Mutations in *sfdA* and *sfdB* Suppress Multiple Developmental Mutations in *Aspergillus nidulans*

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ABSTRACT

Conidiophore morphogenesis in *Aspergillus nidulans* occurs in response to developmental signals that result in the activation of *brlA*, a well-characterized gene that encodes a transcription factor that is central to asexual development. Loss-of-function mutations in *flbD* and other fluffy loci have previously been shown to result in delayed development and reduced expression of *brlA*. *flbD* message is detectable during both hyphal growth and conidiation, and its gene product is similar to the Myb family of transcription factors. To further understand the regulatory pathway to *brlA* activation and conidiation, we isolated suppressor mutations that rescued development in strains with a *flbD* null allele. We describe here two new loci, designated *sfdA* and *sfdB* for suppressors of *flbD*, that bypass the requirement of *flbD* for development. *sfd* mutant alleles were found to restore developmental timing and *brlA* expression to strains with *flbD* deletions. In addition, *sfd* mutations suppress the developmental defects in strains harboring loss-of-function mutations in *fluG*, *flbA*, *flbB*, *flbC*, and *flbE*. All alleles of *sfdA* and *sfdB* that we have isolated are recessive to their wild-type alleles in diploids. Strains with mutant *sfd* alleles in otherwise developmentally wild-type backgrounds have reduced growth phenotypes and develop conidiophores in submerged cultures.

CONIDIATION of *Aspergillus nidulans* follows a developmental program of cell differentiation to result in the production of multicellular spore-bearing structures called conidiophores (Timberlake 1990; Adams *et al.* 1998). Conidiation does not require that cells be starved for nutrients but does require an air interface, as in the context of a colony growing on an agar surface (Axelrod 1972; Law and Timberlake 1980; Champe *et al.* 1981). Precisely timed, but as yet unknown, signals cause conidiophore morphogenesis to begin in the center of a growing colony and to proceed rapidly and radially to cover its surface except for a small margin of leading hyphae. Wild-type strains do not form conidiophores in liquid-grown cultures of standard media but conidiophores have been observed in liquid cultures of strains grown under conditions of nutrient starvation or in certain developmental mutants (Martinelli 1976; Skromne *et al.* 1995; Rosen *et al.* 1999; Yu *et al.* 1999). A genetic pathway has been identified involving the sequential production of key regulators of conidiophore formation (Boylan *et al.* 1987; Mirabito *et al.* 1989). Initiation of development depends upon activation of *brlA*, which encodes a C2H2 zinc-finger transcription factor. BrlA is always required for spore formation and forced expression of *brlA* in liquid-grown mycelia results in misscheduled development (Adams *et al.* 1988).

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Genes that are important for normal *brlA* activation and development have been identified by mutational analyses, and they include *fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE* (Wieser *et al.* 1994). Loss-of-function mutations in each of these genes result in colonies that have a fluffy phenotype characterized by unregulated aerial hyphae proliferation, a decrease in developmentally induced *brlA* RNA transcripts compared to wild-type strains, and reduced sporulation. The fluffy mutants identified fall into three phenotypic classes: *fluG* mutants are very fluffy and almost entirely aconidial; *flbA* mutants are aconidial and also autolytic, resulting in complete colony lysis after several days; *flbB*, *flbC*, *flbD*, and *flbE* are delayed conidiation mutants since conidiophore formation occurs but with at least a 24-hr delay as compared to wild-type strains (Wieser *et al.* 1994). Tests of epistasis as well as the genetic requirements for development following forced expression of genes allowed a model to be proposed that places these genes in an ordered genetic pathway leading to *brlA* expression and development (for review see Adams *et al.* 1998 and Figure 1).

The *fluG* gene product is similar in amino acid sequence to glutamine synthetase and its extracellular complementation by wild-type strains led to the hypothesis that it is required for the production of a small, diffusible extracellular factor that controls initiation of development (Adams *et al.* 1992; Lee and Adams 1994a). The FluG signal is required to activate *flbE*, *flbD*, *flbB*, and *flbC* whose activities are required for *brlA* activation (Lee and Adams 1996). FluG signal is required to acti-

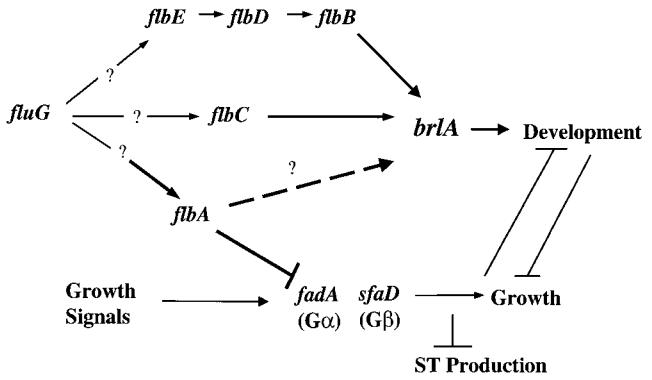

FIGURE 1.—A previously proposed model describes genetic interactions that control growth and development in *A. nidulans* (ADAMS *et al.*1998). FluG is believed to be responsible for generating an extracellular growth signaling factor that feeds into an activation pathway for *brlA*. *flbE*, *flbD*, *flbB*, and *flbC* are required to transmit the *fluG* signal and effect *brlA* activation. Their placement and order in the pathway is based on double-mutant analyses and dependencies for developmental induction by forced expression of several of the genes in the pathway. FluG signals are also required to activate FlbA, a regulator of G protein signaling protein that negatively regulates the activity of FadA. FadA is the Gα-subunit of a heterotrimeric G protein whose downstream signals have been found to promote growth and to antagonize both development and sterigmatocystin (ST) biosynthesis.

vate FlbA, a GTPase-activating protein that negatively regulates a G-protein growth-signaling pathway. FlbA inhibition of FadA, the Gα-subunit of a heterotrimeric G-protein complex, is required to allow both development and secondary metabolite production to occur (YU *et al.* 1996; HICKS *et al.* 1997). Forced expression of *fluG*, *flbA*, or *flbD* individually from an inducible *alcA* promoter causes *brlA* expression and development in submerged cultures, which supports the idea that these genes function in a signaling pathway for *brlA* activation and the initiation of development (LEE and ADAMS 1994b; WIESER and ADAMS 1995).

