The *Aspergillus nidulans* *sfaD* gene encodes a G protein β subunit that is required for normal growth and repression of sporulation

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flbA encodes an *Aspergillus nidulans* RGS (regulator of G protein signaling) domain protein that antagonizes FadA (G α -subunit of heterotrimeric G protein)-mediated growth signaling to allow asexual development. We previously defined and characterized five suppressors of *flbA* (*sfa*) loss-of-function mutations and showed that one suppressor (*sfaB*) resulted from a novel dominant-negative allele of *fadA*. In this report we show that a second suppressor gene (*sfaD*) is predicted to encode the β subunit of a heterotrimeric G protein. Deletion of *sfaD* suppressed all defects resulting from complete loss-of-*flbA* function mutations, caused a hyperactive sporulation phenotype and severely reduced vegetative growth. However, the *sfaD* deletion could not suppress the growth activation caused by dominant-activating *fadA* alleles, indicating that constitutively active FadA can cause proliferative growth in the absence of G $\beta\gamma$ signaling. We propose that SfaD and FadA are both positive growth regulators with partially overlapping functions and that FlbA has an important role in controlling the activities of both proteins. Inactivation of signaling events stimulated by both components of the heterotrimeric G protein is essential for both sexual and asexual sporulation.

Keywords: *Aspergillus nidulans*/conidiophore/growth regulation/heterotrimeric G protein/RGS domain

Introduction

The balance between growth, sporulation and mycotoxin (sterigmatocystin) production in the filamentous fungus *Aspergillus nidulans* is controlled by the genes *flbA* and *fadA*. FlbA is an RGS (regulator of G protein signaling) domain protein that is required to suppress growth signaling by FadA, the α subunit for a heterotrimeric G protein. This FlbA-dependent inhibition of FadA signaling is required for sporulation and sterigmatocystin (ST) production to occur (Yu *et al.*, 1996; Hicks *et al.*, 1997; Adams *et al.*, 1998). Both genetic and biochemical experiments using several different RGS domain proteins have demonstrated that the major role for this conserved domain is to regulate G protein signaling negatively by facilitating

the intrinsic GTPase activity of the G α subunit (Dietzel and Kurjan, 1987; Berman *et al.*, 1996; Koelle and Horvitz, 1996; Yu *et al.*, 1996). The three-dimensional structure of a complex between the RGS protein RGS4 and G α 1 (Tesmer *et al.*, 1997), as well as other recent studies (Hepler *et al.*, 1997; Yan *et al.*, 1997), suggest that some RGS proteins also can function as effector antagonists that compete for effector binding to G α .

The role of *flbA* in controlling growth and asexual sporulation was clarified by analyzing epistatic interactions between *flbA* and *fadA* gain- and loss-of-function mutations (Figure 1). Dominant-activating mutations in *fadA* that are predicted to eliminate the intrinsic GTPase activity of this G α subunit (*fadA*^{G42R}, *fadA*^{R178C} and *fadA*^{Q204L}) cause the same phenotype as loss-of-function mutations in *flbA*: a block in both asexual and sexual sporulation accompanied by uncontrolled growth that leads to proliferation of undifferentiated hyphal masses that autolyse as the colonies mature (Lee and Adams, 1994a; Yu *et al.*, 1996, 1999). In contrast, both loss-of-function and dominant interfering mutations in *fadA* (*fadA*^{G203R}) resulted in reduced growth and suppressed the developmental defects of *flbA* deletion mutant strains. These results are consistent with the hypothesis that the normal role of FlbA in controlling sporulation is to inactivate FadA-dependent growth signaling (Yu *et al.*, 1996).

While many aspects of the requirement for FlbA in sporulation could be explained through its role in inactivating FadA, we also found that overexpression of *flbA* could cause inappropriate activation of asexual development and ST production both in wild-type and *fadA* deletion strains. This result raised the possibility that FlbA could have other targets in addition to FadA that must be affected to allow sporulation and ST production (Yu *et al.*, 1996; Hicks *et al.*, 1997). In this respect it is interesting to note that the *fadA*^{G203R} dominant-interfering mutation not only suppressed loss-of-function mutations in *flbA* but also caused hyperactive asexual sporulation and resulted in production of asexual sporulation structures, called conidiophores, even under environmental conditions that blocked wild-type sporulation. Because deletion of *fadA* did not cause hyperactive sporulation and the *fadA*^{G203R} mutation is predicted to inactivate signaling by preventing dissociation of active, GTP-bound, G α protein from the G $\beta\gamma$ subunits, one possible second target for FlbA could be the G $\beta\gamma$ dimer (Yu *et al.*, 1996). However, it is also possible that the secondary role of FlbA is to inactivate G α proteins other than FadA.

In order to understand better the complex role of FlbA in controlling growth, development and ST production, we isolated extragenic suppressors of loss-of-function mutations in *flbA* (Yu *et al.*, 1999). Two genes identified by these mutations, *sfaC* and *sfaD*, were of particular interest, because mutations suppressed loss of FlbA

Fig. 1. FlbA and FadA have a major role in controlling *A. nidulans* growth and development. We have previously proposed that two antagonistic signaling pathways regulate growth and asexual development in *A. nidulans*. Activated FadA G α protein (GTP bound) affects downstream effector molecules to increase growth and repress sporulation. Either constitutive activation of FadA (G42R, Q204L, R178C, R178L) or loss-of-function FlbA result in a fluffy non-sporulating and autolytic phenotype. FlbA is an RGS domain protein that functions as a GTPase-activating protein (GAP) to inactivate FadA (GDP bound). This inactivation of FadA then allows asexual development to occur. However, because forced expression of *flbA* resulted in inappropriate spore formation even in a *fadA* deletion mutant, FlbA must have additional function in activating asexual sporulation. Developmental signals that are necessary for both activation of FlbA and asexual development require the wild-type FluG activities. The relevant mutant phenotypes are described.

function and caused constitutive sporulation in submerged culture similar to that observed with *fadA*^{G203R} dominant-interfering mutants. Here we show that *sfaD* is predicted to encode the β subunit of a heterotrimeric G protein. Characterization of *sfaD* mutations and their interactions with *flbA* and *fadA* support the hypothesis that both SfaD and FadA are positive growth regulators that negatively affect asexual and sexual development in *A. nidulans*. FlbA is required for controlling the activities of both FadA and SfaD.

