Basic-Zipper-Type Transcription Factor FlbB Controls Asexual Development in Aspergillus nidulans

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Received 14 June 2007/Accepted 30 October 2007

The fungal colony is a complex multicellular unit consisting of various cell types and functions. Asexual spore formation (conidiation) is integrated through sensory and regulatory elements into the general morphogenetic plan, in which the activation of the transcription factor BrlA is the first determining step. A number of early regulatory elements acting upstream of BrlA (fluG and flbA-E) have been identified, but their functional relations remain to be further investigated. In this report we describe FlbB as a putative basic-zipper-type transcription factor restricted to filamentous fungi. FlbB accumulates at the hyphal apex during early vegetative growth but is later found in apical nuclei, suggesting that an activating modification triggers nuclear import. Moreover, proper temporal and quantitative expression of FlbB is a prerequisite for brlA transcription, and misscheduled overexpression inhibits conidiation. We also present evidence that FlbB activation results in the production of a second diffusible signal, acting downstream from the FluG factor, to induce conidiation.

The mycelium is a very successful colony form capable of displaying different cell types and functions, and this attribute has led to the realization that it is governed by a complex chemosensitive system (43). The production of asexual spores is an integral part of the mycelial colony plan and the principal means of dispersal among the filamentous fungi (23, 55). Aspergillus nidulans is a homothallic ascomycete which has been used as a model system for molecular genetic studies due to its versatile life cycle, its amenability to genetic manipulation, and the open availability of its genome sequence (13, 24). It is also a reference for basic studies in fungal morphogenesis (12).

After the germination of a spore, an initial period of vegetative growth ensues, consisting of apical extension of the hyphae. Any of a number of suitable environmental stimuli (emergence to the atmosphere, osmotic and nutrient stress) provokes the halting of hyphal extension and subsequent formation of asexual spore-bearing structures called conidio- phores (2, 6). Molecular analysis of this process has revealed that stimuli are sensed and transduced as intracellular signals that converge in the transcriptional activation of brlA, which encodes a C2H2 zinc finger transcription factor. This first common step also marks the start of an irreversible central developmental cascade of downstream regulators leading to sporogenesis (1, 10).

Among the various possible upstream regulatory branches responding to the above-mentioned stimuli, the principal one, involving air exposure, starts with the biosynthesis of an unidentified low-molecular-weight diffusible FluG factor (21). The FluG signal is proposed to inactivate a repressor of conidiation (SfgA) that negatively regulates a number of development-specific functions required to activate brlA (35). It has been proposed that such FluG-mediated derepression leads to the activation of other Flb (fluffy low brlA expression) components: FlbA, a protein which inhibits vegetative growth signaling mediated by Go (FadA) (22, 51, 54), and potential transcription factors (TFs) FlbD, FlbB and FlbC (48, 2, 49). Simultaneous inhibition of vegetative signaling and activation of Flb TFs are required for the conidiation program to progress. However, detailed functional characterization of FlbB and FlbC remains to be done.

In this report, we present the identification and characterization of flbB (first described as vegA [4]) and demonstrate that FlbB is a potential transcriptional activator with a basic leucine zipper (bZIP) (44) and other conserved domains. The deletion of flbB results in the blockage of the synthesis of an extracellular signaling compound required for conidiation. The expression pattern of flbB shows elevated mRNA steady-state levels during the early phases of vegetative growth, which fall with the initiation of asexual development and remain low or undetectable during sexual development. FlbB initially localizes at the hyphal tip and then relocates to the nuclei proximal to the cell apex during vegetative growth. The complex regulatory roles played by FlbB in brlA transcriptional activation and developmental progression are further discussed.


