Coordinate control of secondary metabolite production and asexual sporulation in *Aspergillus nidulans*

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Microbial secondary metabolite production is frequently associated with developmental processes such as sporulation, but there are few cases where this correlation is understood. Recent work with the filamentous fungus *Aspergillus nidulans* has provided new insights into the mechanisms coordinating production of the toxic secondary metabolite sterigmatocystin with asexual sporulation. These processes have been shown to be linked through a common need to inactivate a heterotrimeric G protein dependent signaling pathway that, when active, serves to stimulate growth while blocking both sporulation and sterigmatocystin biosynthesis.

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Abbreviations

AF	aflatoxin
RGS	regulator of G protein signaling
ST	sterigmatocystin

Introduction

Many microorganisms including fungi and bacteria produce compounds described as secondary metabolites (e.g. pigments, alkaloids, toxins, antibiotics, gibberellins, and carotenoids) that are apparently not required for the life of organisms [1–3]. In some cases, as with antibiotics, bacterial toxins, and mycotoxins, these secondary metabolites take on primary importance because of their beneficial or detrimental effects to humans [3]. Although numerous fungal and bacterial secondary metabolites have been extensively characterized, the mechanisms regulating secondary metabolite biosynthesis are poorly understood. There are many exceptions but as a general rule secondary metabolites are made during the stationary phase of growth [2,4,5]. This link to stationary phase can also be associated with sporulation and this has led to the suggestion that control of secondary metabolism and development are intimately associated [4,5]. An excellent example of this phenomenon comes from *Streptomyces griseus* in which A-factor, a γ-butyrolactone molecule, has been shown to act as a signal that initates both sporulation and production of streptomycin [6–9]. In this case, A-factor interacts with a DNA-binding protein called ArpA that binds to DNA in the absence of A-factor to act as a transcriptional repressor [7,8]. When *S. griseus* enters stationary phase, A-factor accumulates to sufficient levels to interact with ArpA. This interaction is proposed to result in derepression of an as yet uncharacterized gene whose product activates pathways required for aerial hyphae formation leading to sporulation and for streptomycin production and resistance [7,8].

This review focuses on another example of secondary metabolism that is in some way coordinated with sporulation. This example occurs in the filamentous fungus *Aspergillus nidulans* which is known to produce several secondary metabolites including the peptide antibiotic penicillin G and the carcinogenic polyketide mycotoxin sterigmatocystin (ST), a precursor of the better known fungal metabolite, aflatoxin (AF) [10–12]. Although the mechanisms controlling penicillin biosynthesis have been shown to be complex, with major controls involving response to alkalinization of the medium, there is no indication that this regulation is in any way coordinated with developmental processes [12–14]. By contrast, numerous observations have suggested that the ability to complete asexual sporulation may be a prerequisite for ST/AF biosynthesis in *Aspergillus spp.* [4,15,16]; however, this link is not absolute. Although sporulation is typically accompanied by ST/AF production, the converse is not always true because ST/AF can be produced at high levels in growth conditions where sporulation is inhibited (i.e. submerged culture).

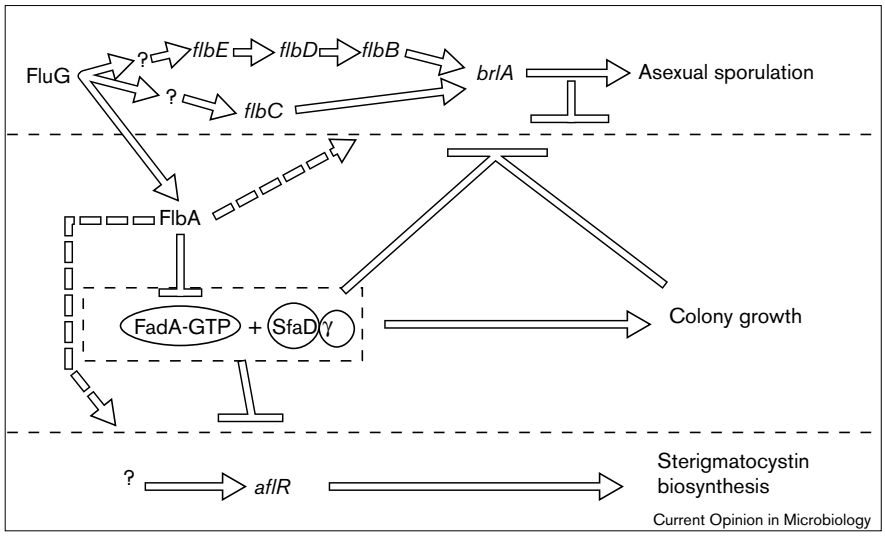
Controlled initiation of asexual sporulation