Results

The *A. nidulans sfaD* gene encodes a G β subunit for a heterotrimeric G protein

Five different genes (designated *sfaA*–*sfaE*) were defined previously as extragenic suppressors of loss-of-function mutations in *flbA* (Yu *et al.*, 1999). We showed that one of these suppressors (*sfaB*) resulted from a novel dominant-negative mutation in *fadA* (*fadA*^{R205H}) and proposed that one target for other suppressor mutations could be genes encoding the β or γ subunits that associate with FadA. Because a previously defined dominant-negative *fadA* mutation (*fadA*^{G203R}) caused the abnormal phenotype of asexual sporulation during growth in submerged culture, we were particularly interested in further characterizing two *flbA* suppressor mutations, *sfaC* and *sfaD*, which caused a similar submerged sporulation phenotype. Both *sfaC* and *sfaD* mutations were dominant, which led us to attempt to clone the mutant genes by constructing cosmid libraries with genomic DNA from either *sfaC* or *sfaD* mutants and using these to transform an *flbA*98; *argB2* mutant strain (fluffy autolytic), followed by visual screen-

ing to identify transformants that conidiated like wild type. However, after examining >3000 transformants each with *sfaC*67 and *sfaD*82 libraries, no suppressed *flbA* transformants were identified.

In order to test the hypothesis that *sfaC*, *sfaD*, or one of the other suppressor loci might encode the G β subunit for a heterotrimeric G protein, we identified a gene encoding a G β subunit by using PCR with degenerate primers designed around conserved sequences in known G β proteins (Materials and methods). This approach resulted in the isolation of a gene with an open reading frame (ORF) predicted to encode a 352 amino acid polypeptide (Figure 2A) that shares 60% identity with mammalian G β proteins. This *A. nidulans* ORF was most similar (82% identity) to Cpgb-1 from *Cryphonectria parasitica* (Kasahara and Nuss, 1997), the only other G β subunit so far reported from a filamentous fungus, but shared much less similarity (~40% identity) with the *Saccharomyces cerevisiae* G β homologue Ste4p. As with other G β subunits (Wall *et al.*, 1995; Sondek *et al.*, 1996), the *A. nidulans* ORF (Figure 2B and C) included seven highly conserved ~40 amino acids repeats terminated by a Trp–Asp dipeptide (so-called ‘WD40 repeats’).

Because we initiated these experiments to learn whether any of the mutations we identified based on their ability to suppress loss-of-function *flbA* mutations corresponded to a gene encoding the G β subunit, it was of interest to learn whether a G β deletion mutation could suppress a *flbA*-null mutation. To test this, we first constructed an *A. nidulans* strain (TSRB1.16) lacking the G β -encoding gene (Δ G β ; see Materials and methods). TSRB1.16 was then meiotically crossed with a Δ *flbA* mutant to generate Δ *flbA*; Δ G β double mutant strains. As shown in Figure 3, the Δ *flbA*; Δ G β mutant sporulated almost as wild type, indicating that the G β deletion suppressed the sporulation defect of Δ *flbA* mutants.

The question of whether a previously defined *flbA* suppressor mutation could represent an allele of the G β -encoding gene was addressed by sexually crossing a Δ *flbA*; Δ G β mutant strain (RSRF1.31) with *flbA*[−] strains having *sfaA*^s, *sfaC*^s, *sfaD*^s or *sfaE*^s suppressor mutant alleles. Approximately 25% of the progeny from crosses with RSRF1.31 and *flbA*[−];*sfaC*^s or *flbA*[−];*sfaE*^s had the *flbA*[−] phenotype (fluffy autolytic non-sporulating progeny) indicating that these *sfa* mutations were not linked to the G β -encoding gene. We were unable to cross RSRF1.31 with the *flbA*[−];*sfaA*^s; however, no fluffy progeny were recovered from crosses between RSRF1.31 and various *sfaD*^s mutant strains, indicating that these *sfaD* suppressor mutations could represent alleles of G β .

To address further the question of equivalence for *sfaD* and the G β -encoding gene, we next constructed genomic DNA libraries from each of five different *sfaD* mutant strains (*sfaD*82, *sfaD*123, *sfaD*125, *sfaD*126 and *sfaD*127) and isolated the G β -encoding genes by hybridization (Materials and methods). Because we had previously shown that each of these *sfaD*^s alleles was dominant to wild type under normal growth conditions at 37°C, each G β clone from the different *sfaD*^s mutants was used to transform a *flbA*[−] mutant strain. More than half of the transformants using the G β clones from the *sfaD*123 and *sfaD*127 mutant strains sporulated like wild type, supporting the hypothesis that *sfaD* encodes the putative

Fig. 2. The *sfaD* gene structure. (A) A restriction map of a 3.2 kb *PstI* fragment containing the *sfaD* gene is shown. The direction of *sfaD* transcription was deduced by analysis of cDNA clones and is indicated by the arrow positioned above the map. Approximate intron positions are indicated by discontinuities in the arrow. The location of the *sfaD* coding region is represented by the black box. The *XhoI* site just upstream of initiation ATG and downstream of stop codon was introduced by site-directed mutagenesis and used for constructing the *sfaD* deletion plasmid, pSRBD1. Restriction sites are abbreviated: A, *ApaI*; Bg, *BglIII*; K, *KpnI*; P, *PstI*; R, *EcoRI*; S, *SmaI*; V, *EcoRV*; X, *XhoI*. (B) The deduced amino acid sequence of SfaD. The figure shows the alignment of the seven WD repeats in SfaD with amino acids predicted to be members of a structural triad of amino acids and the trademark tryptophan, indicated by plus signs (Sondek *et al.*, 1996). Mutations in the different alleles of *sfaD* are underlined and introduced amino acids after conversion are in bold. An asterisk indicates an introduced stop codon; fs, frame shift. (C) A schematic representation of the different mutant alleles of the SfaD protein compared with wild-type SfaD. The seven WD repeats are boxed. Corresponding amino acid changes and nucleotide changes are shown where A of the ATG is indicated as 1. The DDBJ/EMBL/GenBank accession No. for the *sfaD* nucleotide and peptide sequences is AF056182.

heterotrimeric G protein β subunit. In contrast, all of the transformants using G β clones from *sfaD82*, *sfaD125* and *sfaD126* had a *flbA*⁻ phenotype, indicating that either the mutant alleles were not dominant in a merodiploid or *sfaD* does not encode the putative G β protein. Finally, we sequenced the G β -encoding gene from each *sfaD*^s mutant strains and found that each strain had different mutations in the G β -encoding gene, indicating that *sfaD* indeed encodes the putative G β protein (Figure 2).

