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NosA, a transcription factor important in *Aspergillus fumigatus* stress and developmental response, rescues the germination defect of a *laeA* deletion

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Abstract

Aspergillus fumigatus is an increasingly serious pathogen of immunocompromised patients, causing the often fatal disease invasive aspergillosis (IA). One *A. fumigatus* virulence determinant of IA is LaeA, a conserved virulence factor in pathogenic fungi. To further understand the role of LaeA in IA, the expression profile of *laeA* was compared to wild type, and several transcription factors were found significantly misregulated by LaeA loss. One of the transcription factors up-regulated over 4 fold in the *laeA* strain was Afu4g09710, similar in sequence to *A. nidulans* NosA, which is involved in sexual development. Here we assessed loss of *nosA* (*nosA*) and over expression of *nosA* (*OE::nosA*) on *A. fumigatus* in both a wild type and *laeA* background. Based on the multiple alterations of physiological development of single and double mutants, we suggest that NosA mediates the decreased radial growth and delayed conidial germination observed in *laeA* strains, the former in a light dependent manner. The *ΔnosA* mutant showed increased virulence in the *Galleria mellonella* larvae model of disseminated aspergillosis, potentially due to its increased growth and germination rate. Furthermore, the *A. fumigatus nosA* allele was able to partially remediate sexual development in an *A. nidulans ΔnosA* background. Likewise, the *A. nidulans nosA* allele was able to restore the menadione sensitivity defect of the *A. fumigatus ΔnosA* strain, suggesting conservation of function of the NosA protein in these two species.

Keywords

LaeA; NosA; virulence; germination; vegetative growth; menadione; reactive oxygen species

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1. Introduction

Aspergillus fumigatus is an increasingly serious pathogen which causes the majority of filamentous fungal infections of immunocompromised patients (reviewed in Willger et al., 2009). Unfortunately, the mechanisms governing the infection process of *A. fumigatus* are largely unknown. It is therefore of considerable interest to identify which fungal genes are responsible for successful colonization of the host, so to provide potential therapeutic targets.

A 2004 study identified a gene, *laeA*, involved in regulating numerous secondary metabolites in several species of *Aspergillus*, including *A. fumigatus* (Bok et al., 2004). Additional research demonstrated the *A. fumigatus laeA* deletion mutant (Δ*laeA*) to be compromised in infection of a murine model of invasive aspergillosis (Bok et al., 2005, Sugui et al. 2007, Ben-Ami et al. 2009). Several phenotypes of the *laeA* mutant were suggested to be responsible for this decreased virulence, including alterations of the cell surface which led to increased uptake by pulmonary macrophages in an *in vitro* assay (Bok et al., 2005; Dagenais et al., 2010), decreases in iron uptake and storage (Perrin et al., 2007), and decreases in secondary metabolite production (Bok et al., 2005, Perrin et al., 2007, Sugui et al. 2007, Ben-Ami et al. 2009). The variety of phenotypes described above were supported by microarray analysis of the Δ*laeA* mutant, where transcription of approximately 10% of the genome was altered (Perrin et al., 2007), 25% of which was also correlated with induction during murine infection (McDonagh et al., 2008).

Among those genes with significantly altered expression in the Δ*laeA* mutant were dozens of transcription factors (Perrin et al., 2007). Modulation of the transcriptional program of Δ*laeA* is likely a major contributor to its pleiotropic effects on fungal development and virulence (reviewed in Yin and Keller, 2011; Bok et al., 2006). One of the transcription factors found to be upregulated 4.4 fold in the Δ*laeA* mutant was Afu4g09710, a homolog to *A. nidulans* NosA, which has previously been characterized in its requirement for normal sexual development in that species (Vienken and Fischer, 2006).

NosA (number of sexual spores) in the homothallic *A. nidulans* was originally identified as a homolog of the *Sodaria macrospora* developmental regulator Pro1 (Vienken and Fischer, 2006). Deletion of this gene was shown to impair development of sexual primordia, drastically reducing the number of sexual spores produced when self-mating. This deletant also showed greatly reduced expression of several genes associated with sexual development, including the glucose transporter *hxtA* and the catalase peroxidase *cpeA* (Vienken and Fischer, 2006). Little else is known about this protein in any other fungus.

Here we examined the impact of *nosA* deletion and overexpression in *A. fumigatus* as well as its ability to complement an *A. nidulans ΔnosA* strain. We show that heterologous complementation with *A. fumigatus nosA* is able to partially restore *A. nidulans* sexual sporulation defects, suggesting a conservation of function between these two species. In *A. fumigatus* loss of *nosA* increased vegetative growth and germination rates. This latter property was observed even in a *ΔlaeA* background where *nosA* loss restored germination of *ΔlaeA* to wild type levels. The *OE::nosA* strain mimicked the light dependent slow growth of *ΔlaeA* and further decreased growth and delayed germination was observed in the double *ΔlaeA OE::nosA* mutant. Together, this work suggests that several growth and developmental aberrancies found in *ΔlaeA* are mediated by NosA. Finally the *ΔnosA* mutant yields a more virulent strain than wild type in the *Galleria* model of IA.

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2. Material and methods

2.1 Fungal strains, plasmids, and growth conditions

Strains and plasmids used in this study are described in Table 1. All the strains were cultured at 37 °C on glucose minimal medium (GMM) (Shimizu and Keller, 2001) unless otherwise indicated. Where necessary, 5 mM uridine and 5 mM uracil was used to supplement auxotrophs. Phylogenetic tree was constructed using the ClustalW module of MegAlign software (DNAStar Version 9) using cDNA sequences from the Aspergillus Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/).

2.2 Construction of *A. nidulans nosA* mutants

Genomic DNA sequence of *A. nidulans* FGSCA4 AN1848 (*A.n. nosA*) gene was obtained from the Aspergillus Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). Constructs to delete and overexpress *nosA* were created using double joint PCR (Yu et al., 2004). Primers used in this study are listed in Table 2. For the deletion cassette, 1 kb flanking regions of the *nosA* gene of *A. nidulans* were amplified using primers An nosA 5′ flank for, An nosA 5′ flank rev pyrG, An nosA 3′ for pyrG, and An nosA 3′ rev and fused to the *pyrG* gene of *A. parasiticus* from pJW24, which was previously amplified by PCR using A.p. pyrG For and A.p. pyrG Rev. The fusion PCR deletion construct was amplified with An nosA 5′ flank for and An nosA 3′ rev.

The resulting product was used to transform the auxotrophic *A. nidulans* strain TJMP1.1 (Table 1) to create *nosA* (AN1848) knockout (*ΔnosA*) strain TAAS157.13. Standard DNA transformation protocol was used as described previously (Szewczyk et al., 2006). Transformants were confirmed by PCR screening using An nosA 5′ flank ext and Ap pyrG int 3′, followed by Southern analysis (Sanbrook and Russell, 2001).

