EUKARYOTIC CELL, May 2010, p. 774–783 Vol. 9, No. 5
1535-9778/10/$12.00 doi:10.1128/EC.00375-09

Conserved Regulators of Mating Are Essential for *Aspergillus fumigatus* Cleistothecium Formation▽†

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Received 15 December 2009/Accepted 17 March 2010

Sexual reproduction of the human pathogen *Aspergillus fumigatus* (teleomorph: *Neosartorya fumigata*) was assumed to be absent or cryptic until recently, when fertile crosses among geographically restricted environmental isolates were described. Here, we provide evidence for mating, fruiting body development, and ascosporogenesis accompanied by genetic recombination between unrelated, clinical isolates of *A. fumigatus*, and this evidence demonstrates the generality and reproducibility of this long-time-undisclosed phase in the life cycle of this heterothallic fungus. Successful mating requires the presence of both mating-type idiomorphs *MAT1-1* and *MAT1-2*, as does expression of genes encoding factors presumably involved in this process. Moreover, analysis of an *A. fumigatus* mutant deleted for the *nsdD* gene suggests a role of this conserved regulator of cleistothecium development in hyphal fusion and hence heterokaryon formation.

For decades, aspergilli have served as model organisms in genetic studies, based on their multifaceted life cycle. The characteristic asexual reproductive mode of *Aspergillus*, during which conidiospores are abundantly formed on highly characteristic structures, the conidiophores (synonym, aspergillum) (2), defines the genus morphologically. Fundamental aspects of the genetics, such as heterokaryosis and parasexuality, were first studied in the model system *Aspergillus nidulans* (38). Moreover, about one-third of all known *Aspergillus* species are able to form fruiting bodies, the so-called cleistothecia, in which recombinant ascospores are generated by meiosis (8, 15, 48). Again, most insights into cleistothecium formation stem from studies on the homothallic ascomycete *A. nidulans* with its teleomorph *Emericella nidulans*, for which a variety of environmental as well as genetic determinants for successful fruiting were identified: surface growth in darkness and with restricted aeration generally favors sexual development, which might correspondingly be the dominant reproductive mode in the soil or within substrates. Nuclear identity is presumably achieved by a bipolar mating-type system comprising the high-mobility group (HMG) factor MatA and the α-box protein MatB (36, 46). Among regulators of cleistothecium formation that have been characterized at the molecular level, the GATA-type transcription factor NsdD plays a prominent role, as it determines the balance between asexual and sexual propagation and positively supports fruiting (18).

For *Aspergillus fumigatus*, the predominant human pathogen among aspergilli, sexuality had been only supposed for a long time, based on several lines of reasoning. The very first hints were deduced from the preliminary genome sequence, in which several genes likely to encode a pheromone and respective pheromone receptors could be identified (37). Moreover, the presence of a putative mating-type locus was pointed out in these primary studies (12, 53). A more comprehensive inspection of the complete and annotated genome sequence retrieved several more homologues that are involved in mating or ascosporogenesis of ascomycetous fungi (14, 32), and phylogenetic analyses also suggested the existence of a sexual reproductive mode (39). Strong evidence for the presence of an *A. fumigatus* sexual cycle was provided by identification of a bipolar mating-type system resembling that of *A. nidulans* (32) and equal distributions of either of the mating-type idiomorphs *MAT1-1* and *MAT1-2* among worldwide isolates (35). Gene-swapping experiments for both *A. fumigatus* idiomorphs accompanied by overexpression studies for a conserved regulator of fruiting body development in a fertile *Aspergillus* species demonstrated their functional conservation (16, 41). Ultimate proof, however, for the existence of a sexual phase within the *A. fumigatus* life cycle was given recently, when isolates of a limited regional sample set from Dublin City, Ireland, were successfully paired on self-made oatmeal agar plates after 6 months of incubation under specific conditions (33). Along the barrage zone of compatible pairings, cleistothecia were formed that harbored ornamented and viable ascospores, from which typical *A. fumigatus* colonies were grown under standard conditions. Segregation patterns of genetic markers provided evidence of meiotic recombination during the developmental process. Following the dual nomenclature, the corresponding teleomorph was coined *Neosartorya fumigata*.

Besides this seminal but solitary description, no further evidence for sexual reproduction among *A. fumigatus* isolates has been reported. In order to test the general practice of *A. fumigatus* mating and to characterize genetic determinants required for this process, we undertook efforts to verify cleistothecium formation among established, unrelated clinical isolates of *A. fumigatus*. Furthermore, by crossing mutants that carry deletions of mating-type genes or lacking the conserved regulator of cleistothecium development NsdD, the role in fruiting body formation was assessed. Noticeably, a role of NsdD in cell wall stress resistance could be established, which

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† Supplemental material for this article may be found at http://ec.asm.org/.

▽ Published ahead of print on 26 March 2010.

TABLE 1. *A. fumigatus* strains used in this study

Strain	Description	Reference
D141	*A. fumigatus* wild-type strain (syn. NRRL 6585), clinical isolate (*MAT1-1*)	50
Af293	*A. fumigatus* genome sequence reference strain, clinical isolate (*MAT1-2*)	32
AfS41	*pabaA*::*loxP riboB*::*loxP* derivative of D141	16
AfS45	*pabaA*::*loxP pyroA*::*loxP* derivative of Af293	16
AfS14	*nsdD*Δ deletant in D141 background (*nsdD*::*loxP*-Phleo^r/*tk-loxP*)	This study
AfS69	Reconstituted isolate of AfS14 (^{p}*niaD*::*nsdD ptrA*)	This study
AfS71	*mat1-1*::*ptrA* deletion strain, D141 derivative	This study
AfS72	*mat1-2*::*ptrA* deletion strain, Af293 derivative	This study
AfS81	D141 derivative expressing GFP-H2A (^{p}*gpdA*::*gfp2-5*::*his2A ptrA*)	This study
AfS82	Af293 derivative expressing mCherry-H2A (^{p}*gpdA*::*mCherry*::*his2A ptrA*)	This study
AfS83	AfS14 expressing GFP-H2A (*nsdD*Δ; ^{p}*gpdA*::*gfp2-5*::*his2A ptrA*)	This study
AfS84a/b	Independent *mat1-1*::*ptrA* deletants, D141 derivatives	This study
AfS85a/b	Independent *mat1-2*::*ptrA* deletants, Af293 derivatives	This study