The products of *flbB*, *flbC*, and *flbD* have similarity to major classes of transcription factors, supporting their predicted roles as developmental regulators (WIESER and ADAMS 1995; G. MEYER and T. ADAMS, unpublished results; J. WEISER and T. ADAMS, unpublished results). *flbD* is the best characterized of these genes and is similar to the Myb family of transcription factors, many of which control developmental gene expression in other organisms (LIPSICK 1996; MARTIN and PAZ-ARES 1997; JIN and MARTIN 1999). To obtain a greater understanding of the regulatory network that leads to *brlA* activation and development we performed a mutagenic screen for suppressor mutations that restored the timing of conidiation to a Δ*flbD* mutant. We have previously used this approach to identify suppressors of *flbA* and *fluG* mutations (YU *et al.* 1999; D'SOUZA *et al.* 2001). In this article, we describe the isolation and characterization of a novel class of suppressor mutations that affect the temporal and spatial activation of *brlA* and conidiophore development. Mutations in two unlinked loci, *sfdA* and *sfdB*, individually bypass the defects in *brlA* expression and the timing of conidiophore formation in a Δ*flbD* strain. Moreover, phenotypic suppression of conidiation defects was observed for *sfd* mutations present in combination with all of the fluffy mutations tested to date. Strains harboring *sfd* mutant alleles (*sfd*^{S}) in an otherwise wild-type developmental background conidiate normally but have a reduced growth phenotype and develop condiophores in liquid cultures. These data show that *sfdA* and *sfdB* activities are normally required for inhibition of developmental pathways and that mutations in *sfdA* or *sfdB* can rescue several classes of conidiation defects and can also cause the production of condiophores under inappropriate conditions.

MATERIALS AND METHODS

Aspergillus strains and genetic techniques: The *A. nidulans* strains used in this study are listed in Table 1. Standard *A. nidulans* culture, transformation, and genetic techniques were used (PONTECORVO *et al.* 1953; KAFER 1977; MILLER *et al.* 1985; YELTON *et al.* 1985). Two deletion alleles of *flbD* were constructed by replacement of the *flbD* coding sequences with selectable markers *argB*$^+$ or *trpC*$^+$. Strain TEK1003 was constructed by transformation of PW1 with pEK47 and strain TEK1050 was constructed by transformation of FGSC237 with pEK55. The presence of the deletion alleles Δ*flbD::argB* and Δ*flbD::trpC* in TEK1003 and TEK1050, respectively, was determined by Southern analysis.

To examine the segregation of *sfd*^{S} mutations the suppressed isolates (Δ*flbD*, *sfd*^{S}) were crossed to wild-type strain FGSC23. Progeny were scored for the wild-type or deletion allele of *flbD* by Southern analysis. The dominance/recessiveness of *sfd*^{S} alleles was tested through construction of diploid strains that were heterozygous for a given *sfd*^{S} mutant and wild-type allele and homozygous for either Δ*flbD* or *flbD*$^+$ (see Table 1 for strains DEK001–DEK038, DEK2004, and DEK2006). The conidiation phenotype was used to score the *sfd*^{S} dominance relationships. For Δ*flbD* homozygous strains, a fluffy phenotype was taken as evidence that the *sfd*^{S} allele present in the strain was recessive to its wild-type allele. Likewise, in diploids that were homozygous for *flbD*$^+$, a wild-type phenotype was taken as evidence that the *sfd*^{S} allele present in the strain was recessive to its wild-type allele. Meiotic crosses between suppressed strains (Δ*flbD*, *sfd*^{S}) were made to access linkage of *sfd*^{S} alleles. Where these sexual crosses were unproductive, linkage between *sfd*^{S} alleles was determined using parasexual genetics as described previously (KAFER 1958; YU *et al.* 1999). The placement of *sfd*^{S} alleles into the unlinked *sfdA* and *sfdB* complementation groups was determined through pairwise diploid analysis between suppressed strains.

Media and growth conditions: All Aspergillus strains were propagated on appropriately supplemented minimal medium as described by KAFER (1977). Screening of mutagenized spores was done using minimal medium containing 0.5% yeast extract. Developmental induction experiments were performed as described in HAN *et al.* (1993). Briefly, developmental inductions were performed by growing cultures for 20 hr in liquid minimal media containing 0.5% yeast extract after which development was synchronously induced upon filtration of mycelia onto a sterile pad of filter paper placed on an agar plate containing media of the same composition.

TABLE 1

***A. nidulans* strains used in this study**

Strain	Genotype[a]	Source
DEK015[b]	$\frac{+,\ pabaA1,yA2;\ +;\ +;\ +;\Delta flbD::trpC,trpC801}{biA1+\ +;argB2;methG1;sfdA15;\Delta flbD::argB}$	This study
DEK038[b]	$\frac{+,\ pabaA1,yA2;\ +;\ +;\ +;\Delta flbD::trpC,trpC801}{biA1\ +\ +;sfdB38;argB2;methG1;\Delta flbD::argB}$	This study
DEK2004	$\frac{+,\ pabaA1,yA2;\ sfdA15}{biA1,\ +,\ +;\ +}$	This study
DEK2006	$\frac{+,\ pabaA1,yA2;sfdB38;methG1}{biA1,\ +,\ +;\ +;\ +}$	This study
FGSC23	*pabaA1,yA2;chaA1*	FGSC[c]
FGSC26	*biA1*	FGSC
FGSC237	*pabaA1,yA2;trpC801*	FGSC
FGSC288	*suA1,ade20,yA2;wA3;galA1;pyroA4 facA303;sB3;nicB8;riboB2*	This study
MEK015[d]	*biA1;argB2;methG1;sfdA15;ΔflbD::argB*	This study
MEK038[e]	*biA1;sfdB38;argB2;methG1;ΔflbD::argB*	This study
PW1	*biA1;argB2;methG1*	P. Weglenski
REK65.1	*biA1;methG1;sfdA15;ΔflbD::argB*	This study
REK65.13	*biA1;methG1;sfdA15*	This study
REK65.16	*pabaA1,yA2;sfdA15*	This study
REK88.10	*biA1;sfdB38;methG1;ΔflbD::argB*	This study
REK88.14	*pabaA1,yA2;sfdB38;methG1*	This study
REK88.23	*biA1;sfdB38;methG1*	This study
REK100.8	*biA1;fluG::trpC;methG1;sfdA15*	This study
REK100.12	*biA1;methG1,flbB::argB;sfdA15*	This study
REK100.28	*biA1;sfdB38;methG1,flbB::argB*	This study
REK100.35	*biA1,flbE::argB;methG1;sfdA15*	This study
REK100.46	*biA1,flbE::argB;sfdB38;methG1*	This study
REK100.65	*biA1,ΔflbA::argB;pyroA4;sfdA15*	This study
REK100.73	*biA1,ΔflbA::argB;sfdB38;pyroA4*	This study
REK102.8	*biA1;methG1;sfdA15;flbC::argB*	This study
REK102.23	*biA1;sfdB38;methG1;flbC::argB*	This study
REK102.43	*biA1;sfdB38;fluG::trpC;methG1*	This study
REK105.2	*pabaA1;sfdA15;ΔbrlA*	This study
REK105.8	*pabaA1;sfdB38;ΔbrlA*	This study
RGM612	*pabaA1;methG1;flbC::argB*	G. Meyer and T. H. Adams (unpublished results)
RJH046	*biA1,ΔflbA::argB;argB2;pyroA4*	J. K. Hicks and T. H. Adams (unpublished results)
RSH94.4	*methG1;ΔbrlA*	S. T. Han
SEK065	*pabaA1,yA2;sfdA15*	This study
SEK088	*pabaA1;sfdB38;chaA1*	This study
TEK1003	*biA1;argB2;methG1;ΔflbD::argB*	This study
TEK1050	*pabaA1,yA2;ΔflbD::trpC,trpC801*	This study
TJW110	*biA1,flbE::argB;methG1*	J. Weiser and T. H. Adams (unpublished results)
TJW113	*biA1;methG1,flbB::argB*	J. Weiser and T. H. Adams (unpublished results)
TTA127.4	*pabaA1,yA2;fluG::trpC*	Lee and Adams (1994a)