In order to construct complementation strains, *A. fumigatus nosA* (*A.f. nosA*) and *A. nidulans nosA* (*A.n. nosA*) genes with 1 kb flanking regions were cloned into pJW53 using NotI to create pAAS149 and pAAS150, respectively. pJW53 contains a ¾ length fragment of the *pyroA* gene, allowing for targeted integration of the plasmid at the *pyroA* locus. Strains TAAS149.4 (+ *A.f. nosA*) and TAAS150.11 (+*A.n. nosA*) were confirmed by Southern blot (Fig. S1). These strains were then crossed with either TAAS157.13 (*ΔnosA*) or RJMP101.19 to obtain the prototrophic strains RAAS158.6 (*ΔnosA + A.f. nosA),* RAAS159.8 (*ΔnosA + A.n. nosA),* RAAS160.4 (*ΔnosA)*, RAAS161.7 (+ *A.f. nosA*), and RAAS162.2 (+ *A.n. nosA*).

2.3 *A. fumigatus nosA* mutant construction

Genomic DNA sequence of *A. fumigatus* AF293 Afu4g09710 (*nosA*) gene was obtained from the Aspergillus Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/). To investigate the role of *nosA* in *A. fumigatus* physiology, constructs to delete and overexpress *nosA* were created using double joint PCR (Yu et al., 2004). Primers used in this study are listed in Table 2. For the deletion cassette, 1.0 kb flanking regions of the *nosA* gene of *A. fumigatus* were amplified using primers NosA 5′ flank for, NosA 5′ flank Rev, NosA 3′ for, and NosA 3′ rev and fused to the *pyrG* gene of *A. parasiticus* from pJW24, which was previously amplified by PCR using A.p. pyrG For and A.p. pyrG Rev. The fusion PCR deletion construct was amplified with NosA 5′ flank for and NosA 3′ rev. In order to construct an over-expression construct for *nosA*, an *A. parasticus pyrG – A. nidulans gpdA* promoter fusion from pJMP9.1 was amplified using primers pyrG inv for pJMP9 and gpdA(p) 3′ and linked to the open reading frame (ORF) and 5′ flanking region of *nosA,* previously amplified using NosA 5′ flank for, NosA 5′ flank rev for pyrG & gpdA, NosA OE 3′ flank for and NosA 3′ for pyrG & gpdA. The final

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overexpression cassette was amplified with NosA 5′ flank for and NosA 3′ for pyrG & gpdA.

The resulting products were used to transform the auxotrophic *A. fumigatus* strains CEA17 KU80 *pyrG*- and TFYL19.3 (Table 1) to create *nosA* (AFUB_066820) knockout (*ΔnosA*) strains TAAS104 and TAAS107 and over-expression (*OE::nosA*) strains TAAS105 and TAAS108. These CEA10 derivatives were used as they lacked the *KU80* gene thus allowing for higher probability of homologous recombination. Standard DNA transformation protocol was used as described previously (Szewczyk et al., 2006). Transformants were confirmed by PCR screening using either Af nosA 5′ flank ext and Ap pyrG int 3′ (KO) or Af nosA 3′ int 2 and gpdA(p) int 5′ (OE), followed by Southern and northern analysis (Sanbrook and Russell, 2001). Transcript intensity was quantified using ImageJ software. In order to create complementation plasmids for the prototrophic *A. fumigatus* strains, *hph* from pUCH2.8 was subsequently subcloned into pAAS149 and pAAS150 using BamHI and KpnI to create pAAS155 and pAAS156, respectively. These were transformed into TAAS104 to create strains TAAS155.3 and TAAS156.5, respectively.

2.3 Physiology tests

Conidiation and radial growth measurement tests were performed for all the strains by standard methods described previously (Tsitsigiannis et al., 2004), with either constant illumination with white light (light), or constant darkness (dark). For conidial counts, 5 x 10^6 conidia of designated strains were mixed in 5 mL molten GMM medium at a concentration of 0.7% agar and then overlaid on 25 mL, 1.5% agar GMM medium. Conidia from three cores (1 cm, diameter) for each plate were counted after 6 days incubation at 37 °C. Colony diameter was measured of point inoculated colonies on GMM medium every 24 h for three days 37 °C. Both assays were performed with four replicates.

For northern analysis under carbon starvation conditions, 4 replicate cultures of each strain were inoculated at 1 x 10^6 spores/mL in 25 mL of GMM. After 20 hours of shaking incubation at 37°C under constant light, cultures were collected and transferred to either fresh GMM, or MM (GMM – glucose) and cultured for an additional 5 hours. Mycelia were harvested, lyophilized overnight, and total RNA was extracted using Isol-RNA Lysis Reagent (5 Prime) according to manufacturer's recommendations.

Germination was assessed in GMM liquid medium containing 0.1 % yeast extract at 37°C with a total number of 10^6 conidia/mL for each strain at the stationary phase (Dagenais et al, 2008). The rate germination was measured at 4, 5, 6, 7, and 8 hours by microscope examination. Spores were considered germinated when the germ tube was equal in diameter to the conidia. A total number of 100 spores were examined for each strain, time point, and replicate, with four replicates performed.

Hyphal resistance to ROS producers was tested by spotting 5 μl of a 10^6 conidia/mL suspension onto GMM plates containing 40 μM menadione or 4 mM H_2O_2. Colony diameter was observed after 72 hours in constant light at 37° C.

2.4 Sexual analysis of *A. nidulans* strains

Quantification was performed on overlay inoculated cultures set up by pipetting 1 x 10^6 conidia into CHAMPS medium with 0.75% molten agar that was subsequently poured over 1.5% solid agar petri dishes. Cultures were incubated at 37ºC in the dark for 7 days and imaged at 40X resolution. Agar cores were taken from the plates with a 1 cm cork borer. After homogenization, ascospores were quantified using a hemacytometer and represented as ascospores per square millimeter. Four replicates were performed for each strain and condition.

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2.5 Virulence test using an insect model (*Galleria mellonella*)

Larvae of the Greater Wax Moth, *G. mellonella* (Northern Wax Worm Company; 250–300 mg) were used within 15 days of delivery. Larvae were inoculated with 1×10^6 conidia in 2.5 μl PBS through the last left pro-leg into the haemocoel (Fallon et al., 2011). Larvae were kept in petri dishes filled with wood shavings and incubated at 37ºC under dark condition. Mortality rate was recorded daily and confirmed by observing lack of movement, dehydration, and severe melanization of larvae. A solvent (PBS) control was used in all experiments. Three independent experimental replicates were performed using 30 larvae/strain.

2.6 Macrophage assay

2.6.1 Macrophage purification from human blood—Macrophage purification from human blood: Human primary macrophages were purified from human blood after informed consent in Dr. Anna Huttenlocher's lab (University of Wisconsin – Madison); the human subject protocol was approved by the University of Wisconsin Center for Health Sciences Human Subjects Committee. 30 mL of whole blood was diluted in an equal amount of PBS (phosphate buffered saline). 10 mL of lymphoprep density gradient media (Axis-shield, Norway) was placed in 50 mL conical tubes and 20 mL of diluted blood was gently layered on the top of liquid. The suspension was centrifuged at 600 RCF for 30 minutes. The buffer coat layer was collected and immediately diluted into 50 mL conical tubes containing PBS in a ratio of no more than 10 mL of sample to 40 mL of PBS. The conical tubes were centrifuged for 3 min at 600 RCF and the cells were washed once more with 15 mL PBS and two times with 15 mL of macrophage culture media (RPMI 1640 [Invitrogen/Gibco®; Cat. No. 31870-025], 10% fetal calf serum (FCS), 100 μg/mL streptomycin, 100 U/mL penicillin, 2mM glutamine, 1% Na-pyruvate, 1% NEAA (Non-Essential Amino Acids). The washed cells were re-suspended in 15 mL culture media and placed in 10 cm diameter cell culture treated petri dishes (NUNC, Rochester NY). After 4–8 hours the non-adherent cells were aspirated and 15 mL of fresh culture media was added containing 10 μL of rh-MCSF (recombinant human macrophage colony stimulating factor) for a final concentration of 50 ng/mL. The treated petri dishes were incubated in 5% CO_2 incubator at 37° C for 6–7 days. This process was repeated on days 2 and 4 (Starnes *et al.* 2011).

