Aspergillus fumigatus flbB Encodes Two Basic Leucine Zipper Domain (bZIP) Proteins Required for Proper Asexual Development and Gliotoxin Production

Peng Xiao,1,2 Kwang-Soo Shin,3 Tianhong Wang,1 and Jae-Hyuk Yu2*

State Key Laboratory of Microbial Technology, Shandong University, Jinan, Shandong, People’s Republic of China; Departments of Bacteriology and Genetics, University of Wisconsin, Madison, Wisconsin; and Department of Microbiology and Biotechnology, Daejeon University, Daejeon, Republic of Korea

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The opportunistic human pathogen Aspergillus fumigatus reproduces asexually by forming a massive number of mitospores called conidia (19). Under appropriate conditions, the conidium germinates and undergoes vegetative growth that leads to the formation of a network of undifferentiated interconnected hyphae known as the mycelium. After a certain period of hyphal proliferation, in response to appropriate cues (e.g., exposure to air, nutrient deprivation, or osmotic stress) some of the vegetative cells initiate asexual development (conidiation) and go through a series of morphological changes, which result in the formation of conidiophores consisting of foot cell, stalk, vesicle, phialides, and (up to 50,000) conidia (19, 26). The deletion of AfuflbB results in delayed/reduced sporulation, precocious cell death, the lack of conidiophore development in liquid submerged culture, altered expression of AfuflbA and AfuabaA, and blocked production of gliotoxin. While introduction of the wild-type (WT) AfuflbB allele fully complemented these defects, disruption of the ATG start codon for either one of the AfuflbB polypeptides leads to a partial complementation, indicating the need of both polypeptides for WT levels of asexual development and gliotoxin biogenesis. Consistent with this, Aspergillus nidulans flbB* encoding one polypeptide (426 aa) partially complements the AfuflbB null mutation. The presence of 0.6 M KCl in liquid submerged culture suppresses the defects caused by the lack of one, but not both, of the AfuflbB polypeptides, suggesting a genetic prerequisite for AfuFlbB in A. fumigatus development. Finally, Northern blot analyses reveal that both AfuflbB and AfuflbE are necessary for expression of AfuflbD, suggesting that FlbD functions downstream of FlbB/FlbE in aspergilli.

The opportunistic human pathogen Aspergillus fumigatus (reviewed in reference 9) propagates in the environment by producing a massive number of mitotic spores called conidia (19). Under appropriate conditions, the conidium germinates and undergoes vegetative growth that leads to the formation of a network of undifferentiated interconnected hyphae known as the mycelium. After a certain period of hyphal proliferation, in response to appropriate cues (e.g., exposure to air, nutrient deprivation, or osmotic stress) some of the vegetative cells initiate asexual development (conidiation) and go through a series of morphological changes, which result in the formation of conidiophores consisting of foot cell, stalk, vesicle, phialides, and (up to 50,000) conidia (19, 26). Conidia (2 to 3 μm in diameter) are then released into the environment and are small enough to reach the alveoli after being inhaled by humans (31). In immunocompromised hosts the conidia are able to germinate into invasive hyphae, which penetrate the vasculature and migrate to distal sites (reviewed in reference 26).

We have been investigating the mechanisms underlying asexual development and gliotoxin biosynthesis in A. fumigatus, primarily focusing on understanding the functions of a number of developmental regulators identified in the model fungus Aspergillus nidulans (21, 30, 40). A key step for conidiation in A. nidulans is the activation of brlA encoding a C2H2 zinc finger transcription factor (TF) (1). Together with abaA and wetA, these three elements define a central regulatory pathway that coordinates the temporal and spatial control of sporulation-specific gene expression during asexual development in A. nidulans (2, 4, 22, 23). Our previous studies have revealed that the roles of BrlA, AbaA, and WetA in developmental regulation are essentially identical in two Aspergillus species (20; also L. Tao and J.-H. Yu, unpublished data).

Identification and characterization of six upstream genes (flaG, flbA, flbB, flbC, flbD, and flbE) required for proper expression of brlA in A. nidulans have illuminated genetic regulatory cascades leading to the activation of asexual development (2, 23, 33). Among these, flbB, flbC, flbD, and flbE were defined by the fluffy delayed-conidiation mutants (33). FlbB, FlbC, and FlbD are putative TFs containing a basic leucine zipper domain (bZIP), two C2H2 zinc fingers, and a c-Myb–DNA binding domain, respectively, and have been shown to act on the activation of brlA expression (10, 11, 14, 16, 32). Recent studies demonstrated that the bZIP TF FlbB is necessary for the expression of flbD and that FlbB and FlbD activate brlA in a supportive manner (14). The A. nidulans flbE (AnflbE) gene is predicted to encode a 201-amino-acid (aa)-long polypeptide with two conserved yet uncharacterized domains, and it was demonstrated that FlbE and FlbB are functionally interdependent, physically interact in vivo, and colocalize at the hyphal tip in A. nidulans (13).