[a] All strains are *veA1*.

[b] Diploid strains DEK001–038 were constructed from MEK001–038 and TEK1050. Their genotypes are presumed to be identical except for the sfd^S allele present.

[c] Fungal Genetics Stock Center.

[d] MEK001, -007, -015, -016, -023, -025, -026, -028, -031, -032, -033, and -035 are all $sfdA^S$ alleles that were isolated in the original mutagenesis of TEK1003.

[e] MEK004, -006, -009, -024, -029 and -038 are all $sfdB^S$ alleles that were isolated in the original mutagenesis of TEK1003.

The agar plates were incubated at 37° and RNA for Northern analyses was prepared from mycelia removed from the filter paper surface.

Liquid growth experiments to follow development in submerged culture were done by inoculation of 2×10^8 conidia into 200 ml of minimal media supplemented with 0.1% yeast extract in 500-ml culture flasks. The flasks were shaken at 275 rpm at 37° and development was followed through examination of hyphal samples using a Zeiss Axioskop 2 compound microscope and differential interference contrast optics. The

images shown in Figure 3A are at ×200 magnification. The colony images in Figure 2 were generated using a Zeiss Stemi SV11 Apo stereomicroscope at ×6 magnification. All images were captured using a Zeiss Progress 3008 digital camera.

Mutagenesis and isolation of *flbD* suppressor mutations: Conidiospores of strain TEK1003 were mutagenized with NQO (4-nitroquinoline-1-oxide) as previously described (Wieser *et al.* 1994). The percentage of survival after NQO treatment ranged from 0.1 to 95%, depending upon the concentration of NQO used and the length of treatment. Two survival batches with 1 and 10% survival were used for further screening. Strain TEK1003 is largely aconidial at 48 hr after inoculation onto an agar surface whereas wild-type strains begin to conidiate at 24 hr postinoculation and after 48 hr their colony surfaces were completely conidial. Survivors were visually screened for highly conidiating isolates among a background of fluffy isolates after 48 hr incubation on plates at 37°. Among 270,000 survivors, 20 highly conidial isolates were identified. These 20 mutants conidiated strongly over the entire surface of isolated colonies after 48 hr incubation at 37° on minimal medium in contrast to the conidiation delay and fluffy phenotype of the starting strain for mutagenesis, TEK1003. All isolates were smaller in size than TEK1003 and retained some fluffy aerial hyphae at the colony edges after 48 hr incubation at 37°. These fluffy suppressed strains were designated MEK001, -004, -006, -007, -009, -015, -016, -023, -024, -025, -026, -028, -029, -030, -031, -032, -033, -035, -036, and -038. The genetic analyses described above indicated that these 20 mutations identify two loci with 7 and 13 alleles each. We believe this mutagenesis was saturating because multiple alleles of each locus were identified.

Colony growth rate and conidiation: Conidiospores from each of the strains in Table 2 were point inoculated into the center of five agar plates containing appropriately supplemented minimal medium. Strains RSH94.4, REK105.2, and REK105.8, which are entirely aconidial, were inoculated using a small piece of hyphal material. Plates were incubated at 37° and colony diameters were measured at 30 hr, 43 hr, 51 hr, and 72 hr. Average growth rates were determined by measuring the growth rate for each colony in millimeters per hour for each of the three time intervals above and then averaging the measurements across the entire time interval. Standard deviations were determined and the growth rates shown in Table 2 are expressed as a percentage of the wild-type (FGSC26) growth rate, which was 0.35 (±0.022) mm/hr. For most strains the number of independent growth rate measurements was 15 with the exceptions of REK102.48 where $N = 12$ and TTA127.4 and RGM612 where $N = 10$ due to slow initial growth making measurements at 30 hr impossible.

The undifferentiated colony edge is defined as the difference in radius between the leading hyphal tips and the first conidiophores seen in a colony as determined visually using a stereomicroscope. This measurement was taken in four independent regions in two colonies at 72 hr, thus giving $N = 8$ for each measurement. Colony area at 72 hr was also calculated for each strain. The number of extractable conidia per colony was determined at 72 hr by scraping the conidia from the entire colony into 5 ml of sterile water with 0.01% Tween 20 detergent, vortexing, and counting in a hemocytometer. Due to the variation in growth rates, and thus colony sizes at 72 hr, the extent of conidiation was expressed as total extractable conidia/colony area in square millimeters. The average conidia per square millimeter was determined from five colonies of each strain ($N = 5$), except REK102.43 and REK100.35, where four colonies were used ($N = 4$). The presence of conidiophores in colonies was scored at 30 and 43 hr for each strain.

Nucleic acid isolation and manipulation: Total RNA was isolated by addition of 0.6 ml of silica/zirconium beads (BioSpec Products, Bartlesville, OK) and 1 ml of Trizol and mixing in a Mini Bead beater (BioSpec Products) for 2 min followed by RNA purification as outlined for Trizol (GIBCO-BRL, Gaithersburg, MD). Ten micrograms of RNA was separated by electrophoresis through a 1.0% agarose gel with 6% formaldehyde and transferred to Biodyne B nylon membrane (GIBCO-BRL). Hybridization with ^{32}P-labeled random prime probes was performed as in Current Protocols in Molecular Biology (Asubel *et al.* 1993). A 2.5-kb *Bam*HI-*Sal*I fragment from pSH5 containing *brlA* coding sequences was used as a probe for *brlA* RNA in RNA blot analysis. Hybridization was visualized using autoradiographic exposure to film (Kodak XOMAT AR). Figures 3 and 4 each show scanned images of autoradiographs from a single gel and a single exposure.