**TABLE 1. Aspergillus nidulans strains used in this study**

| Strain       | Genotype                     | Source                  |
|--------------|------------------------------|-------------------------|
| FGSCA4       | Wild type (veA<sup>a</sup>)  | 31                      |
| FGSC26       | biA1 veA1                    | 17                      |
| FGSCA68      | suca1iade20 yA2 ade20 acrA1 phenA2 pyroA4 hsbB5 sb3 nic8B riboB2 veA1 | 17                      |
| FGSCA283     | suca1iade20 yA2 ade20 acrA1 galA1 pyroA4 (ssh<sup>a</sup>) facA303 sb3 nic8B riboB2 veA1 | Fungal Genetics Stock Center |
| FGSCS33      | biA1 pyroA4                  | Fungal Genetics Stock Center |
| FGSC777      | pyrg98 wa3 pyroA4            | Fungal Genetics Stock Center |
| TTA127.4     | pabaA1 yA2 ΔflaG:traC veA1   | 21                      |
| RMS011       | pabaA1 yA2 ΔargB::traCAB trpC801 veA1 | 39                      |
| GR5          | pyrg98 wa3 pyroA4 veA1       | 46                      |
| MAD782       | pyrg98 pabaA1 biA1 yA2 veA1  | Eduardo Espeso (CIB-CSIC, Madrid, Spain) |
| TN02A3       | pyrg98Δ::argB argB2 pyroA4 veA1 | 27                      |
| TN02A1       | ΔflaA::argB2 pyroA4 riboB2 veA1 | 27                      |
| TN02A5       | pyrg98 pabaB2 ΔflaA::argB argB2 riboB2 veA1 | 27                      |
| BD14 (flbB100) | biA1 flbB100 veA1             | This study               |
| BD12 (flbB101) | biA1 flbB101 veA1             | This study               |
| BD11 (flbB102) | biA1 flbB102 veA1             | This study               |
| BD109        | pyrg98 pabaA1 yA2 argB2 flbB101 veA1 | This study               |
| BD143        | pyrg98Δ::argB argB2 flbB::pyrg G pyroA4 veA1 | This study               |
| BD164        | pyrg98 pabaA1 argB2 ΔflbB::pyrg G pyroA4 | This study               |
| BD167        | pyrg98Δ::argB argB2 flbB::zpyg G pyroA4 veA1 | This study               |
| BD183        | pyrg98 ΔflaA::argB argB2 gfp::flbB pyroA4 veA1 | This study               |
| TN16.2       | biA1 alcA::pyrg98::pyroA4     | This study               |
| RN124.5      | biA1 alcA::pyrg98::pyroA4<sup>b</sup> | This study               |
| TN122.1      | biA1 nts53::pyroA4<sup>b</sup> | This study               |
| TJW113       | biA1 methylG1::ΔflbB::argB<sup>b</sup> | 18                      |

**MATERIALS AND METHODS**

Strains, oligonucleotides, media, and culture conditions. The strains of *A. nidulans* employed in this study are listed in Table 1. Plasmids were amplified in *Escherichia coli* strain DH5α or DH1 grown in Luria-Bertani medium with ampicillin (75 μg ml<sup>-1</sup>). Purification was carried out using GenElute plasmid miniprep or maxiprep kits (Sigma). Oligonucleotides used in this study are listed in Table 2.

The strains were cultivated in minimal medium (MMA [17]) or complete medium (MMA plus 5 g liter<sup>-1</sup> yeast extract) that was adequately supplemented in case of auxotrophy. Nutrient depletion experiments in solid medium involved dilution of glucose or sodium nitrate to one-fifth of the original concentration.

Salt stress experiments involved the addition of KCl (0.6 M) and MES (2-[N-morpholino]ethanesulfonic acid, 0.05 M). Experiments in liquid medium with nutrient limitation were conducted as described by Skromme et al. (37). Briefly, strains were cultivated for 18 h, at 37°C and 250 rpm, and then transferred to minimal medium with KCl and MES, with or without glucose or sodium nitrate.

The morphology of the mycelium was examined after 10 and 20 h of culture.

Extracellular complementation experiments were conducted by point inoculation of two strains onto the solid medium at a distance of 2 cm in the same petri dish. After 3, 4, and 5 days of cultivation, the contact zone was examined and photographed under a binocular microscope.

Time course experiments for the induction of development were conducted essentially as described previously (3, 30). After 18 h of culture in liquid MMA as described above, mycelium was filtered onto nitrocellulose membranes (0.45 μm; MicronSep; GE Water and Process Technologies), placed on solid medium, and cultured for 6, 12, 24, and 48 h before being processed for Northern blot analysis.