Our recent studies have demonstrated that A. fumigatus FlbE (AfuFlbE) is crucial for proper conidiation and is functionally conserved in two aspergilli (17). In this study, we characterize AfuflbB and present evidence that FlbB is vital for A. fumigatus morphological development and gliotoxin produc-
tion. Unlike flbB in A. nidulans, AfufbB produces two transcripts predicted to encode two bZIP polypeptides, AfuFlbB6 (420 aa) and AfuFlbB8e (390 aa). The deletion of AfufbB results in multiple defects in development, cell viability, and gliotoxin biosynthesis. A series of complementation studies indicate that both AfuFlbB polypeptides are necessary for proper asexual and chemical development. Supporting this, A. nidulans flbB encoding one polypeptide (426 aa) only partially complements the AfufbB null mutation. High KCl concentration suppresses the defects caused by the absence of one, but not both, of the AfuFlbB polypeptides, suggesting a genetic prerequisite for AfuFlbB in A. fumigatus conidiation and gliotoxin production. Finally, we show that both AfufbB and AfufbE are necessary for expression of AfufbD, and we present a model depicting developmental regulation in A. fumigatus.

**MATERIALS AND METHODS**

Strains, media, and culture conditions. Aspergillus strains used in this study are listed in Table 1. Except for the A. fumigatus auxotrophic strains, all fungal strains were routinely grown on solid or liquid glucose minimal medium (MMM) with 0.1% (or 0.5%) yeast extract (YE) at 37°C, as previously described (20). For A. fumigatus conidia (AfupyrG1) 0.1% arginine. uridine-uracil and arginine auxotrophic mutants (strains AF293.1 and AF293.6) with 0.1% (or 0.5%) yeast extract (YE) at 37°C, as previously described (20). For A. fumigatus conidia, the conidia (10^5/ml) in 50 ml of liquid MMG with 0.1% YE and incubated at 250 rpm at 37°C for a period of 4 days. The mycelial aggregates of each strain were observed microscopically starting at 6 h of liquid culture and every 6 h thereafter. To check development and salt stress, the conidia (10^6/ml) of wild-type (WT) and mutants of the liquid submerged culture and post-asexual developmental induction were squeezed dried and stored at –80°C until subjected to total RNA isolation.

**Generation of AfufbB mutants.** The AfufbB gene was deleted in A. fumigatus AF293.1 (pyrG1) and AF293.6 (pyrG1, argB1) strains (34) employing double-joint PCR (DJ-PCR) (37). Oligonucleotides used in this study are listed in Table S1 in the supplemental material. Briefly, approximately 1.4 to 1.7 kb of the 5’ and 3’ flanking regions of the AfufbB gene was amplified from A. fumigatus AF293 genomic DNA with the primer pairs KS31/KS32 (~1.74 bp) and KS34/KS36 (~1.63 bp with the AnigrG tail) and the pairs KS31/KP76 (~1.43 kb) and KP71/KS36 (~1.63 bp with the AmargB tail), respectively. The A. nidulans selective markers were amplified from FGSC4 genomic DNA with the primer pairs oXS0/oXS9 (AnigrG) and oXS16/oXS105 (AnigrG). The 5’ and 3’ flanking regions of AfufbB were fused to each relevant marker and further amplified by the nested primer pairs oXS2/oXS5 and oXS17/oXS35, yielding the final gene deletion constructs. The gene deletion constructs were introduced into recipient strains AF293.1 and AF293.6, respectively. The ΔAfufbB mutants (e.g., strains TKSS1.01 and TPX2.01) were isolated and confirmed by PCR, followed by restricted enzyme digestion (37). At least three deletion strains in each case were isolated.

To complement ΔAfufbB, the wild-type AfufbB allele (open reading frame [ORF] with 0.73 kb of the 5’ and 1 kb of the 3’ regions) was amplified with the primer pair oP5X0/oP5X2, digested with PvuII and HindIII, and cloned into pNZ25 (28). The resulting plasmid pPX1 was then introduced into the recipient ΔAfufbB strain TPX2.01 (ΔAfufbB::AnigrG; AfupyrG1), where preferentially a single-copy AfufbB1 gets inserted into the AfupyrG locus. Multiple complemented mutants were isolated and confirmed by PCR and Southern analysis. Similarly, the AfufbB ORF with a 0.73-kb 5’ flanking region was amplified using the primer pair oPXS0/oPXS6, digested, and cloned between PvuII and HindIII sites in pNZ25 to generate plasmid pPX2, carrying the chimeric AfufbB gene comprised of the AfufbB native promoter, AfufbB coding region, and the AnigrC terminator (35, 36). The resulting plasmid was introduced into the recipient ΔAfufbB strain TPX2.01, and several strains displaying phenotypes identical to those of AfufbB1 with the native terminator were isolated and confirmed.

To generate the mutants producing only AfuFlbB or AfuFlbE, the plasmids pPX3 and pPX4 were constructed and introduced into the ΔAfufbB strain TPX2.01. Briefly, the mutagenic primer pairs oPX101/oPX97 and oPX98/oPX102 and the pairs oPX101/oPX99 and oPX100/oPX102 were used to amplify different parts of AfufbB, see Table S1 in the supplemental material, where base underlines are positioned in the joint parts of each ampiclon conferring ATG-to-GCC mutations in each predicted ATG start codon. After separate conventional PCRs, the amplicons with complementary joint tails were mixed in a 1:1 ratio and subjected to fusion reactions. Then, the fused PCR products were amplified using the primer pair oPX80/oPX26, yielding amplicons with 0.73 kb of 5’ flanking sequence with the mutated AfufbB ORF; the +1 position ATG or +480 position ATG (Met) was replaced with GCC (Ala). The final PCR products were digested and cloned between PvuII and HindIII sites in pNZ25 and sequence verified transducing in pPX3 and pPX4 carrying the mutated AfufbB gene comprised of the AfufbB native promoter, the AfuFlbB coding region from which only one polypeptide is predicted to be translated, and the AnigrC terminator. Then, these two plasmids were introduced into the recipient ΔAfufbB strain TPX2.01 separately. Multiple transformants were isolated and