As shown in Figure 2, the *sfaD123* and *sfaD127* mutant alleles, which each behaved as dominant mutations in the transformation assay described above, result from missense

mutations. For *sfaD123*, the highly conserved aspartate at position 258 in WD-repeat number five was converted to an asparagine, while for *sfaD127*, a cysteine at position 176 in WD-repeat three was changed to tyrosine. In contrast, the *sfaD82*, *sfaD125* and *sfaD126* mutant alleles that behaved as recessive mutations in the transformation assay all represent nonsense or frameshift mutations. The *sfaD82* and *sfaD125* mutations introduced stop codons at amino acid 138 (TCA to TAA) and 109 (TGG to TAG), respectively. The *sfaD126* had an insertion of a cytosine converting arginine 312 to proline and introducing a frameshift in the ORF that leads to early termination at amino acid 333. This allele was distinct in that its ability to suppress the *flbA*⁻ phenotype was cold sensitive; it could suppress the *flbA*⁻ phenotype at 37°C but not at 25°C.

Because previous data indicated that mutations in all of the different *sfaD* alleles described here were dominant in diploids that were heterozygous for *sfaD* and homozygous for Δ *flbA* (e.g. *sfaD82*; Δ *flbA*/*sfaD*⁺; Δ *flbA*) when grown at 37°C (Yu *et al.*, 1999), yet *sfaD82*, *sfaD125* and *sfaD126* behaved as recessive mutations in transformation experiments, it was of interest to investigate the dominance relationships of Δ *sfaD* mutations in diploid strains. Diploids were generated from heterokaryons formed between RSRF1.31 (Δ *sfaD*; Δ *flbA*) and RJH046 (*sfaD*⁺; Δ *flbA*) and the resulting diploid strain (Δ *sfaD*; Δ *flbA*/*sfaD*⁺; Δ *flbA*; DSRGF1.1) was found to sporulate, although it was partially fluffy. This indicates that deletion of *sfaD* is partially dominant, perhaps due to altered ratios of G protein α , β and γ subunits.

SfaD and *FadA* both mediate growth signaling

As shown in Table I, the radial extension rate for *sfaD* deletion mutant strains grown on solid medium was similar to that observed for wild type, although the hyphae were less dense (Figure 3). In submerged cultures, the Δ *sfaD* mutant grew significantly slower than wild type with biomass accumulating to only 20% of wild-type levels (Table I). Biomass accumulation for submerged cultures of Δ *fadA* strains was also reduced significantly, to 63% of wild-type levels. The biomass of a Δ *sfaD*; Δ *fadA* double mutant strain was nearly the same as a Δ *sfaD* mutant, ~20% of wild-type levels. While the conidiation defect of Δ *flbA* mutants was suppressed by either Δ *sfaD* or Δ *fadA*, Δ *flbA* suppressed the growth defect observed in Δ *fadA* but not Δ *sfaD* mutants. These results support the hypothesis that both *FadA* and *SfaD* are required for normal growth but that these activities are at least somewhat distinct in their requirements for *FlbA*.

The *fadA* gene was originally identified by a dominant-activating mutation that is predicted to inactivate its intrinsic GTPase activity and lock *FadA* in a constitutive signaling GTP-bound state. Such mutations result in a fluffy phenotype much like that observed in Δ *flbA* mutants and characterized by increased hyphal growth with a complete absence of sexual and asexual sporulation. To test whether this phenotype was dependent on the presence of an intact G protein β subunit, we transformed a Δ *sfaD* mutant with *fadA*^{R178C} or *fadA*^{Q204L} dominant-activating alleles. Several strains containing the wild-type *fadA* gene and one or more copies of either of these dominant-activating alleles were recovered. In addition, one strain was isolated where the wild-type *fadA* gene had been

Fig. 3. Phenotypes of *sfad* mutant strains. (A) The wild-type (FGSC26), (B) $\Delta flbA$ (RJH046), (C) $\Delta sfad$ (RSRB1.15) and (D) $\Delta sfad; \Delta flbA$ (RSRF1.34) strains were point inoculated onto minimal medium (Käfer, 1977) and allowed to grow for 3 days at 37°C. (E and F) Wild-type (FGSC 26) and $\Delta sfad$ (RSRB1.15) strains after 10 days of growth. At this time point the $\Delta sfad$ deletion strain (F) showed a dramatically increased number of Hülle cells formed compared with wild type (E). These Hülle cells, which are structures proposed to function in supporting fruiting body formation in *A.nidulans*, formed white fluffy aggregates on the surface of the colony. Lower panels represent a 30-fold magnification of the colonies in upper panels. Arrowheads in the lower panel in (B) indicate areas where a $\Delta flbA$ strain has started to autolyse.

replaced with the *fadA*^{R178C} allele. All strains that had acquired a dominant-activating *fadA* allele were fluffy, non-sporulating and autolytic, as was expected if loss of *sfad* did not influence the mutant phenotype. The inability to isolate asexual spores from these mutants prevented analysis of biomass accumulation in submerged culture.

***SfaD*-mediated growth signaling negatively affects sporulation**

Wild-type *A.nidulans* strains do not sporulate in submerged culture under normal growth conditions (Adams *et al.*, 1998). However, our previous results showed that *sfad82* and *sfad125* mutant strains were able to sporulate in submerged culture, and Figure 4 shows that $\Delta sfad$ mutant strains also elaborated nearly complete conidiophores during growth in submerged culture by between 19 and 22 h post-inoculation. This phenotype was not dependent on either *fadA* or *flbA*, because $\Delta sfad; \Delta fadA$ and $\Delta sfad; \Delta flbA$ double mutants also sporulated with identical timing under submerged growth conditions (Figure 4; Table I). As shown previously, *sfad123*, *sfad126* and *sfad127* mutant strains did not produce conidiophores in submerged cultures.

