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# *acon-3*, the *Neurospora crassa* ortholog of the developmental modifier, *medA*, complements the conidiation defect of the *Aspergillus nidulans* mutant

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ABSTRACT

*Aspergillus nidulans* and *Neurospora crassa* are ascomycetes that produce asexual spores through morphologically distinct processes. MedA, a protein with unknown function, is required for normal asexual and sexual development in *A. nidulans*. We determined that the *N. crassa* ortholog of *medA* is *acon-3*, a gene required for early conidiophore development and female fertility. To test hypotheses about the evolutionary origins of asexual development in distinct fungal lineages it is important to understand the degree of conservation of developmental regulators. The amino acid sequences of *A. nidulans* MedA and *N. crassa* ACON-3 shared 37% identity and 51% similarity. *acon-3* is induced at late time points of conidiation. In contrast, *medA* is constitutively expressed and MedA protein localizes to nuclei in all tissue types. Nonetheless, expression of *acon-3* using its native promoter complemented the conidiation defects of the *A. nidulans* Δ*medA* and *medA15* mutants. We conclude that the biochemical activity of the *medA* orthologs is conserved for conidiation.



## 1. Introduction

Conidia are asexual spores produced by fungi including important species that impact agriculture, industry, and medicine (Adams et al., 1998; Ebbole, 2010). Conidia serve as propagules for infection of plants and animals. Moreover, conidiation is a critical reproductive mode in more than 15,000 described fungal species not known to undergo sexual development (Cole, 1986).

*Aspergillus nidulans* and *Neurospora crassa* are model organisms for molecular genetic studies. They are estimated to have diverged at least 215 million years ago (Taylor and Berbee, 2006), and morphological events in conidiation have been extensively documented. The developmental pathways for these two fungi are fundamentally different in their morphogenesis. Conidiophore formation in *A. nidulans* initiates from a specialized foot cell with a thickened cell wall. The foot cell gives rise to an aerial stalk that swells at its tip to form a vesicle (Suppl. Fig. 1). Two layers of sterigmata, the metulae and phialides, form sequentially from the vesicle, and chains of conidia emerge from each phialide. The conidia are generated basipetally, with the youngest conidium emerging closest to the phialide apex (Adams et al., 1998; Cole, 1986; Ni et al., 2010). Asexual development in *N. crassa* includes production of multinucleate macroconidia and uninucleate microconidia. Macroconidiation follows a blastic, and acropetal developmental pattern, while microconidiation produces phialidic spores (Maheshwari, 1999). Macroconidiation proceeds by transition from apical extension of aerial hyphae to budding growth, which results in the formation of short proconidial chains, termed minor constriction chains. At later stages, the budding pattern of growth leads to formation of major constriction chains with more pronounced constrictions (Suppl. Fig. 2). Double cross-walls between the proconidia become cleaved and eventually conidia are separated from conidiophores (Bailey and Ebbole, 1998; Springer and Yanofsky, 1989).

Clutterbuck generated *A. nidulans* mutants that showed defects in conidiation, but not in vegetative growth (Clutterbuck, 1969). A number of later studies with the conidial mutants proposed a central regulatory pathway of *A. nidulans* development. Three genes, *brlA* (bristle), *abaA* (abacus), and *wetA* (wet-white) were identified as central regulators governing conidiation. BrlA in particular plays a key role in *A. nidulans* conidiation, and the *brlA* null mutant failed to transition from the conidiophore stalk to vesicle formation (Timberlake, 1990).

Important regulatory genes have been identified for both fungi. Fluffy (*fl*), the key regulatory gene in *N. crassa* conidiation (Bailey and Ebbole, 1998; Springer and Yanofsky, 1989), plays an analogous role to *brlA* in *A. nidulans* (Boylan et al., 1987), yet they are not homologs. Furthermore, the *A. nidulans* genome does not contain an obvious ortholog of *fl* nor does the *N. crassa* genome contain an obvious ortholog of *brlA*. Orthologs to *fl* do appear in the Sordariomycetes *Sordaria macrospora* and *Podospora anserina*, however,

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it is noteworthy that these fungi do not produce macroconidia. Orthologs to *brlA* are found in the Eurotiomycetes *Aspergillus* sp., and *Penicillium* sp. Considered alone, the lineage specificity of *brlA* and *fl* suggests that regulators of conidiation may not be conserved in different lineages of fungi and that these developmental pathways may have evolved independently.

Conversely, another gene that regulates conidiation in *A. nidulans*, *flbD* (fluffy low *brlA*), has an ortholog in *N. crassa, rca-1* (regulator of conidiation in *Aspergillus*). Transformation with a DNA fragment containing the *N. crassa rca-1* gene complemented the conidiation phenotype of the *A. nidulans flbD* mutant (Shen et al., 1998). The FlbD and RCA-1 proteins display 75% similarity in the putative DNA-binding domain and 42% similarity in other regions. Deletion of *rca-1* does not affect macroconidiation, microconidiation, or ascospore formation in *N. crassa*. Thus, this is an example where the ortholog to a regulator of conidiation does restore the function in conidiation, however, the role of *rca-1* in *N. crassa* is distinct from the role of *flbD* in *A. nidulans* (Shen et al., 1998). Therefore, during the divergence of these fungal lineages there has been either a gain of the function in conidiation for *flbD* or loss of the conidiation function for *rca-1*.