| Strain name | Relevant genotype | Reference and/or source | 
|-------------|------------------|------------------------|
| AF293 | A. fumigatus wild type | 5 |
| AF293.1 | AfupyrG1 | 34 |
| AF293.6 | AfupyrG1, AfuargB1 | 34 |
| A1176 | AfupyrG1, ΔAfufbB::AnigrG* | 20; FGSC |
| TPX2.01 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| TPX5.10 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| TPX6.01 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| TPX7.06 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| TPX8.02 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| TPX9.03 | AfupyrG1, ΔAfufbB::AnigrG* | This study |
| FGSC4 | A. nidulans wild type | 17 |

* Fungal Genetic Stock Center.

**TABLE 1. Aspergillus strains used in this study**
confirmed by PCR. Cross-complemented strains were obtained by introducing the pNJ25-derived plasmid pPX5 carrying *AfuflbB* (amplified using primer pair oPX90/oPX104 and cloned between *Pvu*II and *Xba*I sites in pNJ25) into a Δ*afluB* strain (TPX2.01).

**Nucleic acid isolation and manipulation.** Genomic DNA and total RNA isolation were carried out as described previously (20, 37). Approximately 10 μg (per lane) of total RNA isolated from individual samples was separated by electrophoresis using a 1.5% agarose gel containing 3% formaldehyde and ethidium bromide and blotted onto a Hybond-N membrane (Amersham, NY). The 32P-labeled hybridization probes for *AfuflbB*, *AfuflbC*, *AfuflbD*, *AfuflbE*, *AfubaA*, *AfubacA*, *AfubetA*, and *AfuxcA*, and glz2 were prepared by PCR amplification of individual ORFs from the genomic DNA of AF293 by using specific oligonucleotides (listed in Table S1 in the supplemental material). DNA blots were hybridized with individual probes using modified Church buffer (1 mM EDTA, 0.25 M Na2HPO4, 7H2O, 1% hydrolyzed casein, and 7% sodium dodecyl sulfate, adjusted to pH 7.4 with 85% H3PO4) as previously described (39).

**Assessment of cell viability.** The Alamar blue (AB) assay was used to evaluate the cell viability by the percent reduction of Alamar blue as described previously (27, 29). Briefly, the amount of Alamar blue cell proliferation indicator (AbD Serotec, NC), equal to 10% of the final volume in the wells, was added into each well test of a 24-well plate (Nunc) containing 1 ml of fresh MMG–0.1% YE and 0.5 ml of individual cultures. After 6 h of incubation at 37°C, the absorbance of each well was measured at 570 and 600 nm. The percent reduction of AB was calculated as described previously (29). In addition, to further assess the levels of apoptotic-like cell death, the hyphal cells were stained with Evans blue as previously described (29). The mycelia of relevant strains grown for certain periods were collected and treated with Evans blue staining solution (1% Evans blue [Sigma, St. Louis, MO] in PBS) for 5 min at room temperature, and then excessive dye was washed out with 10 ml of phosphate-buffered saline (PBS) three times. The samples were analyzed using a bright-field microscope.

**Gliotoxin analysis.** Gliotoxin production was analyzed by thin-layer chromatography (TLC) as described previously (3, 28). To examine gliotoxin production in liquid culture, conidia (~106/ml) from individual strains were inoculated into 50 ml of liquid MMG with 0.1% YE in 250-ml flasks and incubated at 37°C and 250 rpm for 48 h. The liquid cultures of relevant strains were filtered by Miracloth, 50 ml chloroform was added to the filtered liquid medium, and the mixture was agitated at 250 rpm for 30 min on a rotary shaker at room temperature (28). The aqueous layer was removed, and the chloroform extract was then air dried at room temperature and suspended in 200 μl of methanol. Approximately 15 μl of each sample was loaded onto the silica TLC plates containing a fluorescent indicator (Kiesel gel 60; E. Merck, Germany). Gliotoxin standard was purchased at the N terminus (amino acids 35 to 69) of this protein. These results suggest that Afu2g14680 is likely the *A. fumigatus* homolog of FlbB, and it was designated *AfuflbB*. Additional reciprocal BLASTP searches using the predicted protein sequence of *AfuflbB* against other *Aspergillus* genomes revealed that the best match is *Ani* in *A. nidulans*. With orthologues of *AfuflbB* found in other *Aspergillus* species including *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus clavatus*, *Aspergillus terreus*, and *Aspergillus flavus*, as reported for *AniFlbB* (11). Sequence alignments of FlbB demonstrated that FlbB is highly conserved in aspergilli; however, the annotated FlbBs in *A. fumigatus* and *A. clavatus* appear to lack 20 to 30 amino acids compared to the sequence of *AniFlbB* (data not shown).