The intracellular localization of FlbB was analyzed by inoculating 40 μl of a conidiospore suspension onto a coverslip submerged in a petri dish containing adequately supplemented liquid minimal medium. After incubation at room temperature for 16, 24, and 36 h, samples were fixed with 4% (wt/vol) p-formaldehyde in phosphate-buffered saline at 16 h at 4°C, washed three times with phosphate-buffered saline, and stained with DAPI (4',6'-diamidino-2-phenylindole) essentially as described in references 7, 30, and 38. The coverslip was inverted onto a slide, and FlbB and nucleus localizations were observed by fluorescence microscopy.

Fluorescein staining experiments for confirmation of autolysis in liquid starvation cultures were performed as described by Roncal et al. (33).

Cloning of *flbB*. The gene library contained in the self-replicating plasmid pRG3-AMA-Not1 (29) was amplified using competent *E. coli* DH5α library efficiency cells (Invitrogen). Preparation and transformation of protoplasts were carried out as previously reported (42) with the following adaptations: after 20 to 24 h of culture at 37°C, in order to avoid conidiation induced by osmotic stress, the protoplast regeneration cultures (MMA plus 1 M sucrose) were overlaid with 7 ml of complete medium. The cultures were then incubated for 24 h. Conidiation colonies which emerged through the overlay were selected as positive transformants, and plasmids were isolated and amplified for sequencing. Inserts were amplified using oligonucleotides pRG3up and pRG3down. In order to localize and define the three mutations, genomic DNA was extracted from the three auxotrophic mutants and FGSC26. Each *flbB* open reading frame (ORF) was amplified using oligonucleotides 7542-1, 7542-2, and 7542-3 and sequenced.

**Genetic techniques and extraction and manipulation of DNA.** Meiotic crosses were performed according to Pontecorvo et al. (32). DNA extractions were carried out from 24-h liquid-culture-grown mycelium that had been filtered, washed, and lyophilized. The extraction process involved a GenElute plant genomic DNA extraction kit (Sigma), with the addition of 50 units of RNase (Sigma), Southern blot analyses were carried out using a digoxigenin High Prime II DNA labeling and detection starter kit (Roche). The cDNA sequence of *flbB* was PCR amplified using specific primers and a cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Invitrogen). A 24-h wild-type total RNA sample was used as the template.

**RNA isolation and analysis.** A mycelium sample (100 mg [dry weight]) was frozen in liquid nitrogen, and 1 ml of TRReagent (Fluka) was added. RNA extraction from these samples was performed according to the Invitrogen protocol for RNA extraction using the TRIZol reagent. RNA concentrations were calculated with a QuBit assay system (Invitrogen). Northern blot analysis of RNA was carried out with a digoxigenin Northern blot starter kit (Roche).

**Fusion PCR.** The DNA constructions for the generation of null mutants as well as green fluorescent protein (GFP) tagging were conducted as described by Yang et al. (50), using the Triplemaster PCR system (Eppendorf). Null mutants were generated using a modified version of the protocol of Yang et al. (50), by constructing a DNA fragment of 5.1 kb with 5' and 3' regions of *flbB* Banking the auxotrophic marker pyrg G of *Aspergillus fumigatus*. GFP tagging at the C-terminal region of FlbB was done as described by Yang et al. (50). In the case of N-terminal tagging, the GFP coding sequence was added behind the start codon using the overlapping PCR technique, and no marker was included. In this latter
case, selection of transformants was conducted using 2 mg/ml fluoroorotic acid in an fbb-null strain, selecting for gene replacement by the A. fumigatus pyrG gene, which replaced the fbb coding sequence.

Construction of alcA(p)::fbb strain. The alcA(p)::fbb construct was created as described previously (53) and cloned into the BamHI site of pJW53 (J. W. Bok and N. P. Keller, unpublished data) to generate pJW53::alcA(p). pJW53 was introduced into FGSC33 to give rise to the control strain TNI16.2. TNI16.2 was crossed with FGSC773 to yield strain TNI24.5. pJW53 was introduced into FGSC33 to yield strain TNI24.5. pJW53 was introduced into FGSC33 to give rise to the control strain TNI22.1.