implies a role of this transcriptional regulator in hyphal fusion accompanying heterokaryon formation.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strain *Escherichia coli* DH5α was used for general cloning procedures and cultivated in LB medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5). Fungal strains used in this study are listed in Table 1. *Aspergillus fumigatus* isolates were typed for their *MAT* idiomorph by a multiplex PCR approach as described by Paoletti et al. (35). Growth of *A. fumigatus* strains was carried out at 37°C on minimal medium prepared according to the methods of Barrat et al. (4) or for mating purposes at 30°C on purchased Difco oatmeal agar in the absence of light with restricted aeration (33). More specifically, strains were spot inoculated on oatmeal plates and allowed to germinate overnight before sealing the plates with parafilm to restrict aeration and wrapped in aluminum foil to prevent illumination. Parafilm seals were removed after 1 week to avoid conditions of excessive hypoxia. Phenotypes of recombinant strains, such as sterility of mating-type deletants or *nsdD*-related phenotypes, were validated by verification in independent mutants or by suppression analysis of reconstituted isolates, respectively.

Antibiotic concentrations were 100 μg/ml for ampicillin, 30 μg/ml for phleomycin, and 0.1 μg/ml for pyrithiamine; nutritional auxotrophies were supplemented with 1 μg/ml *p*-aminobenzoate, 0.5 μg/ml pyridoxine-HCl, or 2.5 μg/ml riboflavin-HCl.

Transformation procedures. Calcium/manganese-treated *E. coli* cells were used for transformation (19); *A. fumigatus* recipients were transformed by polyethylene glycol-mediated fusion of protoplasts as described previously (40).

Nucleic acids manipulations and plasmid construction. Standard protocols of recombinant DNA technology were carried out (45). Phusion high-fidelity DNA polymerase (Finnzymes) was generally used in PCRs (44), and essential cloning steps were verified by sequencing by the GATC Biotech Company (Konstanz, Germany). Sequence analyses were carried out using the Lasergene Biocomputing software package from DNAStar. Fungal genomic DNA was prepared according to the methods of Kolar et al. (23), and Southern analyses were carried out essentially as described previously (49). Recombinant plasmids used during the course of this study are listed in Table 2, with relevant oligonucleotides specified in Table S1 of the supplemental material.

In detail, replacement cassettes and expression constructs were generated as follows. Deletion cassettes for the mating-type idiomorphs were obtained by applying the fusion PCR approach (52). 5′ and 3′ regions of about 2 kb flanking the *MAT1-1* locus were amplified from genomic DNA of *A. fumigatus* strain D141 with the oligonucleotide pairs ES001/ES017 and ES022/ES004, respectively, and fused to the pyrithiamine resistance cassette from pSK275 amplified with primers ES019/ES020. The resulting 6-kb amplicon was used directly to transform *A. fumigatus* D141 and also cloned into pJET1.2 to result in plasmid pSK491. The *MAT1-2* deletion construct of pSK492 was isolated in a similar fashion from genomic DNA of strain Af293 using primers ES005/ES018 (5′ flank) and ES023/ES008 (3′ flank). In order to generate a replacement cassette for targeting *nsdD*, a 7.8-kb BamHI/EcoRI fragment comprising the *nsdD* gene was isolated from *A. fumigatus* genomic DNA and inserted into pBluescript II KS to generate plasmid pSK245, from which the 5′ and 3′ flanking regions were isolated as a 2.2-kb HindIII and 3.3-kb FspI fragment, respectively. Both homology arms were inserted sequentially into pSK259 via HindIII and HpaI to result in construct pSK263, from which an *nsdD* replacement cassette was released via NdeI digestion prior to *A. fumigatus* transformation. Expression plasmids for fluorescent labeling of *A. fumigatus* nuclei in live cells were constructed by fusing synthetic coding sequences for the fluorescent proteins green fluorescent protein (GFP) and mCherry (pSK494 and pSK496, respectively; custom synthesized by GeneArt, Germany) followed by a $(GA)_4$ linker region to a sequence comprising the coding region and 579 bp of its 3′-untranslated region of the histone 2A-

TABLE 2. Plasmids used in this study

Name	Description	Reference or source
pBluescript II KS	General cloning plasmid (*bla*, MCS)[a]	Stratagene
pJET1.2	Positive selection vector, pUC19 derivative (*bla*, MCS in *eco47IR*)	Fermentas
pSK275	*ptrA* resistance gene flanked by SfiI sites in pBluescript II KS (syn. pME3024)	27
pSK259	Recyclable phleomycin resistance cassette flanked by *loxP* attachment sites (syn. pME2891)	25
pSK393	Integrative plasmid for expression of *A. fumigatus nsdD* (^{p}*niaD*::*nsdD*::*his2A*^{t} *ptrA*)[b]	16
pSK379	Integrative *A. fumigatus* expression plasmid carrying ^{p}*gpdA-his2A*^{t} cassette and *ptrA*	This study
pSK245	*nsdD* genomic locus (EcoRI/BamHI) from *A. fumigatus* D141 in pBluescript II KS	This study
pSK263	*nsdD*::*loxP*-Phleo^r/*tk-loxP* replacement cassette	This study
pSK491	*mat1-1*::*ptrA* deletion cassette	This study
pSK492	*mat1-2*::*ptrA* deletion cassette	This study
pSK494	Synthetic, codon-optimized *gfp2-5* allele for expression in *A. fumigatus*	This study
pSK496	Synthetic, codon-optimized mCherry allele for expression in *A. fumigatus*	This study
pSK505	Integrative plasmid for expression of GFP-H2A (^{p}*gpdA*::*gfp2-5*::*his2A ptrA*)	This study
pSK507	Integrative plasmid for expression of mCherry-H2A (^{p}*gpdA*::*mCherry*::*his2A ptrA*)	This study

[a] MCS, multiple cloning site.
[b] *p*, promoter; *t*, terminator.

encoding gene. Chimeric fragments were generated by fusion PCRs with primer pairs Sv648/Sv649 (GFP-GA_4), Sv653/Sv654 (mCherry-GA_4), and Sv557/Sv652 (GA_4-H2A), and the resulting amplicons were inserted into expression vector pSK379 after removal of a 2.0-kb PmeI/AleI fragment to yield integrative plasmids pSK505 and pSK507.

qRT-PCR. Quantitative real-time PCRs (qRT-PCRs) were performed as described recently (6). Sequences of priming oligonucleotides are given in Table S1 of the supplemental material, and gene-specific combinations were as follows: TubFw/TubRev (AFUA_1G10910; encoding β-tubulin), ES077/78 (AFUA_6G06360; α-like pheromone precursor PpgA), ES079/80 (AFUA_5G07880; pheromone receptor PreA), ES081/82 (AFUA_3G14330; pheromone receptor PreB), ES083/84 (AFUA_3G13870; GATA-type transcriptional activator NsdD), and ES085/86 (AFUA_1G12490; sexual development regulator VeA). Melting curves were plotted to confirm specificity of each reaction, and results were quantified according to the comparative threshold cycle method (1).

