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# *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway

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**The filamentous fungus *Aspergillus nidulans* contains a cluster of 25 genes that encode enzymes required to synthesize a toxic and carcinogenic secondary metabolite called sterigmatocystin (ST), a precursor of the better known fungal toxin aflatoxin (AF). One ST Cluster (*stc*) gene, *aflR*, functions as a pathway-specific transcriptional regulator for activation of other genes in the ST pathway. However, the mechanisms controlling activation of *aflR* and synthesis of ST and AF are not understood. Here we show that one important level for control of *stc* gene expression requires genes that were first identified as early acting regulators of asexual sporulation. Specifically, we found that loss-of-function mutations in *flbA*, which encodes a RGS domain protein, or dominant activating mutations in *fadA*, which encodes the α subunit of a heterotrimeric G protein, block both ST production and asexual sporulation. Moreover, overexpression of *flbA* or dominant interfering *fadA* mutations cause precocious *stc* gene expression and ST accumulation, as well as unscheduled sporulation. The requirement for *flbA* in sporulation and ST production could be suppressed by loss-of-function *fadA* mutations. The ability of *flbA* to activate *stc* gene expression was dependent upon another early acting developmental regulator, *fluG*, and AflR, the *stc* gene-specific transcription factor. These results are consistent with a model in which both asexual sporulation and ST production require inactivation of proliferative growth through inhibition of FadA-dependent signaling. This regulatory mechanism is conserved in AF-producing fungi and could therefore provide a means of controlling AF contamination.**

*Keywords*: aflatoxin/fungal development/G protein/RGS domain/secondary metabolism

## Introduction

Several species of the fungal genus *Aspergillus*, including the genetically well studied *Aspergillus nidulans*, produce the mycotoxin sterigmatocystin (ST), which also serves as the penultimate precursor in the aflatoxin (AF) biosynthetic pathway (Aucamp and Holzapfel, 1970; Cole and Cox, 1981). Both AF and ST are among the most toxic, carcinogenic and mutagenic compounds produced in nature and contamination of agricultural crops by these toxins has significant health and economic costs. We have been interested in understanding the mechanisms regulating ST/AF gene expression with the long-term goal of providing potential means of eliminating production of these toxic secondary metabolites. Examination of the *A.nidulans* ST pathway has led to identification of a 60 kb gene cluster (the ST Cluster; *stc*) that includes 25 co-regulated genes, many of which have been shown to function in ST biosynthesis (Brown *et al.*, 1996). Transcription of all of these genes is dependent upon the activity of *aflR*, a pathway-specific regulatory gene found within the ST cluster that encodes a zinc binuclear cluster-type DNA binding protein (Woloshuk *et al.*, 1994; Yu *et al.*, 1996a). *aflR* expression is regulated during the life cycle such that *aflR* mRNA begins to accumulate early in the stationary phase and activation of other genes required for ST biosynthesis quickly follows (Yu *et al.*, 1996a).

Numerous observations have supported the hypothesis that microbial secondary metabolite production and sporulation are intimately associated. The best known example is found in *Streptomyces* spp., in which there are common elements that regulate sporulation and antibiotic production (for a review see Hopwood, 1988). Similarly, earlier observations suggested that the ability to complete wild-type asexual sporulation could be a prerequisite for ST/AF biosynthesis in the filamentous fungus genus *Aspergillus* (Kale *et al.*, 1994, 1996). To study the genetic basis of the relationship between asexual development and secondary metabolism and the possible common elements in regulation of these two processes, we decided to examine the effects of various early acting developmental mutations on ST production in *A.nidulans*. We previously proposed that there are two antagonistic signaling pathways regulating *A.nidulans* growth and development (see Figure 1; Yu *et al.*, 1996b). In this model, growth signaling is mediated by *fadA* (Fluffy Autolytic Dominant), which encodes the α subunit of a heterotrimeric G protein. Mutations in *fadA* that are predicted to interfere with its intrinsic GTPase activity and therefore lock FadA in its active, GTP bound state result in a proliferative phenotype and block sporulation. Developmental activation requires that this FadA signaling pathway be at least partially inactivated and this requires the product of another gene, called *flbA* (Fluffy Low *brlA*; Lee and Adams, 1994b). The FlbA protein has a 120 amino acid C-terminal region termed an RGS domain (for Regulator of G protein Signaling) that is shared by a family of proteins found in organisms ranging from yeast to man. These proteins are all implicated in negatively regulating G protein-mediated signaling pathways (Dietzel and Kurjan, 1987; De Vries *et al.*, 1995; Dohlman *et al.*, 1995; Druey *et al.*, 1996; Koelle and Horvitz, 1996; Yu *et al.*, 1996b; Dohlman and

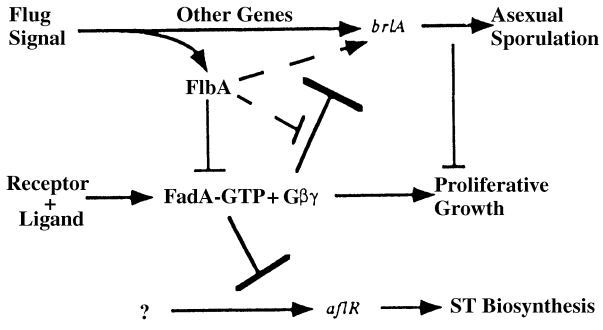


**Fig. 1.** Proposed model for *flbA* and *fadA* control of *A.nidulans* growth, development and secondary metabolism. As described in the text, we propose that two antagonistic signaling pathways regulate development and secondary metabolism (Lee and Adams, 1996; Yu *et al*., 1996b). When FadA is GTP bound it regulates downstream effectors to activate proliferation and repress both sporulation and ST biosynthesis. The RGS domain protein FlbA responds to FluG-dependent developmental signals to inactivate FadA and allow activation of both sporulation and ST biosynthesis.