2.6.2 Macrophage harvesting and preparation—The adherent macrophages were washed with 10 mL PBS and 5 mL of trypsin-EDTA was subsequently added and incubated for 5 min at 37°C in order to lift off the cells. When all the cells were detached, trypsin was diluted in 10 mL of culture media, the cell density was measured and cell suspensions were centrifuged at 600 RCF for 3 min. The cells were re-suspended to 0.2 million cells per mL and 500 uL of cell suspension was placed in each well of a 24 well-plate for the final assay.

2.6.3 Macrophage assay for fungal spores—After allowing the macrophages to adhere for 2 hours in the incubator, the culture media was aspirated and 500 μL of fungal spore suspension was added to each well. To obtain the spore suspension, the spores were harvested in distilled water without tween, and diluted down to 1 million spores per mL in serum-free RPMI media. The spore suspension density was chosen in order to present 5 spores per macrophage. The macrophages were left for 1 hour in the incubator at 37°C in 5% CO_2 to perform the phagocytosis stage of the assay. Subsequently, the media in each well was collected in an eppendorf tube, and the well was washed thoroughly twice with PBS, which was added to the collected media. For the spore survival stage of the assay, the macrophages were then incubated in an incubator at 37° C with 5% CO_2 for 5 hours in 500 μl of culture media containing FBS 10% (Fetal Bovine Serum). Finally, the cell media containing FBS was collected in an eppendorf tube and the macrophages were lysed in 500 μL of lysis buffer (DI water +1% Tween 20) for 10 min in an incubator. Lysis buffer was

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collected into the respective tubes and the wells were washed once with 500 μL PBS, which was similarly collected. The collected suspensions at the different time points (1 and 5 hours) were diluted at a 1:20 ratio and 20 μL of the solution was plated on two plates of solid GMM medium and grown at room temperature for 4 days in order to quantify the number of colonies originated from live single spores.

2.7 Secondary metabolite analysis

Secondary metabolite production was assessed by thin-layer chromatography (TLC). For TLC, 1×10^6 spore/mL was inoculated into was point-inoculated on the center of glucose minimal medium (GMM) and cultured for 5 days at 37°C under constant light. An agar plug of the center of colonies was removed and metabolites extracted with chloroform according to the Smedsgaard's method (Smedsgaard, 1997). Extracts (10 μl/sample) were loaded onto silica TLC plates (Whatman, PE SIL G/UV, Maidstone, Kent, England) and metabolites were separated in the developing solvent toluene: ethyl acetate: formic acid (TEF, 5:4:1). Images were taken following exposure to UV radiation at 254 and 366 nm.

2.8 Statistical analysis

Data were analyzed using Graphpad Prism software (La Jolla, CA) according to the Tukey Multiple Comparison test with a p value < 0.05.

3 Results

3.1 *A. fumigatus* Afu4g09710 encodes a homolog of *A. nidulans* NosA

Microarray analysis of *A. fumigatus* AF293 *laeA* versus wild type strains under liquid stationary culture for 60 hours at 25°C showed the putative transcription factor Afu4g09710 to be 4.4 fold upregulated in the *laeA* strain (Perrin et al., 2007). BLAST searches demonstrated this protein to be a homolog of *A. nidulans* NosA (Vienken and Fischer, 2006), with 71% amino acid identity, and 47.4% identity to the *Sordaria macrospora* developmental regulator Pro1 (Masloff et al., 1999). As *A. nidulans* has previously been shown to encode two Pro1 paralogs with divergent functions (NosA and RosA; Vienken et al., 2005; Vienken and Fischer, 2006), ClustalW analysis of Aspergillus *pro1* paralog cDNAs was performed. As shown in Figure 1, phylogenetic analysis suggests that this *pro1* duplication is common to the Aspergilli, with *nosA* and *rosA* homologs each belonging to separate clades (also see Vienken and Fischer, 2006). This suggests that the function of these respective genes may be conserved within this genus.

In *A. nidulans*, deletion of *nosA* results in several characteristic phenotypes, including inability for sexual primordia to develop, as well as defects under carbon starvation conditions in transcription of two genes associated with sexual development, *hxtA* and *cpeA* (Vienken and Fischer, 2006). We therefore sought to determine if *A. fumigatus nosA* could restore these defects through a heterologous complementation assay. Six strains, wild type, RAAS158.6 (*ΔnosA + A.f. nosA),* RAAS159.8 (*ΔnosA + A.n. nosA),* RAAS160.4 (*ΔnosA)*, RAAS161.7 (+ *A.f. nosA*), and RAAS162.2 (+ *A.n. nosA*) were assessed for completion of sexual development.

Analysis of fruiting body development showed the expected defect in the *ΔnosA* strain (Vienken and Fischer, 2006), yielding underdeveloped cleistothecia which appear to be arrested at the primordial stage (Fig. 2A). Addition of an ectopic copy of *A.n. nosA* is able to increase cleistothecia diameter to near wild type levels, as well as restore normal pigmentation. *A.f. nosA* addition also increases both size and pigmentation of cleistothecia in a *ΔnosA* background, but is unable to approach wild type levels of either. Addition of ectopic copies in a wild type background did not drastically alter physical development.

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In order to determine whether the apparent partial progression of sexual development in the *ΔnosA + A.f. nosA* strain also increased production of sexual spores, ascospore density for all strains was then determined. As shown previously (Vienken and Fischer, 2006), the *ΔnosA* strain is characterized by an approximately 1000 fold decrease in the number of ascospores produced (Fig. 2B). Introduction of *A.n. nosA* nearly completely restored development, as expected from cleistothecial morphology. Although unable to fully compensate for the sexual defect, *A.f. nosA* introduction increased ascospore production by approximately 10 fold in the double mutant.

In addition to development of sexual structures themselves, NosA has also been shown to be required for transcription of several genes induced during sexual development. Both the hexose transporter *hxtA* and the catalase peroxidase *cpeA* require NosA for full induction under carbon starvation conditions (Vienken and Fischer, 2006). In the presence of glucose, only very low levels of these transcripts are present (Fig. 2C). After incubation with no carbon source, these transcripts are upregulated in the wild type, but only slightly induced in the *nosA* mutant. Addition of either *A.n. nosA* or *A.f. nosA* was able to restore full induction of these transcripts under the conditions tested, displaying a conservation of function between these two transcription factors.

