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Role of Nitric Oxide and Flavohemoglobin Homolog Genes in *Aspergillus nidulans* Sexual Development and Mycotoxin Production^{∇†}

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Flavohemoglobins are widely distributed in both prokaryotes and eukaryotes. These proteins are involved in reducing nitric oxide levels. Deletion of the *Aspergillus nidulans* flavohemoglobin gene *fhbA* induced sexual development and decreased sterigmatocystin production. Supplementation with a nitric oxide-releasing compound promoted cleistothecial formation and increased *nsdD* and *steA* expression, indicating that nitric oxide induces sexual development. This is the first study on the effect of nitric oxide on morphogenesis and secondary metabolism in fungi.

Nitric oxide (NO) is a signaling compound of great importance in biological systems (7, 10, 21). This molecule can also cause nitrosative stress which is potentially remediated by the widely distributed flavohemoglobins (FHbs) found in both eukaryotes and prokaryotes (4, 7). Extensive research on FHb proteins from bacteria and yeast revealed their structure, function, and mechanism of action (3, 7, 17). Earlier studies have shown that FHbs in organisms such as *Saccharomyces cerevisiae*, *Alcaligenes eutrophus*, and *Escherichia coli* share similar steady-state NO dioxygenation kinetics (7). Through a dioxygenase-mediated reaction, FHbs, in the presence of molecular O₂, converts NO into nontoxic nitrate ions. FHb proteins contain a hemoglobin-like domain with a noncovalently bound heme B protein and a reductase domain with binding sites for FAD and NAD(P)H. It is known that *fhb* genes are activated by various agents, such as nitrate, nitrite, NO, and NO-releasing agents (5, 7, 8, 11, 20, 24). In aspergilli, the conversion of NO to NO₃⁻ by FHbs, Fhb1 and Fhb2 in *Aspergillus oryzae* (30) and FhbA and FhbB in the filamentous fungus model *Aspergillus nidulans*, has been demonstrated (24). The present work involves the study of the role of FHbs and NO in fungal development and secondary metabolism.

Fungal strains and growth conditions. *Aspergillus nidulans* Cib08 (*biA1 yA2*), CibA (*biA1 yA2 ΔfhbA::argB*), CibB (*biA1 yA2 ΔfhbB::argB*) (described in reference 24), and FGSCA4 were used in this study. The strains were cultured on glucose minimum medium (GMM) plus the appropriate supplements for the corresponding auxotrophic markers (13). Medium was supplemented with 1.5 mM diethylenetriamine-NoNoate (Sigma), a NO-releasing compound, after sterilization when indicated. Solid medium was prepared by adding 10 g/liter agar. Strains were stored as 30% glycerol stocks at -80°C.

Morphological studies. Plates containing 25 ml of solid GMM plus the appropriate supplements were top agar inoculated with 5 × 10⁶ spores per plate of medium. The cultures were wrapped and incubated at 37°C in the dark. Cores (8-mm diameter) were collected from each spread plate and examined for cleistothecium production. Ethanol (70%) was sprayed on the core surface to facilitate the visualization of cleistothecia under the microscope. The experiments included five replicates and were repeated twice, with similar results.

mRNA analysis. Total RNA was isolated from mycelia at 48 h and 72 h after inoculation as previously described (23). Five micrograms of total RNA was treated with DNase I RQI (Promega) and reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Quantitative reverse transcription-PCR (qRT-PCR) was performed with an Mx3000P thermocycler (Stratagene) using SYBR green JumpStart Tag Ready mix (Sigma). The primer pairs used for qRT-PCR are listed in Table 1.

ST analysis. Three cores (16-mm diameter) from each replicate of cultures grown on GMM were harvested after 5 days of incubation and placed in a 50-ml Falcon tubes. Sterigmatocystin (ST) was extracted by adding 5 ml of CHCl₃. Extracts were fractionated by thin-layer chromatography using toluene-ethyl acetate-formic acid (60:30:10 [vol/vol]) as a solvent system. The thin-layer chromatography (TLC) silica plates were sprayed with aluminum chloride (12.5% in ethanol) to intensify fluorescence upon exposure to long-wave (375-nm) UV light and baked for 10 min at 80°C prior to viewing.