Thorner, 1997). The mechanisms of RGS domain protein action are not fully understood, but apparently include activation of the intrinsic GTPase activity of specific heterotrimeric G protein α subunits (Berman *et al.*, 1996; Watson *et al.*, 1996). Strains with *flbA* loss-of-function mutations have a proliferative phenotype resembling *fadA*-activating mutations, as expected if FlbA functions by inactivating FadA (Lee and Adams, 1994b). This hypothesis is further supported by the finding that mutations which inactivate *fadA* can suppress *flbA* loss-of-function mutations, resulting in a nearly wild-type developmental phenotype. However, it is also apparent that FlbA has additional functions, because overexpression of *flbA* has been shown to cause inappropriate activation of asexual development in both wild-type and *fadA* deletion mutant strains (Lee and Adams, 1994b, 1996; Yu *et al.*, 1996b).

Development-specific signaling requires the *fluG* gene product, which is apparently necessary for synthesis of a small diffusible factor that functions extracellularly to signal developmental initiation (Lee and Adams, 1994a, 1996). As with *flbA*, overexpression of *fluG* can cause vegetative cells to differentiate sporulating structures, even under conditions that do not favor wild-type sporulation. This FluG-induced sporulation requires the wild-type activities of other early acting developmental regulatory genes, including *flbA* (Lee and Adams, 1996). Because *flbA*-activated sporulation also requires wild-type *fluG* activity, we have proposed that there are two primary consequences of responding to FluG factor: (i) activation of FlbA, which then interferes with FadA signaling of proliferation; (ii) activation of development-specific functions that require the products of other genes, including *flbB*, *flbC*, *flbD*, *flbE* and *brlA* (Adams *et al.*, 1988; Wieser *et al.*, 1994; Lee and Adams, 1996). Both of these processes must occur if development is to proceed.

In this manuscript we present evidence that inactivation of the FadA signal transduction pathway is required for ST biosynthesis, as well as asexual sporulation. We found that loss-of-function mutations in *fluG* or *flbA* or dominant activating mutations in *fadA* block both sporulation and *stc* gene activation. In the *flbA* loss-of-function mutants the block on *stc* gene activation was due to the absence of *aflR* transcript accumulation. In contrast, overexpression of *flbA* (but not *fluG*) or dominant interfering mutations in *fadA* caused inappropriate sporulation (Lee and Adams, 1996; Yu *et al.*, 1996b), precocious *stc* gene activation and ST biosynthesis. Finally, *fadA* loss-of-function mutations suppressed the need for either *flbA* or *fluG* in activating ST production but only suppressed the *flbA* requirement for sporulation. These results are consistent with a model in which the proposed FluG factor stimulates both development-specific events and activation of FlbA, which in turn inactivates FadA-dependent signaling. Because FadA signaling antagonizes both sporulation and ST production, FadA inactivation by FlbA is a prerequisite for both processes.

**Table I.** Developmental mutations affect *stc* gene transcript accumulation and ST production

| Relevant genotype | *stc* transcript[a] | ST production[b] |
|---|---|---|
| Δ*flbA* | – | – |
| *flbB*$^-$ | + | + |
| *flbC*$^-$ | + | + |
| *flbD*$^-$ | + | + |
| *flbE*$^-$ | + | + |
| Δ*fluG* | – | – |
| Δ*brlA* | + | + |
| *fadA*$^{G42R}$ | –[c] | – |
| Wild-type | + | + |

[a]A + indicates that transcripts were detected by 62 h growth in standard liquid minimal medium. A – indicates that *stc* transcripts were not detected even after 86 h growth. Relative levels of *stc* transcript accumulation were approximately equal in all cases.
[b]A + indicates that ST could be detected via TLC by 7 days growth in stationary liquid culture. Relative levels of ST production were approximately equal in all cases.
[c]Lysis of mutant hyphae prevented isolation of RNA after more than 36 h growth in liquid shake culture. No *stc* transcript was detected at this time.

## Results

### *flbA and fluG are necessary for ST production*

Our genetic analysis of asexual sporulation has led to the identification of numerous genes that are required at early steps in activation of the asexual sporulation pathway (Lee and Adams, 1996; Wieser *et al.*, 1994). We first examined the possibility that *A.nidulans* shared controls for ST production and for activation of asexual sporulation by determining whether various early acting developmental mutant strains could activate *stc* gene expression and produce ST. As summarized in Table I, *flbB*, *flbC*, *flbD*, *flbE* and *brlA* mutant strains all produced ST and expressed the *stc* genes similarly to the wild-type. In contrast, strains with loss-of-function mutations in *flbA* or *fluG* or dominant activating mutations in *fadA* (*fadA*$^{G42R}$; Yu *et al.*, 1996b) completely eliminated *stc* gene expression and ST accumulation and blocked sporulation.