3.2 *A. fumigatus nosA* mutants are altered in development

To ascertain whether a subset of the differences seen in *A. fumigatus ΔlaeA* deletion strains are due to downstream actions of this transcription factor, deletion (*ΔnosA*) and overexpression (*OE::nosA*) alleles were constructed in both wild type and Δ*laeA* backgrounds. To facilitate mutant construction, *A. fumigatus* CEA17 *ΔakuB* strains were used, containing a deletion of the KU80 homolog required for non-homologous end joining (da Silva Ferreira et al., 2006). PCR and Southern analysis confirmed an approximately 50% success rate for transformants with the correct integration (Fig. S2). A representative strain from each background was selected and *nosA* expression determined using northern analysis (Fig. S3), also confirming increased expression in a *ΔlaeA* background relative to wild type (Fig. S3). Complementation of the *ΔnosA* deletion was achieved by transformation of plasmids containing a hygromycin resistance cassette (*hph*) and either *A.n. nosA* + 1 kb flanks or *A.f. nosA* + 1 kb flanks. Strains were confirmed by Southern analysis (Fig. S4).

Examination of *nosA* mutants grown on solid media showed multiple differences relative to wild type (Fig. 3). Under both light and dark conditions, in a wild type background, the *ΔnosA* strain exhibited statistically significant increased radial growth. Interestingly, all *ΔlaeA* strains exhibited a light dependent growth defect, with decreased radial growth under constant light incubations. Overexpression of *nosA* in a wild type background mimicked this effect, with the *OE::nosA* mutant showing relatively less radial growth in light compared to dark.

As the *ΔlaeA* mutant had previously been shown to have various defects in spore development (Bok et al., 2005, Dagenais et al., 2010; reviewed in Bayram and Braus 2012), both spore production and germination of the *nosA* mutants were assessed (Fig. 3C and 4). Under dark but not light conditions, the *ΔnosA* strains showed a slight decrease in production of conidia. Neither overexpression nor deletion of *nosA* restored wild type conidiation to the *ΔlaeA* mutant.

In contrast to the above, deletion of *nosA* restored normal germination in the *ΔlaeA* background. As seen in Figure 4, an assessment of germination rates of the seven strains indicated that deletion of *nosA* increased the rate of germination compared to wild type. This increase in germination rate was abolished by introduction of either ectopic *A.f. nosA* or *A.n. nosA* (data not shown). Furthermore, *nosA* loss was able to remediate the

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germination defect of the *ΔlaeA* mutant. Overexpression of *nosA* in the wild type background was not sufficient to delay germination, but the double *OE::nosA ΔlaeA* strain showed an even further drop in germination rate compared to *ΔlaeA* alone. After 15 hours incubation, these differences are no longer significant, confirming a delay in germination of these strains, rather than a decrease in spore viability (data not shown).

3.2 NosA impacts ROS resistance

A. nidulans nosA is required for normal induction of the catalase-peroxidase *cpeA* and the hexose transporter *hxtA* (Vienken and Fischer, 2006), which are induced during sexual development along with the NADPH oxidase *noxA* (Scherer et al., 2002; Lara-Ortiz et al., 2003). As we showed *A.f. nosA* to functionally complement the induction defect of both *cpeA* and *hxtA* in *A. nidulans* (Fig. 2C), we chose to determine whether *A. fumigatus* also showed the induction of these orthologs (the former called *cat2* in *A. fumigatus,* Takasuka et al., 2009) under carbon limiting conditions, and whether this induction was *nosA* dependent, as in *A. nidulans*. As might be expected from the differences in timing of the sexual cycle between these two species (O'Gorman et al., 2009) transcript levels of these genes in *A. fumigatus* do not mirror those in *A. nidulans* under identical conditions (Fig. S5). A slight induction of transcript levels in medium lacking glucose is seen for all strains, but lacks both the amplitude and *nosA* dependence of that seen in *A. nidulans*.

Although *nosA* dependent differences in *cat2* expression were not detected under the condtions tested above, we next chose to examine whether *A. fumigatus nosA* mutants could exhibit an altered resistance to exogenous sources of ROS by other means, using hydrogen peroxide and menadione as sources. Neither deletion nor overexpression of *nosA* affected radial growth of fungal strains in either a wild type or *ΔlaeA* background when treated with 4 mM hydrogen peroxide (Fig. S6). However all *ΔlaeA* backgrounds were slightly less reduced in radial growth than their controls suggesting a role for *laeA* in protection against ROS generation by hydrogen peroxide.

Treatment with different levels of menadione, however, indicated a need for *nosA* and *laeA* for resistance to this chemical. At 20 μM, whereas there was no difference in growth of *ΔnosA*, *OE::nosA* showed a slight increase in growth compared to wild type (Fig. S6). At 40 μM menadione, deletion of *nosA* in a wild type background resulted in increased susceptibility to this chemical, the strain displaying no growth at this concentration (Fig. 5). Both complementation strains (*ΔnosA* + *A.f. nosA* or + *A.n. nosA*) restored growth. The *OE::nosA* strain again showed a mild resistance relative to wild type, as displayed by a significnantly decreased growth inhibition relative to wild type. All *ΔlaeA* strains displayed increased sensitivity to 40 μM menadione, including the *ΔlaeA OE::nosA* strain. Together, this data suggests that NosA contributes to resistance to menadione but cannot remediate the defect of a *ΔlaeA* allele. Semi-quantitative RT-PCR of transcript levels of identified catalase and superoxide dismutase genes (Takasuka et al., 2009; Lambou et al., 2010) failed to display expression patterns correlating with these differences in resistance (Fig. S6), suggesting the differences in sensitivity are due to modulation of alternative pathways.

3.3 NosA deletant is more virulent in the *Galleria mellonella* model of IA

The properties exhibited by the *nosA* mutants, e.g. increased growth and germination in the *ΔnosA* strain and increased resistance to ROS in the *OE::nosA* strain could both be hypothesized to increase virulence in an animal model. Several insect models have recently been successfully used to screen for alterations in virulence in *A. fumigatus* mutants (Slater et al., 2011) and we therefore chose to examine virulence of *nosA* mutant strains using the *Galleria* model of IA. We found an increase in virulence of the *nosA* deletion strain, but no change in virulence of the overexpression *nosA* strain (Fig. 6). Nevertheless, loss of *nosA*

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did not increase virulence in a *ΔlaeA* strain (data not shown). These differences in virulence do not appear to be due to differential regulation of secondary metabolites, as few differences are seen by TLC analysis (Fig. S7).

As a further investigation on the potential contribution of NosA to *A. fumigatus* virulence, we next examined engulfment and survival of conidia by human primary alveolar macrophages (Fig. S8). However, there was no significant difference in either parameter, suggesting that the increased mortality of the *ΔnosA* strain in the *Galleria* model may not translate to changes in macrophage encounters in other systems.