The *flbD* deletion allele was constructed using plasmid pEK47, which contained a replacement of the *flbD* coding region with *argB*$^+$. pEK47 is a pBluescript SK(−)-based vector that was created through ligations of two PCR-derived fragments, one containing the 1.3-kb region upstream of *flbD* and a second that had a 1.1-kb region downstream of *flbD* with a 1.9-kb *Xho*I fragment isolated from pJW88 (J. Wieser and T. Adams, unpublished results) that contains the *argB* gene and regulatory region. This places the *argB* gene internal to the flanking regions with orientation opposite to that of the original *flbD* gene. The upstream and downstream PCR products were amplified from pJW18 (Wieser and Adams 1995) using primer combinations FlbDup (5′ TTTTCTCGAGGCGAAA CTGTGTTGGTGATG 3′) and the universal T7 primer and FlbDdwn (5′ TTTTGAGCTCCGATCACACGACTCTCTTCC 3′) and the universal T3 primer. FlbDup and FlbDdwn contained *Xho*I sites flanking the regions complementary to sequences directly upstream and downstream of the *flbD* coding sequences. PCR products were cloned as blunt fragments into pBluescript SK(−) and reisolated as *Xho*I/*Bam*HI (downstream) and *Xho*I/*Kpn*I (upstream) fragments. These two fragments were ligated to the *Xho*I fragment from pJW88 and *Bam*HI/*Kpn*I-digested pBluescript SK(−). Plasmid pEK55 was created by replacement of the *Xho*I fragment of pEK47 with a 4-kb *Xho*I fragment from pTA127, which contained the *trpC* sequence and regulatory region. Strains TEK1003 and TEK1050 had identical phenotypes to strain TJW30.1, which has been described previously (Wieser and Adams 1995).

RESULTS

Isolation of suppressor mutations that bypass the requirement for *flbD*: To identify new factors in the initiation pathway for *brlA* activation and conidiophore development, conidiospores of strain TEK1003 (Δ*flbD*; see material and methods and Table 1) were mutagenized using NQO and 250,000 survivors were screened for conidial colonies. Twenty independent strains that harbored mutations that bypassed the need for *flbD* were isolated and these strains were conidiated with timing and levels similar to wild-type strains. These mutations were designated *sfd*^{S} for *s*uppressor of *flbD* and Southern analysis was performed to confirm that all 20 *sfd*^{S} mutant isolates maintained the Δ*flbD* allele. The suppressor mutants (Δ*flbD::argB*; *sfd*^{S}) and wild-type strains were point inoculated onto minimal agar plates and the timing of conidiophore formation was compared. Both the suppressor strains and wild-type strains formed conidiophores in the colony centers after 24–30

TABLE 2

***sfd* mutations suppress conidiation defects in several fluffy mutant strains**

Strain genotype[a]	Growth rate (% of wild type)	Undifferentiated colony edge at 72 hr (mm)	Conidia/mm^2 at 72 hr (% of wild type)	Conidiophores by:[b]	
				30 hr	48 hr
Wild type	100	2 ± 0	100	+	+
sfdA15	65 ± 8.8	2.3 ± 0.27	140 ± 28	+	+
sfdB38	40 ± 8.9	1.4 ± 0.32	16 ± 3.1	+	+
Δ*flbD*	90 ± 8.0	5.5 ± 0.76	41 ± 2.8	−	+
Δ*flbD*, *sfdA15*	60 ± 5.8	2.9 ± 0.18	260 ± 49	+	+
Δ*flbD*, *sfdB38*	68 ± 8.5	2.2 ± 0.37	120 ± 16	+	+
fluG::trpC	94 ± 5.5	5.8 ± 0.53	13 ± 3.7	−	+
fluG::trpC, *sfdA15*	66 ± 6.5	1.6 ± 0.32	120 ± 28	+	+
fluG::trpC, *sfdB38*	50 ± 15	1 ± 0	110 ± 22	+	+
Δ*flbA*	66 ± 8.9	ND	0	−	−
Δ*flbA*, *sfdA15*	37 ± 8.4	2.1 ± 0.18	10 ± 5.2	+	+
Δ*flbA*, *sfdB38*	29 ± 8.1	1.5 ± 0.38	11 ± 4.2	+	+
flbB::argB	95 ± 17	9.1 ± 0.35	21 ± 10	−	+
flbB::argB, *sfdA15*	65 ± 6.3	1.8 ± 0.27	230 ± 28	−	+
flbB::argB, *sfdB38*	60 ± 15	1.8 ± 0.26	84 ± 12	+	+
flbC::argB	86 ± 15	5.1 ± 0.86	28 ± 13	−	+
flbC::argB, *sfdA15*	58 ± 8.5	2.8 ± 0.27	190 ± 75	+	+
flbC::argB, *sfdB38*	56 ± 14	2.0 ± 0.0	85 ± 7.6	+	+
flbE::argB	87 ± 13	9.5 ± 0.53	24 ± 2.6	−	+
flbE::argB, *sfdA15*	59 ± 11	2.8 ± 0.46	300 ± 100	+	+
flbE::argB, *sfdB38*	40 ± 7.4	1.8 ± 0.26	83 ± 18	+	+
Δ*brlA*	88 ± 7.5	ND	0	−	−
Δ*brlA*, *sfdA15*	66 ± 6.7	ND	0	−	−
Δ*brlA*, *sfdB38*	38 ± 7.9	ND	0	−	−

sfdA15 and *sfdB38* mutations suppress conidiation defects when present in several fluffy mutant backgrounds. Five colonies of each genotype shown were point inoculated onto minimal media and grown at 37° for 72 hr. Colonies were measured at several time points and the presence or absence of conidiophores was noted (see MATERIALS AND METHODS). The table shows the growth rate, undifferentiated colony edge at 72 hr growth, the conidia/mm^2 colony area at 72 hr growth, and whether conidiophores were present in colonies after 30 hr and 48 hr growth. Growth rate and conidia/mm^2 are expressed as the percentage of wild type that had a growth rate of 0.35 ± 0.022 mm/hr and 3.0 (±0.35) × 10^5 conidia/mm^2. ND, no data.

[a] The mutations Δ*flbD*, Δ*flbA*, *flbB::argB*, *flbC::argB*, *flbE::argB*, and Δ*brlA* are all null alleles.

