produced hydrophobic grey-green conidia. Similarly, MedA nuclear localization was required for the formation of adherent fungal biofilms, while strains expressing cytoplasmic MedA formed non-adherent

**Figure 6. Relative gene expression of cytoplasmic and nuclear medA measured by RT-PCR.** Af293 is the A. fumigatus wild type strain; MedA, MedA<sup>NLS1</sup>, MedA<sup>NLS2</sup>, MedA<sup>NLS3</sup>, and MedA<sup>NLS4</sup> indicate expression of the corresponding construct under the control of the medA(p) in the ΔmedA strain, normalized to medA expression in strain Af293. Error bars represent the standard error of three triplicates for every strain.

doi:10.1371/journal.pone.0049959.g006

**Figure 7. Phenotypic analysis of ΔmedA strain expressing MedA<sup>346–557</sup> domain.** (A) Conidia hydrophobicity and biofilm formation of the indicated strains. (B) Survival assay of G. mellonella larvae. 28 worms/strain were infected with 10<sup>5</sup> swollen conidia. Af293 indicates the A. fumigatus wild type strain; MedA<sup>346–557</sup> indicates expression of this construct under the control of the medA(p) in the ΔmedA strain. Analysis of survival data was performed using the log rank test. Statistically significant differences are indicated by asterisk (P value ≤0.05).

doi:10.1371/journal.pone.0049959.g007
mycelia mats similar to those formed by the ΔmedA mutant (Figure 5B). Finally, the virulence of the mutant strains in an invertebrate model was also found to be dependent on the subcellular localization of MedA. Galleria larvae infected with either ΔmedA strain or ΔmedA strains complemented with cytoplasmic MedA survived longer compared with those infected with either A. fumigatus wild type strain, or strains expressing nuclear MedA (Figure 5C).

Importantly, the failure of the cytoplasmic MedA constructs to complement the medA mutant was not likely a consequence of inadequate expression, as real time RT-PCR revealed that cytoplasmic MedA strains produced higher levels of medA mRNA than did nuclear MedA expressing strains (Figure 6). Collectively these results suggest that nuclear localization is required for the function of MedA.

Expression of medA\textsuperscript{346–557}, Encoded by the medA Endogenous Promoter, is Sufficient to Complement the Function of MedA

The results of our truncation analysis suggested that amino acids 346–557 are sufficient to mediate nuclear localization of MedA. To determine if this region is also sufficient to generate functional MedA, or if additional upstream or downstream sequences are required to mediate MedA dependent phenotypes, we tested the ability of MedA\textsuperscript{346–557} to complement the ΔmedA mutant. This construct was expressed under the control of medA endogenous promoter as described above and expressed in the ΔmedA mutant strain. Complementation of the ΔmedA mutant strain with the MedA\textsuperscript{346–557} domain restored the wild type phenotype with respect to conidiation, conidial hydrophobicity, the formation of adherent biofilms and the virulence in invertebrate model (Figure 7A–B). Thus, the minimal nuclear localization fragment (MedA\textsuperscript{346–557}) is both necessary and sufficient to govern conidiation, adherence and virulence in A. fumigatus.

Discussion

MedA is a developmental regulator that governs the expression of diverse biological processes in filamentous fungi [37,38]. In order to begin to unravel the mechanism by which this protein regulates development and virulence, we performed a deletion analysis of MedA in A. fumigatus. These studies demonstrate that nuclear localization of MedA in A. fumigatus is mediated by a C-terminal domain (MedA\textsuperscript{346–557}) conserved among MedA orthologues. Surprisingly, this region contained no canonical NLS sequences. Indeed, a bioinformatic analysis of A. fumigatus MedA using PSORT II revealed the presence of a single NLS sequence (NLS1) that was located outside of the conserved domain MedA\textsuperscript{346–557}. However, NLS analysis tools utilize the protein (NLS1) that was located outside of the conserved domain using PSORT II revealed the presence of a single NLS sequence sequences. Indeed, a bioinformatic analysis of (MedA\textsuperscript{346–557}) is both necessary and sufficient to govern conidiation, conidial hydrophobicity, the formation of adherence and virulence in A. fumigatus.

RT-PCR analysis revealed that cytoplasmic MedA strains exhibited by these strains was higher than that observed with strains expressing nuclear MedA. In addition, over-expression of the cytoplasmic MedA\textsuperscript{NLS2}, MedA\textsuperscript{NLS3}, or MedA\textsuperscript{NLS4} in the A. fumigatus wild type strain AF293 under the inducible alcA promoter did not result in a MedA deficient phenotype, arguing that a dominant negative effect of cytoplasmic MedA constructs is unlikely [42]. These results are most consistent with MedA playing a role in governing gene expression either directly, or indirectly. The conserved MedA\textsuperscript{346–557} domain was not only sufficient to mediate nuclear localization, but also to recover the A. fumigatus wild type phenotype when expressed in the ΔmedA background. Thus, it is likely that this region contains not only the nuclear localization signal, but also the necessary sequences to govern gene expression either through directly binding DNA or interacting with other proteins. A bioinformatics analysis of MedA\textsuperscript{346–557} using DNABIND [25] and NCBI BLAST revealed no DNA-binding domain in MedA, although it is possible that MedA\textsuperscript{346–557} contains novel amino acid sequences for the recognition of DNA. A more plausible hypothesis however, is that MedA\textsuperscript{346–557} functions through interaction with other regulatory proteins. MedA\textsuperscript{346–557} contains 13 glutamic acid and 9 aspartic acid within its 212 amino acid sequence. These two negatively charged polar amino acids are frequently involved in protein active or binding sites [43]. This protein interaction hypothesis is also supported by the fact that the secondary structure of this region, analyzed using HHpred and Jpred3, is predicted to be composed of numerous alpha helices and beta strands which are necessary for protein architecture. Unfortunately, homology modeling tools such as PHYRE failed to identify robust structural similarities between MedA and other known proteins, limiting this bioinformatic approach. Future studies focused on identifying the MedA-protein interactome may help better characterize the exact function of MedA\textsuperscript{346–557}.

Despite the clear ability of MedA\textsuperscript{346–557} to complement the ΔmedA phenotype, a role for the N- and C-terminal amino acids upstream and downstream of MedA\textsuperscript{346–557} in MedA function should not be discounted. Our study was limited in that only MedA dependent conidial hydrophobicity, biofilm adherence and virulence in invertebrates were tested. It remains possible that the N- and C-terminal domains of MedA are required for the regulation of other MedA-dependent biological processes that were not examined. Further, these sequences may play an important regulatory role in the expression or kinetics of MedA, that was not apparent in our static assays. Indeed, the observations that full-length MedA and the C-terminal deletion constructs MedA\textsuperscript{557} and MedA\textsuperscript{549} required two days of alcA induction for high level fluorescence while N-terminal deletions produced visible fluorescence after only 24 hours might suggest that the N-terminal sequences of MedA play a role in protein stability or degradation.
Acknowledgments

We would like to thank A. Brakhage (Leibniz Institute for Natural Product Research and Infection Biology - HKI, Germany) for providing the pAL4 plasmid which was used to construct several plasmids in this study.

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