**RESULTS**

**Identification and analyses of *A. fumigatus* FlbB.** The putative *A. fumigatus* FlbB gene was identified by a BLASTP search in the *Aspergillus* genome database (Broad Institute Aspergillus Comparative Database [http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html]) with *A. nidulans* FlbB (GenBank accession number CAM35586.1). Among four matches returned, Afu2g14680 shows 65% identity, 77% similarity, and an expected value of 0. Moreover, a conserved bZIP (pam00170) domain found in *A. nidulans* FlbB was present at the N terminus (amino acids 35 to 69) of this protein. These results suggest that Afu2g14680 is likely the *A. fumigatus* homolog of FlbB.
To understand the nature of these two transcripts, various regions of Afuflb cDNAs were amplified from the A. fumigatus AF293 Uni-ZAP cDNA library (25) (kindly provided by G. S. May at the University of Texas MD Anderson Cancer Center). Direct sequencing of those PCR amplicons combined with comparison to the genomic sequence of A. fumigatus AF293 led to the identification of two overlapping transcription units with identical 3′ ends (Fig. 1B). The 2.2-kb Afuflbβ transcription unit contains four introns (positions –220 to –152, +53 to +421, +512 to +574, and +1233 to +1282) (Fig. 1B) and initiates at –589 with the start codon at +1, resulting in a 420-amino-acid polypeptide (accession number HM582656). The 1.6-kb Afuflbα transcription unit contains two introns (positions +512 to +574 and +1233 to +1282) (Fig. 1B), initiates at +421 with the first ATG at +460, and is predicted to encode a 390-aa polypeptide, which is essentially identical to AfuFlbBβ except for the first 30 amino acids at the N terminus (Fig. 1C). AfuFlbα was identical to the predicted protein sequence originally annotated (XP_755800.2). One polypeptide encoded by the AnilfbB locus is shown for the comparison (Fig. 1C).

**Role of AfuFlbB in asexual development.** To understand the roles of AfuFlbB in growth and development in A. fumigatus, we first generated ΔAfufβB strains by replacing the coding region of the AfuFlbB genomic locus with the A. nidulans pryG or argB marker. Complemented strains were subsequently generated by directly integrating the WT AfuflbB allele into the AfupyrG locus of the ΔAfufβB mutant (TPX2.01). Several ΔAfufβB strains displaying identical phenotypes were isolated and examined. When point inoculated on solid medium, the ΔAfufβB mutant exhibited enhanced accumulation of undifferentiated hyphal mass with delayed/reduced conidiation (Fig. 2A; see also Fig. S1a in the supplemental material), whereas the WT and ΔAfufβB ΔAfufβB− complemented (hereinafter called C′) strains formed conidiophores abundantly. Moreover, the colony edge of WT and C′ strains showed the presence of abundant conidiophores, whereas the ΔAfufβB mutant exhibited only extended hyphae (Fig. 2A, middle and bottom panels). Quantitative analyses of conidia per fungal colony grown on solid medium further demonstrated that the asexual spore production in the ΔAfufβB mutant was dramatically decreased, approximately 50% to 70% of the levels of the WT and C′ strains (see Fig. S1a). These results suggest that AfuFlbB is necessary for normal levels of conidiation on air-exposed solid culture.

We further examined the effects of ΔAfufβB during asexual developmental induction. As shown in Fig. 2B, upon induction, the ΔAfufβB mutant exhibited enhanced proliferation of vegetative cells with delayed progression of conidiation. Moreover, whereas the WT strain began to show accumulation of AfubrlA mRNA at 6 h, with a peak at 12 h postinduction, the ΔAfufβB mutant started to express AfubrlA at 12 h, with a peak at 24 h; i.e., there was about a 6- to 12-h delay in AfubrlA expression. Consequently, AfuabaA expression was delayed in the null mutant as well (Fig. 2C). However, expression patterns of AfuwetA and AfuvosA, which are mainly required for the late phase of conidiation, were not different between WT and the null mutant. These results indicate that AfuFlbB is necessary for proper expression of AfubrlA and AfuabaA during the initiation and progression of conidiation.

**Effects of ΔAfufβB in liquid submerged culture.** To further investigate the roles of AfuFlbB, the conidia of WT, ΔAfufβB, and C′ (ΔAfufβB ΔAfufβB−) strains were inoculated into liquid MMG with 0.1% YE, and morphologies and cell viabilities were examined. As shown in Fig. 3A, whereas WT and C′ strains started to produce simple conidiophores within 18 h, the ΔAfufβB mutant failed to sporulate even after 96 h in submerged liquid culture (data not shown), indicating that AfuFlbB is required for development under these conditions. Furthermore, in accordance with these observations, the WT accumulated AfubrlA mRNA at 12 to 24 h of vegetative growth, whereas no AfubrlA expression was detected in the AfuflbB null mutant (Fig. 2C).

To examine whether the absence of AfuflbB influences cell viability, Alamar blue reduction assays were carried out (Fig. 3B). While all strains examined maintained equally high levels of cell viability until day 3, the ΔAfufβB mutant exhibited drastically reduced cell viability at days 4 and 5. In particular, at day 4 the ΔAfufβB mutant exhibited 52% of cell viability, whereas WT and C′ strains showed 94% and 92% of cell viability, respectively. All strains tested continued to lose cell viability up to day 6, when no differences in the viability were observable (data now shown). To further test whether the reduced cell viability was due to cell death, the hyphal cells of the WT, ΔAfufβB, and C′ strains were stained with Evans blue and microscopically examined. As shown in Fig. 3C, compared to the WT and C′ strains, most of the ΔAfufβB cells were intensely stained with the dye, indicating extensive cell death at day 4. However, when mycelial dry weights of these strains were measured, no obvious differences among WT, C′, and ΔAfufβB strains were observable even at days 4 and 5 (data not shown). These results indicate that the absence of AfuflbB results in accelerated cell death but not autolysis in liquid submerged culture, and they support the idea that fungal autolysis and cell death are separate processes (27).