### *Overexpression of flbA induces stc gene expression*

Earlier experiments demonstrated that while deletion of *flbA* or *fluG* resulted in a loss of sporulation, overexpression of either gene in vegetative hyphae was sufficient to induce conidiophore development inappropriately (Lee and Adams, 1994b, 1996). Because *fluG* and *flbA* were

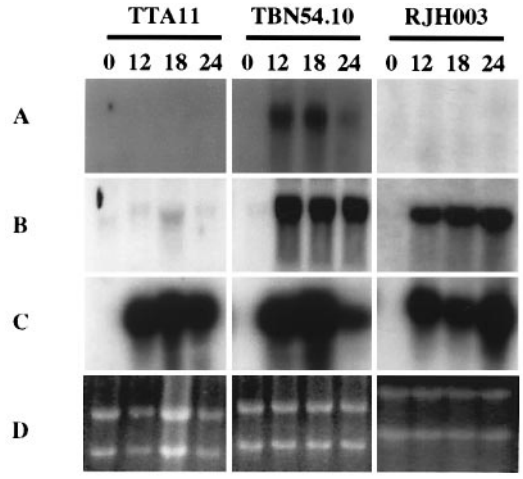


**Fig. 2.** Induced expression of *flbA* activates *stc* gene expression. RNA from TTA11 (wild-type), TBN54.10 (*alcA*[*p*]::*flbA*) and RJH003 (*alcA*[*p*]::*flbA*, Δ*aflR*) was isolated from cultures 0, 12, 18 and 24 h after shifting to *alcA*-inducing medium and analyzed for *stcU* gene expression. A 0.75 kb *Sst*II–*Sma*I fragment from plasmid pRB7 (Yu *et al*., 1996a) was used as a *stcU*-specific probe (**A**); a 2.5 kb *Eco*RI fragment from pBN30 (Lee and Adams, 1994b) was used as a *flbA*-specific probe (**B**); plasmid pJA1 (Adams and Timberlake, 1990) was used as an *alcA*-specific probe (**C**). Equal loading of total RNA was evaluated by ethidium bromide staining (**D**). *stcU* was only detected following activation of *flbA* overexpression in the wild-type strain. The *flbA* transcript observed in TTA11 results from constitutive expression of the wild-type *flbA* gene.

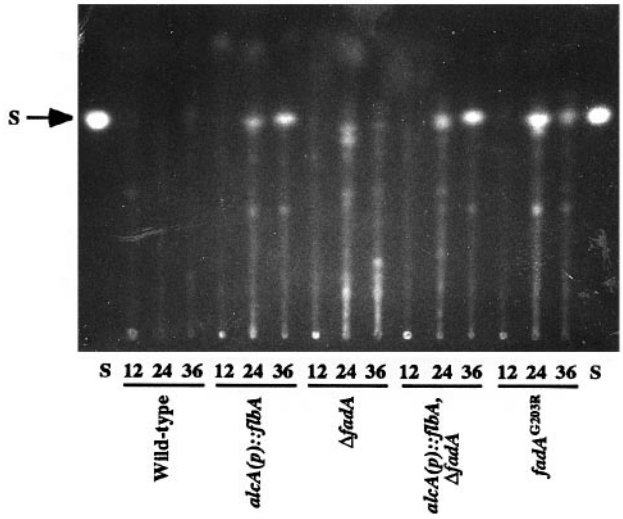


**Fig. 3.** Induced *flbA* expression activates ST biosynthesis. Organic extracts from TTA11 (wild-type), TBN54.10 (*alcA*[*p*]::*flbA*), RJY918.6 (Δ*fadA*), RJYA21 (*alcA*[*p*]::*flbA*, Δ*fadA*) and RJY115.4 (*fadA*$^{G203R}$) were obtained from cultures 12, 24 and 36 h after shifting to *alcA*-inducing medium and separated by TLC as described in Materials and methods. Samples labeled S represent sterigmatocystin standards (Sigma). ST was detected only at 24 and 36 h in TBN54.10, RJYA21 and RJY115.4 (see text). Compounds with different $R_f$ values from ST (e.g. in Δ*fadA* lanes) are not ST but uncharacterized substances.

apparently required for *stc* gene expression, we tested to see if overexpression of *flbA* or *fluG* could also cause early activation of *stc* gene transcription and ST accumulation. As shown in Figure 2, forced activation of *flbA* caused accumulation of the *stcU* (Keller *et al.*, 1994) and other *stc* transcripts (not shown) within 12 h, at least 24 h before *stc* transcripts normally appear in wild-type cultures grown under the same conditions. The *stcU* gene encodes a ketoreductase responsible for converting versicolorin A to demethylsterigmatocystin (for details of the ST/AF pathway genes see Brown *et al.*, 1996). To see whether early *stc* gene activation caused by *flbA* overexpression results in precocious ST production, we also examined organic extracts from *flbA*-induced cultures. As shown in Figure 3, *flbA* overexpression caused ST accumulation by 24 h post-induction. This is at least 24 h before ST begins to accumulate in the wild-type strain (as indicated by the lack of ST in the wild-type lanes even at 36 h post-induction; Figure 3). Perhaps surprisingly, while *fluG* overexpression did activate sporulation, it did not cause premature *stc* transcript accumulation (Table II). Finally, because we had previously observed that two later acting developmental regulators, *brlA* and *flbD*, could also activate sporulation when overexpressed (Adams *et al.*, 1988; Wieser and Adams, 1995), we tested to see if developmental activation by these genes was coupled with ST gene activation. As shown in Table II, neither *brlA* nor *flbD* overexpression caused *stc* gene activation, as expected if these genes have only sporulation-specific functions.

**Table II.** Overexpression of *flbA* activates ST gene expression

| Relevant genotype | *stc* transcripts[a] | | | Submerged conidiation[b] |
|---|---|---|---|---|
| | 0 h | 12 h | 24 h | |
| *alcA*[*p*]::*flbA* | – | + | + | + |
| *alcA*[*p*]::*flbD* | – | – | – | + |
| *alcA*[*p*]::*brlA* | – | – | – | + |
| *alcA*[*p*]::*fluG* | – | – | – | + |
| *alcA*[p]::*flbA*, *flbB*$^-$ | – | – | + | – |
| *alcA*[p]::*flbA*, *flbC*$^-$ | – | + | + | + |
| *alcA*[p]::*flbA*, *flbD*$^-$ | – | + | + | + |
| *alcA*[p]::*flbA*, *flbE*$^-$ | – | – | + | + |
| *alcA*[p]::*flbA*, Δ*fluG* | – | – | – | – |
| *alcA*[p]::*flbA*, Δ*fadA* | – | + | + | + |
| *alcA*[*p*]::*flbA*, Δ*aflR* | – | – | – | + |
| Δ*fadA*[c] | – | – | – | – |
| *fadA*$^{G203R}$ | – | + | + | + |
| Wild-type[c] | – | – | – | – |