HAMF assay. Heterokaryon formation of *A. fumigatus* strains was monitored by histone-assisted merged fluorescence (HAMF) (42) from strains of opposite mating type that expressed fluorescent protein-labeled histone H2A (51). Cultivations were carried out in μ-Slides (ibidi, Germany) to assess the distribution of nuclear fluorescence within hyphae on a Zeiss Axiolab fluorescence microscope equipped with an AxioCam ICc1 digital camera and the AxioVision LE software.

RESULTS

Unrelated clinical isolates of *Aspergillus fumigatus* are able to mate. The first hard evidence for a sexual phase in the *A. fumigatus* life cycle was recently provided by successful crossings among environmental isolates that had been collected in Dublin City, Ireland (33). We were interested in whether this capacity for cleistothecium formation was restricted to members of this collection, which had been limited to one geographic area, or whether mating and ascosporogenesis could be observed between completely unrelated clinical isolates of *A. fumigatus*. In order to put this to the test, established strains carrying opposite mating types were inoculated on growth plates containing purchased oatmeal agar. After prolonged incubation under restricted aeration at 30°C in the dark, a clear barrage zone was evident after 6 weeks for the *MAT1-1*/*MAT1-2* combination of clinical isolates D141 (syn. NRRL 6585) (*MAT1-1*) (50) and Af293 (*MAT1-2*) (32, 34) (Fig. 1A). This junction is characterized by a cotton wool-like appearance, and closer inspection revealed the presence of numerous cleistothecial balls (Fig. 1B). Upon further incubation for 3 to 4 months, the fluffy fruiting bodies matured to become more rigid, with a size of 300 to 400 μm (Fig. 1C). Squeezing these cleistothecia released ascospores of typical size and appearance (Fig. 1D and E).

To confirm recombination between the compatible clinical isolates during mating, mutant strains carrying defined deletions in metabolic marker genes—AfS41 (*MAT1-1*; *pabaA*::*loxP riboB*::*loxP*) and AfS45 (*MAT1-2*; *pabaA*::*loxP pyroA*::*loxP*) as descendants of D141 and Af293, respectively (16)—were crossed. Ascospores were randomly isolated from several of the developing cleistothecia and inoculated for single colonies on defined growth medium. Auxotrophies of clonal isolates were determined by replica plating on appropriately supplemented medium to reveal different genotypes that were in agreement with recombination, such as *riboB*Δ *pyroA*Δ or $riboB^+$ $pyroA^+$ (Fig. 2A). Mating types of the recombinants were determined by multiplex PCR (35), and their segregation patterns in correlation to the auxotroph markers also supported genetic recombination during ascosporogenesis (Fig. 2B).

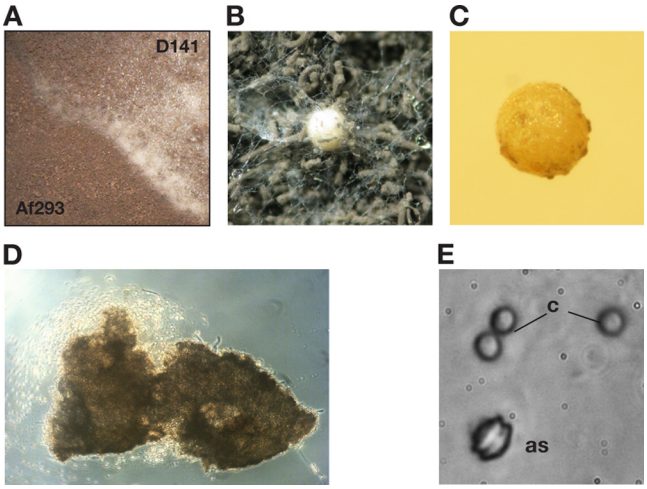

FIG. 1. Cleistothecium development and ascosporogenesis by crossing of unrelated clinical isolates of *A. fumigatus*. (A) Section from the crossing plate with clinical isolates D141 (*MAT1-1*) and Af293 (*MAT1-2*) on oatmeal agar. (B) These strains of opposite mating types develop a cotton-like barrage zone, in which cleistothecia develop upon further incubation. (C to E) From mature fruiting bodies (C), ascospores can be released by squeezing (D), which results in a characteristic appearance with two equatorial crests (E); for comparison, conidia (c) are shown beside one ascospore (as).

Mat1-1 and Mat1-2 determine *A. fumigatus* fruiting body formation and regulate expression of key elements of a putative mating pathway. In order to assess the role of mating-type gene products in *A. fumigatus* cleistothecium formation, we created mutant strains deleted for either locus by targeted gene replacement. The correct genotypes of the corresponding deletants AfS71 (*mat1-1*Δ) and AfS72 (*mat1-2*Δ) were confirmed by Southern analyses (Fig. 3A), and strains were inspected for noticeable deviations from their wild-type progenitors. No obvious phenotype became evident under standard culture conditions, such as growth in or on minimal medium at 37°C (data not shown). However, in crossing experiments, neither deletant supported formation of mature fruiting bodies when confronted with a strain carrying the opposite mating-type idiomorph and incubated under the established conditions for 6 months (Fig. 3B). Although evidence of early stages of mating, such as the cotton wool-like hyphal structures, was noted in pairings of AfS71 with Af293 or of AfS72 with D141, no further development to mature, ascospore-containing cleistothecia could be observed. Testing additionally and independently generated mutants deleted for either mating-type gene confirmed the associated phenotype of sterility: when crossing strains AfS84 or AfS85 with the clinical isolates of the opposite mating type under appropriate conditions, no signs of cleistothecium formation became evident. Accordingly, both products of the mating-type locus are essential for successful fruiting of *A. fumigatus*.