Both AfuFlbB polypeptides are required for proper asexual development. The presence of two overlapping AfuflbB transcripts with distinct expression patterns led us to test whether both are required for proper development in A. fumigatus. This was accomplished by constructing two specific AfuflbB mutant alleles driven by the native AfuflbB promoter carrying a change from ATG (Met) to GCC ( Ala) at each of the predicted start codons (Fig. 4A). Along with the WT allele, each mutant allele was integrated into the AfupyrG locus of the AfuflbB deletion mutant (TPX2.01), and the following three strains were generated (Fig. 4A): the ΔAfufβB AfuflbB− strain (C′; strain TPX6.01), the ΔAfufβB AfuflbBα strain (hereinafter called α; TPX8.02), and the ΔAfufβB AfuflbBβ strain (hereinafter called β; TPX7.06).

To test the requirement for the two polypeptides, we first examined the ability of WT, ΔAfufβB, C′, α, and β strains to produce conidia by point inoculation on solid medium. As shown in Fig. 4B, whereas the WT AfuflbB allele fully complemented the ΔAfufβB strain, the AfuflbBα or AfuflbBβ allele restored conidiation to ~50% of the levels of the WT and C′ strains (see Fig. S1b in the supplemental material), indicating that both polypeptides are required for normal conidiation. To further examine the requirement for two polypeptides, the mycelia of these strains were transferred onto solid medium, and progression of conidiation was examined (Fig. 4C). As
shown, the WT and C’ strains started to produce conidiophores at 6 h, whereas the ΔAfufB strain began to form conidiophores at 12 h postinduction. Similar to the ΔAfufB strain, the AfuFibBα and AfuFibBβ strains showed enhanced hyphal proliferation at 6 h and began to sporulate at 12 h postinduction. However, levels of AfubrlA mRNA in these strains were somewhat inconsistent with the delayed conidiation phenotype. As shown in Fig. 4C, it appears that the α
strain started to express *AfubrlA* from 6 h, with a peak at 12 h, followed by a gradual decrease, whereas the Δ/\text{H9252} strain hardly expressed AfubrlA at 6 h, with a peak at 12 h, followed by a dramatic decrease. These results suggest that both polypeptides are necessary for proper asexual development in *A. fumigatus* and that expression of AfubrlA might be one of many required events for proper conidiation.

The above-mentioned strains were further examined in liquid submerged culture. As shown in Fig. 4D, while the WT and Δ/\text{H9251} strains started to produce conidiophores within 18 h, whereas the Δ/\text{H9004} strain failed to develop. (B) Viability of WT, Δ/\text{H9004}, and Δ/\text{H9251} strains grown in liquid MMG with 0.1% YE at 37°C and 250 rpm for the period of 5 days. Data represent the mean values (± standard deviations) of three independent experiments. Note that the Δ/\text{H9004} strain exhibits accelerated cell death at days 4 and 5. (C) Apoptotic cell death levels of WT, Δ/\text{H9004}, and Δ/\text{H9251}; strains grown in liquid MMG with 0.1% YE at 37°C for 4 days were examined by Evans blue staining. Note the clear differences in the levels of staining among the mycelia.

**FIG. 3.** Effects of Δ/\text{AfubrbB} in liquid submerged culture. (A) Photomicrographs of the mycelium of WT (AF293), Δ/\text{AfubrbB} (Δ; TKSS1.01) and complemented (C'; TPX5.10) strains grown in liquid MMG with 0.1% YE for 18 h at 37°C and 250 rpm. The arrows indicate conidiophore structures. Note that WT and C' strains started to produce vesicles at 18 h, whereas the Δ/\text{AfubrbB} mutant fails to develop. (B) Viability of WT, Δ/\text{AfubrbB} (Δ), and C' strains grown in liquid MMG with 0.1% YE at 37°C and 250 rpm for the period of 5 days. Data represent the mean values (± standard deviations) of three independent experiments. Note that the Δ/\text{AfubrbB} mutant exhibits accelerated cell death at days 4 and 5. (C) Apoptotic cell death levels of WT, Δ/\text{AfubrbB} (Δ), and C'; strains grown in liquid MMG with 0.1% YE at 37°C for 4 days were examined by Evans blue staining. Note the clear differences in the levels of staining among the mycelia.

**Differential requirement of developmental regulators for proper gliotoxin production.** During the cultivation of these strains in liquid submerged culture, we noticed the differences in pigmentation (Fig. 5A). We carried out TLC examination of the chloroform extracts of culture filtrates of various strains grown in liquid MMG with 0.1% YE at 37°C for 2 days. As shown in Fig. 5B, whereas gliotoxin was clearly detectable in the WT and C' strains, it could not be detected in the chloroform extract of the Δ/\text{AfubrbB} mutant. On the other hand, a small amount of gliotoxin was detectable in the chloroform extracts of the Δ/\text{AfubrbB} and Δ/\text{AfubrlA} mutants. Northern blot analyses indicate that gliZ mRNA (3) is absent in the Δ/\text{AfubrbB} mutant but present in the WT, C', α, and β strains (Fig. 5C). We further examined the Δ/\text{AfubrbE} and Δ/\text{AfubrlA} mutants for the ability to produce gliotoxin. These mutants were defective in biosynthesis of gliotoxin in liquid submerged culture (Fig. 5D, left
However, under air-exposed solid culture conditions, the WT and mutant strains tested, except for the ΔAfubrlA mutant, produced similar amounts of gliotoxin (Fig. 5D, right panel). These results indicate that AfuflbB is (likely indirectly) associated with gliotoxin biosynthesis and that both polypeptides are required for proper production of gliotoxin in liquid submerged culture. Furthermore, the results suggest a potential key role of AfubrlA in gliotoxin biosynthesis (see Discussion).