The *fhbA* and *fhbB* gene products are highly conserved in aspergilli (see Fig. S1 and S2 in the supplemental material) and are part of a specific phylogenetic group (25). In *A. nidulans*, *fhbA* has been shown to be AreA independent and a NirA target, which allows FhbA to avoid nitrogen metabolite repression (24). On the other hand, *fhbB* was found constitutively expressed (24). A report by te Biesebeke et al. (25) has suggested a role in mycelial branching. However, whether NO and related FHbs have a function in regulating development or secondary metabolism in the fungal kingdom was completely unknown. In this report, we describe for the first time that NO

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TABLE 1. Primers used in this study

Primer	Sequence
actin-F.....	5'-ATGGAAGAGGAAGTTGCTGCTCTCGTTATCGACAATGGTTC-3'
actin-R.....	5'-CAATGGAGGGGAAGACGGCACGGG-3'
<i>nsdD</i> -F.....	5'-CATCTCACCAGCCACAATTACAGGCCGGAACCATCAC-3'
<i>nsdD</i> -R.....	5'-TTGCGAGCCAGACACAGAGGTCATAACAGTGCTTGC-3'
<i>steA</i> -F.....	5'-TCCAGCAAATGGAACCGTGGAAATCAGGTGCTC-3'
<i>steA</i> -R.....	5'-GAAGGGATGGGGCAAGAATGAGACTTCTGCGGGTAA-3'
<i>affR</i> -F.....	5'-ATGGAGCCCCAGCGATCAGCCAG-3'
<i>affR</i> -R.....	5'-TTGGTGATGGTGCTGTCTTTGGCTGCTCAAC-3'

and FHbs are regulators of sexual development and ST toxin production in *A. nidulans*.

NO has been shown to regulate multiple physiological processes, particularly in mammals (6, 12, 15, 18, 22, 31). These reports reflect that one of the important roles of NO in higher eukaryotes is the regulation of reproduction in males and females. Our study revealed that NO also is involved in the control of sexual development in fungi. We found that the *A. nidulans fhbA* deletion ($\Delta fhbA$) mutant presented an increase in Hülle cell production with respect to the control strain. Hülle cells are postulated to be nursing cells for the formation of fruiting bodies called cleistothecia (27). This result suggests that NO accumulation in the absence of *fhbA* might have a positive effect on the induction of sexual development (Fig. 1A). $\Delta fhbA \Delta fhbB$ presented the same phenotype as $\Delta fhbA$; however, $\Delta fhbB$ did not show this effect (data not shown). This is in agreement with the higher NO stress susceptibility observed in the $\Delta fhbA$ mutant (24). The increase in Hülle cell numbers observed in the $\Delta fhbA$ strain at early time points was

found independent of *nsdD* and *steA* expression, both genes encoding transcription factors necessary for sexual development (9, 26) (data not shown). In older cultures, $\Delta fhbA$ (and $\Delta fhbA \Delta fhbB$) resulted in a slight increase in cleistothecia with respect to the level for the wild type (Fig. 1B). Cleistothecia produced by $\Delta fhbA$, $\Delta fhbB$, or $\Delta fhbA \Delta fhbB$ produced viable ascospores (data not shown). With respect to asexual development, we did not find significant differences under the experimental conditions assayed (data not shown).