Asexual sporulation in *A. nidulans* involves formation of complex multicellular structures called conidiophores that produce chains of uninucleate mitotically derived spores called conidia. The asexual reproductive cycle can be simplistically divided into three stages that begin with a growth phase, which is required for cells to become competent to respond to unknown induction signals [17•,18,19]. Growth continues indefinitely in the absence of sporulation as long as cells are maintained in submerged culture with nutritionally sufficient medium. Next comes the initiation phase, which begins when cells that have acquired the ability to respond to unknown inducing signals are exposed to air. Finally, the execution phase occurs, involving activation of developmentally specific events and culminating in conidiophore and conidium development [17•]. Within the context of a developing colony all three of these phases occur simultaneously with vegetatively growing hyphae at the leading edge of the colony and developmental initiation and execution of conidiophores bearing conidia beginning 1–2 mm behind the margin. Our genetic studies of early events leading to sporulation have led us to propose that a major determinant in the transition from growth to sporulation involves coordination of two antagonistic signal transduction pathways, one for growth and one for sporulation [17•,20].

In our model describing control of growth and sporulation (Figure 1) we propose that growth signaling is mediated through the activity of *fadA* (fluffy autolytic dominant) and *sfaD* (suppressor of *flbA*) that encode the α and β subunits of a heterotrimeric G protein, respectively ([20,21]; S Rosen, J-H Yu, TH Adams, unpublished data). Mutations in *fadA*, which are predicted to interfere with the intrinsic GTPase activity of the α subunit of this G protein and, therefore, lock it in a constitutively active, GTP-bound state, cause a block in sporulation and result in a proliferative growth phenotype described as fluffy [20]. For developmental activation to occur it is necessary to at least partially inhibit this FadA-dependent signaling pathway and this requires the activity of another gene called *flbA* (fluffy low *brlA*) [20,22]. The FlbA protein belongs to the RGS (regulator of G protein signaling) domain family of proteins found in all eukaryotes [23–25]. RGS domain proteins have been implicated in negatively regulating G-protein mediated signaling pathways by activating the intrinsic GTPase activity of specific G protein α subunits [26]. Strains with *flbA* loss-of-function mutations have proliferative growth phenotypes like the *fadA*-activation mutants (i.e. loss of GTPase), as would be predicted if FlbA inactivates FadA signaling by converting FadA–GTP to FadA–GDP [20,22]. The proliferative growth phenotype of FlbA loss-of-function mutants can be suppressed by loss-of-function mutations in either *fadA* or *sfaD*, as expected if the major role of FlbA is inactivation of the FadA-dependent signaling pathway ([20,21]; S Rosen, J-H Yu, TH Adams, unpublished data). Although this model explains most of the activities of FlbA, it is likely that FlbA has additional functions. This realization stems from the observation that overexpression of *flbA* can activate inappropriate asexual sporulation in both wild-type strains and *fadA*-deletion mutants [20].

Development-specific signaling apparently requires the *fluG* gene product [27]. *fluG* mutants result in a fluffy proliferative growth phenotype like that seen with *flbA* and *fadA* mutants but differ in that wild type and other fluffy mutants are able to rescue this defect extracellularly [27]. We have proposed that FluG is required for production of an extracellular sporulation-specific signal and that other gene products are required for responding to this signal [27]. This hypothesis is supported by the finding that *fluG* overexpression can cause inappropriate developmental activation and that this activity requires wild-type functions of other early-acting developmental regulators including *flbA* [27,28]. Because *flbA*-induced sporulation also requires wild-type *fluG* function, we have proposed that there are two distinct effects of responding to the FluG sporulation-inducing factor [28]. First, FlbA is activated which interferes with the FadA-dependent signaling of proliferation [28]. Second, a development-specific pathway is activated that requires numerous other genes including *flbB*, *flbC*, *flbD*, *flbE*, and *brlA* to result in elaboration of conidiophores [28–31]. Because the sporulation defects of *flbA*-null mutants are suppressed by *fadA* (or *sfaD*) loss-of-function mutations but the defects in *fluG* mutants are not, we have concluded that the development-specific role of FluG is FadA independent ([20]; S Rosen, J-H Yu, TH Adams, unpublished data). Both FluG-dependent functions, however, must occur if development is to proceed.

Figure 1



Genetic interactions controlling *Aspergillus* growth, asexual sporulation, and sterigmatocystin (ST) biosynthesis. Two antagonistic signaling pathways control *A. nidulans* growth, asexual development, and production [20,36••]. One pathway requires the product of FluG activity, which is proposed to work as an extracellular signal to activate a sporulation-specific pathway that requires *flbB*, *flbC*, *flbD*, *flbE,* and *brlA* [27–31]. When the FadA Gα protein is GTP bound (active) it is expected to be dissociated from the SfaD(Gβ)–Gγ heterodimer ([20]; S Rosen, JH Yu, TH Adams, unpublished data). Then, both FadA and SfaD–Gγ control downstream effectors to enhance proliferation and repress both asexual sporulation and ST production (controlled by *aflR*) [20,36••]. This FadA (and SfaD) dependent growth signaling pathway is modulated by FlbA and FluG activities [20]. The FluG signal causes inactivation of FadA by activating FlbA which functions as a GTPase activating protein to turn off FadA-dependent signaling [20]. Inactivation of FadA allows both asexual sporulation and ST biosynthesis to occur [20,36••]. It is important to note that the main role of FluG in controlling ST biosynthesis is not direct but instead works through activation of FlbA [36••]. Finally, *brlA* activation has also been shown to cause growth inhibition [30,38]. The dashed arrows describe possible Gα (FadA)-independent FlbA functions that can activate ST production and asexual sporulation [20,36••].