While $\Delta sfad$ mutants always sporulated in submerged culture, we found that the composition of the growth medium had interesting effects on the timing and extent of conidiophore development by $\Delta sfad$ mutant strains. During growth in standard minimal nitrate medium (Käfer, 1977), the *sfad* deletion strains occasionally developed complete conidiophores between 19 and 22 h post-inoculation (Table II). Addition of 10 or 100 mg/l of either yeast extract or casein hydrolysate (Difco Laboratories) to the minimal medium resulted in increased growth and caused a 30- to 300-fold increase in the number of conidiophores produced (Table II). Because yeast extract and casein hydrolysate have in common an abundance of amino acids that could serve as a complex nitrogen source, we examined the potential for better defined nitrogen

Table I. Phenotypic characteristics of *fadA*, *flbA* and *sfad* mutant strains

Relevant genotype ^a	Growth (%)		Submerged conidiation ^d
	Radial extension ^b	Dry weight ^c	
WT	100	100	–
$\Delta sfad$	108 ± 2.8	21 ± 2.1	+
$\Delta sfad; \Delta flbA$	96 ± 3.8	34 ± 2.2	+
$\Delta sfad; \Delta fadA$	66 ± 2.9	19 ± 1.9	+
$\Delta flbA$	98 ± 2.9	119 ± 4.6	–
$\Delta fadA$	84 ± 2.6	63 ± 2.0	–
$\Delta fadA; \Delta flbA$	99 ± 1.5	114 ± 3.1	–
<i>fadA</i> ^{G203R}	75 ± 2.9	72 ± 12.2	+
$\Delta sfad; fadAG203R$	60 ± 2.3	26 ± 8.8	+
<i>fadA</i> ^{R178L}	101 ± 2.1	ND	–
$\Delta sfad; fadAR178C$	101 ± 0.7	ND	–
$\Delta sfad; fadAQ204L$	102 ± 1.9	ND	–

^aAll strains are *veA1*.

^bRadial extension rate of colonies grown on minimal medium (Käfer, 1977) at 37°C was determined as millimeters per hour and values reported as percent (mean ± SE) of wild-type extension rate (wild type: 0.43 ± 0.003 mm/h). For each relevant genotype *n* = 4.

^cThe dry weight of each strain was determined following 21 h growth in minimal liquid medium as described in Materials and methods. Values reported represent percent (mean ± SE) of wild-type dry weight (wild type: 0.26 ± 0.01 g). The experiment was repeated six times with one to three cultures from each strain. For each relevant genotype *n* = 6–12.

^d‘+’ indicates strains that formed conidiophores in submerged cultures after 16–18 h of incubation in minimal medium supplemented with 1.0 g/l of yeast extract. ‘–’ indicates strains that had not formed conidiophores by 26 h growth.

sources to enhance submerged growth and development. As shown in Table II, we found that inclusion of 6 mM ammonium tartrate enhanced both growth and sporulation. In contrast, both glutamine and asparagine provided enhanced growth but did not stimulate conidiophore formation. For all media tested, the pH of the growth medium varied between 6.2 and 6.7 at 19 h post-inoculation. To

Fig. 4. A complete loss-of-SfaD function eliminates the need of air and FlbA activity for conidiophore development. Conidia (1×10^6 conidia/ml) from (A) wild type (FGSC26), (B) $\Delta flbA$ (RJH046), (C) $\Delta sfaD$ (RSRB1.15) and (D) $\Delta sfaD; \Delta flbA$ (RSRF1.31) were inoculated into 100 ml of supplemented liquid minimal media (Käfer, 1977) containing 1 g yeast extract per liter in 250 ml flasks and incubated at 37°C shaking at 300 r.p.m. Mycelia from each culture were observed microscopically every hour starting at 12 h of growth (final observation at 26 h) to assess development. Micrographs were taken at 17 h when *sfaD* deletion (C) and $\Delta sfaD; \Delta flbA$ (D) strains formed conidiophores (arrowheads). Wild-type (A) and $\Delta flbA$ (B) strains never produced conidiophores under these conditions. All strains tested had a mutation in the *veA* gene (*veA1*). The scale bar shown in (A) is 20 μ m.

Table II. Media composition affects submerged conidiation and growth of *sfaD* deletion strains

Medium ^a	$\Delta sfaD$		Wild type
	Conidiophores per field ^b	Dry weight (g) ^c	Dry weight (g) ^c
Minimal medium	0.05 $\pm$ 0.03	0.03 $\pm$ 0.003	0.15 $\pm$ 0.002
+ 10 mg/l of yeast extract ^d	2.05 $\pm$ 0.46*	0.08 $\pm$ 0.003*	0.17 $\pm$ 0.007*
+ 100 mg/l of yeast extract	17.7 $\pm$ 1.93*	0.10 $\pm$ 0.006*	0.23 $\pm$ 0.006*
+ 10 mg/l of casein hydrolysate ^e	1.73 $\pm$ 0.16*	0.06 $\pm$ 0.003*	0.16 $\pm$ 0.004*
+ 100 mg/l of casein hydrolysate	12.6 $\pm$ 3.81*	0.09 $\pm$ 0.007*	ND ^f
+ 0.5 mM ammonium tartrate	0.16 $\pm$ 0.09	0.04 $\pm$ 0.004	ND
+ 6 mM ammonium tartrate	6.62 $\pm$ 2.22*	0.07 $\pm$ 0.006*	ND
+ 6 mM glutamine	0.04 $\pm$ 0.03	0.12 $\pm$ 0.010*	0.25 $\pm$ 0.005*
+ 6 mM asparagine	0.10 $\pm$ 0.05	0.13 $\pm$ 0.007*	ND

^aCultures of RSRB1.15 ($\Delta sfaD$) and FGSC 26 (wild type) were grown in minimal nitrate medium (Käfer, 1977) or minimal nitrate medium supplemented as described.

^bConidiophores were quantified after 19 h of growth using an Olympus BH2 compound microscope at 400 $\times$ magnification and numbers represent the mean of 10 randomly chosen fields. The experiment was repeated three times with duplicate samples (mean $\pm$ SE, $n = 6$).

^cDry weight was determined after 19 h of growth as described in Materials and methods.

The experiment was repeated three times with duplicate samples (mean $\pm$ SE, $n = 6$).

^dYeast extract from Difco Laboratories, MI.

^eEnzymatic hydrolysate of casein (Sigma; C-0626).

^fNot determined.

*Indicates a significant difference ($p < 0.05$; Welch two-tailed *t*-test) in conidiophore number or dry weight comparing supplemented with unsupplemented minimal medium.

ensure that differences in development did not result from differences in pH, we altered the initial pH of the medium between pH 5.7 and 7.2, but did not observe any significant effect on the number of conidiophores formed.