FIG. 4. Both polypeptides are required for proper asexual development. (A) Schematics of the three AfuflbB alleles used for complementation. The predicted start codons (ATG) of the AfuflbB<sup>−</sup> allele were replaced with GCC, leading to the AfuflbB<sup>α</sup> and AfuflbB<sup>β</sup> alleles. (B) Photographs of the colonies of WT (AF293), ΔAfuflbB (Δ; TKSS1.01), C<sup>′</sup> (TPX6.01; ΔAfuflbB AfuflbB<sup>−</sup>), α (TPX8.02; ΔAfuflbB AfuflbB<sup>α</sup>), and β (TPX7.06; ΔAfuflbB AfuflbB<sup>β</sup>) strains grown on solid MMG with 0.1% YE for 3 days. (C) Progression of synchronized asexual development of the WT, ΔAfuflbB (Δ), C<sup>′</sup>, α, and β strains on solid MMG with 0.1% YE. Numbers indicate the time (hours) postinduction. The right panel shows the corresponding Northern blot of the samples taken from developmental induction. Equal loading of total RNA was demonstrated by ethidium bromide staining of rRNA. (D) Photomicrographs of the mycelium of the WT, ΔAfuflbB (Δ), C<sup>′</sup>, α, and β strains grown in liquid MMG with 0.1% YE at 37°C and 250 rpm for 18 h. Note that the ΔAfuflbB, α, and β strains do not produce any conidiophore structures, whereas the WT and C<sup>′</sup> strains start to form vesicles (marked by arrows) at 18 h.
KCl can suppress the developmental defects caused by the lack of one, but not both, of the *AfuFlbB* polypeptides. As previous studies demonstrated that unfavorable conditions including salt stress (11) can induce conidiation in *A. nidulans*, we investigated whether addition of KCl affects development of the above-mentioned strains. We first tested the effects by point inoculating the WT, /H9004* AfuflbB*,/H11032, /H9251, and /H9252 strains on solid MMG containing 0.6 M KCl and examining the morphologies and conidiation levels. We found that while the /H9004 *AfuflbB* mutant still exhibited delayed/reduced conidiation, the WT, /H11032, /H9251, and /H9252 strains all formed a normal layer of conidiophores (Fig. 6A). Moreover, quantitative analyses of conidia produced by these strains indicate that while the /H9004 *AfuflbB* mutant produced only 70% conidia compared to WT and C’ strains, the α and β strains produced the same levels of conidia as the WT and C’ strains (data not shown), suggesting that 0.6 M KCl can suppress the developmental defects caused by the absence of one, but not both, of the *AfuFlbB* polypeptides.

We further examined the suppressive effect of high KCl concentrations in liquid submerged culture. These strains were first cultured in liquid MMG with 0.1% YE for 14 h, and the mycelium was then collected and transferred into liquid MMG–0.6 M KCl and further examined for phenotypic changes. As shown in Fig. 6B, the WT and C’ strains began to form conidiophores at 12 h posttransfer, whereas the /H9004 *AfubrlA* (A1176) mutants failed to produce any conidiophore-like structures up to 48 h. As observed in solid culture, the α and β strains produced conidiophores abundantly, just like the WT and C’ strains, further suggesting that KCl can replace one, but not both, of the two *AfuFlbB* polypeptides for asexual development.

We further investigated whether KCl can suppress the defective gliotoxin production caused by mutations in *AfuflbB*.
TLC analyses of the chloroform extracts of the WT, C\textsuperscript{−}, α, and β strain cultures at 24 h after transfer to KCl-supplemented medium (Fig. 6C) showed equal levels of gliotoxin, whereas the \( \Delta\text{AfuflbB} \) mutant appeared to be still impaired in gliotoxin production. These results further suggest that KCl can suppress the gliotoxin defect caused by the absence of one, but not both, of the polypeptides and that asexual development and gliotoxin production in \( A.\text{fumigatus} \) might be closely associated through \( \text{AfuBr}A \) (see Discussion).

\textit{AniFlbB} partially complements \( \Delta\text{AfuflbB} \). As \( A.\text{nidulans} \) \textit{flbB} is predicted to encode only one polypeptide similar to \( \text{AfuFlbB} \), we hypothesized that \( \text{AniFlbB} \) cannot fully complement the \( \Delta\text{AfuflbB} \) strain. This was tested by introducing \( \text{AniFlbB}^+ \) to the \( \text{AfupyrG} \) locus of the \( \Delta\text{AfuflbB} \) mutant and examining its conidiation potential. As shown in Fig. 7A, when grown on solid medium, the \( \Delta\text{AfuflbB} \) \( \text{AniFlbB}^+ \) (hereinafter called Ani) strain partially restored asexual sporulation to the \( \beta \) strain (compare strains in Fig. 4B). Northern blot analysis confirmed that the introduced \( \text{AniFlbB} \) gene is highly expressed (Fig. 7B). Quantitative analyses of sporulation indicate that the Ani strain produced conidia to a level similar to the levels of the \( \alpha \) and \( \beta \) strains (see Fig. S1b in the supplemental material). Moreover, similar to the \( \alpha \) and \( \beta \) strains in liquid submerged culture, the Ani strain failed to develop up to...