Yeast strains and plasmids for the transactivation assay. The Saccharomyces cerevisiae reporter strain L40 [MATa his3-D200 trp1-901 leu2-3,112 ade2-101 lys2-801 ade2::HIS3 URA3::LEXA(p)::GAL1-LEU2] was obtained from Invitrogen. L40 was grown in synthetic dropout minimal medium (36) with various supplements.

The medium composition (per liter) is as follows: 20 g glucose, 6.7 g of yeast nitrogen base (MP Biomedicals), 50 ml of 20/8 dropout solution (300 mg L-arginine, 1,500 mg L-lysine, 500 mg L-phenylalanine, 200 mg L-asparagine, 300 mg L-tyrosine, 300 mg L-threonine, 300 mg L-tyrosine, 200 mg L-tryptophan, and 500 mg L-adenine hemisulfate salt) with or without 10 ml of 100/8 nutrient solution (10 g leucine, 2 g tryptophan, and 2 g histidine per liter). The plasmid pTLexA (11) (kindly provided by Suhn-Kee Chae at Paichai University, Daejeon, Korea) carrying the LexA DNA binding domain (8) was generated by the modification of pHybLex/Zeo (Invitrogen) by inserting the TRP1 marker (obtained from pPGT-V, Clontech).

Construction and transformation for testing FlbB transactivation activity. The cDNA-derived full-length FlbB (amino acids [aa] 1 to 426) and N-terminal (aa 1 to 172) and C-terminal (aa 166 to 462) regions of FlbB were PCR amplified using the primer pairs ONK20-ONK21, ONK20-ONK69, and ONK68-ONK21, respectively, from an A. nidulans cDNA library provided by Kwang-Yeop Jahng (Chonbuk University, Jeonju, Korea). Individual amplicons were digested with EcoRI and Sall and cloned into pTlexA, resulting in pJW53::alcA(p), pJW53::alcA(p) 5/8 nested with BamHI tail.

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TABLE 3. Mutations characterized in this study

| Allele | DNA change(s) | Mutant proteina | Change(s) in proteinb |
|--------|---------------|-----------------|----------------------|
| flbB100 | G456A | aa 1→426 | G70R |
| flbB101 | G1143A | aa 1→267 + YVLR | L267 fs |
| flbB102 | C125ST, ΔL1257 | aa 1→304 + L-35 | P305L, P306 fs |

a YVLR and L-35 are residues added out of frame.
b fs, frameshift followed by early termination.

Sequence accession numbers. Nucleotide and corresponding amino acid sequences for flbB were deposited in the EMBL database (http://www.ebi.ac.uk/emi/Submission/webin.html) under accession number AM494477.

RESULTS

Three aconidal mutants define distinct flbB mutant alleles. Via chemical (nitrosoguanidine) or UV mutagenesis followed by visual screening of more than 64,000 survivors, we isolated three nonsporulating (fluffy) mutants. Conidiation of these mutant strains (BD11, BD12, and BD14) could be induced by growing them in contact with a conidiating strain. In turn, these mutant strains induced conidiation of fluffy nonconidial mutant strains. The extracellular complementation of the flbG mutant strains. The extracellular complementation of the flbG strains, in which the corresponding diffusible Flug factor is absent, suggested that the mutations were not within the flbG gene.

Meiotic crosses and diploidy analyses revealed that these three mutations were recessive and allelic and mapped to chromosome IV (results not shown). The recessive nature of the mutations allowed us to use an autoreplicative plasmid (pRGC/AMA/NotI)-based genomic DNA library (29). We constructed strain BD109 (Table 1), carrying the pyrG89 mutation from strain BD12, and used it for complementation-based gene cloning. All the plasmids isolated from conidiating (complemented) transformants contained inserts including the AN7542.3 gene (Broad Institute; http://www.broad.mit.edu/annotation/fungi/aspergillus).

Subsequent genomic DNA sequence comparisons of the AN7542.3 region from the three mutants and a wild-type strain confirmed that the aconidal phenotype was caused by mutations in this gene (Table 3). The prediction for the coding region of AN7542.3 showed coincidence with preliminary DNA binding domain only between residues 79 and 113 (Pfam database searches pointed to the presence of a bZIP-type DNA binding domain only between residues 79 and 113 (Pfam accession number PF00170) (Fig. 1B).