***SfaD* and *FadA* are required for normal sexual development**

Aspergillus nidulans reproduces sexually by forming ascospores in fruiting bodies called cleistothecia, which typically are surrounded by specialized globular cells, called Hülle cells, that are proposed to function in supporting cleistothecial development (Yager, 1992; Navarro-Bordonaba and Adams, 1994). As shown in Table III, we found that mutations in *fadA* and *sfaD* had major effects on sexual development. Deletion of *sfaD* resulted in an ~22-fold increase in Hülle cell formation relative to wild type (Figure 3F; Table III). However, no cleistothecia were ever produced. Similarly, the *fadA*^{G203R} dominant-negative mutation had an ~9-fold increase in Hülle cell

formation and cleistotheciation was completely blocked. In contrast, deletion of *fadA* did not result in increased Hülle cell formation compared with a wild-type strain, although these mutants did fail to form cleistothecia. $\Delta sfaD; \Delta fadA$ double mutants had an intermediate phenotype producing a 13-fold increase in Hülle cells and blocking cleistothecia. Finally, strains with dominant-activating *fadA* mutant alleles did not form Hülle cells or cleistothecia, regardless of *sfaD* genotypes, and the colonies were completely autolysed after prolonged incubation.

Although little is known about what controls sexual reproduction in *A.nidulans*, one gene that has been shown to influence cleistothecium formation is *veA*. Commonly used laboratory strains of *A.nidulans*, including those described here, have the *veA1* mutation, which has the effect of removing a light requirement that is normally present for asexual development and leading to enhanced asexual sporulation while reducing cleistothecium produc-

Table III. Mutations in *sfaD* and *fadA* affect sexual development

Relevant genotype	Hülle cells (per cm ²) ^a	Cleistothecia (per cm ²) ^a
WT; <i>veA1</i> (FGSC26)	91 500 ± 7 800	95 ± 14
Δ <i>sfaD</i> ; <i>veA1</i>	2 000 000 ± 101 000 ^b	0
Δ <i>fadA</i> ; <i>veA1</i>	116 000 ± 16 300	0
Δ <i>sfaD</i> ; Δ <i>fadA</i> ; <i>veA1</i>	1 230 000 ± 95 800 ^b	0
<i>fadA</i> ^{G203R} ; <i>veA1</i>	800 000 ± 96 300 ^b	0
WT; <i>veA</i> ⁺ (FGSC4)	363 000 ± 27 500	781 ± 75
Δ <i>sfaD</i> ; <i>veA</i> ⁺	7 800 000 ± 415 000 ^b	0

^aThe strains were grown on minimal medium (Käfer, 1977) for 10 days at 37°C. To promote sexual development the plates were sealed with tape 2 days after inoculation. The number of cleistothecia formed were quantified using a dissecting microscope. To quantify the number of Hülle cells an agar plug from the colony surface (1 cm²) was vortexed for 90 s in an Eppendorf tube containing 1 ml water with 0.2% (v/v) Tween 20 and 150 µl of glass beads (diameter = 0.4–0.6 mm) and quantified using a hemacytometer. Two different agar plugs were analyzed from each colony and three to five different colonies from each strain were analyzed. The experiment was repeated once. The significance of the difference in the number of Hülle cells formed between wild-type strain and mutant strains was determined calculating *t* (Welch two-tailed *t*-test). Mutant strains with *veA1* or *veA*⁺ were compared with FGSC26 and FGSC4 wild-type strains, respectively. Means and standard errors are presented in the table.

^bMutant strains that formed significantly different (*p* < 0.0001) number of Hülle cells compared with respective wild-type strains. The number of Hülle cells formed by the other mutant strains were not significantly different (*p* > 0.05) from respective wild-type strains.

tion (Mooney and Yager, 1990). To examine the effect of the wild-type *veA* gene on sexual and asexual sporulation by *sfaD* mutants, Δ *sfaD*;*veA*⁺ strains were generated and compared with Δ *sfaD*;*veA1* mutants (see Materials and methods). We generally found that asexual sporulation was reduced in Δ *sfaD*;*veA*⁺ strains and that mutation in the *veA* gene (*veA1*) is required for hyperactive sporulation in *sfaD* deletion strains. No conidiophores were observed in Δ *sfaD*;*veA*⁺ strains in submerged cultures, regardless of illuminating conditions. Furthermore, the biomass increased in Δ *sfaD*;*veA*⁺ strains compared with Δ *sfaD*;*veA1* strains from biomass 19 ± 1.4% (mean ± SE) to 61 ± 2.2% of wild-type (*sfaD*⁺) biomass (*n* = 12). The biomass for the *sfaD*⁺;*veA1* (FGSC26) and the *sfaD*⁺;*veA*⁺ (FGSC4) wild-type strains did not differ significantly from each other. The radial growth rates for Δ *sfaD*;*veA*⁺ strains were similar (0.44 ± 0.08 mm/h using minimal medium, *n* = 4) to Δ *sfaD*;*veA1* strains (see Table I).

Discussion

We showed previously that *fadA* encodes the α subunit of a heterotrimeric G protein in *A.nidulans*, which upon activation stimulates proliferation and antagonizes multiple processes including conidiation and secondary metabolism (Yu *et al.*, 1996; Hicks *et al.*, 1997). FadA signaling is inactivated by FlbA, an RGS domain protein that is proposed to function by stimulating the GTPase activity of FadA. The data presented in this paper demonstrate that the *sfaD* gene, which was identified as a suppressor of *flbA* loss-of-function mutations, probably encodes the β subunit for a heterotrimeric G protein. As detailed below, we propose that the Gβγ heterodimer containing SfaD is an active participant along with the FadA encoded Gα protein in signaling downstream effectors that deter-

Fig. 5. Coordinate control of *A.nidulans* growth and asexual development. We propose that both SfaDGγ and FadA function in response to unknown growth signals to stimulate growth in liquid submerged culture and for aerial growth (plate). FlbA is required for controlling the activities of both FadA and SfaDGγ. Our study supports that both SfaDGγ and the *veA* gene product mediate signaling that negatively regulate asexual development. In air exposed cultures, red light-dependent derepression of the activity of the *veA* gene product is required for optimum asexual development. In submerged cultures asexual development requires inactivation of both the *veA* and *sfaD* gene products.

mine critical decisions in the *A.nidulans* lifecycle and differentiate multiple processes including growth, asexual and sexual development, and secondary metabolite production (Figure 5).