As both gene products from the *A. fumigatus* mating-type idiomorphs are DNA binding factors putatively involved in regulation of gene expression, we were interested in the levels of selected transcripts that are likely involved in mating and sexual development. More precisely, genes encoding the putative sexual pheromone precursor PpgA, the pheromone recep-

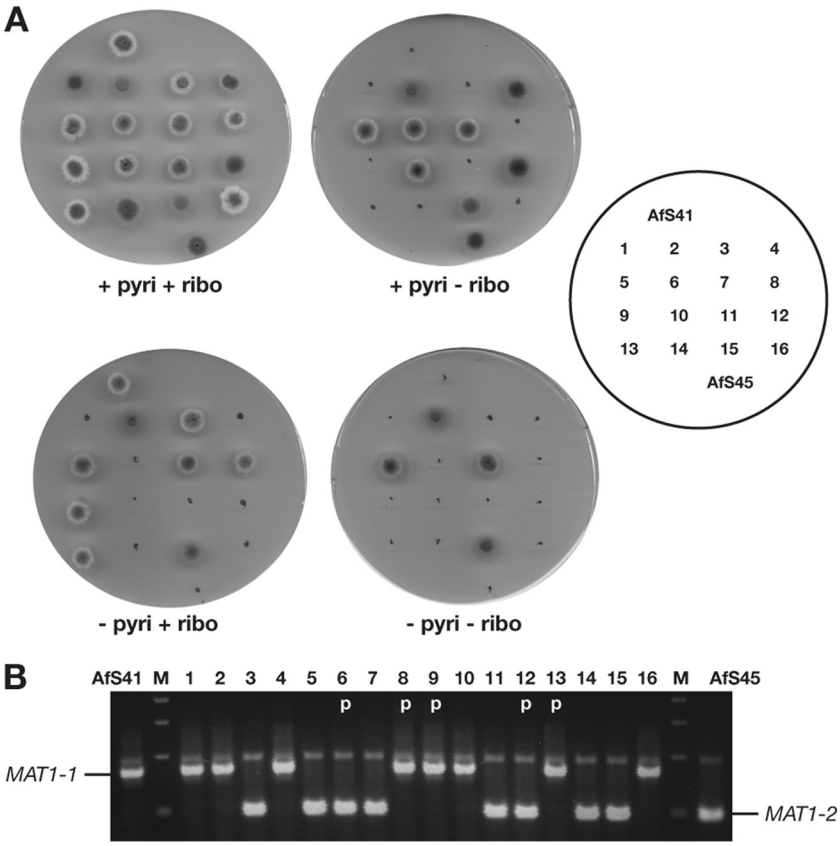

FIG. 2. Distribution of auxotrophies (A) and mating types (B) among 16 descendants from a cross between *A. fumigatus* strains AfS41 (*MAT1-1*; *pabaA*::*loxP riboB*::*loxP*) and AfS45 (*MAT1-2*; *pabaA*::*loxP pyroA*::*loxP*). All growth media were supplemented with *p*-aminobenzoic acid. The presence of the *MAT1-1* or *MAT1-2* idiomorph was verified by diagnostic multiplex PCR (19). Marker combinations and segregation of mating types clearly indicated the genetically recombined nature of the progeny. p, parental genotype.

tors PreA and PreB, and the regulators of cleistothecium formation, NsdD and VeA, were analyzed (Fig. 3C). Transcript levels of the encoding genes were determined from mycelia that had been transferred from minimal medium to oatmeal agar to be further incubated under conditions that favor sexual development. In parallel, split mycelia were further propagated on minimal medium under the same conditions to assess the influence of the culture medium, and message levels in wild-type strains D141 and Af293 under these conditions served as respective benchmarks (Fig. 3C). Distinct differences became evident for the pheromone precursor-encoding gene *ppgA*: on minimal medium, expression of this transcript declined drastically in the absence of the *MAT1-1* gene but increased if the *MAT1-2* gene had been deleted. On oatmeal agar, this trend was less pronounced (D141 versus AfS71) or absent (Af293 versus AfS72), with expression levels being generally lower in either genetic background. These data suggest that expression of the α-factor-like pheromone is regulated reciprocally by the mating-type genes. Furthermore, transcript levels for the pheromone receptor genes *preA* and *preB* were determined to reveal similar patterns of regulation for both loci. On minimal culture medium, transcript levels decreased when either mating type was deleted; on oatmeal agar, this situation was counterbalanced for *preB* to result in transcript levels similar to the ones of either deletant on minimal medium. Accordingly, either mating-type gene product appears to be required for downregulation of *preB* transcription upon a shift from minimal medium to oatmeal agar. When propagated on the oatmeal culture medium, levels of the *preA* transcript in each mutant were slightly higher than in the wild type. In the *mat1-2*Δ background, this elevated level was equal to that on minimal medium, whereas in the *mat1-1*Δ strain a downregulation of *preA* transcript levels upon the shift from minimal to oatmeal medium was still evident, although to a lesser degree than in the parental wild-type strain.

To analyze any influence of Mat1-1 or Mat1-2 on expression of regulators of *Aspergillus* sexual development, transcript levels for the GATA-type transcription factor NsdD and the velvet complex subunit VeA (5, 16) were determined. No significant changes were evident for either transcript in the *mat1-1* deletion strain on either culture medium, except for a reduction of elevated *nsdD* transcript levels in the presence of oatmeal. In the *mat1-2*Δ strain, when grown on minimal medium, an increase in *nsdD* transcription could be detected, whereas elevated transcript levels did not change significantly on the complex medium. Levels of the *veA* transcript did not show any significant regulation with respect to genotype or culture medium.