Protein database searches showed the existence of putative homologues in other Aspergillus species, such as A. terreus, A. fumigatus, and A. oryzae, as well as other filamentous fungi, such as Magnaporthe grisea, Fusarium graminearum, Neurospora crassa, Botrytis cinerea, Coccidioides immitis, Sclerotinia sclerotiorum, and Stagonospora nodorum (Fig. 1B). Bcin and Cinn2 homologues have less similarity with respect to FlbB in the region before the bZIP than in the conserved regions of the C-terminal half (Fig. 1B). Thus, it is possible to differentiate a group of FlbB homologues showing poor sequence conservation in this region. On the other hand, we could not detect similar genes in yeasts, such as Saccharomyces cerevisiae, Candida albicans, and Schizosaccharomyces pombe, or higher eukaryotes, such as Homo sapiens and Arabidopsis thaliana, indicating a restricted phylogenetic distribution of FlbB homologues. To further explore this idea, we searched for all putative bZIP-containing proteins in the genomes of different filamentous fungi (A. nidulans, C. immitis, M. grisea, and N. crassa) and yeasts (S. cerevisiae and S. pombe). We found 23 proteins in A. nidulans, and functions have been described for the following ones: AN1812.3/Ilha (40), AN2911.3/AtfA (5), AN3675.3/CpcA (15), AN4361.3/MetR (26), AN4900.3/MeaB (31), AN8251.3/HapX (41), and AN9397.3/Hac1 (34). The derived phylogenetic tree shows that the FlbB bZIP belongs to a branch where yeasts are excluded (Fig. 1C). Our in silico analyses strongly suggest a highly specialized filamentous fungal function for at least FlbB and probably for other bZIP-containing proteins, such as AN4900.3/MeaB and AN0951.3.

Phenotypic analysis of flbB mutants. To further analyze the phenotypes of the isolated mutations, we constructed an flbB-null strain by gene replacement using the A. fumigatus pyrG gene (see Fig. S1 in the supplemental material). Strains carrying mutant and null alleles of flbB were further analyzed in parallel. Surface cultures (Fig. 2) of the strains carrying the different flbB mutant alleles yielded the aconidal phenotype in solid MMA. We then investigated whether carbon or nitrogen starvation or salt stress might have a suppressing effect on the aconidal phenotype displayed by the flbB mutant alleles (Fig. 2), as it was previously shown to promote conidiation in the wild type (2, 37). A reduction in glucose content provoked low levels of conidiation accompanied by some autolysis at the lesser degree.

The growth and conidiation patterns of the null mutant and the three original mutants were also assessed in liquid cultures...
Tant were some vesicles seen after 20 h of incubation. No conidiation response was shown, while the wild-type strain confirmed by the absence of staining with a vital stain fluorescent, while a wild-type strain generated conidiophores and conidiation response but rather exhibited an autolytic phenotype. In liquid medium with a high salt concentration, neither the mutant nor the three flbB mutants could extracellularly rescue conidiation in a ΔfluG strain. These results indicate that FlbB may function in the synthesis of a diffusible signal compound that is necessary for normal conidiation and distinct from the FluG factor.

FlbB expression precedes and determines brlA activation. Earlier studies had shown that wild-type strains extracellularly restored conidiation in all flb mutants (flbA-E) upon direct physical contact or through a dialysis membrane (21). flb mutants, in turn, unidirectionally restored conidiation in fluG loss-of-function mutants (47). We found that conidiation of flbB mutants was rescued by growing them in contact with a wild-type strain (Fig. 4) or through a dialysis membrane (data not shown). Moreover, all flbB mutants could extracellularly rescue conidiation in a ΔfluG strain. These results indicate that FlbB may function in the synthesis of a diffusible signal compound that is necessary for normal conidiation and distinct from the FluG factor.

FlbB expression precedes and determines brlA activation. The expression of flbB during the A. nidulans life cycle started with vegetative growth and continued into early phases of asexual development (Fig. 5A). The decline of flbB mRNA steady-state levels coincided with the start of brlA transcription. In addition, flbB expression resumed 12 h after asexual induction and was also observed in conidia and ascospores (sexual spores).