While loss-of-function mutations in *fadA* were previously shown to cause significant reductions in hyphal growth, we found that this effect was enhanced by deletion of *sfaD*. Δ *sfaD* mutants were as strongly restricted in growth as Δ *sfaD*; Δ *fadA* double mutants. Although these results are consistent with SfaD having a direct role along with FadA in activating effectors that signal hyphal growth, there are at least three complicating factors clouding this interpretation. First, FadA dominant-activating mutations are not at all affected by Δ *sfaD* mutations (Table I), indicating that FadA can function independently of SfaD in causing uncontrolled proliferative growth that leads to autolysis and cell death. This finding both confirms our earlier conclusion that FadA actively controls proliferation (Yu *et al.*, 1996) and shows that whatever the role of SfaD in proliferative growth, it is not essential.

The second complicating factor in interpreting the effects of Δ *sfaD* mutations on growth results from the knowledge that the *A.nidulans* genome, like that of other filamentous fungi, has at least two additional genes encoding heterotrimeric G protein α subunits (our unpublished data). In contrast, *sfaD* appears to be the only gene encoding a Gβ protein, although there are numerous genes encoding members of the WD40-repeat protein family.

Thus, SfaD probably interacts with other G α proteins, and it is possible that some effects of deleting *sfaD* result from altering activity of distinct signaling pathways. No mutations have been described to date for these additional *A.nidulans* G α -encoding genes but in other filamentous fungi, mutations in homologous genes have little or no phenotypic effect (e.g. Gao and Nuss, 1996; Liu and Dean, 1997; Regenfelder *et al.*, 1997). In any case, the fact that loss-of-function mutations in either *fadA* or *sfaD* caused suppression of *flbA* loss-of-function mutations supports the conclusion that FadA and SfaD function as part of the same heterotrimeric G protein.

The final complication in interpreting the role of SfaD in growth comes from the observation that deletion of *sfaD* (but not *fadA*) resulted in hyperactive sporulation defined by sporulation in submerged culture, a growth condition that normally blocks development. Thus, one role of effectors stimulated by the G $\beta\gamma$ heterodimer could be suppression of the pathway leading to asexual sporulation during submerged growth. This interpretation is further supported by earlier findings that dominant-negative mutations in *fadA*, which are proposed to prevent dissociation of the G α and G $\beta\gamma$ subunits, also caused hyperactive sporulation (Yu *et al.*, 1996). Previous studies have shown that misactivation of genes with specific roles in activating sporulation (e.g. *brlA* or *flbD*) caused a severe reduction in fungal growth (Adams and Timberlake, 1990; Wieser and Adams, 1995). One possible interpretation of these results is that SfaD functions predominantly in activating a signaling pathway that prevents asexual sporulation and the effect of deleting *sfaD* on growth is entirely indirect. This interpretation might explain the different growth responses observed for $\Delta fadA$; $\Delta flbA$ and $\Delta sfaD$; $\Delta flbA$ double mutant strains (Table I). Because deletion of *flbA* did not suppress the hypersporulation phenotype of $\Delta sfaD$ mutants it was not able to suppress the growth defect completely. Finally, it is important to recognize that loss of *sfaD* is not sufficient to allow asexual sporulation to occur. Loss-of-function mutations in *sfaD* were unable to suppress mutations in the development-specific regulator FluG (data not shown; Lee and Adams, 1994b, 1996).

While asexual and sexual development are clearly distinct processes in the life cycle of *A.nidulans*, some earlier studies have indicated that these two important events must have certain features in common. For instance, many mutations that were originally identified as causing defects in conidiophore development also block sexual development (Yager, 1992; Navarro-Bordonaba and Adams, 1994). We have shown here that mutations affecting both *sfaD* and *fadA* cause profound effects on sexual development along with their effects on growth and asexual sporulation. Loss-of-function mutations in *sfaD* and dominant-inactivating mutations in *fadA* both resulted in abundant production of Hülle cells, which are normally produced during formation of sexual fruiting bodies and are thought to have a supporting function in fruiting body formation (Yager, 1992; Navarro-Bordonaba and Adams, 1994). We interpret this result to mean that *sfaD* signaling negatively impacts Hülle cell development in much the same way as we propose it affects asexual sporulation. In contrast, we found that sexual fruiting body formation was blocked by loss-of-function mutations

in *sfaD* or *fadA* or dominant-activating mutations in *fadA*. Thus, controlled signaling by FadA and SfaD is in some way required for further sexual development.

The relationship between *sfaD* and sexual or asexual development is further complicated by our finding that the ability of $\Delta sfaD$ mutations to cause hyperactive sporulation was suppressed by the wild-type allele of a gene called *velvet* (*veA*⁺). Most laboratory strains of *A.nidulans* have the *veA1* mutation, which causes increased asexual sporulation at the expense of sexual sporulation. VeA has been proposed to function as a negative regulator of conidiophore development that prevents asexual sporulation in dark-grown colonies of *A.nidulans*. VeA is inactivated by red light, allowing sporulation to occur in light-grown colonies (Mooney and Yager, 1990). The mechanism by which asexual sporulation is blocked by VeA is not clear, but our results suggest a model in which VeA and SfaD block development through independent but partially redundant mechanisms that monitor environmental conditions. If VeA is absent, SfaD is sufficient to block hyperactive or submerged sporulation although light control has been removed. When SfaD is absent, VeA maintains light control of conidiation by air-exposed colonies and provides an alternative block to hyperactive sporulation during submerged growth. When both SfaD and VeA are absent, conidiation occurs inappropriately both in the dark and in submerged culture.