[a]A + indicates that *stc* transcripts accumulated by the time indicated after transfer to *alcA*-inducing medium. *stc* transcripts were never observed in the wild-type strain earlier than 36 h after transfer to *alcA*-inducing medium.
[b]Submerged conidiation is defined as conidiation occurring in liquid shake culture and does not normally occur in wild-type strains. This differs from aerial conidiation, which occurs in wild-type strains grown on solid medium exposed to air. For more information regarding submerged sporulation phenotypes refer to Adams *et al.* (1988), Lee and Adams (1996), Wieser and Adams (1995) and Yu *et al*. (1996b).
[c]*stc* transcripts cannot be observed in these strains prior to 48 h post-shift.

### *Genetic requirements for flbA activation of stc gene expression*

The ability of *flbA* overexpression to cause inappropriate activation of sporulation has been shown to require the activities of other developmental regulators, including *brlA*, *flbB* and *fluG* (Lee and Adams, 1996). Table II summarizes the genetic requirements of *flbA*-induced activation of *stc* gene expression. We found that, as with development, *fluG* was required for *flbA*-induced activation

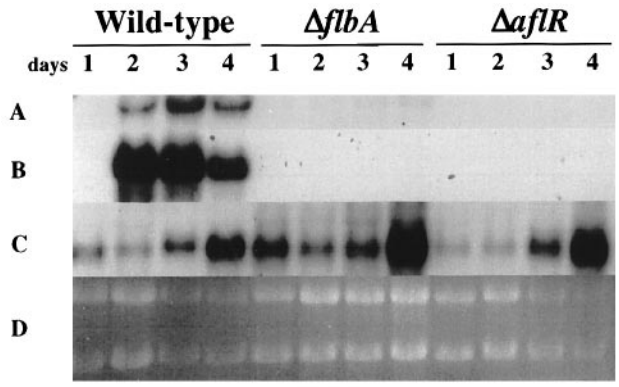


**Fig. 4.** Loss of *flbA* function results in lack of *aflR* transcription. Total RNA was isolated from 1–4-day-old liquid stationary cultures of FGSC26 (wild-type), TBN39.5 (Δ*flbA*) and TMF4.12 (Δ*aflR*) and the resulting blots were hybridized with (**A**) *aflR*-, (**B**) *stcU*- and (**C**) *argB*-specific probes (see Materials and methods). *aflR* (~1.6 kb) and *stcU* (~0.9 kb) transcripts only accumulated in the wild-type strain after 2 days, whereas *argB* transcript was present in all strains at all time points. The minor signal observed in (A) for the Δ*flbA* mutant appears to be due to non-specific hybridization corresponding to the 15S rRNA (~1.8 kb) band, which migrates just above *aflR* mRNA. This minor non-specific signal was observed at the same position with all three probes. Equal loading of total RNA was evaluated by ethidium bromide staining (**D**).

of *stc* transcript accumulation, supporting the hypothesis that FluG is required for FlbA activity. All the other mutants still responded to *flbA* overexpression by activating *stc* gene expression, although the response was somewhat delayed in *flbB* and *flbE* mutants. Finally, we tested to see if the ST cluster-specific transcription factor *aflR* (Yu *et al.*, 1996a) was required for *flbA*-induced activation of other *stc* genes. As shown in Figure 2 and summarized in Table II, we found that overexpression of *flbA* in the *aflR* deletion mutant did not induce *stc* gene expression but did cause sporulation, indicating that *flbA*-induced *stc* gene expression requires AflR. Based on our model (Figure 1), we hypothesized that FlbA relieves FadA-mediated repression of events leading to transcriptional activation of *aflR*. Therefore, we predicted that loss-of-function mutations in *flbA* would result in loss of *aflR* transcription. To test this prediction, we examined *aflR* transcript accumulation in an *flbA* loss-of-function mutant. As expected if FlbA function is required for *aflR* expression, *aflR* mRNA was not detected in the Δ*flbA* mutant strain (see Figure 4 and its legend).

### *FlbA inactivates FadA-mediated repression of stc gene expression*

The results presented above support a model in which FlbA functions in activating ST biosynthesis in a manner similar to how it functions in activating sporulation, by negatively controlling the FadA signaling pathway that blocks sporulation (Yu *et al.*, 1996b). To further test this proposed FlbA function, we examined the effects of various combinations of activating and inactivating *flbA* and *fadA* alleles on ST biosynthesis. As shown in Table III, unlike *fadA*-activating mutations (see $fadA^{G42R}$ in Table I), *fadA* deletion (Δ*fadA*; Table III) and dominant interfering mutations ($fadA^{G203R}$; Table III and Figure 3) did not prevent ST production in $flbA^{+}$ strains. In fact, we observed early activation of *stc* gene expression (Table II) and ST production in the $fadA^{G203R}$ mutant strain (at 24 h post-shift; Figure 3), even without *flbA* overexpression. Moreover, the Δ*fadA* and $fadA^{G203R}$ mutations suppressed both the conidiation (Yu *et al.*, 1996b) and ST biosynthesis defects observed for a *flbA* deletion mutant (Table III), as expected if the primary role of FlbA in activating ST biosynthesis is to negatively control FadA signaling. However, *flbA* overexpression in the Δ*fadA* mutant strain caused sporulation (Yu *et al.*, 1996b), early induction of *stc* gene expression (Table II) and precocious ST biosynthesis (at 24 h post-induction; Figure 3), suggesting some additional role for FlbA in controlling these events (see Discussion). It is important to note that under the growth conditions examined neither the wild-type nor the Δ*fadA* strains accumulated *stc* transcripts (Table II) or ST (Figure 3) until 48 h post-shift.