Interestingly, addition of the NO-releasing compound drastically increased the formation of cleistothecia in *A. nidulans* (Fig. 1B and C). This increase in sexual development was accompanied by a higher accumulation of *nsdD* and *steA* transcripts in the NO-treated cultures than in those cultures without the treatment (Fig. 2). Overall, these results support the role of NO as a positive regulator of sexual development in *A. nidulans*. It is known that the presence of NO at a low concentration acts as a signaling molecule in biological systems (7, 10, 21), while at higher levels this compound causes nitrosative

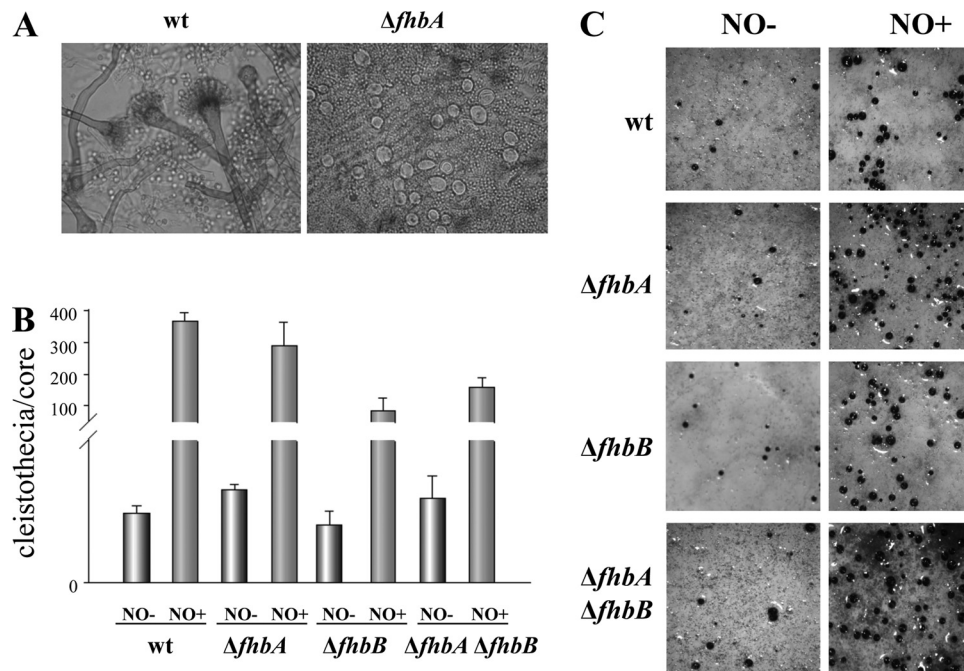


FIG. 1. Deletion in *fhbA* or supplementation of nitric oxide (NO) promotes *A. nidulans* sexual development. (A) Micrographs showing Hülle cells produced by $\Delta fhbA$ 5 days after inoculation on solid GMM (right), while no Hülle cells were detected in cultures of the isogenic control strain (left). wt, wild type. (B) Quantification of cleistothecia produced by the wild type and the *fhb* mutants with and without NO supplementation (core = 16-mm diameter). (C) Micrographs of the wild type and the *fhb* mutants with and without nitric oxide (NO) supplementation (35 \times). Cultures were sprayed with 70% ethanol to facilitate the visualization of cleistothecia.