Cpgb-1 from the chestnut blight fungus *Cryphonectria parasitica* is the only G β subunit that has previously been described for a filamentous fungus and is the closest known relative to SfaD, having 82% identical amino acids. As with *A.nidulans*, *C.parasitica* apparently has a single gene encoding a G protein β subunit, but at least two G protein α subunits (*cpg-1* and *cpg-2*). Cpg-1 is closely related to FadA and like *fadA*, null mutants in *cpg-1* have reduced growth as well as having reduced virulence in *C.parasitica* pathogenesis of chestnut trees (Choi *et al.*, 1995; Gao and Nuss, 1996; Yu *et al.*, 1996). Interestingly, deletion of *cpgb-1* from *C.parasitica* caused the opposite phenotype of *A.nidulans sfaD* deletion: increased growth rate and reduced asexual sporulation (Kasahara and Nuss, 1997). This difference in regulatory outcomes for what is apparently a highly conserved fungal signaling pathway may well reflect innate differences in the lifestyles of *A.nidulans* and *C.parasitica*. The asexual sporulation pathway is normally activated quite early in growth of an *A.nidulans* colony while active growth is still abundant, but in *C.parasitica* asexual sporulation takes place after growth is exhausted. As this pathway is understood in more detail and in more fungi it will be interesting to discover how subtle changes in responses lead to vast differences in timing and regulation of critical life cycle events in different fungi.

Materials and methods

Aspergillus strains, growth conditions and genetics

The *A.nidulans* strains used in this study are described in Table IV. Standard *A.nidulans* transformation and genetic techniques were used (Pontecorvo *et al.*, 1953; Yelton *et al.*, 1984). When appropriate, genotypes of strains generated for this study were confirmed by genomic DNA Southern blot analyses. The *sfaD* deletion strains TSRB1.16 and TJYGV2 were generated by transforming PW1 and VER7, respectively, with pSRBD1. The VER7 strain was a gift from Dong Min Han,

Table IV. *Aspergillus nidulans* strains

Strain	Genotype	Source
FGSC4		FGSC ^a
FGSC26	<i>bia1;veA1</i>	FGSC
FGSC237	<i>pabaA1, yA2;trpC801, veA1</i>	FGSC
PW1	<i>bia1;argB2;methG1;veA1</i>	P.Weglenski
RJY98.22	<i>pabaA1, yA2, bia1, flbA98;argB2;veA1</i>	J.-H.Yu and T.H.Adams, unpublished
RJY115.28	<i>yA2;methG1;fadA^{G203R}, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
RJY115.30	<i>bia1, ya2;fadA^{G203R}, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
RJY115.33	<i>yA2;pyroA4;fadA^{G203R}, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
RJY918.6	<i>argB2;methG1;ΔfadA::argB, veA1</i>	Yu <i>et al.</i> (1996)
RJY918.12	<i>yA2, bia1;methG1;ΔfadA::argB, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
TJYE07.11.1	<i>bia1;ΔflbA::argB;ΔfadA::argB, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
TJYE07.11.2	<i>bia1;ΔflbA::argB;ΔfadA::argB, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
TJYE07.11.3	<i>bia1;ΔflbA::argB;ΔfadA::argB, veA1</i>	J.-H.Yu and T.H.Adams, unpublished
RJH046	<i>bia1, ΔflbA::argB;argB2;pyroA4;veA1</i>	J.K.Hicks and T.H.Adams, unpublished
RJH057	<i>bia1, ΔflbA::argB;argB2;trpC801, veA1</i>	J.K.Hicks and T.H.Adams, unpublished
TTA127.4	<i>pabaA1, yA2;ΔfluG::trpC;trpC801, veA1</i>	Lee and Adams (1994)
RJY82.4	<i>bia1, ΔflbA::argB;sfaD82;veA1</i>	Yu <i>et al.</i> (1999)
SFA82	<i>pabaA1, yA2, flbA98;sfaD82, veA1</i>	Yu <i>et al.</i> (1999)
MSR123	<i>bia1, ΔflbA::argB;argB2;pyroA4;sfaD123, veA1</i>	Yu <i>et al.</i> (1999)
MSR125	<i>bia1, ΔflbA::argB;argB2;pyroA4;sfaD125, veA1</i>	Yu <i>et al.</i> , 1999
RSR126.3	<i>pabaA1, bia1, ΔflbA::argB;sfaD126, veA1</i>	Yu <i>et al.</i> (1999)
MSR127	<i>bia1, ΔflbA::argB;argB2;pyroA4;sfaD127, veA1</i>	Yu <i>et al.</i> (1999)
TSRB1.16	<i>bia1;methG1;argB2;ΔsfaD::argB, veA1</i>	this study
RSRB1.4	<i>pabaA1, bia1;trpC801, ΔsfaD::argB, veA1</i>	this study
RSRB1.15	<i>bia1;ΔsfaD::argB, veA1</i>	this study
RSRB1.17	<i>pabaA1, yA2, methG1;ΔsfaD::argB, veA1</i>	this study
RSRB1.19	<i>pabaA1, yA2;trpC801, ΔsfaD::argB, veA1</i>	this study
RSRF1.31	<i>bia1, ΔflbA::argB;methG1;ΔsfaD::argB, veA1</i>	this study
RSRF1.34	<i>bia1, ΔflbA::argB;methG1;ΔsfaD::argB, veA1</i>	this study
RSRF1.37	<i>bia1, ΔflbA::argB;ΔsfaD::argB, veA1</i>	this study
RSRG1.1	<i>pabaA1, yA2;ΔfluG::argB;methG1;ΔsfaD::argB, veA1</i>	this study
RSRR1.4	<i>yA2;fadA^{G203R}, ΔsfaD::argB, veA1</i>	this study
RSRR1.21	<i>bia1, pyroA4;fadA^{G203R}, ΔsfaD::argB, veA1</i>	this study
RSRFA.1	<i>pabaA1, bia1;methG1;ΔfadA::argB, ΔsfaD::argB, veA1</i>	this study
RSRFA.2	<i>yA2, bia1;ΔfadA::argB, ΔsfaD::argB, veA1</i>	this study
RSRFA.3	<i>pabaA1, bia1;ΔfadA::argB, ΔsfaD::argB, veA1</i>	this study
RSRGD ^b	<i>pabaA1, bia1;trpC::fadA^{R178C}, fadA⁺, ΔsfaD::argB, veA1</i>	this study
RSRGD6	<i>pabaA1, bia1;fadA^{R178C};ΔsfaD::argB, veA1</i>	this study
RSRFG4 ^c	<i>pabaA1, bia1;trpC::fadA^{Q204L}, fadA⁺, ΔsfaD::argB, veA1</i>	this study
VER7	<i>pabaA1, yA2;ΔargB::trpC, trpC801</i>	D.M.Han
TJYGV2	<i>pabaA1, yA2;ΔargB::trpC;trpC801, ΔsfaD::argB</i>	this study
TJYGV3	<i>pabaA1, yA2;ΔargB::trpC;trpC801, ΔsfaD::argB</i>	this study
DSRGF1.1	<i>bia1, ΔflbA::argB;+;methG1, +;ΔsfaD::argB, veA1</i> <i>bia1, ΔflbA::argB;argB2;+, pyroA4;+, veA1</i>	this study

^aFungal Genetics Stock Center.^bThis strain has a single integration of the *fadA^{R178C}* allele at the *trpC801* locus as well as wild-type *fadA⁺*.^cThis strain has approximately two copies of the *fadA^{Q204L}* allele as well as wild-type *fadA⁺*.