Since the above result did not clarify whether FlbB activated or repressed brlA expression, the ΔflbB mutant was examined by Northern blotting (Fig. 5B). The absence of detectable brlA mRNA accumulation demonstrated that FlbB is necessary for brlA expression and formally confirmed the designation of the gene as an flb (fluffy with low bristle expression) gene.

Finally, no significant differences could be detected in the flbB expression pattern between a veA wild-type (FGSC4) strain (Fig. 5A) and a veA mutant (veA1; FGSC26) strain (Fig. 5B), suggesting that flbB expression is not conditioned by the light-dependent conidiation regulator VeA.

Localization of FlbB at the hyphal tip and in the nucleus. In order to determine the subcellular distribution of FlbB, strains expressing either FlbB:GFP or GFP:FlbB proteins were constructed (see Materials and Methods and Fig. S2 in the supplemental material). Microscopic examination of the strains in liquid static cultures revealed that FlbB is in the cytoplasm and accumulates at the hyphal tip at 16 h (Fig. 6A) and then (at 24 to 36 h) into the nuclei, as predicted by the putative transcriptional regulatory function of FlbB, despite the absence of an identifiable nuclear localization signal (Fig. 6A). Interestingly, in most of the screened cells, the nucleus closest to the cell apex exhibited the highest level of fluorescence, indicating the high degree of regulation in localization or compartmentalization of FlbB (see Discussion). The vast majority of the hyphae showed this localization for either C- or N-terminally tagged obtained mutants and their comparisons with the null mutant lead us to conclude that the former bear partial loss-of-function mutations.

FlbB is associated with the production of a diffusible compound needed for conidiation. Earlier studies had shown that wild-type strains extracellularly restored conidiation in all flb mutants (flbA-E) upon direct physical contact or through a dialysis membrane (21). flb mutants, in turn, unidirectionally restored conidiation in fluG loss-of-function mutants (47). We found that conidiation of flbB mutants was rescued by growing them in contact with a wild-type strain (Fig. 4) or through a dialysis membrane (data not shown). Moreover, all flbB mutants could extracellularly rescue conidiation in a ΔfluG strain. These results indicate that FlbB may function in the synthesis of a diffusible signal compound that is necessary for normal conidiation and distinct from the FluG factor.

Fig. 1. General description of flbB. (A) Genome localization and general description of flbB. Black segments represent exons, and black lines designate introns. (B) Sequence alignment of FlbB homologues. Arrows indicate mutations which define changes in the amino acid sequence. Regions with low similarity are excluded. Genedoc software was used (version 2.6.003; wwwpsc.edu/biomed/genedoc). (C) Phylogenetic tree of bZIP domains identified in various fungal and yeast species. The bZIP branch including FlbB bZIP is framed. Abbreviations: Ater, A. terreus; Bein, B. cinerea; Ssc1, S. sclerotiorum; Aory, A. oryzae; Afum, A. fumigatus; Cimm, C. immitis; Snod, S. nodorum; Nera, N. crassa; Fgra, F. graminearum; Mgri, M. grisea; Spom, S. pombe; Scer, S. cerevisiae. Phylogenetic and molecular evolutionary analyses were conducted using MEGA software, version 3.1 (neighbor-joining method, with a bootstrap of 50,000 replicates and amino p-distance substitution model).
FlbB proteins, up to 36 h of culture (Fig. 6A; data not shown for GFP::FlbB). Blast searches using different programs available at www.expasy.org (HMMTOP, PredictProtein, SOSUI, TMAP, TMHMM, Tppred, TopPred, and PSORT) showed an absence of predictable transmembrane domains or cysteine residues susceptible to prenylation (PrePS), suggesting that FlbB is probably not anchored to the membrane and that its apical localization responds to other, as-yet-unknown factors (Fig. 6B).