2 Discussion

Phylogenetic analysis of the LaeA regulated transcription factor Afu4g09710 showed it to be closely related to *A. nidulans nosA* (Fig. 1), which has been characterized for its role in sexual development (Vienken and Fischer, 2006). Heterologous complementation assays showed that *A.f. nosA* is able to partially functionally compensate for *A.n. nosA* loss when introduced in *A. nidulans*. Examination of sexual structures showed a partial compensation of the developmental block seen in Δ*nosA* strains, as demonstrated by an increase in both size of cleistothecia primordia, and amount of ascospores produced (Fig. 2). Although the defect in development of sexual structures was not completely remediated, carbon starvation dependent induction of the sexual cycle induced genes *cpeA* and *hxtA* was restored to wild type levels in the *A. nidulans* Δ*nosA* + *A.f. nosA* strain. This suggests that the function of NosA is considerably conserved between these two species, and the lack of a full complementation maybe be due to either a slight divergence in specialization, or differences in expression, perhaps due to missing elements within the 1 kb promoter used. Both divergence (ex. CflA; Boyce et al., 2001) and promoter differences (ex. StuA; Miller et al., 1991) have previously been shown to influence the degree of complementation in closely related fungi.

We then began dissecting the action of NosA on *A. fumigatus* physiology, both in wild type and *ΔlaeA* backgrounds. As *nosA* is upregulated in a *ΔlaeA* background (Perrin et al., 2007; Fig. S2), we were particularly interested in the consequences of overexpressing *nosA* in a wild type background and deletion of *nosA* in a *ΔlaeA* background, to dissect which portions of the *ΔlaeA* phenotype may be attributed to altered expression of this transcription factor. Initial phenotypic analysis uncovered a role of *nosA* in growth and development under the conditions tested. Mutant response differed between light and dark conditions, further demonstrating the presence of a light sensing program in *A. fumigatus* related to acquiring developmental competence (Sheppard et al., 2005; Ben-Ami et al., 2010). The *OE::nosA* mutant displayed a distinct defect in radial growth when incubated under light conditions, mimicking the defect seen in *ΔlaeA* strains under this condition. Deletion of *nosA*, in contrast, led to increased radial growth of wild type strains under both light and dark conditions, although it failed to restore the light dependent growth defect of *ΔlaeA.* Increased growth of *ΔnosA* corresponded to a defect in production of conidia under dark conditions, suggesting a shift in morphological programming to vegetative growth. Both of these effects were negated by the introduction of an ectopic copy of either *A.f nosA* or *A.n. nosA*.

As conidia production differed among strains in our mutant series, we chose to explore whether other conidial characteristics were also altered, such as rate of germination. *ΔnosA* strains showed increases in germination rate in a wild type background, and loss of *nosA* was able to completely rescue the delayed germination of the *ΔlaeA* strain. Taking the radial growth and germination data together, this suggests that NosA regulates a subset of genes

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downstream of *ΔlaeA,* contributing to some of the *ΔlaeA* abberancies in vegetative growth and spore germination (Bok et al., 2005).

A. nidulans nosA mutants show impaired expression of the catalase-peroxidase *cpeA* (Vienken and Fischer, 2006). As host produced reactive oxygen species play a role in defense against pathogens (Dagenais and Keller, 2009), we examined *A. fumigatus nosA* mutants response to the reactive oxygen species generators H_2O_2 and menadione. Hyphal growth on H_2O_2 was unaffected by *nosA* mutations, although a slight resistance was seen in *ΔlaeA* mutants. In contrast, *ΔlaeA* and *ΔnosA* strains displayed an increased sensitivity to 40 μM menadione. While H_2O_2 produces peroxide radicals, menadione is believed to produce both superoxide and peroxide radicals (Farr and Kogoma, 1991). This suggests that both of these mutants may be more sensitive to superoxide, rather than peroxide radicals, although likely through different pathways. This is emphasized by the slightly increased radial growth of the *OE::nosA* strain on menadione, but the inability of the *OE::nosA ΔlaeA* strain to grow on this medium.

We were curious to test the virulence of the *nosA* mutants as characteristics of both the overexpression (increased resistance to menadione) and deletion (increased vegetative growth and germination rates) *nosA* strains have been correlated with virulence in other mutants (Ejzykowicz et al., 2009; Kim et al., 2009; reviewed in Abad et al., 2010). Because the *ΔlaeA* mutant (with increased *nosA* expression) is hypovirulent (Bok et al., 2005, Sugui et al. 2007, Ben-Ami et al. 2009), we predicted that the *ΔnosA* strain was more likely to show increased virulence than the *OE::nosA* strain. We chose to use the *Galleria mellonella* insect model for our virulence tests as this model has recently been shown to encode homologs of the NADPH oxidase complex required for the generation of superoxide radical ions (Bergin et al., 2005), and has been shown to yield similar results as murine models of pathogenicity for known virulence factors (Slater et al., 2011). Figure 6 shows that *ΔnosA* displayed increased pathogenicity in our model. Although the exact reason(s) for this remains to be elucidated, we propose that this may be due to the increased vegetative growth and germination of the *ΔnosA* mutant. Previous research has shown that virulence of *A. fumigatus* to *G. mellonella* larvae increases along with the germination status of the spore (Renwick et al., 2006). On the other hand, it appears that the enhanced sensitivity of *ΔnosA* and increased resistance of the *OE::nosA* to menadione was of little relevance in the insect/ fungal interaction.

Taken together, our results indicate that *A. fumigatus* Afu4g09710 acts as a functional homolog of *A. nidulans nosA*. The differences in radial growth, sporulation, and germination of *A. fumigatus nosA* mutants show that *nosA* has an impact on additional aspects of fungal development and chemical testing indicates NosA contributes to resistance to menadione. Finally, the slow growth and delayed germination phenotype of *ΔlaeA* appear to be, at least in part, regulated by NosA. We speculate that the *Galleria* results shown here could be primarily attributable to the faster germination and vegetative growth rate of the *ΔnosA* strain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IA	invasive aspergillosis
WT	wild type
ROS	reactive oxygen species

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Highlights

1. LaeA regulated transcription factors effect virulence and development downstream
2. NosA is negatively regulated by LaeA
3. The *ΔlaeA* germination defect is remediated in the double *ΔnosA ΔlaeA* mutant
4. *ΔnosA* has increased pathogenicity in the *Galleria* model of invasive aspergillosis
5. NosA is involved in resistance to menadione

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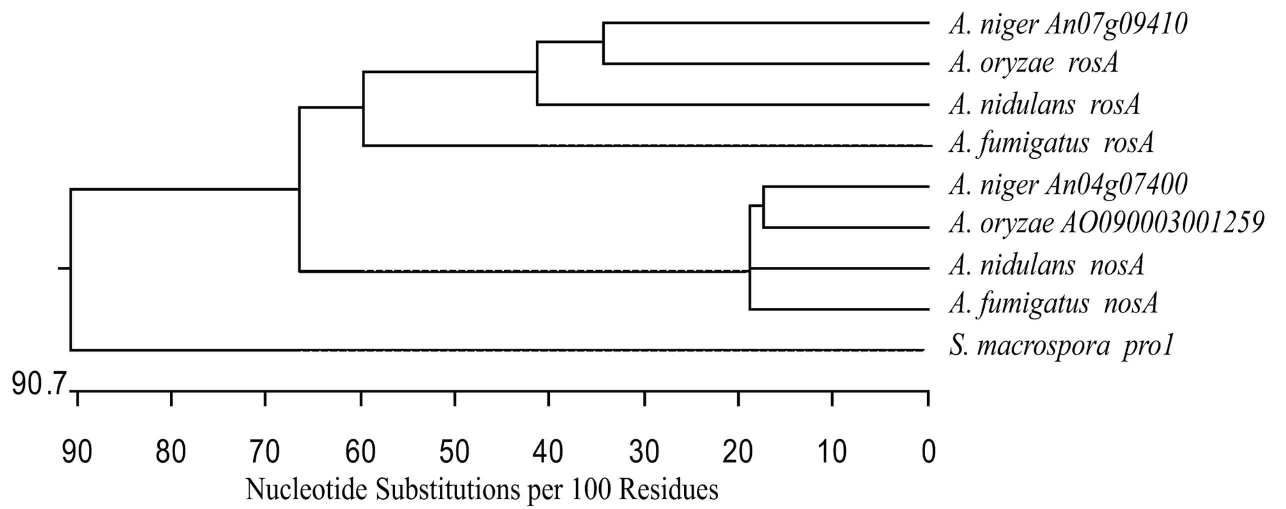

Fig. 1.