**Table III.** Genetic interaction of *fadA flbA* and *fadA fluG* for ST production

| Relevant genotype | Relative ST production[a] | Relative conidiation[b] | |
|---|---|---|---|
| | | Aerial | Submerged |
| Wild-type | ++ | ++ | – |
| Δ*flbA* | – | – | – |
| Δ*fadA* | + | ++ | – |
| $fadA^{G203R}$ | + | +++ | + |
| $fadA^{G42R}$ | – | – | – |
| Δ*fadA* Δ*flbA* | + | ++ | – |
| $fadA^{G203R}$ Δ*flbA* | + | +++ | + |
| Δ*fadA* Δ*fluG* | ++ | – | – |
| $fadA^{G203R}$ Δ*fluG* | + | – | – |

[a]A + indicates that ST was detected via TLC by 7 days growth in stationary liquid culture.
[b]Submerged and aerial conidiation are defined in Table II.

The inability of *fluG* overexpression to cause premature *stc* gene activation raises the possibility that FluG signaling has a specific function in developmental activation and that its only role in activating ST biosynthesis is in determining an uncharacterized post-transcriptional activation of FlbA (Lee and Adams, 1996). We tested this hypothesis by examining the ability of $fadA^{G203R}$ and Δ*fadA* mutations to suppress the loss of ST production phenotype observed for *fluG* deletion mutants. As shown in Table III, both $fadA^{G203R}$ Δ*fluG* and Δ*fadA* Δ*fluG* double mutant strains produced ST but were developmentally defective.

### *FadA function is conserved in Aspergillus parasiticus*

The demonstration that the FadA proliferation pathway blocks both sporulation and ST biosynthesis in *A.nidulans*, coupled with the strong conservation between FadA and Gα proteins from other filamentous fungi (Turner and Borkovich, 1993; Choi *et al.*, 1995), might indicate that the need to regulate FadA-associated signaling is a common requirement for activating development and ST/AF production in other aspergilli. To test this hypothesis, we introduced the $fadA^{G42R}$ dominant activating allele from *A.nidulans* into an *Aspergillus parasiticus* strain (B62, $niaD^{-}$; $br^{-}$; $nor\text{-}1^{-}$; Trail *et al.*, 1994) that accumulates a colored precursor of AF (norsolorinic acid, NOR). Transformants were examined visually for sporulation and

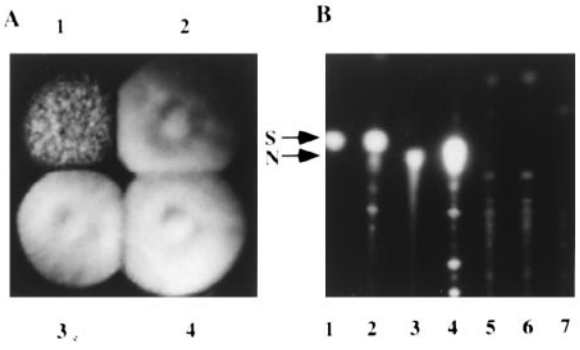


**Fig. 5.** *Aspergillus nidulans fadA*-activating mutations function in *A.parasiticus* to block sporulation and AF production. (**A**) A developmentally wild-type *niaD*$^{+}$ transformant of *A.parasiticus* strain B62 (*niaD*$^{-}$, *br*$^{-}$, *nor-1*$^{-}$) lacking *fadA*$^{G42R}$ (1) and fluffy autolytic *fadA*$^{G42R}$-containing *niaD*$^{+}$ transformants of B62, TJYP1-22 (2), TJYP1-23 (3) and TJYP1-28 (4), were point inoculated on complete medium and incubated for 3 days at 30°C. (**B**) The *A.parasiticus* strains shown in (A) were grown in liquid complete medium for 7 days at 30°C (stationary culture) and organic extracts were separated by TLC as described previously (Yu and Leonard, 1995). Lane 1, ST standard; lane 2, extract from an *A.nidulans* wild-type strain producing ST; lane 3, NOR standard; lane 4, extract from a developmentally wild-type *niaD*$^{+}$ transformant of *A.parasiticus* B62; lanes 5–7, extracts from *fadA*$^{G42R}$ *A.parasiticus* transformants TJYP1-22, TJYP1-23 and TJYP1-28 respectively. Four times as much extract was loaded in lanes 5–7 as compared with lane 4 to determine if trace amounts of NOR could be detected.

production of NOR and by Southern blot analysis to determine if they had acquired the *A.nidulans* gene. Three *A.parasiticus* transformants (TJYP1-22, TJYP1-23 and TJYP1-28) were identified that had fluffy autolytic phenotypes similar to the *A.nidulans fadA*$^{G42R}$ mutant and were each found to have one to three copies of the *A.nidulans fadA*$^{G42R}$ allele, while none of the developmentally wild-type transformants had obtained the *A.nidulans fadA* gene (data not shown). As shown in Figure 5, none of the three fluffy transformants produced detectable NOR, while the developmentally wild-type transformants did. This result indicates that FadA-associated proliferation signaling and its relationship with development and ST/AF biosynthesis is conserved in other species of *Aspergillus*.