FlbB is a potential transcriptional activator. To test whether FlbB can function as a TF likely activating downstream genes necessary for conidiation, the ability of the FlbB protein to activate two reporters was examined. The full-length protein (FlbB-F), the N-terminal region including the bZIP domain (FlbB-N), and the C-terminal region without the bZIP domain (FlbB-C) were fused with LexA<sub>DBD</sub> (DNA binding domain), and individual fusion proteins were expressed in <i>S. cerevisiae</i>. As shown in Fig. 7, when the transformants were tested on the medium lacking two nutrients (uracil and histidine) in the presence of various concentrations of 3-AT for the HIS<sub>3</sub> reporter, those expressing LexA<sub>DBD</sub>::FlbB-F and LexA<sub>DBD</sub>::FlbB-C grew on the medium with 5 mM and 10 mM 3-AT, respectively. However, the strains with pTLexA alone or LexA<sub>DBD</sub>::FlbB-N were unable to form colonies. In the X-Gal test, those expressing LexA<sub>DBD</sub>::FlbB-C showed blue color within 4 h after inoculation, similar to the PJ69-4A strains expressing Gal4<sub>DBD</sub>::AfR (28, 52). The strains expressing LexA<sub>DBD</sub>::FlbB-F began to exhibit blue color after 1 day. Moreover, when multiple transformants for each construct were tested for their β-galactosidase activity (Fig. 7, right), the

![FIG. 3. Mutant characterization in liquid media. Phenotypes of wild-type (WT; TN02A3), ∆flbB (BD143), flbB100, flbB101, and flbB102 strains after 18 h of culture in MMA, followed by transfer to MMA-glucose, MMA-nitrate, or MMA plus KCl and MES for a further 20 h (see Materials and Methods), are shown. Arrowheads indicate conidium-like structures. Bar = 30 μm.](image-url)
strains expressing LexA DBD::FlbB-C and LexA DBD::FlbB-F showed high levels of β-galactosidase activity (489 ± 12 and 248 ± 7 units, respectively). These results indicate that the C-terminal region of FlbB has a transactivation activity and that the N-terminal region may be involved in a modulating capacity, not excluding inhibitory effects on transactivation.

Overexpression of flbB inhibits conidiation. To further examine the regulatory role of flbB in conidiation, we generated an flbB overexpression mutant by fusing the flbB ORF with the inducible alcA promoter (14). Since the above-mentioned data support the positive regulatory roles for FlbB in conidiation, a hyperconidiating phenotype was expected. However, overexpression of flbB resulted in severely reduced conidiation (Fig. 8A). Examination of brlA and flbB mRNA levels revealed that overexpression of flbB abolished the proper accumulation of brlA mRNA (Fig. 8B). This is consistent with the observation that the alcA(p)::flbB mutant showed hyphal growth only after synchronized asexual developmental induction on threonine medium, whereas a control strain produced plenty of conidio- phores (Fig. 8C). These findings suggest that FlbB may be coupled with another factor(s) in a finely balanced stoichiometry, requiring a correct dosage in order to form an activating (heteromeric) complex (see Discussion).

DISCUSSION

In a mycelium, vegetative hyphae extend radially at the periphery, and some rise into the atmosphere. Recent evidence indicates that the recognition of this status is determined by an increase in extracellular levels of the FluG factor (33, 48). At this stage, some hyphae undertake conidiophore morphogenesis (1), but at least one additional signal seems to be required to confirm this particular fate, as previously shown (47) and confirmed in this study. The flbB-D genes appear to intervene at this morphogenetic stage, as a mutation in any one of them blocks the synthesis of the second diffusible signal (47). Epis- tasis studies show that the sequence FluG → FlbD → FlbB → BrlA (FlbC acting in parallel) is operational (55), FlbB being the last reported element in the sequence before the activation of brlA. In addition, FlbB is a transcription factor located at the hyphal apex near the growing point of the cell. Such a location suggests that distinct but as-yet-undetermined changes at the growing tip may be involved in its activation. Finally, FlbB is required for the synthesis of the second signal downstream of FluG, which is necessary for conidiation induction.

Consistent with these observations, flbB displays unique characteristics as a transcription factor, i.e., a highly conserved N-terminal bZIP domain and various conserved regions, which
participate in the function of the protein and could participate in transcriptional activation, as found in this study.