[b] The time that conidiophores were first seen to be present was scored and presented as "+" for any number of conidiophores or "−" for no conidiophores at all, at 30 hr and 48 hr.

hr of incubation at 37° and by 48 hr the entire surface was conidial (Figure 2, A–D); however, colonies of suppressor mutants (Δ*flbD:: argB*; *sfd*^{S}) retained some fluffy character around their edges and had a smaller size than either wild-type or Δ*flbD* mutant strains (Figure 2, C and D). These phenotypic characteristics made colonies of the Δ*flbD::argB*; *sfd*^{S} genotype easily distinguishable from both wild-type and fluffy (Δ*flbD*) colonies.

Each of the 20 suppressor mutant strains (Δ*flbD::argB*; *sfd*^{S}) was backcrossed to wild-type strain FGSC23 to determine segregation patterns and to examine the phenotypes of *sfd*^{S} mutations in *flbD*$^+$ strains. These crosses each yielded equal numbers of progeny with four phenotypes: wild type, fluffy, suppressed, and a new phenotype termed hyperconidial invasive (Figure 2, A–F). These progeny were examined by genetic backcrosses and Southern blot analyses and the following genotypes (in parentheses) corresponded to each of the phenotypic classes: wild type (*flbD*$^+$;*sfd*^{WT}), fluffy (Δ*flbD*; *sfd*^{WT}), suppressed (Δ*flbD*; *sfd*^{S}), and hyperconidial invasive (*flbD*$^+$;*sfd*^{S}). These results indicated that the 20 original *sfd*^{S} mutant strains each harbored a single mutation that was unlinked to *flbD* and that *sfd*^{S} mutations conferred characteristic phenotypes in both *flbD*$^+$ and Δ*flbD* strains. The hyperconidial-invasive phenotype of *sfd*^{S} strains (*flbD*$^+$;*sfd*^{S}) was characterized by abundant conidiation with wild-type timing, reduced colony size, and extensive mycelial growth underneath the agar with some conidiophore-like forms present there. This is in contrast to wild-type colonies that grow largely on the surface of the agar and conidiate almost exclusively from aerial hyphae. The conidiophores produced by strains carrying *sfd*^{S} mutations appeared phenotypically wild type (data not shown).

The dominance/recessiveness of each of the 20 *sfd*^{S}

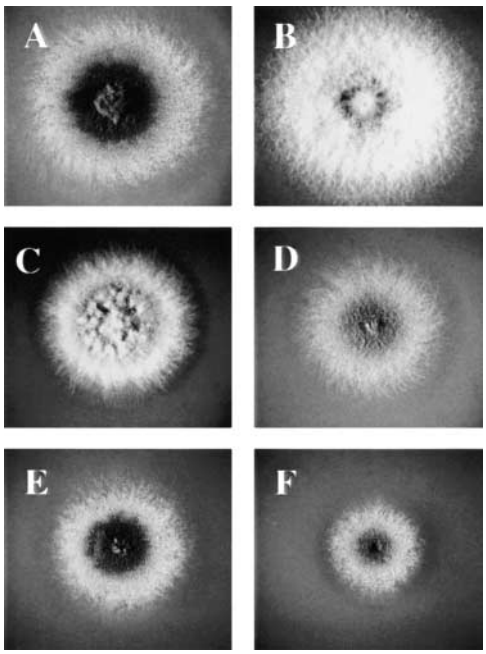

Figure 2.—Colony phenotypes of *sfdA15* and *sfdB38* mutant strains. (A) FGSC26 (wild type); (B) TEK1003 (Δ*flbD*); (C) REK65.1 (Δ*flbD*; *sfdA15*); (D) REK88.10 (Δ*flbD*; *sfdB38*); (E) REK65.13 (*flbD*$^+$; *sfdA15*); and (F) REK88.23 (*flbD*$^+$; *sfdB38*). Colonies were point inoculated onto minimal medium (Kafer 1977) and allowed to grow for 48 hr at 37°.

mutations was tested using diploid strains that were homozygous for Δ*flbD* and heterozygous for each *sfd*^{S} mutation individually (Δ*flbD::argB*; *sfd*^{S}/Δ*flbD::trpC*; *sfd*^{WT}). All of these strains exhibited a fluffy phenotype demonstrating that all of the *sfd*^{S} mutations were recessive to their wild-type alleles in these strains (see Table 1, strains DEK015 and DEK038). Pairwise meiotic crosses between suppressor strains with representative *sfd*^{S} alleles were carried out to determine the number of loci represented by *sfd*^{S} mutations. Many of these crosses were unproductive and additional linkage analysis was performed using parasexual genetics (see materials and methods). Complementation analysis was also performed by construction of pairwise diploids with different *sfd*^{S} mutant alleles in strain backgrounds that were homozygous for Δ*flbD::argB*. Taken together, the results of these analyses indicated that the 20 *sfd*^{S} mutant alleles represented two unlinked complementation groups, designated *sfdA* and *sfdB*, with 13 and 7 alleles, respectively. Two representative alleles, *sfdA15* and *sfdB38*, were chosen for further characterization. Both *sfdA15* and *sfdB38* were also found to be recessive to their wild-type alleles in diploid strains homozygous for the wild-type *flbD* allele (see materials and methods, strains DEK2004 and DEK2006).

To determine the chromosomal linkage of the *sfdA15* and *sfdB38* mutations, a standard parasexual genetic analysis was performed using a mitotic mapping strain, FGSC288 (Kafer 1958; Yu *et al.* 1999). This type of analysis allows a chromosome linkage to be assigned but not a map position, which requires further analyses. Diploid strains were isolated from heterokaryons formed between REK65.13 (*sfdA15*) or REK88.23 (*sfdB38*) and FGSC288. Haploid segregants were isolated after benomyl treatment as yellow (*yA2*) or white (*wA1*) sectors. The segregation of *sfdA15* and *sfdB38* with respect to the known markers was determined. *sfdA15* was found to be linked to chromosome VI and *sfdB38* was found to be linked with chromosome II.