The GATA-type factor NsdD is required at an early stage of mating and contributes to resistance toward cell wall stress. To assess the role of an established determinant of *Aspergillus*

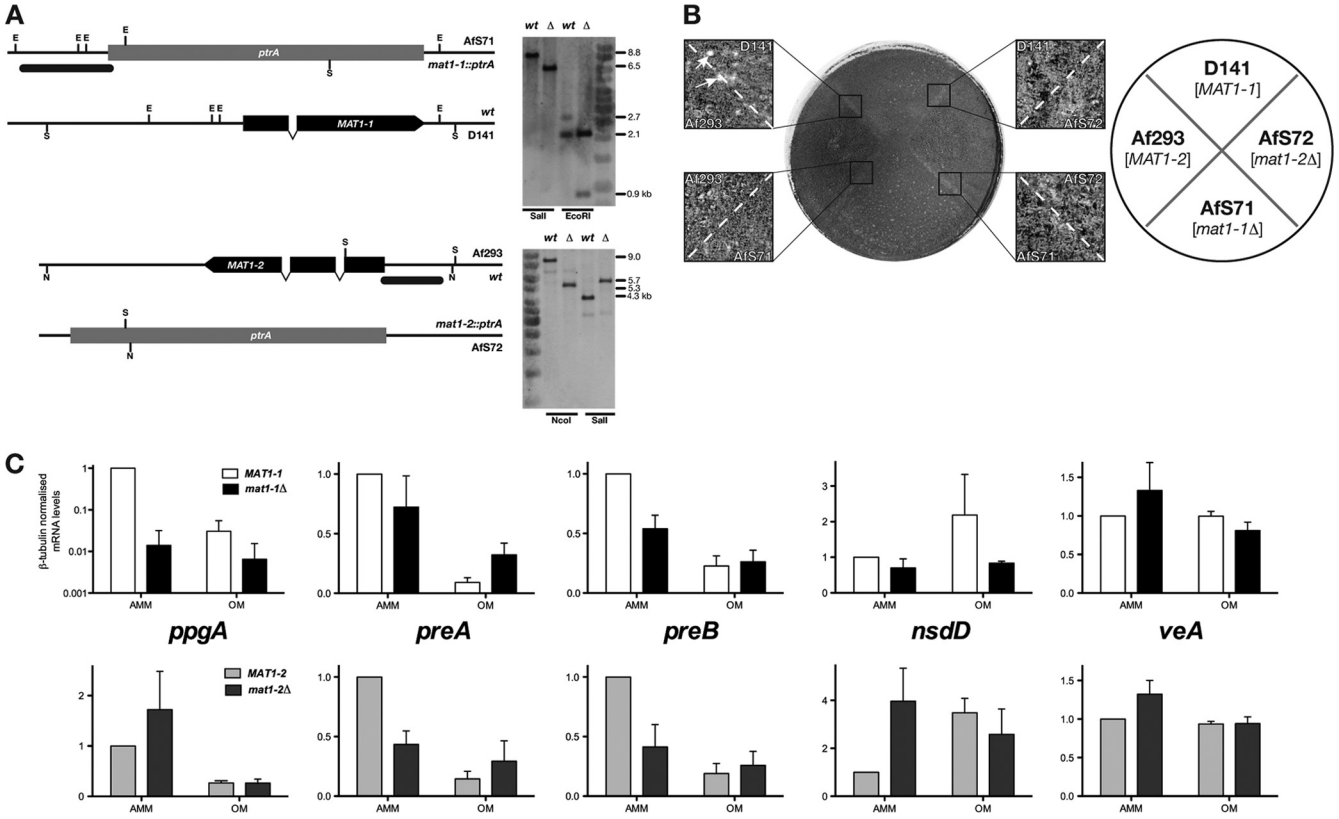

FIG. 3. *MAT1-1* and *MAT1-2* are required for mating of *A. fumigatus*. (A) Schematic representation of deleted mating-type loci and Southern analyses of strains AfS71 (*mat1-1*::*ptrA*) and AfS72 (*mat1-2*::*ptrA*). Black bars correspond to probe positions, and restriction enzyme recognition sites are indicated for EcoRI (E), NcoI (N), and SalI (S), with the resulting fragment sizes displayed on the right. (B) Crossing plate for *mat1* deletants with wild-type progenitors. Close-up images of barrage zones between indicated strains show the presence of mature cleistothecia only for the D141 × Af293 cross (arrows), whereas other combinations failed to produce fruiting bodies. (C) Effects of mating-type deletions on expression of genes involved in pheromone signal transduction and regulation of cleistothecium formation. Mycelia of the indicated strains were propagated at 30°C in the dark under restricted aeration on *Aspergillus* minimal medium (AMM) or oatmeal agar (OA) for 4 days before preparation of total RNA. Transcript levels were determined from three biological replicates by quantitative real-time PCRs with three technical replicates, using β-tubulin transcript levels as the internal standard and assigning the levels for wild-type strains on AMM as 100%.

fruiting body formation for *A. fumigatus* mating, we investigated the GATA-type transcriptional activator NsdD in more detail. Crossing strains that overexpress the *nsdD* gene (16) on oatmeal agar containing the inducing N source nitrate did not speed up cleistothecium development or skew the balance between asexual and sexual sporulation (data not shown), as it is the case in homothallic *A. nidulans* (18). In further mating experiments, the Af293 isolate (*MAT1-2*) was crossed to the mutant strain AfS14 (*MAT1-1*; *nsdD*Δ), which is derived from D141 and carries a deletion of the conserved *nsdD* gene (18), which was introduced by gene targeting and confirmed by Southern hybridization analyses (Fig. 4A). One obvious phenotype of this deletant became evident when the strain was grown on minimal medium, on which reduced hyphal extension and darkening of the colony's reverse side was observed (Fig. 4B). Both phenotypes were revoked by reintroducing the *nsdD* gene at basal levels from the *niaD* promoter (16). To test for its ability to mate and to form cleistothecia, AfS14 (*nsdD*Δ; *MAT1-1*) was crossed with the *MAT1-2* strain Af293 on oatmeal agar. No fruiting bodies could be retrieved from this strain combination when propagated under the validated conditions, even after prolonged incubation for 6 months. When taking a closer look at the barrage zone that is formed between these crossed strains, a difference from pairings with the sterile mating-type deletants became evident: while the latter formed an intimate hyphal mesh from which aerial hyphae emerged, no such structures between AfS14 and Af293 were observed (Fig. 4C). Again, this particular phenotype was reversed when crossing the reconstituted strain AfS69 to a strain of the opposite mating type.