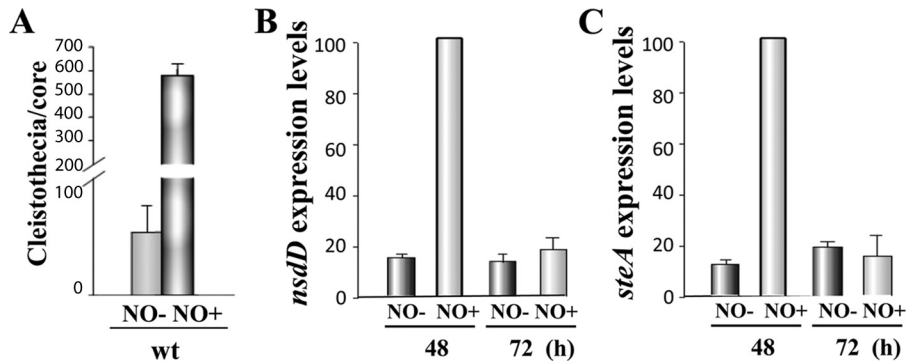


FIG. 2. Addition of nitric oxide increases *nsdD* and *steA* expression. (A) Quantification of cleistothecia produced by the wild type with and without NO supplementation (core = 16-mm diameter). (B) qRT-PCR results showing *nsdD* expression levels in NO-treated versus nontreated cultures 48 h and 72 h after inoculation. (C) *steA* expression levels in NO-treated versus nontreated cultures 48 h and 72 h after inoculation. The relative expression levels were calculated using the method described by Kenneth and Schmittgen (14), and all values were normalized to the expression of the *A. nidulans* actin gene.

stress (4, 7). It is possible that although either *fhbA* deletion or NO supplementation is conducive to an increase of fungal sexual development, only higher nitrosative stress levels lead to the increase of *nsdD* and *steA* expression, resulting in a drastic increase in cleistothecium formation in NO-supplemented cultures at notably higher levels than those in the wild-type or *fhb* mutants. Future studies will examine the effect of NO level in these processes. The same induction effect of NO on sexual development was observed in both a strain carrying the *veA1* allele (Fig. 1 and 2) and also in a strain carrying the *veA*⁺ allele (see Fig. S3 in the supplemental material), indicating that this effect is independent of the *veA* allele.

Recent studies showed that FHbs in aspergilli could also be related to the oxidative stress response (29). It is known that oxidative stress influences sexual development in *A. nidulans* (16). It is possible that the effect of NO on sexual development could also be in part linked to self-inflicted reactive oxygen

species (ROS) production that occurs during the initiation of sexual development (16).

In addition to its effect on fungal morphogenesis, *fhbA* also influences mycotoxin biosynthesis in *A. nidulans* (Fig. 3). $\Delta fhbA$ (or $\Delta fhbA \Delta fhbB$) showed a reduction of ST production. This reduction coincided with a decrease in the expression of *aflR*, a transcription factor necessary for the activation of the ST gene cluster (28) (Fig. 3). However, $\Delta fhbB$ produced the same amount of toxin as the control strain. Surprisingly, supplementation with NO showed a recovery of ST near wild-type level in strains with a $\Delta fhbA$ background (Fig. 4A and B). NO supplementation resulted in an increase of *aflR* expression levels (Fig. 4C). Our experiments suggest that different NO threshold levels could have opposite effects on mycotoxin production. It is also possible that *fhbA* in *A. nidulans* could influence other regulatory mechanisms in addition to those related to NO detoxification. Due to the conservation of FHbs in