Timing of *brlA* mRNA accumulation is restored by mutations in *sfdA* and *sfdB*: The timing and levels of *brlA* transcript accumulation were monitored in RNA prepared from strains induced to develop synchronously as described previously (Axelrod 1972; Law and Timberlake 1980). *brlA* transcript was detected at high levels in RNA prepared from samples of the wild-type strain (FGSC26) at 4 hr postinduction and remained at this high level in samples from 8, 12, and 24 hr (Figure 3). In RNA samples prepared from TEK1003 (Δ*flbD*), the *brlA* message was undetectable in samples harvested at 0, 4, 8, and 12 hr postinduction and only barely detectable at 24 hr. These results are similar to what has been observed previously in a strain containing a mutation in *flbD* that was due to a disruption of the coding sequence (Wieser and Adams 1995). In contrast to TEK1003, strains REK65.1 (Δ*flbD; sfdA15*) and REK88.10 (Δ*flbD; sfdB38*) both contained detectable *brlA* message in RNA prepared from samples harvested at 8 hr postinduction and these message levels were observed to increase at the 12-hr and 24-hr time points, with the 24-hr levels being comparable to the highest levels seen in FGSC26 at any time point. These results demonstrated that mutations in *sfdA15* and *sfdB38* partially restored *brlA* mRNA accumulation to Δ*flbD* strains during conidiophore development. Finally, RNA samples prepared from developmentally induced cultures of strains REK65.13 (*flbD*$^+$; *sfdA15*) and REK88.23 (*flbD*$^+$; *sfdB38*) contained high levels of *brlA* message at 4 hr postinduction and these levels remained high in samples harvested at 8 and 12 hr postinduction and decreased somewhat at 24 hr. This demonstrated that the delay in *brlA* mRNA accumulation observed in strains REK65.1 (Δ*flbD*; *sfdA15*) and REK88.10 (Δ*flbD*; *sfdB38*) is not due to the *sfd*^{S} mutations but to some additive effects of the *sfdA15* or *sfdB38* mutations and Δ*flbD* mutation.

Photographs taken of the surface of developmentally induced cultures at 24 hr postinduction are shown in Figure 3C. The phenotypic characteristics of these strains with respect to the extent of conidiation reflected the observed kinetics of *brlA* mRNA accumulation. Strains FGSC26, REK65.13 (*flbD*$^+$; *sfdA15*), and REK88.23 (*flbD*$^+$; *sfdB38*) conidiated extensively with the entire surface of hyphal material being covered entirely with conidiophores at 24 hr postinduction. Strains REK65.1 (Δ*flbD*; *sfdA15*) and REK88.10 (Δ*flbD*; *sfdB38*) elabo-

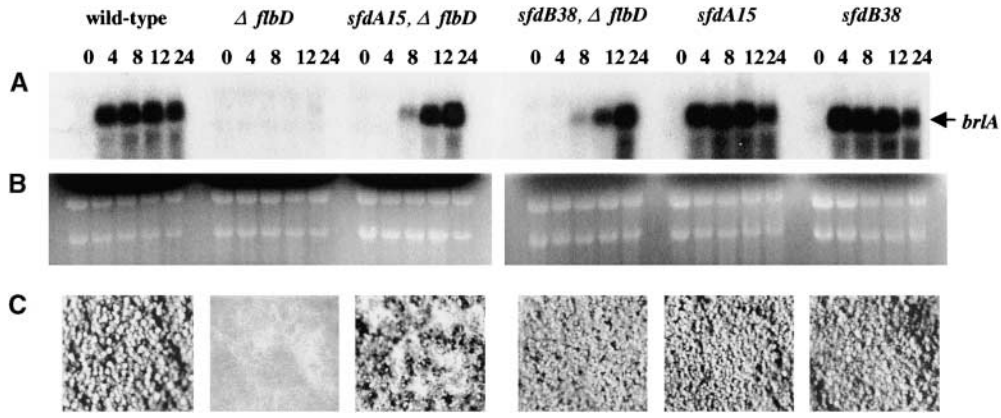

FIGURE 3.—*sfdA15* and *sfdB38* restore the timing and levels of *brlA* mRNA accumulation to Δ*flbD* mutant strains. (A) Total RNA was isolated from vegetative hyphae and from developing cultures of wild-type (FGSC-26) and of Δ*flbD* (TEK1003), Δ*flbD*; *sfdA15* REK65.10), Δ*flbD*; *sfdB38* (REK 88.10), *flbD*$^+$; *sfd15* (REK65.13), and *flbD*$^+$; *sfdB38* (REK88.23) mutant strains. Times shown are immediately prior to developmental induction (t = 0) and postinduction intervals of t = 4, 8, 12, and 24 hr. Total RNA isolated at each time point was fractionated on a formaldehyde-agarose gel and the resultant gel blot was probed with a radiolabeled *brlA*-specific DNA fragment. (B) Equivalent loading of total RNA was confirmed by ethidium bromide staining. (C) Photographs of the surface of developmentally induced cultures at 24 hr postinduction are shown.

rated many conidiophores on the surface of the hyphal material in contrast to TEK1003 (Δ*flbD*), which had no conidiophores present. Strain REK65.1 (Δ*flbD*; *sfdA15*), and to a lesser extent REK88.10 (Δ*flbD*; *sfdB38*), retained some fluffiness due to the formation of long aconidial aerial hyphae consistent with the delay in *brlA* activation in these strains (Figure 3A).

***sfdA15* or *sfdB38* mutations cause precocious development in liquid culture:** Wild-type strains of *A. nidulans* generally do not conidiate in submerged cultures unless starved for glucose or nitrogen although this can be strain and medium dependent (AXELROD *et al.* 1973; MARTINELLI 1976; SKROMNE *et al.* 1995; ADAMS *et al.* 1998). We observed that the colonies of *sfd*^{S} mutant strains exhibited extensive hyphal growth underneath the agar surface and many conidiophore-like structures were also formed by these agar-bound hyphae. This led us to test whether these mutants would form developmental structures in submerged cultures of hyphae grown in liquid media. Liquid cultures were inoculated with spores and incubated shaking vigorously at 37°. Conidiophore formation was monitored by microscopic visualization and *brlA* mRNA accumulation was monitored by RNA blot analysis. We found that stalk cells and vesicles began to form from hyphae in REK65.13 (*flbD*$^+$; *sfdA15*) and REK88.23 (*flbD*$^+$; *sfdB38*) after 15–18 hr of growth and primary sterigmata on these conidiophores were readily detectable by 24 hr (Figure 4B). Strain REK88.23 (*sfdB38*) developed more conidiophores than REK65.13 (*sfdA15*) and neither strain formed morphologically wild-type conidiophores such as produced in response to air. There were clearly stalks, vesicles, primary sterigmata, and conidiospores but the sterigmata were somewhat amorphous and conidiophores were not always symmetrical, especially in REK65.13 (Figure 4B). In contrast, conidiophores were not detected in cultures of FGSC26, TEK1003 (Δ*flbD*), REK65.1 (Δ*flbD; sfdA15*), and REK88.10 (Δ*flbD*; *sfdB38*) at any time through 24 hr (Figure 4B). Strains containing the other *sfdA* and *sfdB* mutant alleles isolated in the original screen have a similar ability to conidiate in submerged cultures (data not shown). The morphological results were reflected in the observation that *brlA* message was detectable at both 12 and 24 hr in strain REK88.23 and at 24 hr in strain REK65.13, whereas FGSC26 (wild type), TEK1003 (Δ*flbD*), REK65.1 (Δ*flbD; sfdA15*), and REK88.10 (Δ*flbD*; *sfdB38*) showed little or no *brlA* message accumulation at 12 or 24 hr (Figure 4A).