Phylogenetic tree depicting relatedness of *nosA* homologs. ClustalW analysis of cDNA sequences of *Aspergillus nosA* homologs and the related *S. macrospora pro1* shows that within the Aspergilli, all *nosA* sequences group within a single clade, distinct from the *rosA* homologs.

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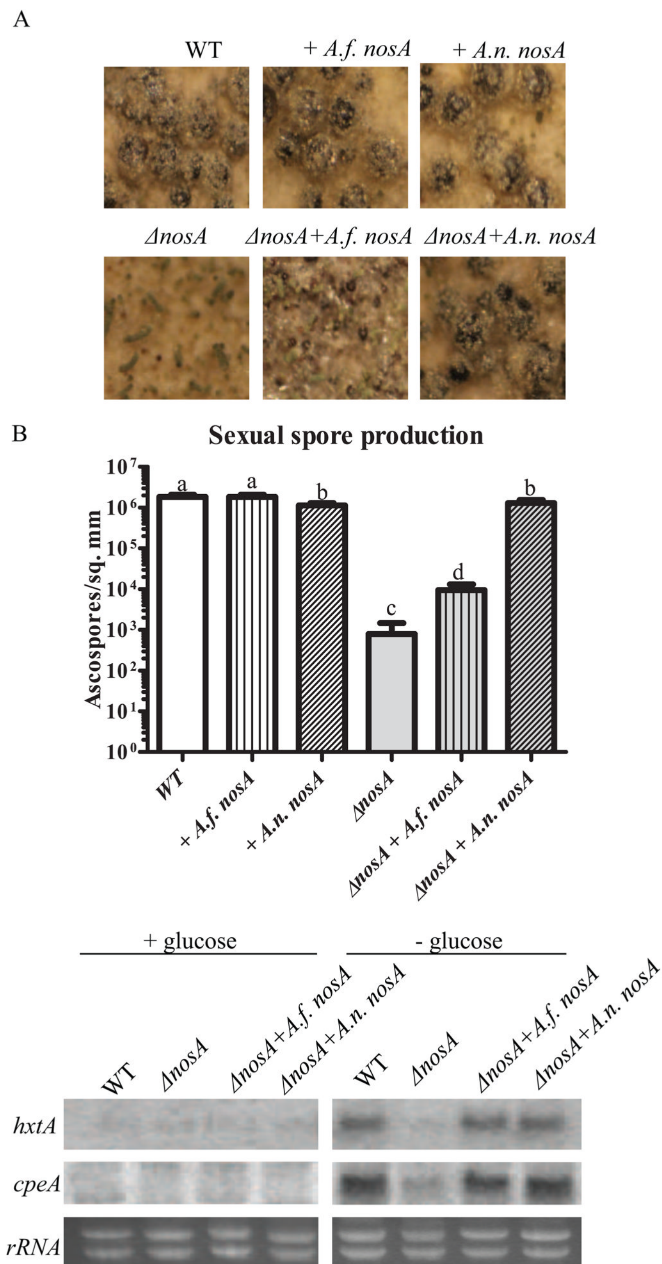

Fig. 2.

A.f. nosA can partially complement *A. nidulans ΔnosA* developmental defects. A. *A. nidulans ΔnosA* is defective in development, showing an arrest at the primordial stage of cleistothecial development (Vienken and Fischer, 2006). Introduction of an ectopic copy (*ΔnosA + A.n. nosA*) of wild type *nosA* is able to complement this defect. Heterologous complementation with *A.f. nosA*, in contrast, is only able to partially restore development, as seen in the increased size of cleistothecia/primordia. B. Analysis of completion of the sexual cycle by determination of ascospores produced. *ΔnosA* strains are severely impaired in development, producing ~1000 fold fewer ascospores than wild type (Vienken and Fischer, 2006). Introduction of an ectopic copy of *A.n. nosA* is able to nearly fully restore sexual spore production. Inclusion of *A.f. nosA* increases sexual spore production ~10 fold, but is not able to fully remediate ascospore production. Different letters denote significant

differences in ascospore numbers at $p < 0.05$. C. *ΔnosA* strains also display a defect in induction of several genes under carban starvation, including the catalase peroxidase *cpeA* and the hexose transporter *hxtA* (Vienken and Fischer, 2006). Complementation with either *A.n. nosA* or *A.f. nosA* is able to restore starvation dependent induction of these genes.

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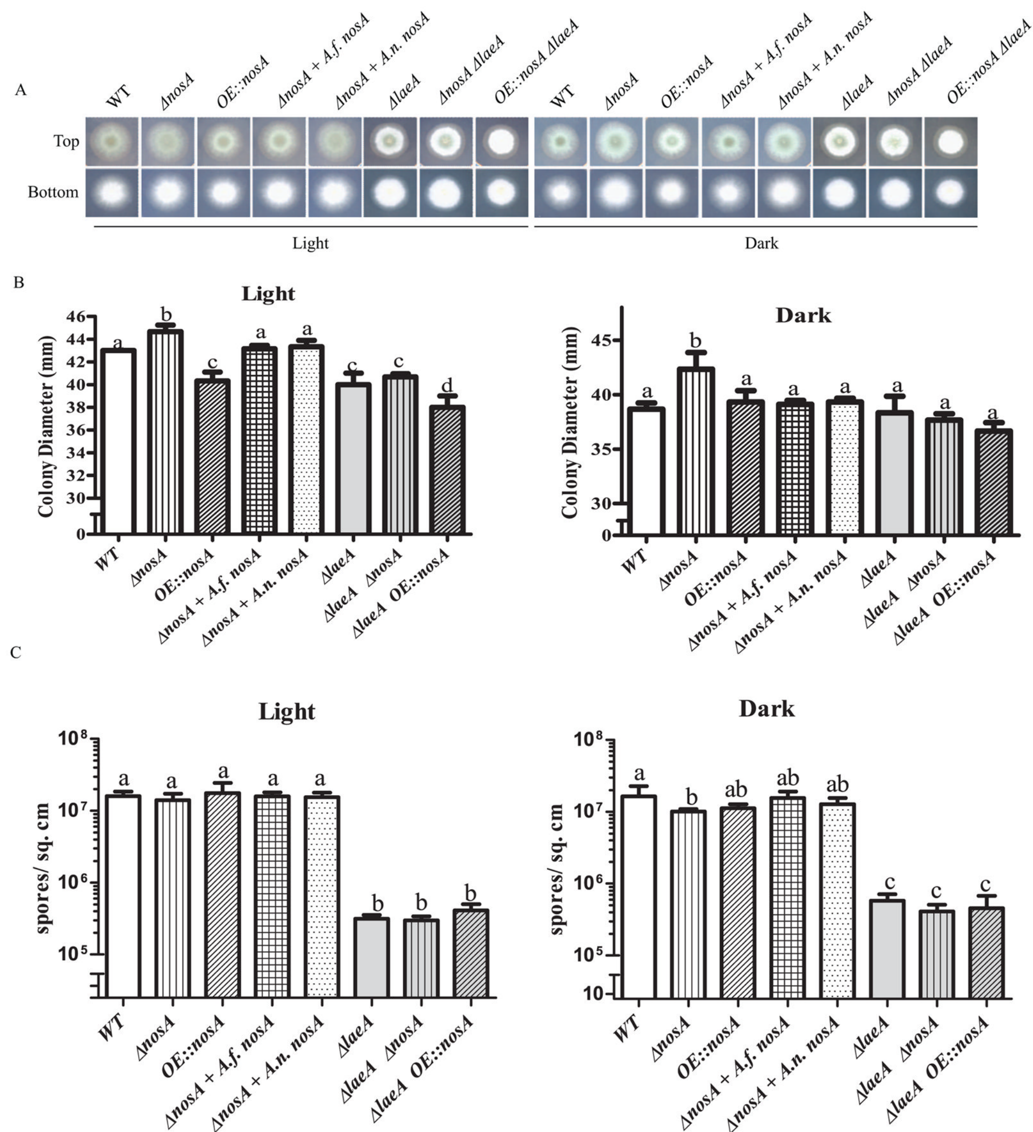