The fact that internucleic complementation of NsdD function is apparently not feasible when crossing the *nsdD*Δ deletion strain to a wild-type mating partner points to impaired heterokaryon formation, a process tightly linked to hyphal fusion and cell wall remodeling. To test this aspect, the *nsdD* deletant AfS14 was subjected to the cell wall-interfering agents calcoflour white and Congo red to reveal a pronounced morphological phenotype in dependence on the *nsdD* gene; whereas the wild-type progenitor displayed straight hyphal extension in the presence of the cell wall stressors, hyphae of the *nsdD*Δ deletion mutant appeared stunted, sometimes with swollen hyphal tips forming bulky structures that occasionally

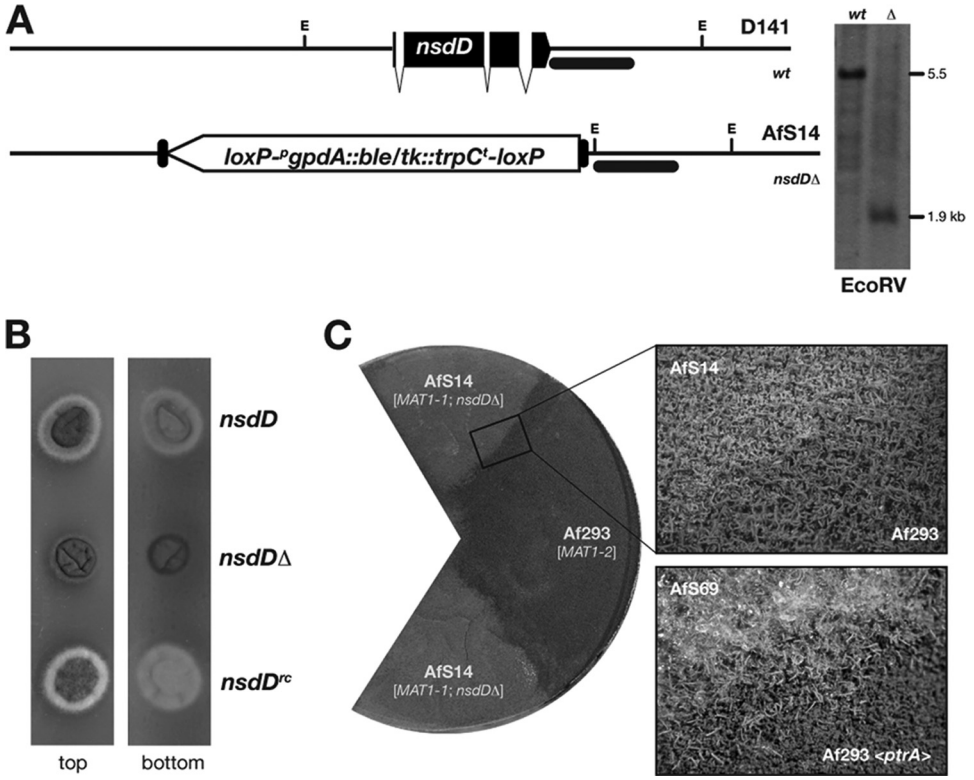

FIG. 4. The conserved GATA factor NsdD is required for mating. (A) Deletion of *nsdD* and Southern analysis after EcoRV (E) digestion; recognition sites are schematically indicated together with calculated fragment sizes, and the black bar corresponds to the hybridization probe position. (B) Growth phenotypes of an *nsdD*Δ strain on minimal medium, characterized by reduced hyphal extension (top view) and darkening of mycelium (bottom view), which is reversed after reconstitution (*nsdD*^{rc}). (C) Mating capacities of the *nsdD*Δ deletant. Neither aerial hyphae nor fruiting bodies were formed in the barrage zone (top close-up image) between the deletant AfS14 (*nsdD*Δ; *MAT1-1*) and the *MAT1-2* strain Af293. For comparison, the barrage zone of the reconstituted strain AfS69 with a *MAT1-2* mating-type partner is shown (bottom close-up image), in which aerial hyphae as early indicators of the mating process are formed.

burst (Fig. 5A). This phenotype was restored to wild type in reconstituted strain AfS69, which carries a plasmid for expression of *nsdD* and thus displays straight hyphae in the presence of cell wall stress-inducing agents (data not shown). To test any associated phenotype with respect to cell wall synthesis-inhibiting agents, susceptibilities for the antimycotics caspofungin, a β-1,3-glucan synthase inhibitor, and nikkomycin Z, a chitin synthase-inhibiting agent, were analyzed. Etests quantifying the sensitivities of strains D141 and AfS14 toward caspofungin did not uncover any significant differences (data not shown), whereas growth tests on minimal medium plates in the presence of nikkomycin Z revealed an increase in resistance of the *nsdD*Δ deletant toward this antibiotic (Fig. 5B). This was also reflected in liquid cultures when increasing concentrations of nikkomycin Z were applied: mutants deleted for the *nsdD* gene were able to germinate and grow profoundly, accompanied by conidiation at concentrations of up to 2,048 μg/ml, in contrast to the wild-type isolate D141 or the reconstituted strain AfS69, which were able to germinate at 512 μg/ml and conidiated at a concentration of 8 μg/ml or less.

In summary, these data indicate that the GATA-type transcription factor NsdD plays a role in cell wall synthesis and that it is required during an early stage of mating. Accordingly, it might influence initial cellular processes, such as formation of anastomoses resulting in heterokaryosis. In order to support this hypothesis we assessed the ability of the *nsdD*Δ strain AfS14 to form heterokarya when cocultured with a mating partner by HAMF (42). Nuclei of AfS14 and its parental strain D141 were labeled with the green fluorescent protein by constitutively expressing a GFP-H2A fusion protein, whereas strain Af293 was accordingly labeled with the red fluorescent variant mCherry. Strains of opposite mating types were inoculated in minimal medium, and the formation of heterokaryotic hyphae that contained both types of fluorescent nuclei was monitored (Fig. 6). Either strain alone displayed patterns of uniformly fluorescing nuclei. When scanning the pairing of the D141 descendant AfS81 with the Af293 derivative AfS82, few hyphae could be spotted in which both kinds of nuclei were present. In comparison, the mixture of AfS82 with the *nsdD*Δ deletion strain AfS83, which expresses the GFP-H2A allele, exclusively yielded homokaryotic hyphae, indicating that deletion of the *nsdD* gene impairs heterokaryon formation.