![TLC analyses of developmental defects by 0.6 M KCl.](image)
48 h (data not shown). In addition, during developmental induction, the Ani strain exhibited delayed conidiation, despite recovery of nearly WT levels of \textit{AfubrlA} expression, as observed in the \(\Delta\) and \(C'\) strains (Fig. 7D). Finally, gliotoxin production was only partially restored by Ani \(\text{FlbB}\) (Fig. 7C).

Taken together, these results further corroborate that both \textit{Afu}\text{FlbB} polypeptides are essential for proper asexual development and gliotoxin production and suggest that \textit{A. fumigatus} has evolved uniquely with two transcription units of \textit{AfuflbB}.

**Genetic interactions between upstream developmental regulators in \textit{A. fumigatus}**. To dissect genetic interactions between key developmental regulators, a series of Northern blot analyses was carried out to test the need for \textit{AfuFlbB} and \textit{AfuFlbE} for proper expression of \textit{AfubrlA}. As shown in Fig. 8A, the absence of \textit{AfuflbB} or \textit{AfuflbE} abolished accumulation of \textit{AfubrlD} mRNA throughout the life cycle, indicating that both \textit{AfuFlbB} and \textit{AfuFlbE} are required for expression of \textit{AfubrlD}. However, expression of \textit{AfuflbB} and \textit{AfuflbE} was not affected by the absence of \textit{AfuFlbC}. These results suggest that the proposed model for genetic interactions among upstream developmental regulators in \textit{A. nidulans} (16, 17) is partially applicable for \textit{A. fumigatus} (Fig. 8B; see also Discussion).
**DISCUSSION**

*A. fumigatus* is an important opportunistic human pathogenic fungus that causes a severe and usually life-threatening systemic mycosis termed invasive aspergillosis in immunocompromised individuals (8). In addition to a massive number of small hydrophobic asexual spores, *A. fumigatus* produces the toxic secondary metabolite gliotoxin, a key virulence determinant contributing to its pathogenesis (18).

Based on the studies of growth and development in the distantly related *A. nidulans*, we have been dissecting the roles of genes associated with development in *A. fumigatus* (17, 20, 40; also Tao and Yu, unpublished). Our previous studies demonstrated that functions of the central regulators BrlA, AbaA, and WetA in conidogenesis are conserved in *A. nidulans* and *A. fumigatus* (20; also Tao and Yu, unpublished). On the other hand, while the upstream developmental regulator *AfuFlbE* plays a crucial role in proper development of *A. fumigatus*, *AfuFluG* is not essential for conidiation (20). In the present study, we further expanded our understanding of upstream regulation in *A. fumigatus* development by characterizing *AfuFlbB*. The deletion of *AfuFlbB* resulted in a phenotype similar to that caused by the *A. nidulans* flbB null mutation but differing in its severity. In contrast to the ΔflbB strain with a proliferation of undifferentiated aerial hyphae and the nearly absent conidiophore formation (11), the *AfuFlbB* null mutant exhibited a certain level of asexual development under air-exposed culture conditions although the process was delayed and the conidiation level was reduced. Conversely, *AfuFlbB* deletion strains did not develop in liquid submerged culture, whereas *A. fumigatus* WT and C strains elaborated conidio- phores abundantly within 24 h. We further asked whether the conidiation defects caused by ΔflbB were associated with altered expression of *AfuBrA* and other conidiation-specific genes. Consistent with a delay in asexual development in the ΔflbB mutant under synchronized developmental induction conditions (Fig. 2B), expression of *AfuBrA* was delayed for 6 to 12 h (Fig. 2C). Furthermore, expression of *AfuAbA*, another key regulator directly activated by *AfuBrA* (Tao and Yu, unpublished), was delayed about 12 h. However, *AfuwetA* and *AfuVosA*, whose transcripts mainly accumulate at the late phases of asexual development (24; also Tao and Yu, unpublished), were not affected. We speculate two possible explanations for this: *AfuWetA* and *AfuVosA* function in the process of conidium maturation (wall formation and trehalose biogenesis) and confer spore viability, which is a separate phase from the initiation of conidiophore formation activated by *AfuBrA* and *AfuAbA* (23), and/or additional components are associated with activation of *AfuwetA* and *AfuVosA* (Tao and Yu, unpublished).

Probably the most important finding of the present study is that two overlapping mRNAs and polypeptides are encoded by the *AfuFlbB* gene and that both are necessary for proper morphological development and gliotoxin biogenesis in *A. fumigatus*. The longer transcript *AfuFlbBβ* is present at a relatively constant level throughout the life cycle, whereas the shorter transcript *AfuFlbBα* specifically accumulates during the progression of conidiation. In *A. nidulans*, only one transcript is encoded by *AnflbB*, which primarily accumulates during asexual development (11). *AnFlbB* (426 aa) shares a high similarity with *AfuFlbBβ* (420 aa). Examination of the mutants that produce only one of the two predicted polypeptides reveals that both *AfuFlbBα* and *AfuFlbBβ* are required for proper asexual development (Fig. 4B; see also Fig. S1b in the supplemental material), indicating that the two proteins likely play an additive and/or complementary role. This is supported by the fact that 0.6 M KCl can suppress the developmental defects caused by the absence of one, but not both, of the polypeptides.