MedA has been characterized as a developmental modifier in *A. nidulans* because it coordinates temporal and spatial organization of the conidiophores. Disruption of *medA* (medusa) alters the temporal expression of *brlA*, and the *medA* mutant alleles produce abnormal conidiophores with multiple layers of sterigmata and occasionally secondary conidiophores (Busby et al., 1996; Clutterbuck, 1969). Although the *medA* orthologs in other filamentous fungi, including *Magnaporthe oryzae* and *Fusarium oxysporum*, have been shown to play a role in conidiation (Lau and Hamer, 1998; Ohara et al., 2004) the biochemical function of MedA in conidial development is not well understood.

Here we studied the functional conservation of the *medA* orthologs in *A. nidulans* (ANID_06230.1) and *N. crassa* (NCU07617.4). Based on our results the *N. crassa* ortholog to *medA* is *acon-3* (*acon*idiate-3). We have expressed *acon-3* in *A. nidulans medA* mutants (*ΔmedA* and *medA15*), and analyzed conidiophore morphology and conidia production compared to the *medA* mutants. Transcript expression was compared between the organisms and the temporal aspects of expression and localization were monitored in *A. nidulans* using a green fluorescence protein (GFP) fusion in live cells.

## 2. Materials and methods

### 2.1. Strains, media, and growth conditions

A list of *A. nidulans* strains, and plasmids and constructs used in this study is shown in Table 1 and Suppl. Table 1, respectively. Minimal medium (MM) and complete medium (CM) for growing *A. nidulans* strains were prepared with appropriate supplements as described (http://www.fgsc.net). In physiological studies, strains were cultured at 28 °C unless otherwise indicated. All reagents for media, supplements, and buffers used were purchased from Sigma (St. Louis, MO) unless otherwise indicated. All plasmids were stored and amplified in *Escherichia coli* XL1-blue (Stratagene, La Jolla, CA).

Conidia production studies were carried out on MM with appropriate supplements. Ten microliters of sterile water containing $10^6$ conidia were placed in the center of a solid MM plate, and incubated at 28 °C for 5 days. Then, conidia were collected with sterile water and the number of conidia was counted using a hemocytometer. This experiment was performed in triplicate.

### 2.2. Identification of orthologs

To find the putative ortholog of *A. nidulans medA*, we used a 705 amino acid sequence of *A. nidulans* MedA as a query sequence against the *N. crassa* genome (http://www.broadinstitute.org/annotation/genome/neurospora) using the BLASTP program. The 693 amino acid sequence of NCU07617.4 was the sole candidate for orthology to *medA*, and this result was confirmed by reciprocal BLAST against the *A. nidulans* database. Additional putative orthologs of *A. nidulans medA* in other fungal species were identified using the same approach. Sequences used for the bi-directional comparison of the putative orthologs in *M. oryzae*, *F. oxysporum*, *Botrytis cinerea*, *Coccidioides immitis*, *Sclerotinia sclerotiorum*, *Ustilago maydis*, and *Cryptococcus neoformans* were obtained from the Fungal Genome Initiative database at the Broad Institute (http://www.broadinstitute.org/science/projects/fungal-genome-initiative). Sequence of the *Penicillium marneffei medA* ortholog (XP_002147078) was obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), and sequence of the *Trichoderma virens* ortholog (Trive1|34415) was obtained from the DOE Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Trive1/Trive1.home.html). Sequence alignment was performed using the ClustalW program online at EMBL-EBI (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and visualized with GeneDoc software (Nicholas et al., 1997).

### 2.3. Molecular cloning procedures

pDC2.1, used to complement the *A. nidulans medA15* mutant and *ΔmedA* with *N. crassa acon-3*, was designed to express *acon-3* using the native *N. crassa* promoter. Oligonucleotides used in this study are summarized in Suppl. Table 2. A DNA fragment of 5426 bp including the 2359 bp coding region, 1490 bp of upstream sequence and 1577 bp of downstream sequence of *acon-3* was amplified using the primers NcmedAF and NcmedAR. The PCR product was digested using *Xba*I, and ligated into the *Xba*I digested pTA-argB vector, that carried the *A. nidulans argB* sequence as a selectable marker.

The plasmid pDC1.1 contained a 4179 bp clone of *A. nidulans medA* including the 2084 bp coding region, 1659 bp upstream and 436 bp downstream of the *medA* coding region was amplified using the primers AnmedAF-SpeI and AnmedAR-SpeI. The PCR product was digested using *Spe*I, and ligated into *Spe*I digested pTA-argB vector.

Strain A586 was crossed to strain ASL91 to generate strain Asum3 (*argB2*; *pyrG89*; *medA15*). The plasmids, pDC1.1, and pDC2.1 were transformed into Asum3 using the protoplast transformation method. This resulted in generation of pDC1.1 transformants TDC6.14 and TDC6.19, and pDC2.1 transformants TDC1.23 and TDC1.29. A single copy insertion of the plasmid pDC2.1 was verified by PCR and Southern blot analysis.

To generate the Δ*medA* strain, the *medA* knock-out cassette from the FGSC (McCluskey, 2003; Program Project Grant GM068087) was transformed into *A. nidulans* wild type TN02A25 using the protoplast transformation method. This resulted in generation of a transformant, Δ*medA*. Homologous replacement of *medA* with *Aspergillus fumigatus pyrG* was verified by PCR and Southern blot analysis. To generate strain RDC20.10, TDC1.23 was crossed to *A. nidulans* wild type PW1, and the cross progeny were screened by PCR. Strain RDC21.1 and RDC22.3 were generated by crossing Δ*medA* to RDC20.10 and TDC6.14, respectively.

### 2.4. Identification of acon-3

The NCU07617.4 locus was amplified from both *N. crassa acon*-3 (FGSC #5074) and the wild type strain 74-OR23-1VA using