DISCUSSION

We were able to demonstrate mating capacities of unrelated clinical *A. fumigatus* strains. Possible differences between environmental and clinical isolates of this saprophytic pathogen have been under debate for a long time (3, 9, 10, 17, 29, 43); as with most studies, our finding argues against such a difference,

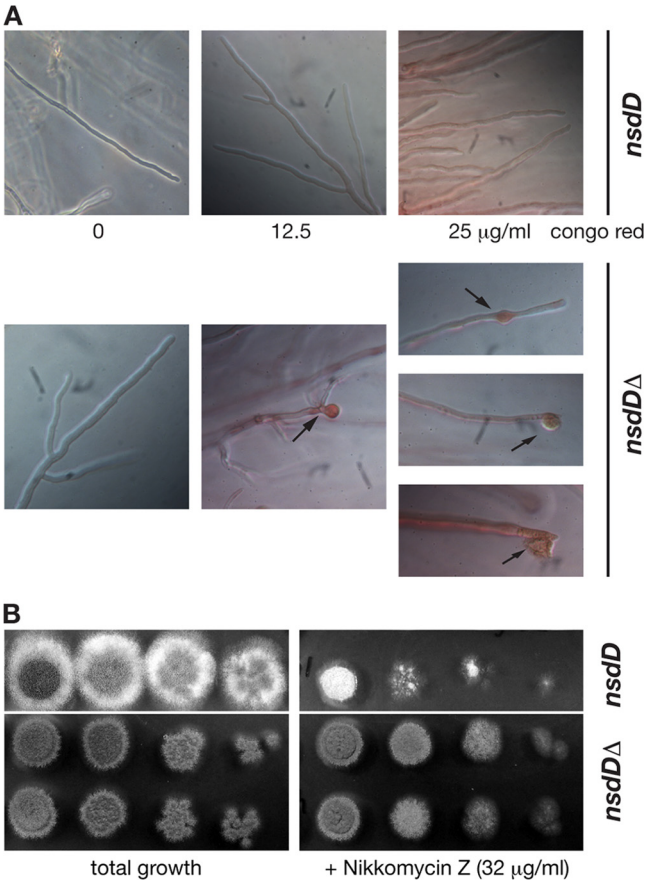

FIG. 5. NsdD is involved cell wall stress resistance. (A) An *nsdD*Δ strain displays hyphal tip swelling (arrows) in the presence of Congo red, which occasionally results in bursting and the apparent release of hyphal contents. (B) The *nsdD* deletion mutant is less sensitive toward nikkomycin Z. Aliquots of 5 μl from serial dilutions of conidial suspensions (4×10^6 to 4×10^3 ml^{-1}) were spotted on minimal medium containing 32 μg/ml nikkomycin Z and allowed to grow at 37°C for 2 days. Two independent *nsdD*Δ isolates are shown.

as we could validate that clinical specimens were able to mate and produce fruiting bodies containing recombinant ascospores in a similar manner as the environmental isolates described in the seminal study of O'Gorman et al. (33). Accordingly, the mating behavior and the existence of a sexual phase in the life cycle of *A. fumigatus* are not restricted to a locally constrained subpopulation but appear to be a general trait. However, Kwon-Chung and Sugui recently described their failed efforts to mate the reference strain Af293 to other established isolates, such as CE10 or B-5233 (30), and further and more comprehensive studies will have to answer the question of how universal mating is among the *A. fumigatus* population. Nutritional requirements of mating partners might have an influence, as they have been described to hamper cleistothecium formation of the homothallic relative *A. nidulans* (13, 26). The fact that we were able to produce recombinant offspring from two genetically marked strains indicates that at least the employed auxotrophies for riboflavin and pyridoxin do not influence fertility of *A. fumigatus* or its ability to produce fruiting bodies. One additional aspect of our mating studies lies in the fact that cleistothecia were already formed after 4 months, which is 2 months sooner than described for the Irish isolates. Given that even in this collection variations in the abilities to mate and produce fruiting bodies were evident, the quicker formation of cleistothecia in our experimental setup seems reasonable. Mating of *N. fumigata* appears to be determined by a variety of genetic determinants and results in differing characteristics of fruiting body formation between different isolates.

A. fumigatus is not the only anamorphic fungus for which a long-time-undisclosed sexual cycle has recently been demonstrated. *Aspergillus parasiticus* as well as *A. flavus* produces recombinant progeny in fruiting bodies within several months when crossed under appropriate conditions (20–22), and for several other fungal species a bipolar mating-type system or even the existence of mating and/or sexual reproduction was

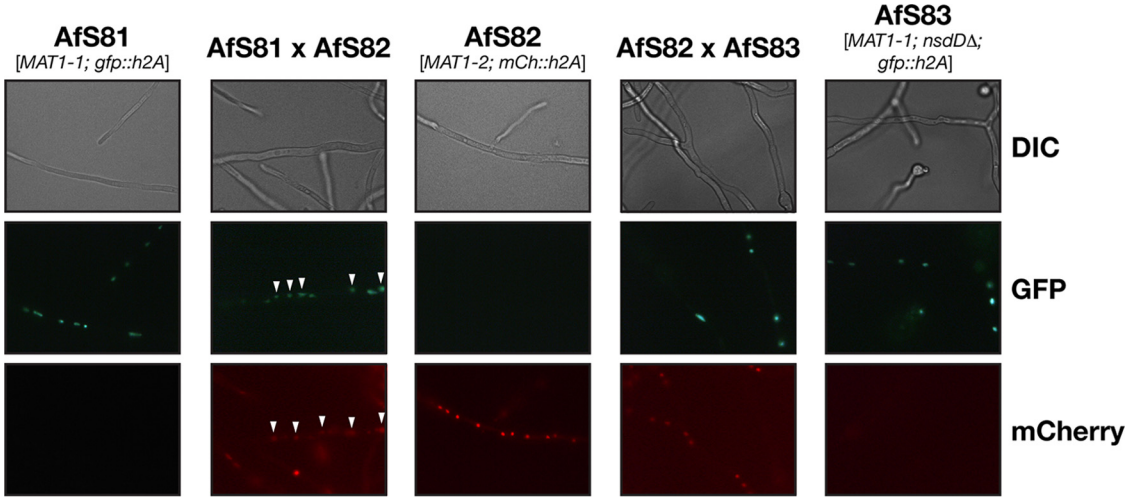

FIG. 6. NsdD is required for heterokaryon formation as assessed by HAMF. Strains of opposite mating types that express fluorescent protein-tagged histone 2A variants were cocultivated to spot mycelial compartments that contained labeled nuclei of either type and, in the case of heterokaryon formation, both kinds. When derivatives of the wild-type strains D141 and Af293 were coincubated, hyphae with both fluorescently labeled nuclei could be identified (AfS81 × AfS82; white arrowheads), whereas exclusively uniform hyphae were detected when a labeled *nsdD*Δ strain was crossed to the Af293 descendant.

confirmed in recent times (28). From a technical point of view, mating competence of the *A. fumigatus* sequence reference strain Af293 assists molecular characterization of its heterothallic breeding system, as mutant generation by means of gene targeting is facilitated in such a defined and established isolate. The existence of isogenic auxotrophs, for instance, in this genetic background (54) will support the generation of recombinant strains and will surely contribute to molecular biology of this fungal pathogen (7, 24). However, given the long period of time that is required to obtain mature cleistothecia harboring recombinant ascospores from *N. fumigata*, further studies are mandatory to enhance feasibility of genetic crosses for routine research purposes. Molecular determinants that support mating and sexual differentiation of *A. fumigatus* need to be defined, and moreover, any relevance of its restricted sexuality for virulence has to be addressed (31).