***sfdA*^{S} and *sfdB*^{S} mutations suppress developmental mutants other than Δ*flbD*:** To examine the relationship between *sfdA*^{S} and *sfdB*^{S} and other developmental mutants, meiotic crosses were performed to isolate double mutants. Analysis of the progeny of these crosses indicated that *sfdA15* and *sfdB38* are unlinked to *fluG*, *flbA*, *flbC*, *flbB*, *flbE*, *fadA*, *sfaD*, and *dsgA* (data not shown). Strikingly, we found that the *sfdA15* and *sfdB38* mutations suppressed null alleles of *fluG*, *flbA*, *flbC*, *flbB*, and *flbE* in addition to *flbD*. As shown in Table 2, the timing of conidiation and the density of conidiophores formed were both restored to near wild-type levels in all of the double mutants except Δ*flbA*; *sfdA15* and Δ*flbA*; *sfdB38*, which have 10–11% the density of conidiophores of a wild-type strain. However, as one would predict, the conidiation defects conferred by a deletion allele of *brlA* were not suppressed by *sfdA15* or *sfdB38*. Table 2 also shows that the growth rates of all *sfd*^{S}-containing strains were reduced from 29 to 68% of the wild-type growth rate.

Finally, a previously characterized phenotype of the delayed fluffy mutants is a larger undifferentiated colony edge that is the distance between the leading edges of hyphae in a colony growing on agar and the radial position where the first conidiophores are found. In a wild-type colony after 72 hr incubation at 37° this distance is about 2 mm. Double mutants of fluffy genes with *sfdA15* or *sfdB38* at 72 hr were found to have undif-

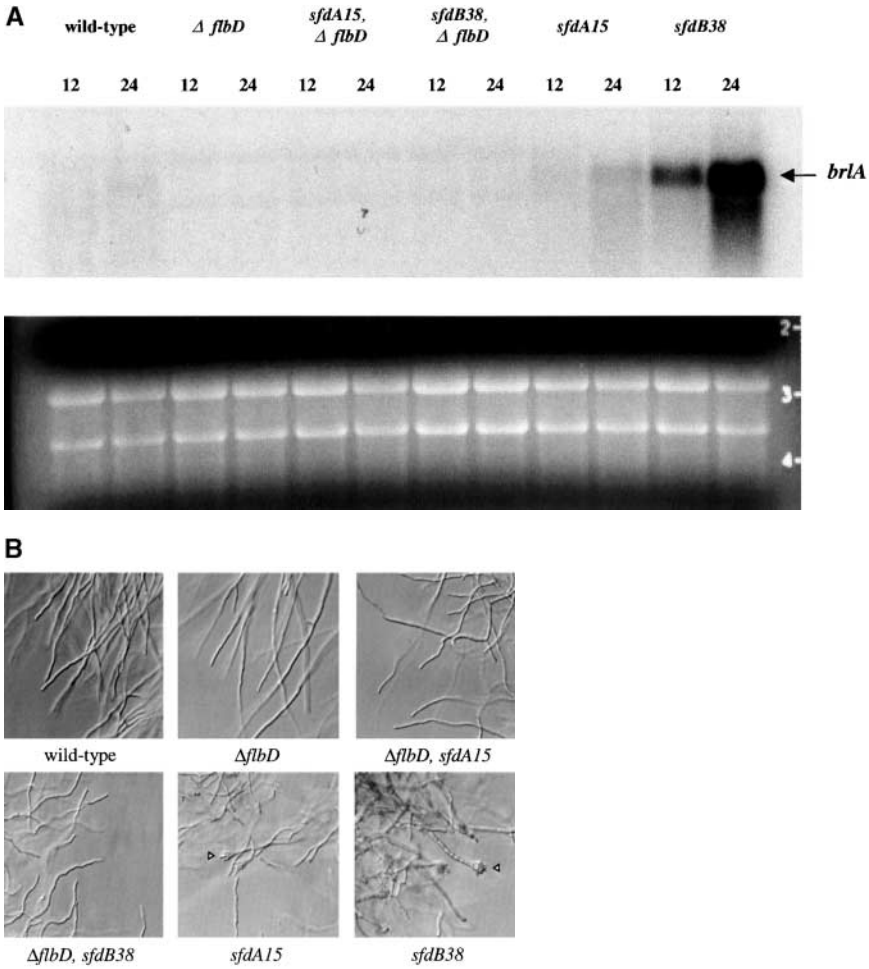

FIGURE 4.—*sfdA15* and *sfdB38* mutations cause *brlA* mRNA accumulation and development in submerged cultures. (A) Total RNA was isolated from liquid-grown cultures of wild-type (FGSC26) and of Δ*flbD* (TEK1003), Δ*flbD*; *sfdA15* (REK65.10), Δ*flbD*; *sfdB38* (REK88.10), *flbD*$^+$; *sfdA15* (REK 65.13), and *flbD*$^+$; *sfdB38* (REK88.23) mutant strains. Conidia were inoculated into minimal media containing 0.1% yeast extract and grown shaking at 37° (see MATERIALS AND METHODS). Total RNA was isolated after 12 and 24 hr and fractionated on a formaldehyde-agarose gel. The resultant gel blot was probed with a *brlA*-specific DNA probe. Equivalent loading of total RNA was confirmed by ethidium bromide staining. (B) Micrographs were taken at 24 hr after inoculation. Only strains containing *sfdA15* or *sfdB38* mutations in an otherwise wild-type developmental background formed conidiophores by 24 hr and these structures were abundant and readily detectable in every microscopic field examined.

ferentiated colony edges that were at or near the wild-type distance in contrast to the fluffy strains analyzed (Table 2).

DISCUSSION

We describe here a new class of *A. nidulans* developmental suppressor mutations that are able to suppress a broad range of conidiation-defective mutations. Recessive mutations in the *sfdA* and *sfdB* genes, *i.e.*, *sfdA15* and *sfdB38*, could suppress the developmental defects for air-induced conidiation in strains with loss-of-function mutations in *fluG*, *flbA*, *flbB*, *flbC*, and *flbE* as well as *flbD*. The ability of *sfdA15* and *sfdB38* mutations to bypass the conidiation defects of strains mutated in both the direct developmental pathway components and growth pathway components, combined with their recessive nature, lead us to propose that they represent a novel class of suppressor mutations. All of the suppressor mutations we have previously described have been dominant or semidominant and, with the exception of *dsgA1*, do not have such a broad ability to bypass multiple defects in positive factors that regulate conidiation (YU *et al.* 1999; D'SOUZA *et al.* 2001).