**FIG. 8.** Genetic interactions between upstream developmental regulators in *A. fumigatus*. (A) Levels of *AfubflbB*, *AfubflbC*, *AfubflbD*, and *AfubflbE* transcripts in WT (AF293), Δ*AfubflbB* (TKSS1.01), and Δ*AfubflbE* (TKSS6.07) strains throughout the life cycle. Numbers indicate the time (hours) in liquid MMG with 0.1% YE (Veg) or of post-axial developmental induction (Asex). A band shown between the two *AfuflbB* transcripts is due to nonspecific binding of the probe to rRNA. (B) A genetic model for upstream regulation of asexual development in *A. fumigatus* (see Discussion).
and that cross-species complementation of ΔAfuflbB with AfuflbB resulted in a partial restoration of asexual development and gliotoxin production, as observed in AfuflbBa and AfuflbBβ strains. Our preliminary data indicate that both AfuflbBβ and AfuflbBa show trans-activation ability in yeast (N. J. Kwon and J.-H. Yu, unpublished data), suggesting that both can act as putative bZIP transcription factors. Our Northern blot analyses of the α and β mutants reveal that only the AfuflbBβ mRNA is produced in both the AfuflbBa and AfuflbBβ strains, indicating that both AfuflbBβ and AfuflbBa are necessary for expression of AfuflbBα, which specifically accumulates during conidiogenesis (data not shown). Additional molecular studies dissecting the complex regulation of AfuflbB gene expression need to be carried out.

Aspergillus secondary metabolite production is a complex process, yielding various compounds including carcinogenic mycotoxins, such as aflatoxins (AFs) and sterigmatocystin (ST), and is closely associated with morphological development (6, 15, 38). Our present studies suggest that gliotoxin production and asexual development might be interconnected through the activities of the key developmental regulator BrlA in A. fumigatus. Whereas the deletion of AfuflbB totally abolished gliotoxin production (Fig. 5B) under liquid submersed culture conditions, strains carrying either AfuflbBβ or AfuflbBa or both produce gliotoxin, although the amount was reduced compared to levels of the WT and C' strains. Indeed, gliotoxin production patterns (Fig. 5B and D) are somewhat consistent with the levels of asexual development (Fig. 4B and D and 5A). This idea was further supported by the fact that under salt stress conditions, where all strains tested except the ΔAfuflbB mutant formed developmental structures (Fig. 6B), the α and β strains restored gliotoxin production and conidiation to the WT levels (Fig. 6C). Importantly, similar to ΔAfuflbB, the absence of AfuflbE abolishes gliotoxin production in liquid submersed culture (Fig. 5D) where the ΔAfuflbE mutant does not develop (see reference 17), and ΔAfuflbEβA eliminates gliotoxin production under liquid and/or solid culture conditions (Fig. 5D). These observations suggest that AfubrlA might play a central role in the cooperative regulation of gliotoxin production and asexual development. Interestingly, multiple BrlA binding sites [BrlA response elements (BREs); 5'-(CA/G)(G/A)AGGG(G/A)-3'] (7) are present in the promoter regions of 10 out of the 12 gliotoxin biosynthetic clustered genes (12, 18; data not shown). It would be of great interest to check whether AfubrlA indeed binds to these regions and exerts direct regulation (activation) on many of the gliotoxin biosynthetic genes.

Recent studies in A. nidulans demonstrated that FlbB is necessary for expression of flbD encoding a C-myb protein and that FlbB and FlbD activate brlA in a cooperative manner (14). Furthermore, it was demonstrated that FlbE and FlbB are functionally interdependent, physically interact in vivo, and are colocalized at the hyphal tip in an actin cytoskeleton-dependent manner in A. nidulans, and expression of these two genes is interdependent (13, 14). The A. fumigatus flbE gene is predicted to encode a 222-aa-length polypeptide and is required for normal conidiation (17) and gliotoxin production (Fig. 5D). Our preliminary study further suggests that AfubflD is essential for proper development in A. fumigatus (P. Xiao and J.-H. Yu, unpublished data). To examine potential genetic interactions among upstream developmental regulators in A. fumigatus, a series of expression studies was carried out, and the results suggest that A. fumigatus possesses an upstream regulatory cascade slightly different from the one proposed for A. nidulans (Fig. 8A and B). The observations that AfuflbB and AfuflbE are each expressed independently of each other and that both are required for proper expression of AfuflbD led to a model that AfuflbB and AfuflbE function upstream of AfuflbD and cooperatively activate the expression of AfuflbD, which in turn results in activation of AfubrlA (Fig. 5B). Although at the transcriptional level AfuflbB and AfuflbE are independent of each other (Fig. 8A), we cannot exclude the possibility that the AfuflbB and AfuflbE proteins interact and form a functional complex, as found in A. nidulans (13). In addition, expression of AfuflbC is independent of AfuflbB and AfuflbE, indicating that AfuflbC functions in a separate pathway, as found in A. nidulans; characterization of AfuflbC and additional developmental controllers is in progress.

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