## Discussion

Many microorganisms produce compounds described as secondary metabolites that are apparently not required by the producing organism but, like the antibiotics and mycotoxins, can have either beneficial or detrimental effects for humans. The results presented in this paper demonstrate that in the filamentous fungus *A.nidulans* asexual sporulation and biosynthesis of a toxic and carcinogenic polyketide secondary metabolite called sterigmatocystin (ST) requires inactivation of a heterotrimeric G protein signaling pathway. As summarized in Figure 1, we propose that this negative control is mediated by the FlbA RGS domain protein, apparently in response to a specific developmental signal produced through the activity of FluG (Lee and Adams, 1996; Yu *et al.*, 1996b). FlbA presumably functions as a GAP, like other RGS domain proteins (Berman *et al.*, 1996; Dohlman *et al.*, 1996; Dohlman and Thorner, 1997), to stimulate the endogenous GTPase activity of the α subunit of a heterotrimeric G protein, encoded by *fadA*, leading to inactivation of FadA-dependent signaling.

This model is supported by the finding that loss-of-function mutations in *flbA* or dominant activating mutations in *fadA* lead to a coordinate block in both sporulation (Yu *et al.*, 1996b) and ST production. Moreover, the requirement for FlbA in both processes can be overcome by mutations that eliminate FadA function, indicating that the primary role for FlbA in activating sporulation and ST biosynthesis is through inactivation of FadA. However, as described previously (Yu *et al.*, 1996b), this model must have further complications in that FlbA must have some FadA-independent function. This follows from the observation that *flbA* overexpression in a *fadA* deletion mutant activated *stc* gene expression, ST production and sporulation just as well as it did in a wild-type strain. Because a dominant interfering *fadA* mutation (*fadA*$^{G203R}$) that is proposed to prevent dissociation of the Gβγ subunits from Gα-GTP (Rens-Domiano and Hamm, 1995) caused both sporulation in submerged culture (Yu *et al.*, 1996b) and precocious ST production, we have proposed that a second target for FlbA action is the Gβγ subunits of the heterotrimeric G protein. However, potential roles for FlbA in directly activating development and ST biosynthesis or in inhibiting a second Gα subunit or downstream effector(s) in the FadA signaling pathway cannot be excluded.

While regulation of secondary metabolite production is poorly understood, these compounds are often produced during the stationary phase of growth and in several organisms a connection between secondary metabolite production and the ability to reproduce by sporulation has been observed (Demain, 1992; Kale *et al.*, 1994, 1996; Betina, 1995). The best characterized relationship between microbial secondary metabolite production and development is in *Streptomyces griseus*, where A-factor, a γ-butyrolactone molecule, has been shown to function as a signal that activates both antibiotic production and sporulation during stationary phase (Horinouchi, 1996). In this case A-factor has been shown to bind to the ArpA DNA binding protein, blocking its ability to function as a negative regulator of genes required for sporulation and antibiotic production. The interaction between ArpA and A-factor is proposed to result in derepression of an unknown gene whose product activates pathways leading to both aerial mycelia production (leading to sporulation) and streptomycin production and resistance (Horinouchi, 1996). However, A-factor is only able to stimulate sporulation and streptomycin production following entry into stationary phase resulting from starvation of the *S.griseus* colony (Beppu, 1992). In contrast, the ability of FlbA to activate sporulation and ST biosynthesis is apparently independent of growth phase. Instead, we propose that FlbA is normally activated when FluG factor accumulates to a sufficient level and that activation of FlbA leads primarily to inactivation of the FadA-dependent proliferation pathway (Yu *et al.*, 1996b). Thus, FlbA-directed inhibition of FadA provides an endogenous growth control mechanism that is necessary for both sporulation and ST production.

Although ST/AF production typically accompanies sporulation in many *Aspergillus* spp. (Kale *et al.*, 1994, 1996), the converse is not true in that ST/AF production