Mating-type loci and their products are considered master regulators of sexual propagation, especially in heterothallic species. *MAT* genes determine sexual (and probably nuclear) identity, and as DNA binding factors they do so by orchestrating transcriptional programs in the respective cellular compartment. The generated deletion strains confirm a presumed role of both mating-type loci in *A. fumigatus* sexuality, as cleistothecium formation was blocked in compatible crosses of mutant strains. This situation clearly resembles findings for other heterothallic and also homothallic ascomycetes (11, 36) and emphasizes the role of *MAT* gene products as master regulators for sexual development. We have gained initial information on the regulatory targets by quantitative analyses of gene transcript levels putatively involved in pheromone signaling and regulation of fruiting body formation. Most interestingly, we were able to demonstrate a role of both Mat proteins in regulation of pheromone expression: whereas Mat1-1 is required for proper *ppgA* gene transcription, Mat1-2 appears to repress its expression. The latter finding is in line with data from the homothallic species *A. nidulans*, for which a negative influence of the HMG protein MatA on *ppgA* transcript levels has been suggested (36). Our finding that *ppgA* transcription is regulated in a reciprocal manner in the different mating-type backgrounds illustrates the identity-establishing function of the Mat proteins and might assist in identifying the so-far-ambiguous *ppgB* pheromone-encoding gene. Both putative pheromone receptor genes, however, appear to be regulated by the *MAT* loci in a similar fashion, being less transcribed in deletants grown on minimal medium but stably transcribed when grown on oatmeal agar. This pattern reveals a pronounced effect of the mating-inducing growth substrate but also implies balanced fine-tuning of receptor expression. The *preA* or *preB* transcript levels are similar in either mating-type deletion strain irrespective of the culture medium, which indicates that environmental regulation of pheromone receptor expression is in part mediated by the mating-type factors. We were unable to detect any significant regulation of the *MAT* gene products on *veA* transcription, an established regulator of *Aspergillus* cleistothecium formation, which suggests that this factor acts upstream of or parallel to any Mat1-directed signal transduction. For the *mat1-2*Δ deletion strain, an increase in *nsdD* transcript levels was evident on minimal medium; any significance of this negative regulation of NsdD expression by Mat1-2 for sexual development, however, remains to be demonstrated. Our quantitative data demonstrate that expression profiles *per se* do not mirror the capacity to propagate sexually: transcript levels of genes involved in the mating process were elevated on minimal medium, which does not support cleistothecium formation, but appeared to be downregulated on the complex and conducive oatmeal culture medium. The differing patterns of transcription reflect the completely dissimilar culture conditions (synthetic minimal versus complex rich medium), a situation that apparently is overcome by an unknown oatmeal component to support cleistothecium formation.

We and others had shown previously that functional determinants of sexual development in the homothallic relative *A. nidulans* are expressed in *A. fumigatus*, among them the GATA-type factor NsdD (18). Phenotypes of the *nsdD*Δ deletant AfS14 reveal conserved functions of this transcription factor in both species, illustrated by accumulation of dark mycelial pigments and the absence of sexual development. Our finding that this conserved activator of cleistothecium formation appears to be required for mating in *A. fumigatus* and that crosses of the deletant with a compatible wild-type isolate do not result in fruiting bodies implies that it plays a role in early steps of heterothallic reproduction. This reflects the situation in homothallic *A. nidulans*, in which an early role for NsdD to act on initiation of fruiting body formation has been proposed (47). Compatible fertile strains usually fuse hyphae at the zone of confrontation. The presence of mixed nuclei within the resulting heterokaryon would allow intergenic complementation and therefore fruiting body formation in an *nsdD*Δ × *nsdD*$^+$ cross. The observation that this is not the case implies a defect in anastomosis for this strain combination. Alternatively, other cellular processes might require NsdD in either mating partner, or levels of NsdD expression might not be sufficient to support fruiting body formation. Our data from histone-assisted merged fluorescence reveal a defect in hyphal fusion of the *nsdD*Δ deletion mutant. In line with this is the observed sensitivity against cell wall stressors and apparently weakened hyphal tips of the *nsdD*Δ deletant. This particular phenotype is in accordance with the one observed when *nsdD* is overexpressed, which results in hyphal curling (16). Hence, NsdD appears to be necessary for balanced cell wall synthesis and may act on proper expression of proteins involved in this fundamental process, such as chitin synthases, α-1,3-glucan synthases, or cell wall mannoproteins. The fact that sensitivity of *A. fumigatus* toward the antimycotic nikkomycin Z, an inhibitor of chitin synthesis, is altered when NsdD is lacking supports this supposed role of the regulator in cell wall maintenance. Accordingly, NsdD appears to affect the cell wall integrity pathway and thus might be required for hyphal fusion events, during which cell wall remodeling has to occur. The scope of the NsdD-dependent regulon, however, awaits determination by comprehensive analyses of its transcriptome to provide further information on the cellular function of this regulator of *Aspergillus* sexual development.

In summary, we were able to demonstrate that determinants of *Aspergillus* mating and fruiting body formation, mainly characterized in the homothallic species *A. nidulans*, are required for *A. fumigatus* mating and are therefore functionally conserved in this heterothallic species.

ACKNOWLEDGMENTS

We thank all members of YIG2 and of the Institute for Molecular Infection Biology for support, suggestions, and discussions. We are indebted to Thomas Hartmann for indispensable help with expression analyses, and highly valuable technical assistance was carried out by Michaela Dümig. Ulrich Kück, Birgit Hoff, and David Löper from the University of Bochum are thanked for stimulating and helpful discussions.

Financial support was granted by the German Research Foundation, the Free State of Bavaria, and the University of Würzburg.

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