Fig. 3.

Colony morphology of *nosA* mutants grown for 3 days on GMM at 37°C. A. Morphology of NosA mutants. B. Measurement of colony diameters reveals small but significant differences among strains. Under light conditions, the *OE::nosA* strain has a defect in radial growth compared to wild type. This decrease is abrogated under dark conditions and in a *ΔlaeA* background. Deletion of *nosA* increased colony diameter in a wild type background, but not in a *ΔlaeA* background. Error bars represent standard deviation of 4 replicates. Letters represent significant differences at $p < 0.05$. C. Conidia production of *nosA* mutants grown for 6 days on GMM at 37°C. The *ΔnosA* strain exhibits a dark dependent defect in conidiation relative to wild type. This difference is not seen under light conditions or in a *ΔlaeA* background. Error bars represent standard deviation of 4 replicates. Letters represent significant differences at $p < 0.05$.

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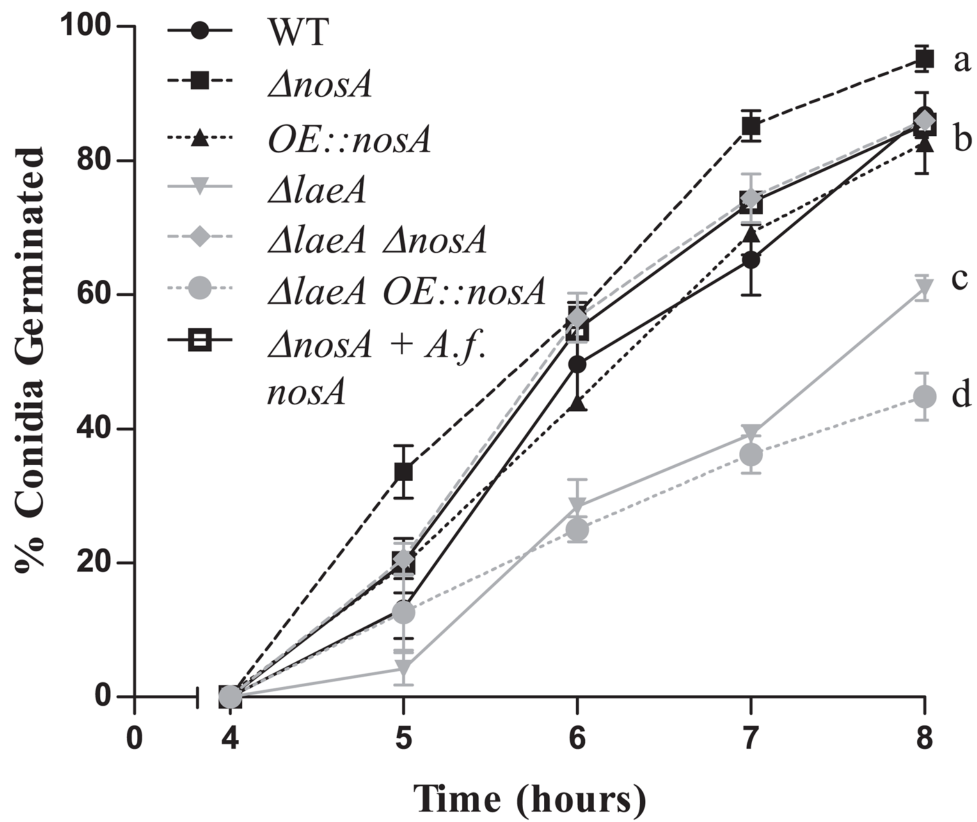

Fig. 4.

Germination rate of *nosA* mutants. *ΔnosA* displays an increased germination rate compared to wild type and restores the rate of germination to wild type levels in the *ΔlaeA* background (*ΔlaeA ΔnosA*). In contrast, overexpression of *nosA* does not affect germination in either a wild type but further decreases germination rate in a *ΔlaeA* background. Four replicates of 100 spores for each strain were counted. Letters represent significant differences at $p < 0.05$.

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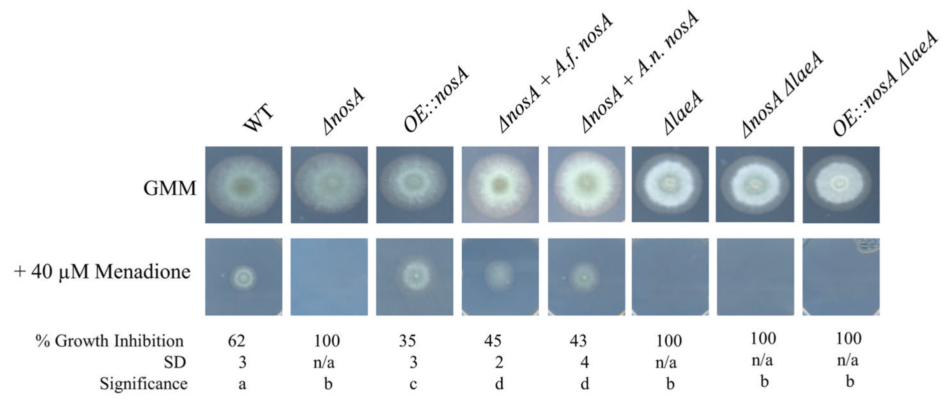

Fig. 5.

Resistance to the ROS generator menadione. Both deletion of *nosA* and *laeA* show an increased sensitivity to menadione, evidenced by no growth on media containing 40 μM menadione. The *OE::nosA* strain shows a slight increase in resistance relative to wild type, as evidenced by a significantly decreased growth inhibition. Letters represent significant differences at $p < 0.05$.