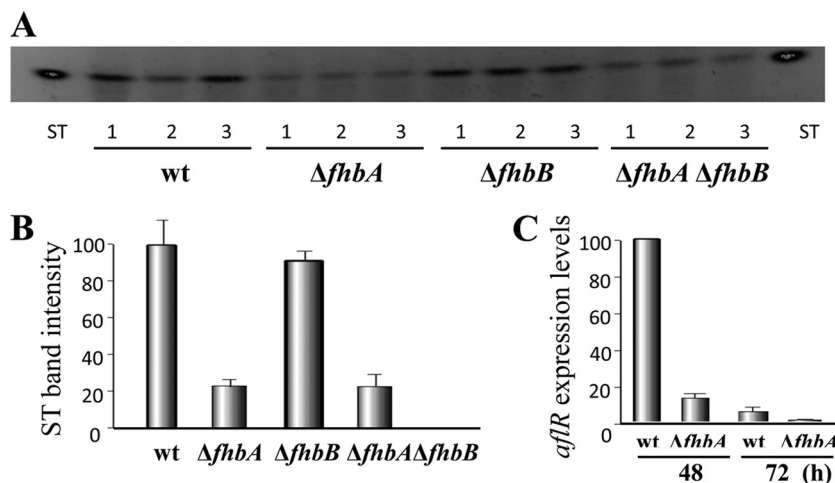


FIG. 3. Production of sterigmatocystin (ST) in *A. nidulans* is regulated by *fhbA*. (A) ST TLC analysis from wild-type, $\Delta fhbA$, $\Delta fhbB$, and $\Delta fhbA \Delta fhbB$ cultures grown on GMM for 5 days. (B) Densitometry of the TLC image in panel A. Band intensity quantification was carried out with Scion Image Beta 4.03 software. The normalized ST band intensity values were normalized with respect to the highest level of intensity, considered 100. (C) *aflR* expression levels in the $\Delta fhbA$ strain and its isogenic control strain at 48 h and 72 h after inoculation on solid GMM. The relative expression levels were calculated using the method described by Kenneth and Schmittgen (14), and all values were normalized to the expression of the *A. nidulans* actin gene.

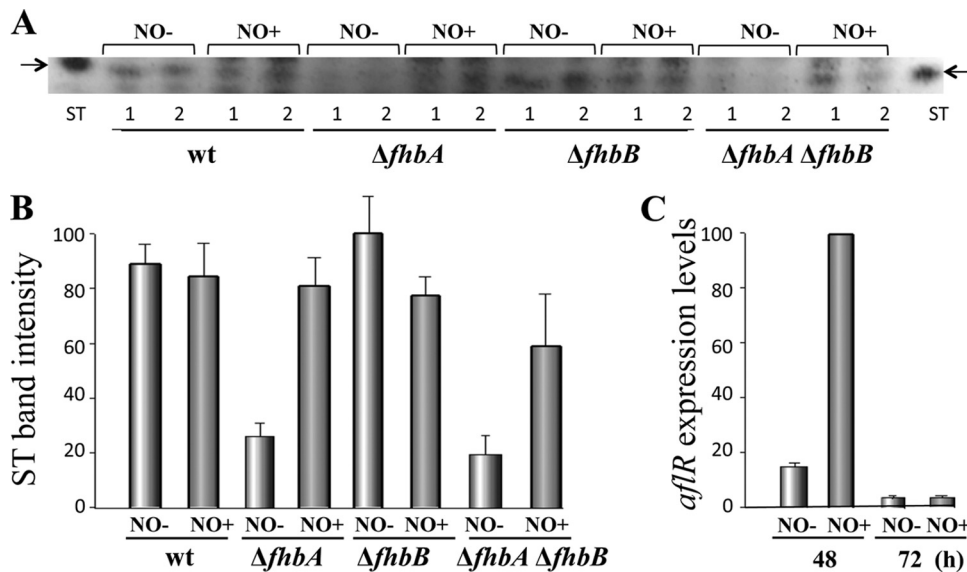


FIG. 4. Supplementation of NO positively affects ST production. (A) ST TLC analysis from wild-type, $\Delta fhbA$, $\Delta fhbB$, and $\Delta fhbA \Delta fhbB$ cultures with and without NO supplementation grown on GMM for 5 days. (B) Densitometry of the TLC image in panel A. Band intensity quantification was carried out with Scion Image Beta 4.03 software. The normalized ST band intensity values were normalized with respect to the highest level of intensity, considered 100. (C) *aflR* expression levels in NO-treated versus nontreated cultures 48 h and 72 h after inoculation. The relative expression levels were calculated using the method described by Kenneth and Schmittgen (14), and all values were normalized to the expression of the *A. nidulans* actin gene.

other aspergilli and due to the fact that ST and aflatoxin are synthesized through the same biosynthetic pathway, it is possible that *fhbA* could also play a role in regulating the biosynthesis of the potent carcinogenic mycotoxin aflatoxin (1, 2, 19).

This is the first study of the effect of NO on sexual development and secondary metabolism in fungi, further revealing NO as a signaling molecule in another kingdom of lower eukaryotes, suggesting an ancient role for this simple molecule in complex regulatory pathways affecting numerous cellular processes, including morphogenesis and metabolism.

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