Wonkwang University, South Korea. RSRB1.4, RSRB1.15 and RSRB1.19 were derived from a sexual cross between FGSC237 and TSRB1.16. *ΔsfaD;ΔflbA* (RSRF1.31), *ΔsfaD;ΔfluG* (RSRG1.1) and *ΔsfaD;fadA^{G203R}* (RSRR1.21) strains were generated by sexual crosses of TSRB1.16 with RJH057 (*ΔflbA*), TTA127.4 (*ΔfluG*) and RJY115.33 (*fadA^{G203R}*), respectively. RSRFA.1 (*ΔsfaD;ΔfadA*) was generated from a sexual cross of RSRB1.4 with RJY 918.12 (*ΔfadA*). The *ΔsfaD;fadA^{Q204L}* strain (RSRFG4) was generated by transforming RSRB1.4 with a plasmid carrying the *fadA^{Q204L}* allele (pJY.26SH) and was shown to have at least two copies of the *fadA^{Q204L}* allele as well as wild-type *fadA⁺*. The *ΔsfaD;fadA^{R178C}* strains (RSRGD2 and RSRGD6) were generated by transforming RSRB1.19 with pJY.27SH and were shown to have a replacement of wild-type *fadA* (RSRGD6) or a single integration of the *fadA^{R178C}* allele at the *trpC* locus (RSRGD2). To test whether a loss-of-function mutation in Gβ could be dominant, diploids were generated between RSRF1.31 (*ΔsfaD;ΔflbA*) and RJH046 (*sfaD⁺;ΔflbA*).

All strains were grown in appropriately supplemented minimal or complete medium (Käfer, 1977) unless otherwise indicated. Radial growth rates were determined as described previously (Wieser *et al.*, 1994). For biomass analyses and developmental timing in submerged cultures, liquid cultures were inoculated at a density of 1×10^6 spores/ml (100 ml medium/250 ml Erlenmeyer flasks). The flasks were incubated on a New Brunswick rotary shaker (300 r.p.m.) at 37°C and mycelia

from each culture were observed microscopically every hour starting at 12 h to assess development. To determine biomass, cultures were harvested by filtering through Miracloth, freeze-dried and weighed.

Nucleic acid manipulations and other methods

Cloning of the gene encoding the Gβ subunit (*sfaD*) was as follows. The degenerate primers pGB5 (5'-ATHTAYGCNATGCAYTGG-3') and pGB3 (5'-RAARTCRTCTANCCNGC-3'), with H = A + C + T, R = A + G, Y = C + T and N = A + T + G + C, were used for PCR amplification of *A.nidulans* DNA from wild-type strain FGSC26. Amplification was achieved in 35 cycles of 0.5 min at 94°C, 1 min at 43°C, and 1.5 min at 72°C. The PCR product was precipitated, run on an agarose gel and a 0.75 kb fragment was excised. The labeled fragment was used to screen a partially ordered genomic cosmid library and three cosmid clones known to be derived from chromosome eight (W08C10, W17E07 and W30E09) were recovered (Brody *et al.*, 1991). A 3.2 kb *PstI* fragment from one of these cosmids (W17E07) was cloned into pBluescript SK⁻ for sequencing (pSRB10). Four incomplete Gβ cDNA clones were isolated by screening the λUNI-ZAP library made from vegetatively grown wild-type tissue (May *et al.*, 1987). The 5' end of Gβ cDNA was PCR amplified using 5'-CGACGCGCTCGTTCCGC-3' and 5'-GGACTTTGTTCTGATGG-3' as primers with λgt10 (Osmani

et al., 1988) cDNA library as a template. A 0.4 kb amplified fragment was isolated, cloned into pPK1 and sequenced.

The plasmid used for deletion of G β , pSRBD1, was constructed from pSRB10. The *XhoI* and *SalI* sites (found in the polylinker region) in pSRB10 were eliminated by digestion with *XhoI* and *SalI* and religation resulting in plasmid pSRB11. Using this plasmid the entire ORF (25 bases upstream and 91 bases beyond G β start and stop codon, respectively) of G β was deleted using site-directed mutagenesis with the synthetic oligonucleotide 5'-CAATGCGGACATCCGGCTCTCGAGCGGCAAT-TGGAGTGGTG-3', as described previously (Kunkel, 1985), introducing a *XhoI* site 25 bases 5' of the G β start codon. The resulting plasmid, designated pSRBD0, was digested with *XhoI* and ligated with a 1.8 kb *XhoI* fragment from pJW88 (J. Wieser, unpublished) containing the *A. nidulans argB* gene to yield pSRBD1 which was used to transform *A. nidulans* PW1 strain. The *fadA*^{Q204L} and *fadA*^{R178C} dominant-activating alleles were generated by site-directed mutagenesis as described previously (Yu *et al.*, 1999) and then moved into pSH96 (Wieser and Adams, 1995) to give pJY.26SH and pJY.27SH, respectively.

To isolate mutant alleles of *sfaD*, we constructed genomic libraries using a plasmid vector that contained the *A. nidulans argB*⁺ gene (pPK1; Wieser and Adams, 1995). The libraries were constructed by cutting genomic DNA from the different *sfaD* strains with *PstI*, *HindIII*, *XhoI*, *XbaI* and *SacII* and then ligated to *PstI*-cut pPK1. To test for dominance or recessives of the *sfaD* allele mutations a *flbA*⁻ strain (RJY98.22) was transformed with pPK1 plasmids containing inserts (3.2 kb *PstI* fragments) encoding the different *sfaD* alleles.

Photomicrographs of hyphal development were taken using an Olympus BH2 compound microscope and differential interference contrast optics. All other microscopy was carried out using an Olympus SZ-11 stereo microscope and transmitted light.

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