**Table IV.** Strains used in this study

| Strain | Relevant genotype | Source |
|---|---|---|
| FGSC26 | *biA1*, *veA1* | FGSC[a] |
| FGSC237 | *pabaA1*; *trpC801*, *veA1* | FGSC[a] |
| h1FAD4 | *biA1*; *fadA*$^{G42R}$, *veA1* | Yu *et al*. (1996) |
| PW1 | *biA1*; *argB2*; *methG1*; *veA1* | Weglenski |
| RBN051 | *biA1*; Δ*fluG*::*trpC; veA1* | Lee and Adams (1994a) |
| RBN138 | *wA3*; *pyroA4*; *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RBN145 | *pabaA1*, *yA2*; Δ*fluG*::*argB*; *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RBN149 | *pabaA1*, *yA2*; *flbD14*; *alcA*[p]*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RBN151 | *pabaA1*; *wA3*; *alcA*[*p*]::*flbA*::*trpC*, *veA1*; *flbC8* | Lee and Adams (1996) |
| RBN155 | *pabaA1*, *yA2*; *flbE58*; *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RBN180 | *biA1*; *flbB1*, *pyroA4*; *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RBN197 | *pyroA4*; *brlA*::*argB*, *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| RJF009 | *biA1*; *methG1*; *veA1*, *flbC8* | J.Fondon and T.Adams |
| RJH003 | *wA3*; *methG1*, *pyroA4*, Δ*aflR*::*argB*; *alcA*[p]::*flbA*::*trpC*, *veA1* | this study |
| RJW012 | *biA1*; *flbB7*; *veA1* | Wieser *et al*. (1994) |
| RJW120 | *flbE58*; *methG1*; *veA1* | Wieser *et al*. (1994) |
| RJYA21 | *biA1*, *wA3*; Δ*fadA*::*argB*, *alcA*[p]::*flbA*::*trpC*, *veA1* | Yu *et al*. (1996) |
| RJYB27 | *pabaA1*, *yA2*; Δ*fluG*::*trpC*; Δ*fadA*::*argB*, *veA1* | Yu *et al*. (1996) |
| RJYC28 | *pabaA1*, *yA2*; Δ*fluG*::*trpC*; *fadA*$^{G203R}$, *veA1* | Yu *et al*. (1996) |
| RJYF01 | *biA1*, Δ*flbA*::*argB*; *fadA*$^{G203R}$, *veA1* | Yu *et al*. (1996) |
| RJY115.4 | *yA2*; *fadA*$^{G203R}$, *veA1* | Yu *et al*. (1996) |
| RJY918.6 | *argB2*; *methG1*; Δ*fadA*::*argB*, *veA1* | Yu *et al*. (1996) |
| TA046 | *biA1*; *argB2*; *pyroA4*; Δ*brlA*::*argB*, *veA1* | Han *et al*. (1993) |
| TBN39.5 | *biA1*, Δ*flbA*::*argB*; *methG1*; *veA1* | Lee and Adams (1994b) |
| TBN54.10 | *pabaA1*, *yA2*; *alcA*[*p*]::*flbA*::*trpC*, *veA1* | Lee and Adams (1996) |
| TBN57.8 | *pabaA1*, *yA2*; *alcA*[*p*]::*fluG*::*trpC*, *veA1* | Lee and Adams (1996) |
| TJW29.2 | *pabaA1*, *yA2*; *alcA*[*p*]::*flbD*::*trpC*, *veA1* | Wieser and Adams (1995) |
| TJW30 | *pabaA1*, *yA2*; Δ*flbD*::*trpC*, *veA1* | Wieser and Adams (1995) |
| TJYE07.1 | *pabaA1*, *biA1*, Δ*flbA*::*argB*; Δ*fadA*::*argB*, *veA1* | this study |
| TMF4.12 | *biA1*; *methG1*, Δ*aflR*::*argB*; *veA1* | M.Fernandes, N.P.Keller and T.H.Adams |
| TTA11 | *pabaA1*, yA2; *veA1* | Adams and Timberlake (1990) |
| B62[b] | *niaD*$^{-}$; *br*$^{-}$; *nor1*$^{-}$ | Trail *et al*. (1994) |
| TJYP1-22[b] | *brn*$^{-}$; *nor1*$^{-}$; *fadA*$^{G42R}$ | this study |
| TJYP1-23[b] | *brn*$^{-}$; *nor1*$^{-}$; *fadA*$^{G42R}$ | this study |
| TJYP1-24[b] | *brn*$^{-}$; *nor1*$^{-}$; *fadA*$^{G42R}$ | this study |

[a]Fungal Genetics Stock Center.
[b]*Aspergillus parasiticus* strains

can readily occur under conditions where no sporulation is observed (i.e. submerged culture). This finding presumably reflects some fundamental difference in the regulation of development versus ST biosynthesis. In this respect it is interesting to note the differing requirements for FluG. While *fluG* deletion mutants are defective in both ST biosynthesis and sporulation, the requirement for FluG in ST production can be suppressed by mutations that inactivate *fadA*. However, inactivation of *fadA* does not suppress the need for FluG in activating sporulation. This implies that the primary role of FluG in activating ST biosynthesis is to activate FlbA, so that it can inactivate FadA. While this function of FluG is also required for sporulation, there is a second, pathway-specific role for FluG that leads to activation of genes needed for sporulation. It is not yet clear if a similar FluG-like signaling pathway is also required to activate *aflR* and *stc* gene expression.

It is important to realize that the model presented to explain the relationship between conidiation and ST/AF production does not necessarily encompass all of secondary metabolism. For instance, it is clear from the many unidentified compounds separated by the TLC assays presented in Figures 3 and 5 that the various developmental mutants produce a variety of unique compounds. These molecules may represent additional secondary metabolites that, in contrast to ST/AF, are positively controlled by FadA-mediated signaling. Similarly, while A-factor stimulates both sporulation and streptomycin production in *S.griseus* (Chater *et al.*, 1989; Horinouchi, 1996), it has been shown that overexpression of the *Streptomyces coelicolor* sporulation gene *whiG* induces sporulation but inhibits actinorhodin biosynthesis. Thus, the intricate interactions between growth, development and secondary metabolite production will likely require specific knowledge of each pathway.

Finally, it is interesting to consider whether or not there is any biological significance to the common regulatory link observed between ST/AF production and asexual sporulation in *Aspergillus* spp. Unfortunately, the importance of this link is difficult to establish because the biological role of ST/AF in the fungus is not known. Although existing evidence is not convincing, Wicklow (Wicklow, 1990; Wicklow *et al.*, 1994) and others (Janzen, 1977) have speculated that ST and AF could serve a protective role in the fungus as a means of defending against insects. The finding that asexual development is usually accompanied by ST production in *A.nidulans* and that AF and ST are frequently found in spores and other differentiated tissues as well as the mycelium lends support to the hypothesis that there is some as yet undetermined evolutionary significance in the linkage of ST/AF biosynthesis with sporulation. In any case, the finding that FadA activation blocks both ST and AF biosynthesis raises the possibility of designing specific control measures to

prevent AF contamination of commercial food crops. As more is learned about the mechanism of action of FluG, FlbA and FadA, it should be possible to identify molecules that either antagonize the effects of FluG or lead to direct stimulation of FadA, prolonging proliferative growth while inhibiting both sporulation and secondary metabolism.