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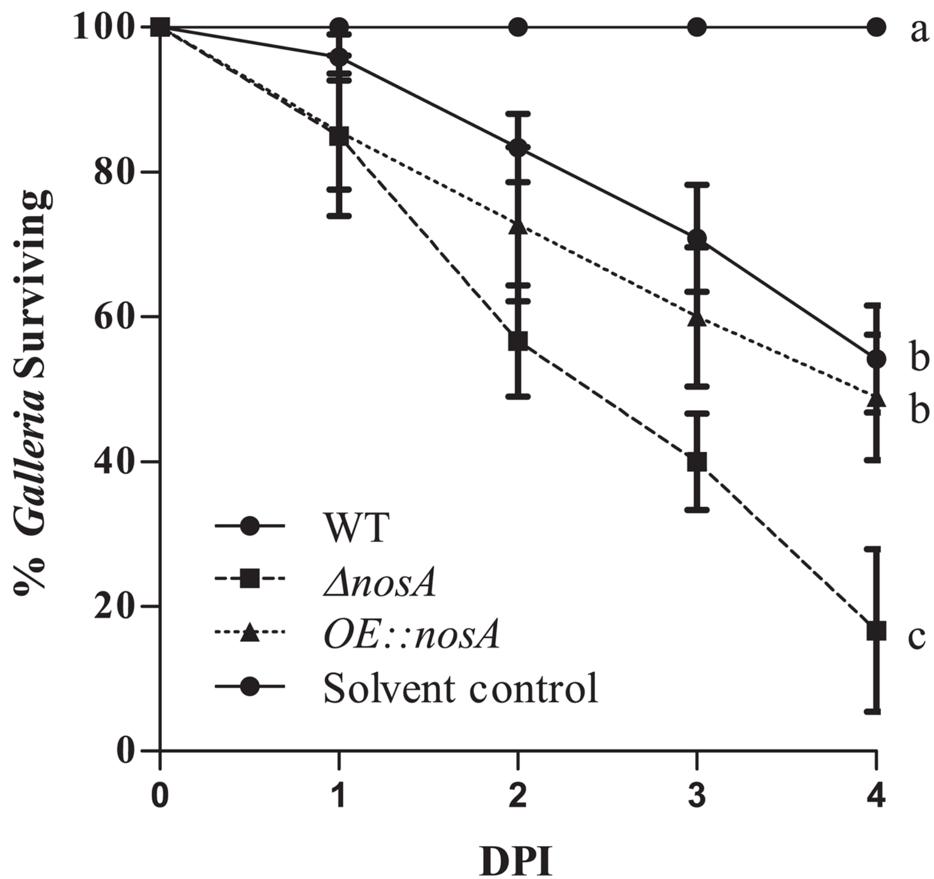

Fig. 6.

Deletion of *nosA* increased virulence in a *Galleria mellonella* insect model. Mortality was significantly increased at 4 days in larvae injected with *ΔnosA* spores. No significance differences are seen between wild type and *OE::nosA* strains. Errors represent SEM of 3 independent experiments of 30 infected larvae/strain. Letters represent significant differences at $p < 0.05$.

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Table 1

Aspergillus fumigatus strains and plasmids used in this study

Strain name	Genotype	Derivative of	Source
CEA17 KU80 *pyrG-*	*ΔnkuB; pyrG-*	*A. fumigatus* CEA17	da Silva Ferreira et al., 2006
TFYL19.3	*ΔnkuB; ΔlaeA::hph; pyrG-*	*A. fumigatus* CEA17	F.Y. Lim and N.P. Keller
CEA17 KU80	*ΔnkuB*	*A. fumigatus* CEA17	da Silva Ferreira et al., 2006
TFYL20.10	*ΔnkuB; ΔlaeA::hph; A parasiticus pyrG*	*A. fumigatus* CEA17	F.Y. Lim and N.P. Keller
TAAS104	*ΔnkuB; ΔnosA::A parasiticus pyrG*	*A. fumigatus* CEA17	This study
TAAS105	*ΔnkuB; nosA(p)::A. parasiticus pyrG::A. nidulans gpdA(p)::nosA*	*A. fumigatus* CEA17	This study
TAAS107	*ΔnkuB; ΔlaeA::hph; ΔnosA::A parasiticus pyrG*	*A. fumigatus* CEA17	This study
TAAS108	*ΔnkuB; ΔlaeA::hph; nosA(p)::A. parasiticus pyrG::A. nidulans gpdA(p)::nosA*	*A. fumigatus* CEA17	This study
TAAS155.3	*ΔnkuB; ΔnosA::A parasiticus pyrG + pAAS155*	*A. fumigatus* TAAS104	This study
TAAS156.5	*ΔnkuB; ΔnosA::A parasiticus pyrG + pAAS156*	*A. fumigatus* TAAS104	This study
RJMP1.1	*pyrG89; pyroA4; riboB2; ΔnkuA::argB; veA1*	*A. nidulans*	J. Palmer and N.P. Keller
RJMP1.59	*pyrG89; pyroA4*	*A. nidulans*	Shaaban et al., 2010
RJMP101.19	*pyrG89*	*A. nidulans*	J. Palmer and N.P. Keller
TAAS157.13	*ΔnosA::A parasiticus pyrG; pyrG89; pyroA4; riboB2; ΔnkuA::argB; veA1*	*A. nidulans* RJMP1.1	This study
TAAS149.4	*pyroA::pAAS149::pyroA4; pyrG89*	*A. nidulans* RJMP1.59	This study
TAAS150.11	*pyroA::pAAS150::pyroA4; pyrG90*	*A. nidulans* RJMP1.59	This study
RAAS160.4	*ΔnosA::A parasiticus pyrG*	TAAS157.13 x RJMP101.19	This study
RAAS158.6	*ΔnosA::A parasiticus pyrG; pyroA::pAAS149::pyroA4*	TAAS157.13 x RTAAS149.4	This study
RAAS159.8	*ΔnosA::A parasiticus pyrG; pyroA::pAAS150::pyroA4*	TAAS157.13 x RTAAS150.11	This study
RAAS161.7	*pyroA::pAAS149::pyroA4*	TAAS149.4 x RJMP101.19	This study
RAAS162.2	*pyroA::pAAS150::pyroA4*	TAAS150.11 x RJMP101.19	This study
RJMP103.5	WT	*A. nidulans*	Soukup et al., 2012
pJW24	*A. parasitcus pyrG* in pBluescript	pBluescript	Calvo et al., 2004
pJMP9.1	*A. parasitcus pyrG – A. nidulans gpdA* in pBluescript	pBluescript	J. Palmer and N.P. Keller
pUCH2.8	*hygR* in pBluescript	pBluescript	Alexander et al., 1998
pJW53	3/4 *A. nidulans pyroA* in pBluescript	pBluescript	Shaaban et al., 2010
pAAS149	*A. fumigatus nosA* and flanks in pJW53	pJW53	This study
pAAS150	*A. nidulans nosA* and flanks in pJW53	pJW53	This study
pAAS155	*hygR* in pAAS149	pAAS149	This study
pAAS156	*hygR* in pAAS150	pAAS150	This study