Three models (Figure 5) are discussed. In Figure 5A, SfdA and SfdB act as direct repressors of *brlA* and one or more *flb* or *fluG* gene products are required to antagonize Sfd activity and allow *brlA* induction and conidiation. In Figure 5B, SfdA and SfdB act to antagonize the activity of one or more of the *flb* or *fluG* products, which act to promote *brlA* induction. And in Figure 5C, SfdA and SfdB act in a previously unknown pathway, independent of the *flb* genes and *fluG*, to repress *brlA* induction, either directly or by acting upon as-yet-unidentified proteins. In support of the models in Figure 5, A and C, in the context of air-induced conidiation, *sfdA15* and *sfdB38* mutant alleles can bypass individual null alleles of each of the *flb* genes and *fluG* to restore *brlA* activation and conidiation. The model in Figure 5B would require that some of the *flb* gene products have overlapping or redundant functions as targets for SfdA/B negative action, since individual null alleles of all *flb* genes and *fluG* are bypassed by *sfdA15* or *sfdB38* mutations.

We have observed that, unlike wild-type strains, strains with *sfdA15* or *sfdB38* mutations accumulate *brlA* mRNA and develop conidiophore-like structures bearing viable spores during growth in liquid medium. Interestingly,

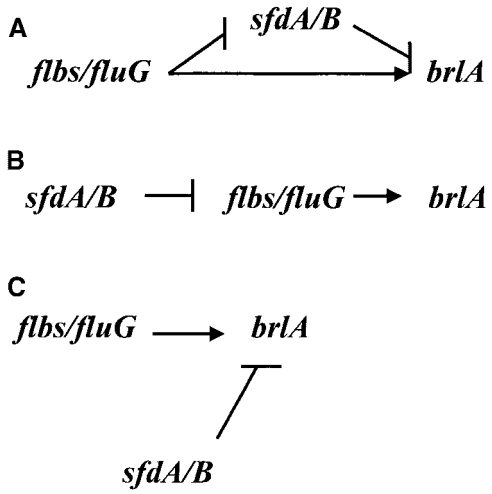

FIGURE 5.—Three models for SfdA and SfdB action during initiation of conidiophore development. (A) SfdA and SfdB act as direct repressors of *brlA* and one or more *flb* or *fluG* gene products are required to antagonize Sfd activity and allow *brlA* induction and conidiation. (B) SfdA and SfdB act to antagonize the activity of one or more of the *flb* or *fluG* products, which act to promote *brlA* induction. (C) SfdA and SfdB act in a previously unknown pathway, independent of the *flb* genes and *fluG*, to repress *brlA* induction.

the *sfdA15* or *sfdB38* mutations caused only submerged conidiation and *brlA* depression in strains with wild-type alleles of *flbD*, *fluG*, *flbA*, *flbB*, *flbC*, and *flbE* (Figure 4B and data not shown). This contrasts with the observation that *sfdA15* and *sfdB38* can bypass null mutations in all of the same developmental loci above for air-induced conidiation on agar plates. These data fit with the model in Figure 5B, which shows that *sfdA* and *sfdB* function to antagonize the activity of *flb/fluG* gene product since *sfdA15* and *sfdB38* mutant alleles cause only liquid development in strains with wild-type copies of *flb* genes and *fluG*. Alternatively, it may be that *sfdA/B* function in a new pathway in an entirely *flb*-independent manner (Figure 5C). The difference in requirement for the *flb/fluG* genes in liquid *vs.* agar growth for *sfdA/B* mutations to cause *brlA* induction could be due to the presence of positive signals from air. During growth on an agar surface, loss of any individual *flb/fluG* gene function can be bypassed by *sfdA15* or *sfdB38* mutations; however, in the absence of positive signals from air, during liquid growth both *sfdA15* or *sfdB38* mutations and an intact *flb* gene pathway are required to allow *brlA* activation and conidiation.

The liquid development phenotype has been observed previously in strains with developmental suppressor mutations (ROSEN *et al.* 1999; YU *et al.* 1999; D'SOUZA *et al.* 2001), in mutations in *brlA* regulatory regions (HAN *et al.* 1993), and in strains where the expression of specific developmental regulators (*brlA*, *flbA*, *fluG*, or *flbD*) is ectopically induced under the control of the alcohol dehydrogenase promoter (*alcA*; ADAMS *et al.* 1988; LEE and ADAMS 1994b, 1996; WIESER and ADAMS 1995). *sfdA15* and *sfdB38* mutations differ from the previously isolated dominant suppressors of *fluG* and *flbA* mutations in their inability to cause liquid conidiation in combination with *fluG* or *flbA* null mutations. Mutations in upstream *brlA* regulatory regions that cause its misactivation also cause precocious development in liquid culture (HAN *et al.* 1993). Because *brlA* is on chromosome VIII, and *sfdA15* and *sfdB38* map to chromosomes VI and II, respectively, it is unlikely that the liquid conidiation phenotype observed is related to defects in the *brlA* regulatory regions. Lastly, although liquid development is observed in strains with increased levels of *flbD*, *flbA*, or *fluG*, achieved through fusion of these genes to the *alcA* promoter, *sfdA15* and *sfdB38* do not appear to cause development through increased production of one of these regulators since their transcript levels in RNA isolated from liquid-grown sfd^S mutants were not found to be at levels required to drive development (data not shown). Heterologous expression of *flbC*, *flbE*, or *flbB* from similar fusions to the *alcA* promoter has not been demonstrated to cause *brlA* activation or conidiation in liquid cultures (J. WIESER and T. ADAMS, unpublished results).

Like *sfdA15* and *sfdB38*, mutations in *rco-1* of *Neurospora crassa* allow aberrant expression of the asexual developmental gene *con-10* in vegetative hyphae. However, unlike *sfdA15* and *sfdB38* mutant strains, *rco-1* mutants are defective in conidiation (YAMASHIRO *et al.* 1996). Similarly, it has been found that a deletion of the *A. nidulans* Rco-1 homolog, *rcoA*, results in a conidiation-defective phenotype (HICKS *et al.* 2001). Although we favor a model in which *sfdA* and *sfdB* function as repressors of *brlA*, it is also unknown whether the derepression of gene expression due to mutations in *sfdA* and *sfdB* is specific to *brlA* or more generalized.

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