The Gly residue, where the missense mutation FlbB100 (G70R) lies, is situated nine residues upstream from the starting Leu of the bZIP domain. Previous reports have shown the presence a similar sequence in HapX, where this Gly is universally conserved (41). This region is necessary for the recruitment of HapX to the Hap complex and consequent recognition of CCAAT sequence-containing promoter regions (25). The apparent necessity of this flanking region for the functionality of FlbB suggests that the protein may form a (heterogenic) complex to activate conidiation, although an altered function of the bZIP DNA binding domain cannot be excluded.

The conserved FlbB67-78 and C-terminal regions were all required for normal conidiation in solid air-exposed cultures. However, in liquid culture, where stimuli can be studied separately, a differential response was revealed. Under carbon source-limiting conditions, which are known to induce conidiation in liquid cultures (37), a functional FlbB67-78 region is essential. In contrast, the C-terminal region does not appear to play a fundamental role. Under nitrogen starvation, however, all domains are required for conidiation in liquid culture, whereas in solid medium, truncating the protein from residue 267 onwards can result in notable conservation of activity. A more restricted truncation, affecting only residues 305 onwards, has a considerably greater effect, however, and this paradox raises the possibility of complex interactions between domains and indeed other interacting proteins. Under salt stress, mutants affected in the C-terminal region still showed a substantial conidiation response in solid medium, in sharp contrast to the mutant affected in Gly70, which resembled the practically fluffy phenotype of the null mutant. This indicates that the conserved region adjacent to the DNA binding domain, and possibly affecting bZIP function, appears to be essential for the response to salt stress. In contrast, in liquid medium all the regions characterized are necessary for conidiation to occur.

_expression starts just when \textit{flbB} expression ends, and this could lead to the conclusion that FlbB could act as a \textit{brlA} transcriptional repressor. However, no \textit{brlA} expression was detected in the \textit{flbB}-null mutant, showing that FlbB is required, directly or indirectly, for proper \textit{brlA} transcription regulation. This suggests that the regulation of \textit{flbB} expression must be precisely controlled for a normal asexual life cycle. The observation that \textit{flbB} expression starts again at 48 h after asexual development induction suggests that FlbB could act in a cyclic way, having a sensory role, in order to confirm or remind that conidiation should continue. To confirm this, \textit{flbB} and \textit{brlA} expression should be analyzed for a longer period after asexual induction. Finally, \textit{flbB} expression is not conditioned by VeA.
Function, resulting in an aconidial phenotype. Since hypofunctional \textit{flbB} mutants are not affected in growth pattern or kinetics, the role of this regulatory mechanism appears to be strictly limited to the control of conidiation.

The nature of the stimulus triggering FlbB relocalization to the nucleus was not clarified in this study. Among the various possible cues is reactive oxygen species, which is associated with the cessation of growth and has been reported to have a role in fungal morphogenesis (19). Moreover, FlbB shares a number of conserved cysteines in its C-terminal region with another bZIP transcription factor, Pap1 of \textit{S. pombe}, which is capable of sensing moderate levels of oxidative stress through these residues (9). This and other likely possibilities are under examination.

**ACKNOWLEDGMENTS**

We express our admiration and gratitude to T. H. Adams, J. Aguirre, and J. K. Wieser for their pioneering work in the field of conidiation induction, and especially with \textit{flb} mutants, which served as a basis for the findings of this investigation. We are also grateful to J. Clutterbuck for provision of strains and advice and T. Roncal and S. Cordobés for their assistance with mutant screening.

The work carried out at UW-Madison was supported by Hatch (WIS04667) and National Science Foundation (MCB-0421863 and IOS-0640067) grants to J.H.Y. E.A.E thanks Ministerio de Educación y Ciencia for support through grant BFU2006-04185. U.U. thanks Ministerio de Educación y Ciencia for grant BFU2004-03499/BMC and UPV/EHU for grant GIU05/36. O.E received a doctoral grant from UPV/EHU, and A.G. was a contract researcher under the re- search program of Gipuzkoako Foru Aldundia/Diputación Foral de Gipuzkoa. The work was also supported by the Max-Planck-Institute for Terrestrial Microbiology, Marburg, Germany.

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