Coordination of ST production with growth control and sporulation

Examination of the genetic mechanisms underlying ST production in *A. nidulans* led to identification of a 60 kb cluster of 25 co-regulated genes (called *stc* genes), many of which are known to function in ST biosynthesis [32–34]. Whereas most of these genes encode enzymes in the complex biosynthetic pathway, one ST cluster gene, *aflR*, encodes a zinc binuclear cluster DNA-binding protein that acts as a pathway specific transcriptional activator of other genes in the ST pathway [34,35•]. *aflR* mRNA accumulation is regulated during the lifecycle such that accumulation begins in early stationary phase with activation of the other genes needed for ST production quickly following [34]. The activity of *aflR* is required for expression of *stc* genes and misexpression of *aflR* during growth is sufficient to activate *stc* gene expression under inappropriate conditions [34]. These *aflR* mutants, however, sporulate normally and misexpression of *aflR* has no developmental consequences.

To begin to address the mechanistic aspects of the relationship between asexual sporulation and ST production, we examined the consequences of early acting developmental mutations on regulation of ST biosynthesis. We found that null mutations in *flbA* or *fluG*, or dominant activating mutations in *fadA*, blocked *stc* gene activation and ST production just as they had blocked asexual sporulation [36••]. Moreover, overexpression of *flbA* (but not *fluG*) or dominant inactivating *fadA* mutations caused early activation of *stc* genes and ST biosynthesis [36••]. Finally, *fadA* loss-of-function mutations suppressed the need for either *flbA* or *fluG* in ST production [36••]. This is an important contrast to observations with sporulation where *fadA* loss-of-function suppressed *flbA* but not *fluG* mutants [20].

These results are consistent with a model in which asexual sporulation and ST biosynthesis are linked by a common need to slow growth by inactivation of a growth signaling pathway (Figure 1). FluG is required for both ST production and development only because of its dual role in stimulating development-specific events and activating FlbA, which then inactivates FadA [36••]. Because active FadA signals events that antagonize both sporulation and ST biosynthesis, FadA inactivation is essential for both processes. As with sporulation, however, it is important to recognize that FlbA must have additional abilities in that forced overexpression of *flbA* in a *fadA* deletion strain stimulates both sporulation and ST production just as in the wild type [36]. An important question remaining to resolve is whether this role for FlbA is distinct from its activity with FadA or whether it stems from stimulating GTPase activity of another G α protein.

Although the model presented above explains why many *Aspergillus* mutants defective in sporulation have also lost the ability to produce ST/AF, it in no way encompasses all of secondary metabolism. Although *flbA* and *fluG* loss-of-function mutants and *fadA* dominant-activating mutants fail to produce ST, they produce a variety of unique compounds in its place [36••,37] and have not lost the ability to make penicillin G (JK Hicks, NP Keller, TH Adams, unpublished data). It is possible that these other metabolites represent molecules that in contrast to ST/AF are stimulated by FadA-mediated signaling. Similar results have been observed in *Streptomyces* where A-factor stimulates both sporulation and streptomycin production but overexpression of the developmental regulatory gene *whiG* in *Streptomyces coelicolor* causes sporulation while blocking production of the antibiotic actinorhodin [6,7]. These results would seem to indicate that a general rule will not define the intricate interactions between growth, development, and secondary metabolism.

Conclusions

The finding that activation of ST/AF production and asexual sporulation is coordinated through a common need to control growth raises some important questions. For instance, it would be interesting to know if coordination of these apparently dissimilar pathways provides any biological advantage to *Aspergillus*. Given that no biological role for ST/AF has been determined this is a difficult question to resolve. It is known that ST and AF are frequently found in spores and other differentiated tissues of the producing fungi lending some support to the idea that there is an as yet undetermined significance to coordinating these processes but this could as easily be a coincidence. Of greater practical importance is the question of whether the finding that active FadA blocks ST production in *A. nidulans* could be exploited in designing strategies to control AF contamination of crops. The observation that dominant activating *A. nidulans fadA* alleles cause a block in AF production when stably transformed into *Aspergillus parasiticus* indicates that this is a broadly conserved regulatory strategy and not simply an oddity of *A. nidulans* [36••]. Identifying ligands, receptors, and effector molecules involved in FluG and FadA dependent signaling pathways could provide means of prolonging stimulation of FadA to extend proliferative growth phases while blocking conidiation and AF/ST accumulation.

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