## Materials and methods

### *Aspergillus strains, growth conditions and genetics*

The *Aspergillus* strains used in this study are described in Table IV. Standard *A.nidulans* genetic and transformation techniques were used (Pontecorvo *et al.*, 1953; Yelton *et al.*, 1984). When appropriate, relevant genotypes of strains generated for this study were confirmed by genomic DNA Southern blot analyses. The *A.nidulans aflR* deletion strain TMF4.12 was created by transforming PW1 with pMF4 (M.Fernandes, N.P.Keller and T.H.Adams). RJH003 is the meiotic progeny of a cross between RBN138 and TMF4.12. RJF009 is the meiotic progeny of a cross between RBN026 (Wieser *et al.*, 1994) and PW1. RJYE07 (*biA1*, Δ*flbA*::*argB*; Δ*fadA*::*argB*, *trpC801*, *veA1*) was transformed with pSH96 (Wieser and Adams, 1995) to give the *trpC*$^{+}$ strain TJYE07.1. *Aspergillus parasiticus* transformants containing the *A.nidulans fadA*$^{G42R}$ mutation were generated by co-transforming *A.parasiticus* strain B62 (Trail *et al.*, 1994) with pGAPN-2 (*niaD*-containing vector; kindly provided by Dr G.Payne, North Carolina State University) and pJYSM3 (*A.nidulans fadA*$^{G42R}$; Yu *et al.*, 1996b). *Aspergillus parasiticus* was transformed following the standard *A.nidulans* protocol (Yelton *et al.*, 1984) except that the time required for protoplast formation was increased to 3 h.

All *A.nidulans* strains were grown in and maintained on appropriately supplemented minimal medium (Käfer, 1977). *Aspergillus parasiticus* strain B62 was maintained on modified minimal medium with 88 mM sucrose (as a carbon source), nitrate-free minimal salts, trace elements for *A.parasiticus* (Abdollahi and Buchanan, 1981) and 10 mM ammonium tartrate (as a nitrogen source). *Aspergillus parasiticus* transformants were selected on the same medium as above but with 1.2 M sorbitol and 70 mM $NaNO_3$ (as a selective nitrogen source) instead of ammonium tartrate.

*stc* gene expression in *alcA*[*p*] fusion strains (shown in Table II and Figure 2) was examined by inoculating 500 ml minimal liquid nitrate medium with $1\times10^6$ spores/ml and shaking for 14 h at 300 r.p.m. at 37°C. Mycelium was then collected, washed once with minimal medium lacking glucose, divided into equal parts, transferred to flasks containing 100 ml minimal medium with 100 mM L-threonine (as a sole carbon source) and shaken for 24 h at 300 r.p.m. at 37°C. Samples were harvested at the time of the medium shift (0 h) and 12, 18 and 24 h after the shift. Cultures to examine ST production in *alcA*[*p*]–*flbA* fusion strains (shown in Figure 3) were grown as above except that samples were taken at 12, 24 and 36 h after shifting to threonine medium.

The timing of *stc* gene expression in strains with loss-of-function mutations in individual developmental genes (shown in Table I) was determined by inoculating liquid minimal medium with $1\times10^6$ spores/ml and growing for 14, 38, 62 and 86 h (300 r.p.m. at 37°C) before harvesting. Because only negligible amounts of ST accumulated under these growth conditions, it was not possible to accurately determine whether or not mutants produced ST. ST production (shown in Table III) and stationary culture transcript level (for Figure 4) was determined by inoculating $1\times10^5$ spores in 2 ml liquid complete medium (minimal medium with 2% glucose, 2% peptone, 1% yeast extract and 1% casamino acids) in an 8 ml vial and allowing to grow for 7 days (or 1–4 days for transcripts; Figure 4) at 30°C without shaking (stationary culture) as described previously (Yu and Leonard, 1995).

### *Thin layer chromatography (TLC) analysis*

ST was extracted from stationary cultures by adding 1 ml $CHCl_3$ directly to the 2 ml culture, vortexed thoroughly and allowed to stand for 5 min at room temperature before collecting the organic phase and centrifuging for 2–3 min to remove residual aqueous material. For 100 ml liquid shake cultures, 20 ml $CHCl_3$ was added directly, shaken for 30 min at 300 r.p.m. and then centrifuged for 10 min at 2000 r.p.m. to separate the organic phase. All samples were dried down and resuspended in 50 μl $CHCl_3$. Fifteen microliters of each extract were separated on a silica gel TLC plate in benzene:acetic acid (90:10) and, when necessary, toluene:ethyl acetate:acetic acid (80:10:10) solvent systems. The TLC plates were treated with aluminum chloride to enhance ST fluorescence upon exposure to longwave (365 nm) UV light (Stack and Rodricks, 1971).

### *Nucleic acid manipulations*

Total RNA was isolated from lyophilized mycelia using Trizol according to the manufacturer's instructions (Gibco BRL) and 20 μg each RNA were separated on formaldehyde gels. RNA blots were hybridized with appropriate probes: a 0.75 kb *Sst*II–*Sma*I *stcU*-specific fragment from plasmid pRB7 (Yu *et al.*, 1996a); a 2.5 kb *Eco*RI *flbA*-specific fragment from pBN30 (Lee and Adams, 1994b); a 1.3 kb *Eco*RV–*Xho*I *aflR*-specific fragment from pAHK25 (Brown *et al.*, 1996); a 1.8 kb *Bam*HI *argB*-specific fragment from pSalArgB (Berse *et al.*, 1983); the *alcA*-containing plasmid pJA1 (Adams and Timberlake, 1990).

The *aflR* disruption vector was generated by first eliminating the *Sma*I site in the multiple cloning site of pAHK25 (Brown *et al.*, 1996) by cutting with *Eco*RI and *Bam*HI in the vector, filling in the ends with Klenow fragment and religating. The resulting plasmid, pMF3, was digested with *Sma*I to remove a 0.85 kb fragment from the *aflR* coding region. pSalArgB (Berse *et al.*, 1983) was digested with *Bam*HI and the resulting 1.8 kb fragment containing the wild-type *argB* gene was treated with Klenow fragment to generate blunt ends that were in turn ligated with pMF3 digested with *Sma*I, creating pMF4 (M.Fernades, N.P.Keller and T.H.Adams), which was then used to transform PW1 to arginine prototrophy. *aflR* deletion mutants were identified through Southern blot analysis.

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